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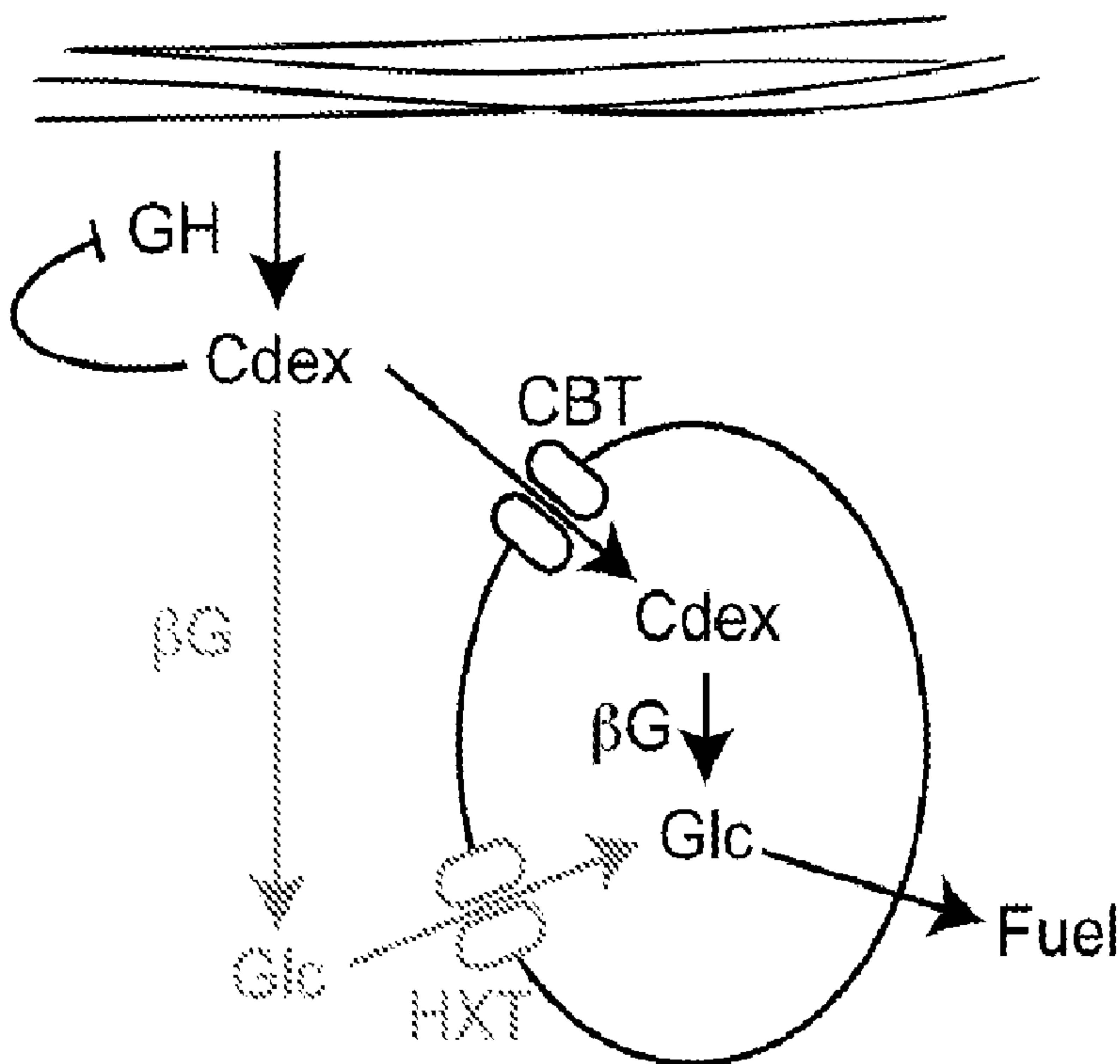
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(54) Title: METHODS AND COMPOSITIONS FOR IMPROVING SUGAR TRANSPORT, MIXED SUGAR
FERMENTATION, AND PRODUCTION OF BIOFUELS

Fig. 25



(57) Abrégé/Abstract:

The present disclosure relates to host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports cellodextrin into the cell. The present disclosure further relates to methods of increasing transport of cellodextrin into a

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(57) **Abrégé(suite)/Abstract(continued):**

cell, methods of increasing growth of a cell on a medium containing cellodextrin, methods of co-fermenting cellulose-derived and hemicellulose- derived sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports cellodextrin into the cell. The present disclosure relates to host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell, methods of increasing transport of a pentose into a cell, methods of increasing growth of a cell on a medium containing pentose sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell.

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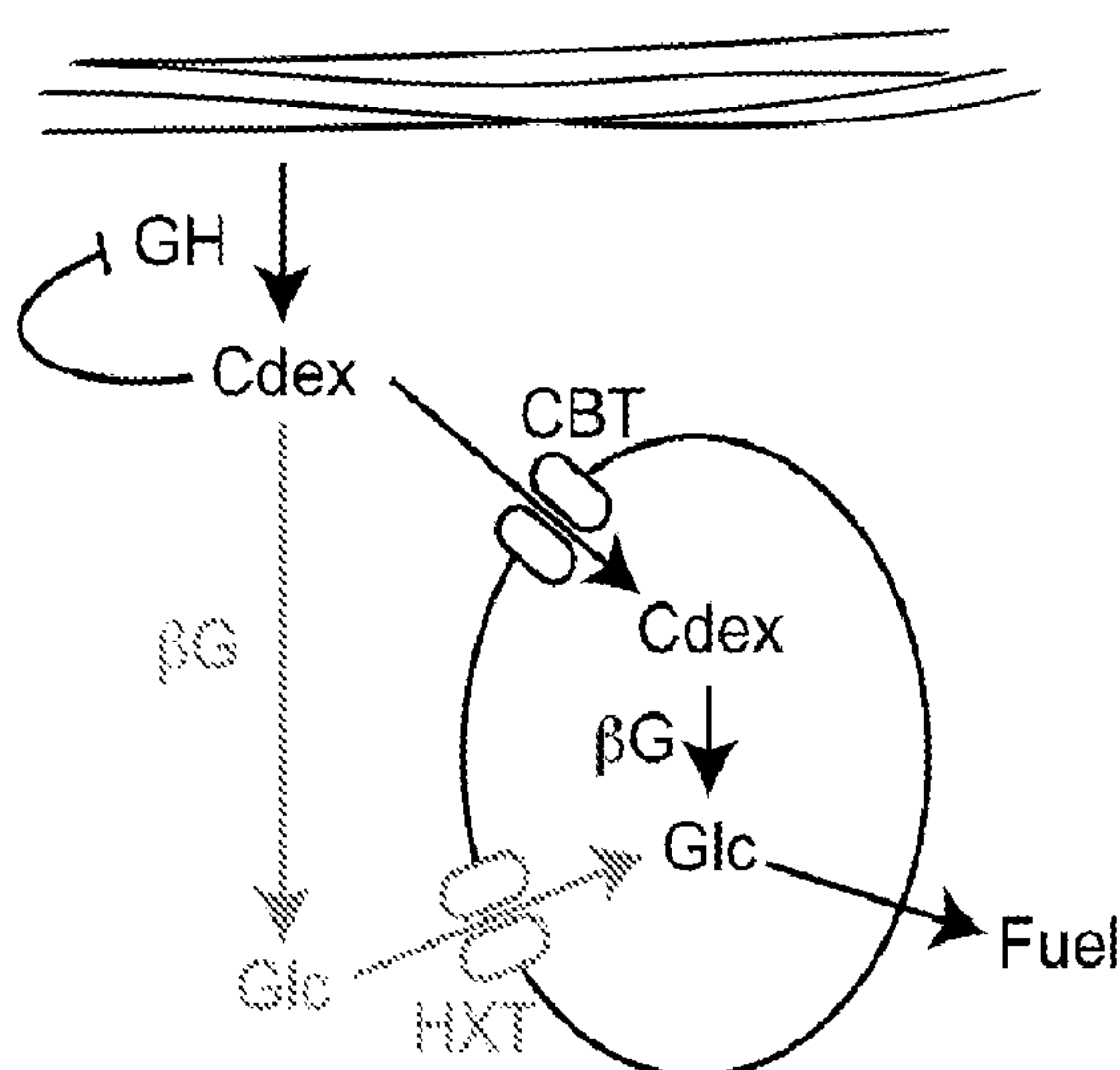
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(54) Title: METHODS AND COMPOSITIONS FOR IMPROVING SUGAR TRANSPORT, MIXED SUGAR FERMENTATION, AND PRODUCTION OF BIOFUELS

Fig. 25



(57) Abstract: The present disclosure relates to host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports celldextrin into the cell. The present disclosure further relates to methods of increasing transport of celldextrin into a cell, methods of increasing growth of a cell on a medium containing celldextrin, methods of co-fermenting cellulose-derived and hemicellulose-derived sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports celldextrin into the cell. The present disclosure relates to host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell, methods of increasing transport of a pentose into a cell, methods of increasing growth of a cell on a medium containing pentose sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell.

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METHODS AND COMPOSITIONS FOR IMPROVING SUGAR TRANSPORT, MIXED SUGAR FERMENTATION, AND PRODUCTION OF BIOFUELS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/285,526, filed December 10, 2009, and U.S. Provisional Application No. 61/271,833, filed July 24, 2009, both of which are hereby incorporated by reference in their entirety.

FIELD OF THE INVENTION

The present disclosure relates to methods and compositions for increasing the transport of sugars into cells, for increasing growth of cells, for increasing synthesis of hydrocarbons and hydrocarbon derivatives, and for co-fermenting cellulose-derived and hemicellulose-derived sugars.

BACKGROUND OF THE INVENTION

Biofuels are under intensive investigation due to the increasing concerns about energy security, sustainability, and global climate change (Lynd *et al.*, 1991). Bioconversion of plant-derived lignocellulosic materials into biofuels has been regarded as an attractive alternative to chemical production of fossil fuels (Lynd *et al.* 2008; Hahn-Hagerdal *et al.* 2006). Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin.

The engineering of microorganisms to perform the conversion of lignocellulosic biomass to ethanol efficiently remains a major goal of the biofuels field. Much research has been focused on genetically manipulating microorganisms that naturally ferment simple sugars to alcohol to express cellulases and other enzymes that would allow them to degrade lignocellulosic biomass polymers and generate ethanol within one cell. However, an area that has been less well studied is that of sugar transporters. An understanding of the regulation of sugar transport and the genetic engineering of microorganisms to have improved sugar-uptake ability will greatly improve efficiency (Stephanopoulos 2007). Furthermore, other types of proteins involved in the regulation of cellulase expression and activity remain to be fully explored.

Saccharomyces cerevisiae, also known as baker's yeast, has been used for bioconversion of hexose sugars into ethanol for thousands of years. It is also the most widely used microorganism for large

scale industrial fermentation of D-glucose into ethanol. *S. cerevisiae* is a very suitable candidate for bioconversion of lignocellulosic biomass into biofuels (van Maris *et al.*, 2006). It has a well-studied genetic and physiological background, ample genetic tools, and high tolerance to high ethanol concentration and inhibitors presented in lignocellulosic hydrolysates (Jeffries 2006). The low fermentation pH of *S. cerevisiae* can also prevent bacterial contamination during fermentation.

Unfortunately, wild type *S. cerevisiae* cannot utilize pentose sugars (Hector *et al.*, 2008). To overcome this limitation, pentose utilization pathways from pentose-assimilating organisms have been introduced into *S. cerevisiae*, allowing fermentation of D-xylose and L-arabinose (Hahn-Hagerdal *et al.*, 2007; Brat *et al.*, 2009; Wisselink *et al.*, 2007, 2009; Wiedemann and Boles 2008; Karhumma *et al.*, 2006). However, efficient conversion of pentose sugars into biofuels is limited by multiple issues including cellular redox imbalance, low influx of pentose phosphate pathway, and lack of efficient pentose transport into the cell (Hector *et al.*, 2008).

In addition, both natural and engineered microorganisms show reduced ethanol tolerance during xylose fermentation as compared to glucose fermentation (Jeffries and Jin 2000). Combined with the lower fermentation rate, the reduced ethanol tolerance during xylose fermentation poses a significant problem in fermentation of sugar mixtures containing the high concentrations of glucose (~ 70-100 g/L) and xylose (~ 40-60 g/L) present in cellulosic hydrolysates. Since microorganisms utilize glucose preferentially, at the time of glucose depletion (when cells begin to use xylose), the ethanol concentration is already high enough (~ 35-45 g/L) to further reduce the xylose fermentation rate. As a result, sequential utilization of xylose after glucose depletion because of “glucose repression” is a significant challenge to be overcome in order to successfully utilize mixed sugars in cellulosic hydrolysates.

Thus, a need exists for the identification of additional genes that are critical for the degradation of lignocellulose and for their use in the engineering of microorganisms for improved growth on lignocellulose and uptake of compounds resulting from lignocellulose degradation. A further need exists for improved methods of efficient conversion of pentose sugars into biofuels and of mixed sugar fermentation for the production of biofuels.

BRIEF SUMMARY OF THE INVENTION

In order to meet these needs, the invention described herein provides methods of increasing transport of cellodextrin into a cell, methods of increasing growth of a cell on a medium containing cellodextrin, methods of co-fermenting cellulose-derived and hemicellulose-derived sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports cellodextrin into the cell. Further described are host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports cellodextrin into the cell. Further described herein are host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell, methods of increasing transport of a pentose into a cell, methods of increasing growth of a cell on a medium containing pentose sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell.

As used herein, cellodextrin refers to glucose polymers of varying length and includes, without limitation, cellobiose (2 glucose monomers), cellotriose (3 glucose monomers), cellotetraose (4 glucose monomers), cellopentaose (5 glucose monomers), and cellohexaose (6 glucose monomers).

Thus one aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 contains SEQ ID NO: 1, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 contains SEQ ID NO: 2, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of

the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 contains SEQ ID NO: 3, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 contains SEQ ID NO: 4, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 contains SEQ ID NO: 5, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 contains SEQ ID NO: 6, and culturing the cell in a medium such that the

recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 contains SEQ ID NO: 7, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of cellodextrin into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them contains SEQ ID NO: 8, and culturing the cell in a medium such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide.

In certain embodiments that may be combined with any of the preceding aspects, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments that may be combined with any of the preceding embodiments, the host cell contains a second recombinant polynucleotide encoding at least a catalytic domain of a β -glucosidase. In certain embodiments that may be combined with the preceding embodiments having a host cell containing a second recombinant polynucleotide encoding at least a catalytic domain of a β -glucosidase, the β -glucosidase is from *Neurospora crassa*. In certain embodiments that may be combined with the preceding embodiments having a host cell containing a second recombinant polynucleotide encoding at least a catalytic domain of a β -glucosidase from *Neurospora crassa*, the β -glucosidase is encoded by

NCU00130. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. In certain embodiments that may be combined with the preceding embodiments having a host cell further containing one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization, the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains a third recombinant polynucleotide where the third recombinant polynucleotide encodes a pentose transporter. In certain embodiments that may be combined with the preceding embodiments having the host cell further containing a third recombinant polynucleotide where the third recombinant polynucleotide encodes a pentose transporter, the pentose transporter is selected from the group consisting of NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, and XUT3.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 contains SEQ ID NO: 1, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 contains SEQ ID NO: 2, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 contains SEQ ID NO: 3, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 contains SEQ ID NO: 4, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 contains SEQ ID NO: 5, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 contains SEQ ID NO: 6, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 contains SEQ ID NO: 7, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them contain SEQ ID NO: 8, and the polypeptide is a cellodextrin transporter, and culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

In certain embodiments that may be combined with any of the preceding aspects of increasing growth of cells, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments that may be combined with any of the preceding embodiments, the host cell contains an endogenous or a second recombinant polynucleotide where the polynucleotide encodes at least a catalytic domain of a β -glucosidase. In certain embodiments that may be combined with the preceding embodiments having a host cell containing an endogenous or a second recombinant polynucleotide where the polynucleotide encodes at least a catalytic domain of a β -glucosidase, the β -glucosidase is from *Neurospora crassa*. In certain embodiments that may be combined with the preceding embodiments having a host cell containing an endogenous or a second recombinant polynucleotide where the polynucleotide encodes at least a catalytic domain of a β -glucosidase from *Neurospora crassa*, the β -glucosidase is encoded by NCU00130.

Another aspect includes methods of co-fermenting cellulose-derived and hemicellulose-derived sugars, containing providing a host cell, where the host cell contains a first recombinant polynucleotide

encoding a cellodextrin transporter and a second recombinant polynucleotide encoding a catalytic domain of a β -glucosidase, and culturing the host cell in a medium containing a cellulose-derived sugar and a hemicellulose-derived sugar, where expression of the recombinant polynucleotides enables co-fermentation of the cellulose-derived sugar and the hemicellulose-derived sugar. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 contains SEQ ID NO: 1. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 contains SEQ ID NO: 2. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 contains SEQ ID NO: 3. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 contains SEQ ID NO: 4. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 contains SEQ ID NO: 5. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 contains SEQ ID NO: 6. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 contains SEQ ID NO: 7. In certain embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them contain SEQ ID NO: 8. In certain embodiments that may be combined with any of the preceding embodiments, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at

least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments that may be combined with any of the preceding embodiments, the β -glucosidase is from *Neurospora crassa*. In certain embodiments that may be combined with the preceding embodiments having a host cell containing a second recombinant polynucleotide encoding a catalytic domain of a β -glucosidase from *Neurospora crassa*, the β -glucosidase is encoded by NCU00130. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. In certain embodiments that may be combined with the preceding embodiments having a host cell further containing one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization, the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains a third recombinant polynucleotide where the third recombinant polynucleotide encodes a pentose transporter. In certain embodiments that may be combined with the preceding embodiments having the host cell further containing a third recombinant polynucleotide where the third recombinant polynucleotide encodes a pentose transporter, the pentose transporter is selected from the group consisting of NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, and XUT3. In certain embodiments that may be combined with any of the preceding embodiments, the cellulose-derived sugar is selected from the group consisting of cellobiose, cellotriose, and celltetraose, and the hemicellulose-derived sugar is xylose.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 contains SEQ ID NO: 1, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, containing providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 contains SEQ ID NO: 2, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 contains SEQ ID NO: 3, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 contains SEQ ID NO: 4, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant

polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 contains SEQ ID NO: 5, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 contains SEQ ID NO: 6, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 contains SEQ ID NO: 7, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and

transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them contain SEQ ID NO: 8, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

In certain embodiments that may be combined with any of the preceding aspects increasing the synthesis of hydrocarbons or hydrocarbon derivatives, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains a second recombinant polynucleotide where the polynucleotide encodes at least a catalytic domain of a β -glucosidase. In certain embodiments that may be combined with preceding embodiments having the host cell further containing a second recombinant polynucleotide where the polynucleotide encodes at least a catalytic domain of a β -glucosidase, the β -glucosidase is from *Neurospora crassa*. In certain embodiments that may be combined with preceding embodiments having the host cell further containing a second recombinant polynucleotide where the polynucleotide encodes at least a catalytic domain of a β -glucosidase from *Neurospora crassa*, the β -glucosidase is encoded by NCU00130. In certain embodiments that may be combined with any of the preceding embodiments, the source of the cellodextrin contains cellulose. In certain embodiments that may be combined with any of the preceding embodiments, the hydrocarbons or hydrocarbon derivatives can be used as fuel. In certain embodiments that may be combined with the preceding embodiments having the hydrocarbons or hydrocarbon derivatives used as fuel, the hydrocarbons or hydrocarbon derivatives contain ethanol. In certain embodiments that may be combined with the preceding embodiments having the hydrocarbons or hydrocarbon derivatives used as fuel, the hydrocarbons or hydrocarbon derivatives contain butanol.

In certain embodiments that may be combined with any of the preceding aspects, the medium contains a cellulase-containing enzyme mixture from an altered organism, where the cellulase-containing mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism. In certain embodiments that may be combined with any of the preceding aspects, the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces*

monacensis, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*. In certain embodiments that may be combined with any of the preceding aspects, cellodextrin is selected from one or more of the group consisting of cellobiose, cellotriose, and cellotetraose.

Another aspect includes host cells containing a recombinant polynucleotide encoding a polypeptide having transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, an intracellular N-terminus, an intracellular C-terminus, and a sequence selected from the group consisting of SEQ ID NO: 1 in transmembrane α -helix 1, SEQ ID NO: 2 in transmembrane α -helix 2, SEQ ID NO: 3 in a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3, SEQ ID NO: 4 in transmembrane α -helix 5, SEQ ID NO: 5 in transmembrane α -helix 6, SEQ ID NO: 6 in the sequence between transmembrane α -helix 6 and transmembrane α -helix 7, SEQ ID NO: 7 in transmembrane α -helix 7, and SEQ ID NO: 8 in transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them, where the polypeptide is a cellodextrin transporter. In certain embodiments, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments that may be combined with either of the preceding embodiments, the host cell further contains a second recombinant polynucleotide where the second recombinant polynucleotide encodes a catalytic domain of a β -glucosidase. In certain embodiments that may be combined with preceding embodiments having the host cell further containing a second recombinant polynucleotide where the second recombinant polynucleotide encodes a catalytic domain of a β -glucosidase, the β -glucosidase is from *Neurospora crassa*. In certain embodiments that may be combined with the preceding embodiments having the host cell further containing a second recombinant polynucleotide where the second recombinant polynucleotide encodes a catalytic domain of a β -glucosidase from *Neurospora crassa*, the β -glucosidase is encoded by NCU00130. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains one or more recombinant polynucleotides where the one or

more polynucleotides encode one or more enzymes involved in pentose utilization. In certain embodiments that may be combined with the preceding embodiments having the host cell further containing one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization, the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase. In certain embodiments that may be combined with any of the preceding embodiments, the host cell further contains a third recombinant polynucleotide where the third recombinant polynucleotide encodes a pentose transporter. In certain embodiments that may be combined with the preceding embodiment having the host cell further containing a third recombinant polynucleotide where the third recombinant polynucleotide encodes a pentose transporter, the pentose transporter is selected from the group consisting of NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, and XUT3.

In certain embodiments that may be combined with any of the preceding aspects, the host cell further contains one or more inducible promoters operably linked to the one or more recombinant polynucleotides.

Another aspect includes a host cell containing a recombinant polynucleotide encoding a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, where the polypeptide transports xylose into the cell.

Another aspect includes a host cell containing a recombinant polynucleotide encoding a XUT1 polypeptide, where the polypeptide transports arabinose into the cell.

Another aspect includes a host cell containing a recombinant polynucleotide encoding an NCU06138 polypeptide, where the polypeptide transports arabinose and glucose into the cell.

Another aspect includes a host cell containing a recombinant polynucleotide encoding a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, where the polypeptide transports xylose and glucose into the cell.

Another aspect includes a host cell containing a recombinant polynucleotide encoding an NCU04963 polypeptide, where the polypeptide transports xylose, arabinose, and glucose into the cell.

In certain embodiments that may be combined with any of the preceding aspects having a host cell containing a recombinant polynucleotide encoding a pentose transporter, the host cell further contains one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. In certain embodiments that may be combined with the preceding embodiment having the host cell further containing one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization, the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.

Another aspect includes methods of increasing transport of xylose into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of xylose into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of arabinose into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a XUT1 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of arabinose into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of arabinose or glucose into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU06138 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of arabinose or glucose into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of xylose or glucose into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in

increased transport of xylose or glucose into the cell compared with a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing transport of xylose, arabinose, or glucose into a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU04963 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of xylose, arabinose, or glucose into the cell compared with a cell that does not contain the recombinant polynucleotide.

In certain embodiments that may be combined with any of the preceding aspects of increasing transport of xylose, arabinose, or glucose into cells, the method further includes one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. In certain embodiments that may be combined with the preceding embodiments having the method further including one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization, the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, and the polypeptide transports xylose, and culturing the host cell in a medium containing xylose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a XUT1 polypeptide, and the polypeptide transports arabinose, and culturing the host cell in a medium containing arabinose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes method of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes an

NCU06138 polypeptide, and the polypeptide transports arabinose and glucose, and culturing the host cell in a medium containing arabinose or glucose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, and the polypeptide transports xylose and glucose, and culturing the host cell in a medium including xylose or glucose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a NCU04963 polypeptide, and the polypeptide transports xylose, arabinose, and glucose, and culturing the host cell in a medium containing xylose, arabinose, or glucose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

In certain embodiments that may be combined with the preceding aspects of increasing growth of cells by culturing a host cell containing a recombinant polynucleotide encoding a polypeptide that transports xylose and/or arabinose and/or glucose, the host cell further contains one or more endogenous or recombinant polynucleotides encoding one or more enzymes involved in pentose utilization. In certain embodiments that may be combined with the preceding embodiments having the host cell further containing one or more endogenous or recombinant polynucleotides encoding one or more enzymes involved in pentose utilization, the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, where the polypeptide transports xylose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing xylose or a source of

xylose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of xylose into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a XUT1 polypeptide, where the polypeptide transports arabinose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing arabinose or a source of arabinose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of arabinose into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding an NCU06138 polypeptide, where the polypeptide transports arabinose or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing arabinose or glucose or a source of arabinose or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of arabinose or glucose into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, where the polypeptide transports xylose or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing xylose or glucose or a source of xylose or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of xylose or glucose into the cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, including providing a host cell, where the host cell contains a recombinant polynucleotide encoding an NCU04963 polypeptide, where the polypeptide transports xylose, arabinose, or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing xylose, arabinose, or glucose or a source of xylose, arabinose, or

glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of xylose, arabinose, or glucose into the cell is increased upon expression of the recombinant polynucleotide.

In certain embodiments that may combine any of the preceding aspects of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by culturing a host cell containing a recombinant polynucleotide encoding a polypeptide that transports glucose, the source of glucose contains cellulose. In certain embodiments that may combine any of the preceding embodiments, the source of xylose or arabinose contains hemicellulose. In certain embodiments that may combine any of the preceding embodiments, the hydrocarbons or hydrocarbon derivatives can be used as fuel. In certain embodiments that may combine the preceding embodiment having the hydrocarbons or hydrocarbon derivatives used as fuel, the hydrocarbons or hydrocarbon derivatives contain ethanol. In certain embodiments that may combine the preceding embodiment having the hydrocarbons or hydrocarbon derivatives used as fuel, the hydrocarbons or hydrocarbon derivatives contain butanol.

In certain embodiments that may combine any of the preceding embodiments, the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a NCU07705 polypeptide, and culturing the cell in a medium containing cellulose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. In certain embodiments, the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*,

Candida shehatae, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*. In certain embodiments, the host cell further contains an inducible promoter operably linked to the recombinant polynucleotide. In certain embodiments, expression of cellulases is increased in the host cell upon expression of the recombinant polynucleotide.

Another aspect includes methods of increasing growth of a cell on a biomass polymer, including providing a host cell, where the host cell contains an endogenous polynucleotide where the polynucleotide encodes an NCU05137 polypeptide, inhibiting expression of the endogenous polynucleotide, and culturing the cell in a medium containing the biomass polymer, where the host cell grows at a faster rate in the medium than a cell in which expression of the endogenous polynucleotide is not inhibited. In certain embodiments, the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*. In certain embodiments, cellulase activity of the host cell is increased upon inhibiting expression of the endogenous polynucleotide. In certain embodiments, hemicellulase activity of the host cell is increased upon inhibiting expression of the endogenous polynucleotide. In certain embodiments, inhibiting expression of the endogenous polynucleotide contains mutating or deleting a gene containing the endogenous polynucleotide. In certain embodiments, the biomass polymer is cellulose. In certain embodiments, the biomass polymer is hemicellulose.

Another aspect includes methods of increasing growth of a cell, including providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a polypeptide selected from the group consisting of NCU01517, NCU09133, and NCU10040, and culturing the cell in a medium containing hemicellulose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. In certain embodiments, the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces*

monacensis, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*. In certain embodiments, the host cell further contains an inducible promoter operably linked to the recombinant polynucleotide. In certain embodiments, hemicellulase activity of the host cell is increased upon expression of the recombinant polynucleotide.

Another aspect includes methods of degrading cellulose, including providing a composition containing cellulose, and contacting the composition with a cellulase-containing enzyme mixture from an altered organism, where the cellulase-containing mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism, and where the cellulose is degraded by the cellulase-containing mixture. In certain embodiments, the organism is altered by mutation of a gene encoding a β -glucosidase. In certain embodiments, the organism is altered by reducing the expression of a β -glucosidase. In certain embodiments that may be combined with any of the preceding embodiments, the organism is selected from the group consisting of a fungus and a bacterium. In certain embodiments that may be combined with any of the preceding embodiments having the organism selected from the group consisting of a fungus and a bacterium, the organism is a filamentous fungus. In certain embodiments that may be combined with any of the preceding embodiments, the cellulose is from plant material. In certain embodiments that may be combined with the preceding embodiments having the cellulose from plant material, the plant material is selected from the group consisting of switchgrass, *Miscanthus*, rice hulls, bagasse, flax, bamboo, sisal, abaca, straw, leaves, grass clippings, corn stover, corn cobs, distillers grains, legume plants, sorghum, sugar cane, sugar beet pulp, wood chips, sawdust, and biomass crops.

Yet another aspect includes methods of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell comprising providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a polypeptide encoded by a sequence selected from the group consisting of NCU00801, NCU00988, NCU01231, NCU04963, NCU05519, NCU05853, NCU05897, NCU06138, NCU00809, NCU08114, NCU10021, and any of the genes listed in

Table 15 and culturing the host cell in a medium comprising a source of a compound to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein the compound is a substrate for the synthesis of the hydrocarbons or hydrocarbon derivatives, and wherein transport of the compound into the cell is increased upon expression of the recombinant polynucleotide. In certain embodiments, the host cell is selected from the group consisting of *Saccharomyces cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, *Neurospora crassa*, *Candida shehatae*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Moorella thermocetica*, *Thermoanaerobacterium saccharolyticum*, *Klebsiella oxytoca*, and *Pichia stipitis*. In certain embodiments, the host cell further comprises an inducible promoter operably linked to the recombinant polynucleotide. In certain embodiments, the recombinant polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by a sequence selected from the group consisting of NCU00801, NCU00988, NCU01231, NCU04963, NCU05519, NCU05853, NCU05897, NCU06138, NCU00809, NCU08114, NCU10021, and any of the genes listed in Table 15. In some embodiments, the hydrocarbons or hydrocarbon derivatives can be used as fuel. In certain embodiments, the medium comprises cellulose. In other embodiments, the medium comprises hemicellulose. In certain embodiments, the compound is a sugar. In certain embodiments that may be combined with the preceding embodiments, the sugar is a pentose. In certain embodiments that may be combined with the preceding embodiments, the sugar is a hexose. In certain embodiments that may be combined with the preceding embodiments, the sugar is a disaccharide. In certain embodiments that may be combined with the preceding embodiments, the sugar is an oligosaccharide. In other embodiments, the compound is a plant phenol. In certain embodiments that may be combined with the preceding embodiments, the plant phenol is quinic acid. In certain embodiments that may be combined with the preceding embodiments, the plant phenol is nicotinamide. In other embodiments, the compound is pyruvate or lactate.

Another aspect includes methods of increasing growth of a cell on a biomass polymer comprising providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a polypeptide encoded by any of the *Neurospora* or *Pichia stipitis* genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, *PNAS*, 2009, vol. 106, no. 52, 22157-22162, the disclosure of which is hereby incorporated by reference, in Table 15, or NCU01517, NCU09133, or NCU10040 and culturing the cell in a medium comprising the biomass polymer, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide. In certain embodiments, the polynucleotide encodes a polypeptide encoded by any of the

sequences NCU00130.2, NCU00248.2, NCU00326.2, NCU00762.2, NCU00810.2, NCU00890.2, NCU03328.2, NCU03415.2, NCU03731.2, NCU03753.2, NCU04197.2, NCU04249.2, NCU04287.2, NCU04349.2, NCU04475.2, NCU04997.2, NCU05057.2, NCU05159.2, NCU05493.2, NCU05751.2, NCU05770.2, NCU05932.2, NCU06009.2, NCU06490.2, NCU07340.2, NCU07853.2, NCU07997.2, NCU08744.2, NCU08746.2, NCU08760.2, NCU09108.2, NCU09495.2, NCU09680.2, or NCU10045.2. In certain embodiments, the polynucleotide encodes a polypeptide encoded by NCU07705. In certain embodiments, the recombinant polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by any of the *Neurospora* or *Pichia stipitis* genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009, or in Table 15. In certain embodiments, the polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by any of the sequences NCU00130.2, NCU00248.2, NCU00326.2, NCU00762.2, NCU00810.2, NCU00890.2, NCU03328.2, NCU03415.2, NCU03731.2, NCU03753.2, NCU04197.2, NCU04249.2, NCU04287.2, NCU04349.2, NCU04475.2, NCU04997.2, NCU05057.2, NCU05159.2, NCU05493.2, NCU05751.2, NCU05770.2, NCU05932.2, NCU06009.2, NCU06490.2, NCU07340.2, NCU07853.2, NCU07997.2, NCU08744.2, NCU08746.2, NCU08760.2, NCU09108.2, NCU09495.2, NCU09680.2, or NCU10045.2. In certain embodiments, the recombinant polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by NCU07705. In certain embodiments, the biomass polymer is cellulose. In other embodiments, the biomass polymer is hemicellulose. In certain embodiments, the host cell is selected from the group consisting of *Saccharomyces cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, *Neurospora crassa*, *Candida shehatae*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Moorella thermocetica*, *Thermoanaerobacterium saccharolyticum*, *Klebsiella oxytoca*, and *Pichia stipitis*. In certain embodiments, the host cell further comprises an inducible promoter operably linked to the recombinant polynucleotide. In certain embodiments, expression of cellulases is increased in the host cell upon expression of the recombinant polynucleotide. In other embodiments, expression of hemicellulases is increased in the host cell upon expression of the recombinant polynucleotide.

Yet another aspect includes methods of increasing growth of a cell on a biomass polymer comprising providing a host cell, wherein the host cell comprises an endogenous polynucleotide wherein the polynucleotide encodes a polypeptide encoded by any of the *Neurospora* or *Pichia stipitis* genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009, or in Table 15, or, inhibiting expression of the endogenous polynucleotide, and culturing the cell in a medium comprising the biomass

polymer, wherein the host cell grows at a faster rate in the medium than a cell in which expression of the endogenous polynucleotide is not inhibited. In certain embodiments, the endogenous polynucleotide encodes a polypeptide encoded by any of the sequences NCU00130.2, NCU00248.2, NCU00326.2, NCU00762.2, NCU00810.2, NCU00890.2, NCU03328.2, NCU03415.2, NCU03731.2, NCU03753.2, NCU04197.2, NCU04249.2, NCU04287.2, NCU04349.2, NCU04475.2, NCU04997.2, NCU05057.2, NCU05159.2, NCU05493.2, NCU05751.2, NCU05770.2, NCU05932.2, NCU06009.2, NCU06490.2, NCU07340.2, NCU07853.2, NCU07997.2, NCU08744.2, NCU08746.2, NCU08760.2, NCU09108.2, NCU09495.2, NCU09680.2, or NCU10045.2. In certain embodiments, the endogenous polynucleotide encodes a polypeptide encoded by NCU05137. In certain embodiments, the endogenous polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by any of the *Neurospora* or *Pichia stipitis* genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009, or in Table 15. In certain embodiments, the endogenous polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by any of the sequences NCU00130.2, NCU00248.2, NCU00326.2, NCU00762.2, NCU00810.2, NCU00890.2, NCU03328.2, NCU03415.2, NCU03731.2, NCU03753.2, NCU04197.2, NCU04249.2, NCU04287.2, NCU04349.2, NCU04475.2, NCU04997.2, NCU05057.2, NCU05159.2, NCU05493.2, NCU05751.2, NCU05770.2, NCU05932.2, NCU06009.2, NCU06490.2, NCU07340.2, NCU07853.2, NCU07997.2, NCU08744.2, NCU08746.2, NCU08760.2, NCU09108.2, NCU09495.2, NCU09680.2, or NCU10045.2. In certain embodiments, the endogenous polynucleotide encodes a polypeptide having at least 50% amino acid identity to the polypeptide encoded by NCU05137. In certain embodiments, the host cell is selected from the group consisting of *Saccharomyces cerevisiae*, *Escherichia coli*, *Zymomonas mobilis*, *Neurospora crassa*, *Candida shehatae*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Moorella thermocetica*, *Thermoanaerobacterium saccharolyticum*, *Klebsiella oxytoca*, and *Pichia stipitis*. In certain embodiments, the biomass polymer is cellulose. In other embodiments, the biomass polymer is hemicellulose. In certain embodiments, cellulase activity of the host cell is increased upon inhibiting expression of the endogenous polynucleotide. In other embodiments, hemicellulase activity of the host cell is increased upon inhibiting expression of the endogenous polynucleotide. In certain embodiments, inhibiting expression of the endogenous polynucleotide comprises mutating or deleting a gene comprising the endogenous polynucleotide.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the domain structure of the polypeptide encoded by NCU07705.

FIG. 2 shows the phylogenetic analysis of NCU05137. The predicted orthologs of *N. crassa* NCU05137 were retrieved from NCBI and JGI based on amino acid sequences showing significant similarity by BLAST. All identified filamentous fungal orthologs are shown; NCBI E values were 0.0 except for *B. fuckeliana*, which was 9e-175. Homologs of NCU05137 were also identified in a number of bacteria (E value ~ e-30). YP 981875 from *Polaromonas naphthalenivorans* (a beta-proteobacterium) was used as an outgroup. A.=*Aspergillus*; N.=*Neosartorya*; P. *chyrosogenum*=*Penicillium*; S.=*Sclerotinia*; B.=*Botryotinia*; P.=*Pyrenophora*; C.=*Cochliobolus*; N. *haematococca*=*Nectria*; P. *anserina*=*Podospora*; N.=*Neurospora*. The tree was made by MEGA3, NJ. Bar=0.2 substitutions per amino acid site.

FIG. 3 shows an analysis of *N. crassa* FGSC2489 and *T. reesei* QM9414 endoglucanase activity when grown on *Miscanthus* and Avicel as a sole carbon source. Endoglucanase activity in culture filtrates of *N. crassa* WT strain FGSC2489 and *T. reesei* QM9414. *N. crassa* was grown on Vogel's minimal medium containing 2% of either Avicel or *Miscanthus* powder as a sole carbon source at 25 °C. *T. reesei* strain was inoculated in MA medium with either 1% Avicel or *Miscanthus* powder as sole carbon source at 25 °C. Both strains were inoculated with the same amount of conidia (1×10^6 /mL in 100mL culture). The endoglucanase activity in the cultures at different time points were measured at pH 4.5 using Azo-CM-cellulose as a substrate according to the manufacturer's instructions (Megazyme, Ireland).

FIG. 4 shows transcriptional profiling of *N. crassa* grown on *Miscanthus* and Avicel. (A) Hierarchical clustering analysis of 769 genes showing expression differences in *Miscanthus* culture. Dark shading indicates higher relative expression and light shading indicates lower relative expression. Lane 1: Expression profile of a 16 hr Vogel's minimal medium *N. crassa* culture (Vogel 1956). Lane 2: Expression profile of a culture grown on *Miscanthus* as a sole carbon source for 16 hrs. Lanes 3, 4, 5: Expression profiles from cultures grown on *Miscanthus* for 40 hrs, 5 days, and 10 days. The three clusters are shown as C1, C2, and C3. The cluster that showed increased expression levels of most of the cellulase and hemicellulase genes is boxed (C3 cluster). (B) Analysis of the overlap in expression profiles between the *N. crassa* *Miscanthus* versus Avicel grown cultures (Top). Analysis and overlap of proteins

detected in the culture filtrates of *N. crassa* grown on *Miscanthus* and Avicel by tandem mass spectrometry (Bottom). (C) Functional category (FunCat) enrichment analysis (Ruepp 2004) of the 231 genes that showed an increase in relative expression levels in *Miscanthus* cultures. Functional categories that showed significant enrichment ($p < 0.001$), including the unclassified group are shown.

FIG. 5 shows the relative expression levels of *N. crassa* genes encoding cellulases (A) and hemicellulases (B) during growth on minimal medium (MM) and during growth on *Miscanthus* for 16 hr, 40 hr, 5 days and 10 days.

FIG. 6 shows the protein profile and enzymatic activity of culture supernatants from strains containing deletions of genes encoding secreted proteins identified by MS. (A) SDS-PAGE of proteins present in the culture filtrates of 16 deletion strains as compared to wild type when grown on Avicel for 7 days. Deletion strains were chosen based on identification of the protein by mass spectrometry in both *Miscanthus* and Avicel culture filtrates. Strains are ordered based on gene NCU number, the wild-type strain is FGSC 2489. Missing protein bands that correspond to the deleted genes are marked with boxes. (B) Total secreted protein, azo-CMCase, and β -glucosidase activity assays (see Example 5) performed on 16 deletion strains and the wild-type parental strain (FGSC 2489) using the same sample from (A). Activities and protein concentrations were normalized compared to wild type levels and represent the average of triplicate biological measurements. (C) Cellulase activity of the culture filtrates from the 16 deletion strains using the same samples as in (A). Culture filtrates were diluted 10 fold and mixed with 5 mg/mL Avicel (see Example 5) to assess Avicelase activity. Glucose (black) and cellobiose (white) were measured after 8 hours of incubation at 40 °C.

FIG. 7 shows the identity of *N. crassa* secreted proteins based on mutant analysis from a culture grown on Avicel as a sole carbon source. SDS-PAGE of secreted proteins from WT *N. crassa* (FGSC 2489) grown on 2% Avicel in 100 mL shake flasks for 7 days at 25°C. 15 μ L of unconcentrated culture filtrate was loaded onto Criterion 4-15% 26-well gel. Proto Blue Safe (Coomassie) from National Diagnostics was used to stain the gel. The protein bands were identified in this study as shown in Fig. 6A based on analysis of secreted proteins in deletion strains.

FIG. 8 shows the profile of secreted proteins and expression of *cbh-1* (NCU07340) and *gh6-2* (NCU09680; CBHII) in Δ NCU04952 and Δ NCU05137. (A) SDS-PAGE of total secreted proteins in WT, Δ NCU04952, and Δ NCU05137. Cultures were grown on Avicel from conidia, and harvested at 30

hrs, two days (48 hrs) and three days (72 hrs) (see Example 5). Lanes 1-3, 20x concentrated culture filtrates after 30 hrs of growth on Avicel from WT, Δ NCU04952, and Δ NCU05137 strains, respectively. Lanes 4-6, unconcentrated culture filtrates after two days of growth from WT, Δ NCU04952, and Δ NCU05137 strains, respectively. Lane 7-9, unconcentrated culture filtrates after three days of growth from WT and Δ NCU04952 and Δ NCU05137 strains, respectively. (B) RT-PCR of *cbh-1* (NCU07340; CBHI) and *gh6-2* (NCU09680; CBHII) in the WT, Δ NCU04952, and Δ NCU05137 strains during growth on Avicel. The WT and deletion strains were grown on Avicel from conidia, and harvested at 48 hrs and 72 hrs (see Example 5). The minimal medium (MM) culture, with sucrose as a sole carbon source (Vogel 1956), was grown for 16 hrs (similar developmental time point). The fold induction of *cbh-1* and *gh6-2* were relative to the expression of these genes under MM conditions, with actin gene expression used as the control in all samples.

FIG. 9 shows a model of plant cell wall deconstruction in *N. crassa*. Induction: Extracellular enzymes expressed at low levels generate secondary metabolites that signal *N. crassa* to dramatically increase the expression level of genes encoding plant cell wall degrading enzymes, most of which are secreted. Utilization: Extracellular enzymes and transporters specific for translocation of cell wall degradation products enable *N. crassa* to utilize plant cell material for growth. Some extracellular proteins (NCU05137, NCU05057, and NCU04952) may generate metabolites that modulate gene expression of cellulases and hemicellulase during the utilization phase; double hexagon (cellobiose), double pentagon (xylobiose), hexagon (glucose), and pentagon (xylose). The depicted plant cell wall-degrading enzymes include CBH(I), CBH(II), EG2, EG1, EG6, and xylanase. Additional cellulolytic enzymes are not shown. Thickness of arrows indicates relative strength of response.

FIG. 10 shows BLAST results from searching the sequences of *N. crassa* putative transporters against a database of *S. thermophile* protein sequences or from searching the sequences of *S. thermophile* putative transporters against a database of *N. crassa* protein sequences.

FIG. 11 shows the growth phenotype of a *N. crassa* strain lacking NCU08114. (A) Shaker flasks of WT (left) and Δ NCU08114 (right) *N. crassa* strains after 3 days of growth with crystalline cellulose as a carbon source. (B) Mean Alamar Blue[®] fluorescence from *N. crassa* cultures grown with either sucrose or crystalline cellulose as a carbon source for 16 or 28 hours, respectively. Fluorescence was normalized by setting WT to 100%. Error bars were the standard deviation between measurements

from three biological replicates. *N. crassa* lacking NCU00801 did not have an obvious phenotype. *N. crassa* secreted β -glucosidases (Tian *et al.*, 2009) that hydrolyzed cellodextrins to glucose, which was subsequently taken up by monosaccharide transporters (Scarborough 1973). This alternate route of consumption led to an underestimate of the cellodextrin transport defect in these deletion lines.

FIG. 12 shows (A) cellobiose consumption for *S. cerevisiae* strains expressing NCU00801, NCU05853, or NCU08114 along with NCU00130; (B) cellotriose consumption for *S. cerevisiae* strains expressing NCU00801, NCU05853, or NCU08114 along with NCU00130; (C) cellotetraose consumption for *S. cerevisiae* strains expressing NCU00801, NCU05853, or NCU08114 along with NCU00130; and (D) cellohexaose consumption for *S. cerevisiae* strains expressing NCU00801, NCU05853, or NCU08114 along with NCU00130.

FIG. 13 shows cellodextrin consumption by *N. crassa* strains lacking NCU008114 or NCU00801. The indicated *N. crassa* strains were incubated with 90 μ M of the respective sugars for 15 minutes. Bars represent the mean concentration of sugars remaining in the supernatant following the incubation from two independent experiments. Error bars were the standard deviation between these experiments.

FIG. 14 shows cellobiose transport by a *S. cerevisiae* strain expressing NCU00801/*cbt1*. Shown is cellobiose transport by yeast with (○) or without (●) CBT1. Both strains expressed the intracellular β -glucosidase, NCU00130. The initial concentration of cellobiose was 50 μ M. All values were the mean between two measurements, with error bars representing the standard deviation between these measurements.

FIG. 15 shows localization and quantification of GFP fused to CBT1 and CBT2. (A) Images of *S. cerevisiae* strains expressing *cbt1* (left), or *cbt2* (right), fused to GFP at their C-terminus. (B) GFP fluorescence of yeast strains without a cellobiose transporter, or expressing *cbt1* or *cbt2* fused to GFP at their C-terminus. Values were the mean from three biological replicates, and error bars represent the standard deviation between these replicates.

FIG. 16 shows cellodextrin transport by *N. crassa* transport systems expressed in *S. cerevisiae*. (A) Cellobiose-mediated growth of yeast strains expressing the gene NCU00801 (named *cbt1*, ○), NCU08114 (named *cbt2*, ▼), or no transporter (●). All strains also expressed the intracellular β -

glucosidase, NCU00130. A representative experiment is shown. Growth rates from three independent experiments were as follows: *cbt1*, $0.0341 \pm 0.0010 \text{ hr}^{-1}$; *cbt2*, $0.0131 \pm 0.0008 \text{ hr}^{-1}$; no transporter, $0.0026 \pm 0.0001 \text{ hr}^{-1}$. (B) Growth of yeast strains on cellotriose and cellotetraose. Strains expressing the intracellular β -glucosidase, NCU00130, as well as the transporters listed in the legend, were grown with 0.5% (w/v) of cellotriose (G3) or cellotetraose (G4) serving as the sole carbon source. A representative experiment is shown. Growth rates from three independent experiments were as follows: *cbt1* cellotriose, $0.0332 \pm 0.0004 \text{ hr}^{-1}$; *cbt1* cellotetraose $0.0263 \pm 0.0020 \text{ hr}^{-1}$; no transporter cellotriose, $0.0043 \pm 0.0015 \text{ hr}^{-1}$; *cbt2* cellotriose, $0.0178 \pm 0.0005 \text{ hr}^{-1}$; *cbt2* cellotetraose $0.0041 \pm 0.0003 \text{ hr}^{-1}$; no transporter cellotetraose, $0.0031 \pm 0.0008 \text{ hr}^{-1}$. (C) Glucose produced from cellobiose (G2), cellotriose (G3), and cellotetraose (G4) hydrolysis by purified NCU00130. The mean and standard deviation of three independent measurements are shown. Residual glucose in incubations without enzyme (2 nmol) was subtracted from the values shown.

FIG. 17 shows growth of *S. cerevisiae* strains expressing *cbt1* (○), *cbt2* (▼), or no transporter (●) on glucose. All strains expressed the β -glucosidase, NCU00130. A representative experiment is shown.

FIG. 18 shows cellobiose-mediated growth of *S. cerevisiae* strains in 250 mL flasks. Values represent the mean OD between two replicate cultures of yeast strains expressing the β -glucosidase, NCU00130, *cbt1* or *cbt2*, or a strain expression NCU00130, but lacking any transporters. Error bars represent the standard deviation between replicates.

FIG. 19 shows kinetics of cellobiose transport by CBT1 and CBT2. The rate of cellobiose transport was determined as a function of cellobiose concentration by yeast strains expressing either *cbt1* or *cbt2*. The transport rate was normalized for transporter abundance.

FIG. 20 shows the ability of *S. cerevisiae* expressing the combinations of *Neurospora* genes shown on the x-axis to grow on cellobiose, cellotriose, or cellotetraose.

FIG. 21 shows competition by cellodextrins for cellobiose transport in strains carrying *cbt1* or *cbt2*. A 5-fold excess of the respective unlabeled sugar was included during assays of [^3H]-cellobiose transport. Substrates of CBT1 or CBT2 would decrease the [^3H]-cellobiose transport rate by competing for binding. Bars represent the mean from three replicates. Error bars represent the standard deviation

between these replicates. Values were normalized by setting the rate of [^3H]-cellobiose transport without a competing sugar to 100.

FIG. 22 shows the SDS-PAGE gel of purified NCU00130. Lane 1, Protein molecular weight standards, in kDa. Lane 2, NCU00130 after purification over nickel-NTA resin. Molecular weights in kDa are shown to the left.

FIG. 23 shows maximum likelihood phylogenetic analysis of the cellobiose transporters NCU08114 and NCU00801. With the exception of *S. cerevisiae* HXT1 and *K. lactis* LACP, all genes encoding proteins shown are reported to increase in expression level when the fungus comes into contact with plant cell wall material or cellobiose (Tian *et al.*, 2009; Noguchi *et al.*, 2009; Wymelenberg *et al.*, 2010; Martin *et al.*, 2010). *S. cerevisiae* HXT1, a low affinity glucose transporter (Reifenberger *et al.*, 1997), was used as an outgroup.

FIG. 24 shows cellobiose fermentation, and simultaneous saccharification and fermentation of cellulose, by *S. cerevisiae* expressing the cellobiose transport system from *N. crassa*. (A) Cellobiose fermentation to ethanol. Ethanol produced by yeast strains with CBT1 (●), or without CBT1 (○). Cellobiose concentration during the fermentation reaction using yeast strains with CBT1 (▼), or without CBT1 (△). (B) SSF using yeast strains with and without CBT1. Cellobiose (●) and glucose (▼) concentrations in the presence of a strain with CBT1, and cellobiose (○) and glucose (△) concentrations in the presence of a strain lacking CBT1. Note, 0.1 mg/mL cellobiose = 292 μM . (C) Ethanol produced during SSF using a strain with CBT1 (●), or without CBT1 (○). In all panels, values are the mean of 3 biological replicates. Error bars were the standard deviation between these replicates. All strains also expressed the intracellular β -glucosidase, NCU00130.

FIG 25 shows use of cellodextrin transport pathways from filamentous fungi during simultaneous saccharification and fermentation of cellulose by yeast. The cellodextrin (Cdex) transport pathway (black) includes a cellodextrin transporter (CBT) and intracellular β -glucosidase (βG). The sugar catabolism pathway presented in standard yeast includes hexose transporters (HXT). In SSF, both cellulases (GH) and extracellular β -glucosidase (βG) could be used.

FIG. 26 shows residues in NCU00801 and NCU08114 that are critical for function (A) Alascan of cbt1/NCU00801. (B) Polypeptide sequence (important residues marked) of cbt1/NCU00801. (C) Polypeptide sequence (important residues marked) of cbt2/NCU08114.

FIG. 27 shows a comparison of *S. cerevisiae* strains expressing cellobiose transporters from *P. stipitis*. (A) Cell growth of *S. cerevisiae* strains expressing β -glucosidase and orthologs of cellobiose transporters NCU00801, NCU08114, and NCU05853. (B) Comparison of cellobiose transporters from *P. stipitis*: cell growth of *S. cerevisiae* strains expressing β -glucosidase and cellobiose transporters. (C) Comparison of cellobiose transporters from *P. stipitis*: xylose consumption and ethanol production by *S. cerevisiae* strains expressing β -glucosidase and cellobiose transporters.

FIG. 28 shows alignments of cellobiose transporter orthologs. (A) Alignment of cellobiose transporter orthologs including ones that did not appear to have transporter function under the conditions tested. (B) Alignment of cellobiose transporter orthologs that had transport function. (C) Alignment of NCU00801 and NCU08114.

FIG. 29 shows functionally important motifs marked in homology models of NCU00801 and NCU08114. (A) Location of cellobiose transporters motifs on NCU00801 homology model. Motifs are highlighted in black. Motif [LIVM]-Y-[FL]-x(13)-[YF]-D is shown in panels a and b. Motif [YF]-x(2)-G-x(5)-[PVF]-x(6)-[DQ] is shown in panels c and d. Motif G-R-[RK] is shown in panels e and f. Motif R-x(6)-[YF]-N is shown in panels g and h. Motif WR-[IVLA]-P-x(3)-Q is shown in panels i and j. Motif P-E-S-P-R-x-L-x(8)-A-x(3)-L-x(2)-Y-H is shown in panels k and l. Motif F-[GST]-Q-x-S-G-N-x-[LIV] is shown in panels m and n. Motif L-x(3)-[YIV]-x(2)-E-x-L-x(4)-R-[GA]-K-G is shown in panels o and p. Note that in these images, some of the residues connecting transmembrane helices 6 and 7 have been removed for clarity as they occlude the pore. (B) Location of cellobiose transporters motifs on NCU08114 homology model. Motifs are highlighted in black. Motif [LIVM]-Y-[FL]-x(13)-[YF]-D is shown in panels a and b. Motif [YF]-x(2)-G-x(5)-[PVF]-x(6)-[DQ] is shown in panels c and d. Motif G-R-[RK] is shown in panels e and f. Motif R-x(6)-[YF]-N is shown in panels g and h. Motif WR-[IVLA]-P-x(3)-Q is shown in panels i and j. Motif P-E-S-P-R-x-L-x(8)-A-x(3)-L-x(2)-Y-H is shown in panels k and l. Motif F-[GST]-Q-x-S-G-N-x-[LIV] is shown in panels m and n. Motif L-x(3)-[YIV]-x(2)-E-x-L-

x(4)-R-[GA]-K-G is shown in panels o and p. Note that in these images, some of the residues connecting transmembrane helices 6 and 7 have been removed for clarity as they occlude the pore.

FIG. 30 shows the cloning process used in the construction of plasmid expressing: (A) putative transporters and (B) transporter-GFP fusion proteins.

FIG. 31 shows pentose transport activity of putative transporters identified to have glucose-uptake activity.

FIG. 32 shows pentose transport activity of putative transporters identified to not have glucose-uptake activity.

FIG. 33 shows pentose uptake of NCU00821 (AN25), STL12/XUT6 (Xyp29), and XUT1 (Xyp32). Part (A) shows xylose uptake and part (B) shows arabinose uptake.

FIG. 34 shows ^{14}C -labeled sugar uptake by *S. cerevisiae* expressing STL12/XUT6 (Xyp29).

FIG. 35 shows localizations of transporters expressed in *S. cerevisiae* cells as monitored by GFP fluorescence. First row from left to right: NCU00821-GFP fluorescence, NCU00821 nuclei; second row from left to right: STL12/XUT6-GFP fluorescence, STL12/XUT6 nuclei.

FIG. 36 shows the effect on pH upon addition of maltose to un-buffered cell suspension expressing: (a) NCU00821 (AN25), (b) STL12/XUT6 (Xyp29), and (c) XUT1 (Xyp32). The black arrows indicate the time points when maltose was added.

FIG. 37 shows results of a symporter assay of NCU00821, STL12/XUT6, and XUT1. Part (A) shows NCU00821 for xylose, part (B) shows NCU00821 for arabinose, part (C) shows XUT1 for arabinose, part (D) shows XUT1 for xylose, part E shows STL12/XUT6 for arabinose, and part F shows STL12/XUT6 for xylose. The black arrows the time points when maltose was added.

FIG. 38 shows phenotypic analyses of transporter overexpression. Part A shows OD, part B shows xylose concentration, and part C shows xylose consumption in 0.5% xylose-containing media. Part D shows OD, part E shows xylose concentration, and part F shows xylose consumption in 5% xylose-containing media. Part G shows the growth curve of *S. cerevisiae* containing pentose transporters introduced on pRS424, a multicopy plasmid.

FIG. 39 shows maps of the plasmids used for cloning of heterologous transporters.

FIG. 40 shows results of the sugar-uptake assay by *S. cerevisiae* strains expressing pentose transporter orthologs.

FIG. 41 shows sequence alignments of the pentose transporter orthologs by Clustal W (1.81). (a) Alignment of the xylose transporter orthologs. (b) Alignment of the arabinose transporters. (c) Alignment of xylose and arabinose transporters. Consensus key: * - single, fully conserved residue; : - conservation of strong groups; . - conservation of weak groups.

FIG. 42 describes the different *S. cerevisiae* strains engineered to express xylose-utilizing enzymes.

FIG. 43 shows xylose metabolism (as monitored by xylose consumption, ethanol production, etc.) of three *S. cerevisiae* strains of different backgrounds expressing identical cassettes containing xylose utilization pathway enzymes.

FIG. 44 shows xylose-uptake rates and metabolite yields of three *S. cerevisiae* strains of different backgrounds expressing identical cassettes containing xylose utilization pathway enzymes.

FIG. 45 shows xylose fermentation by the *S. cerevisiae* strain DA24 under various conditions. (a) 40 g/L xylose in a shaker flask, (b) 80 g/L xylose in a shaker flask, and (c) 80 g/L xylose in a bioreactor. Symbols: xylose (■), ethanol (◆), and OD₆₀₀ (●).

FIG. 46 shows a comparison of xylose consumption and ethanol production between (a) *S. cerevisiae* DA24 and (b) *P. stipitis*. Symbols: xylose (■), ethanol (◆), and OD₆₀₀ (●).

FIG. 47 describes the experimental design used to test the effect of *XYL2* over-expression levels on xylose metabolism in engineered *S. cerevisiae*.

FIG. 48 shows the effect of additional *XYL2* integration (i.e. increased *XYL2* expression level) into the genome of engineered xylose-fermenting *S. cerevisiae*.

FIG. 49 shows the effect of additional simultaneous over-expression of *XYL2* and *XYL3* on xylose fermentation by engineered *S. cerevisiae*.

FIG. 50 describes *S. cerevisiae* strains expressing different levels of xylose-fermenting enzymes.

FIG. 51 shows the effect of differential *XYL1* expression of fermentation by engineered *S. cerevisiae*.

FIG. 52 describes *S. cerevisiae* strains engineered to over-express identical *XYL2* and *XYL3* but different reductases (*XYL1* vs. *GRE3*).

FIG. 53 shows the effect of over-expressing *XYL1* versus *GRE3* on xylose fermentation by engineered *S. cerevisiae* grown in 40 g/L xylose.

FIG. 54 shows the effect of over-expressing *XYL1* versus *GRE3* on xylose fermentation by engineered *S. cerevisiae* grown in 80 g/L xylose.

FIG. 55 shows the thermal and pH-dependent properties of different wild-type LAD enzymes: anLAD (■), tlLAD (◆), and pcLAD (●). (a) Temperature-dependent catalytic activities, (b) Thermal inactivation at 50 °C over time, and (c) pH-dependent catalytic activities. Error bars indicate standard error of the mean (n = 3).

FIG. 56 shows an alignment of XDH from *N. crassa* (ncXDH) and *P. stipitis* (psXDH).

FIG. 57 show a comparison of pH rate profiles of *N. crassa* LAD and XDH. Data taken from the characterization of LAD was performed in universal buffer MES/Tris/glycine, and overlapped with data for ncXDH (closed triangles) and ncLAD (closed circles) performed in universal buffer acetic acid/MES/Tris for lower pH values.

FIG. 58 shows ethanol production by *S. cerevisiae* strain L2612 transformed with xylose isomerase enzyme from *Bacteroids stercoris* (*BtXI*), *Bifidobacterium longum* (*BfXI*), and *BtXIO* coding for codon-optimized *BtXI*. The XI gene was cloned into the pRs424TEF vector.

FIG. 59 shows xylose consumption and ethanol production by *S. cerevisiae* strain D452-2, which had *BtXI* integrated into its genome by the vector pRS403TEF. Comparison is also made to xylose-fermentation by *S. cerevisiae* strain L2612, which expresses *BtXI* from a plasmid

FIG. 60 shows xylose fermentation by *S. cerevisiae* strain, containing integrated *BtXI* and expressing *XYL2* or *XYL3* or *XYL2* and *XYL3*.

FIG. 61 shows the necessity of *XYL3* expression in *S. cerevisiae* engineered to over-express enzymes, such as GND1, involved in the pentose phosphate pathway in order to efficiently metabolize xylose.

FIG. 62 shows the effect of over-expression of NCU09705 homologs in *E. coli*, *S. cerevisiae*, and *P. stipitis* on fermentation parameters. Over-expression of galM, GAL10-Sc, GAL10-Ps, YHR210C, and YNR071C on (A) cellobiose consumption, growth, and ethanol production; and on (B) ethanol yield and productivity.

FIG. 63 shows the experimental design enabling simultaneous co-fermentation of cellobiose and xylose without glucose repression through integration of a cellodextrin assimilation pathway from filamentous fungi (*N. crassa*) and modified xylose metabolic pathway from the xylose-fermenting yeast *P. stipitis* into *S. cerevisiae*. (a) A strain improvement strategy to engineer yeast strain capable of fermenting two non-metabolizable sugars (cellobiose and xylose). The cellodextrin assimilation pathway consists of a cellodextrin transporter (NCU00801) and an intracellular β -glucosidase (NCU00130) from *N. crassa*. The modified xylose metabolic pathway utilizes xylose reductase isozymes (wild-type XR and mutant XR^{R276H}), xylitol dehydrogenase (*XYL2*), and xylulokinase (*XKS1*). (b) Fermentation profile of a sugar mixture containing glucose and xylose by the engineered *S. cerevisiae* developed in this study. Glucose fermentation repressed xylose fermentation completely so that xylose fermentation begins only after glucose depletion. (c) Fermentation profile of a sugar mixture containing cellobiose and xylose by the engineered *S. cerevisiae* developed in this study. Cellobiose and xylose are simultaneously utilized, as neither carbon source repressed consumption of the other.

FIG. 64 shows the scheme for plasmid construction. The pRS425 shuttle vector was linearized followed by assembly of the cellobiose transporter and β -glucosidase genes using the DNA assembler method (Shao *et al.*, 2009).

FIG. 65 shows the change in concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and biomass (□) during co-fermentation of 4% cellobiose and 5% D-xylose by *S. cerevisiae* strains (a) SL01, (b) SL04, (c) SL02, (d) SL05, (e) SL03, (f) SL06, and (g) SL00 as a function of time.

FIG. 66 shows the change in concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and biomass (□) in *S. cerevisiae* strains SL01 (a, c) and SL00 (b, d) grown in cellobiose-xylose mixtures in shake-flasks (a, b) or bioreactors (c, d) plotted as a function of time.

FIG. 67 shows the change in concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and biomass (□) in *S. cerevisiae* strains SL01 (a, c) and SL00 (b, d) grown in media containing 5 g/L glucose-40 g/L cellobiose-50 g/L xylose mixture (a, b) or 10 g/L glucose-40 g/L cellobiose-50 g/L xylose mixture (c, d) in bioreactors, plotted as a function of time.

FIG. 68 shows a comparison of cellobiose utilizations by β -glucosidase (NCU00130)-containing *S. cerevisiae* strain expressing (a) NCU00801, (b) NCU00809, and (c) NCU08114. Symbols: cellobiose (■), ethanol (◆), and OD₆₀₀ (●).

FIG. 69 shows co-fermentation of cellobiose and xylose by the *S. cerevisiae* strain DA24-16BT3 grown in mixtures containing various concentrations of the two sugars: (a) 20 g/L (each) of cellobiose and xylose, (b) 30 g/L (each) of cellobiose and xylose, and (c) 40 g/L (each) of cellobiose and xylose. Symbols: cellobiose (▲), xylose (■), ethanol (◆), and OD₆₀₀ (●).

FIG. 70 shows the synergistic effects of co-fermentation of cellobiose and xylose by the *S. cerevisiae* strain DA24-16BT3. Symbols: cellobiose (▲), xylose (■), ethanol (◆), and OD₆₀₀ (●): (a) 40 g/L cellobiose, (b) 40 g/L (each) of cellobiose and xylose, and (c) 40 g/L xylose.

FIG. 71 shows co-fermentation of glucose, cellobiose, and xylose by the *S. cerevisiae* strain DA24-16BT3 and the wild-type *P. stipitis* strain. Symbols: cellobiose (▲), xylose (■), ethanol (◆), OD₆₀₀ (●), and glucose (▼). (a) DA24-16BT3 and (b) *P. stipitis*.

FIG. 72 shows HPLC chromatograms from each time point, suggesting cellotriose and cellotetraose accumulation during c-fermentation of cellobiose and xylose by the *S. cerevisiae* strain DA24-16BT3.

FIG. 73 shows HPAEC analysis demonstrating cellodextrin accumulation in fermentation medium after 22 hours fermentation by the *S. cerevisiae* strain DA24-16BT3 during co-fermentation of cellobiose and xylose. (G1: glucose, G2: cellobiose, G3: cellotriose, G4: cellotetraose, and G5: cellopentaose).

FIG. 74 shows a comparison of sugar utilization by *S. cerevisiae* transformants expressing (a) an integrated copy of NCU00801 and (b) NCU00801 on a multi-copy plasmid, during co-fermentation of 40 g/L (each) of cellobiose and xylose. Symbols: cellobiose (▲), xylose (■), ethanol (◆), and OD₆₀₀ (●).

FIG. 75 shows ethanol production by cultivation of two different yeast strains. (a) The two different *S. cerevisiae* strains used in study: DA24-16 and D452BT. A xylose molecule is shown as a pentagon and a cellobiose molecule is shown as two hexagons; and (b) Mixed cultures of xylose-fermenting strain and cellobiose-fermenting strain.

FIG. 76 shows a listing of 354 xylan-induced genes in *N. crassa*.

FIG. 77 shows secreted protein levels, reducing sugar, and azo-xylanase activity for various *N. crassa* knock-out strains. Secreted protein levels were relatively constant for all strains.

FIG. 78 shows (A) total secreted protein and CMC-activity for wild type, ΔNCU05137, and ΔNCU05137/ΔNCU05137-GFP *Neurospora* strains, and (B) a Coomassie stain of total protein in supernatants from cultures of the three different strains.

FIG. 79 shows localization of NCU05137-GFP in conidia.

FIG. 80 shows localization of NCU05137-GFP in the hypha tip.

DETAILED DESCRIPTION OF THE INVENTION

The present disclosure relates to host cells containing a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -

helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, and α -helix 12, where one or more of the following is true: transmembrane α -helix 1 comprises SEQ ID NO: 1, transmembrane α -helix 2 comprises SEQ ID NO: 2, the loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 5 comprises SEQ ID NO: 4, transmembrane α -helix 6 comprises SEQ ID NO: 5, sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, transmembrane α -helix 7 comprises SEQ ID NO: 7, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8, and where the polypeptide transports cellodextrin into the cell. Further described herein are methods of increasing transport of cellodextrin into a cell, methods of increasing growth of a cell on a medium containing cellodextrin, methods of co-fermenting cellulose-derived and hemicellulose-derived sugars, and methods of making hydrocarbons or hydrocarbon derivatives using the host cells. Further described herein are host cells containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell, methods of increasing transport of a pentose into a cell, methods of increasing growth of a cell on a medium containing pentose sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell.

As used herein, cellodextrin refers to glucose polymers of varying length and includes, without limitation, cellobiose (2 glucose monomers), cellotriose (3 glucose monomers), cellotetraose (4 glucose monomers), cellopentaose (5 glucose monomers), and cellohexaose (6 glucose monomers).

As used herein, sugar refers to monosaccharides (e.g., glucose, fructose, galactose, xylose, arabinose), disaccharides (e.g., cellobiose, sucrose, lactose, maltose), and oligosaccharides (typically containing 3 to 10 component monosaccharides).

Polynucleotides of the Invention

The invention herein relates to host cells and methods of using such host cells where the host cells comprise recombinant polynucleotides encoding polypeptides capable of transporting various sugars.

As used herein, the terms "polynucleotide," "nucleic acid sequence," "sequence of nucleic acids," and variations thereof shall be generic to polydeoxyribonucleotides (containing 2-deoxy-D-ribose), to polyribonucleotides (containing D-ribose), to any other type of polynucleotide that is an N-

glycoside of a purine or pyrimidine base, and to other polymers containing non-nucleotidic backbones, provided that the polymers contain nucleobases in a configuration that allows for base pairing and base stacking, as found in DNA and RNA. Thus, these terms include known types of nucleic acid sequence modifications, for example, substitution of one or more of the naturally occurring nucleotides with an analog; inter-nucleotide modifications, such as, for example, those with uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoramidates, carbamates, etc.), with negatively charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), and with positively charged linkages (e.g., aminoalkylphosphoramidates, aminoalkylphosphotriesters); those containing pendant moieties, such as, for example, proteins (including nucleases, toxins, antibodies, signal peptides, poly-L-lysine, etc.); those with intercalators (e.g., acridine, psoralen, etc.); and those containing chelators (e.g., metals, radioactive metals, boron, oxidative metals, etc.). As used herein, the symbols for nucleotides and polynucleotides are those recommended by the IUPAC-IUB Commission of Biochemical Nomenclature (Biochem. 9:4022, 1970).

As used herein, a "polypeptide" is an amino acid sequence comprising a plurality of consecutive polymerized amino acid residues (e.g., at least about 15 consecutive polymerized amino acid residues, optionally at least about 30 consecutive polymerized amino acid residues, at least about 50 consecutive polymerized amino acid residues). In many instances, a polypeptide comprises a polymerized amino acid residue sequence that is a transporter, a transcription factor, a predicted protein of unknown function, or a domain or portion or fragment thereof. A transporter is involved in the movement of ions, small molecules, or macromolecules, such as a carbohydrate, across a biological membrane. A transcription factor can regulate gene expression and may increase or decrease gene expression in a host cell. The polypeptide optionally comprises modified amino acid residues, naturally occurring amino acid residues not encoded by a codon, and non-naturally occurring amino acid residues.

As used herein, "protein" refers to an amino acid sequence, oligopeptide, peptide, polypeptide, or portions thereof whether naturally occurring or synthetic.

Recombinant polynucleotides of the invention include any polynucleotides that encode a polypeptide encoded by any of the genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009; in Tables 14, 15, 16, 29; or in Fig. 76. In preferred embodiments, polynucleotides of the invention include any polynucleotides that encode a polypeptide encoded by any of the sequences

NCU00801, NCU00809, NCU08114, NCU00130, NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, XUT3, NCU07705, NCU05137, NCU01517, NCU09133, or NCU10040.

Table 1 shows polynucleotides of the invention including sequences listed below or sequences encoding polypeptides listed below.

Gene Name/Locus	Alternate Name	NCBI Reference Sequence/GenBank Accession Number	Organism
NCU00801	<i>cbt1</i>	XP_963801.1/EAA34565	<i>N. crassa</i>
NCU00809		XP_964302.1/EAA35116.1	<i>N. crassa</i>
NCU00821	AN25	XP_964364.2/EAA35128.2	<i>N. crassa</i>
NCU00988	Xy33	XP_963898.1/EAA34662.1	<i>N. crassa</i>
NCU01231		XP_961597.2/EAA32361.2	<i>N. crassa</i>
NCU01494	AN49	XP_955927.2/EAA26691.2	<i>N. crassa</i>
NCU02188	AN28-3	XP_959582.2/EAA30346.2	<i>N. crassa</i>
NCU04537	Xy50	XP_955977.1/EAA26741.1	<i>N. crassa</i>
NCU04963	AN29-2	XP_959411.2/EAA30175.2	<i>N. crassa</i>
NCU05519		XP_960481.1/EAA31245.1	<i>N. crassa</i>
NCU05853		XP_959844.1/EAA30608.1	<i>N. crassa</i>
NCU05897		XP_959888.1/EAA30652.1	<i>N. crassa</i>
NCU06138	Xy31	XP_960000.1/EAA30764.1	<i>N. crassa</i>
NCU08114	<i>cbt2</i>	XP_963873.1/EAA34637.1	<i>N. crassa</i>
NCU09287	AN41	XP_958139.1/EAA28903.1	<i>N. crassa</i>
NCU10021		XP_958069.2/EAA28833.2	<i>N. crassa</i>
XP_001387242	Ap26	XP_001387242	<i>P. stipitis</i>
HGT3	Xyp30-1	XP_001386715.1/ABN68686.1	<i>P. stipitis</i>
STL1	Xyp30	XP_001383774.1/ABN65745.1	<i>P. stipitis</i>
STL12/XUT6	Xyp29	XP_001386589.1/ABN68560.1	<i>P. stipitis</i>
SUT2	Ap31	XP_001384295.2/ABN66266.2	<i>P. stipitis</i>
SUT3	Xyp37	XP_001386019.2/ABN67990.2	<i>P. stipitis</i>
XUT1	Xyp32	XP_001385583.1/ABN67554.1	<i>P. stipitis</i>
XUT2	Xyp31	XP_001387242.1/EAZ63219.2	<i>P. stipitis</i>
XUT3	Xyp33	XP_001387138.1/EAZ63115.1	<i>P. stipitis</i>
XUT7	Xyp28	XP_001387067.1/EAZ63044.1	<i>P. stipitis</i>
NCU07705	<i>cdr-1</i>	XP_962291.1/EAA33055	<i>N. crassa</i>
NCU05137		XP_956635.1/ EAA27399	<i>N. crassa</i>
NCU01517		XP_956966.1/ EAA27730	<i>N. crassa</i>
NCU09133		XP_958905.1/ EAA29669	<i>N. crassa</i>
NCU10040			<i>N. crassa</i>

In certain embodiments, the recombinant polynucleotides of the invention encode polypeptides having at least about 20%, or at least about 29%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 92%, or at least about 94%, or at least about 96%, or at least about 98%, or at least about 99%, or at least about 100% amino acid residue sequence identity to a polypeptide encoded by any of the genes listed in genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009; in Tables 14, 15, 16, 29; or in Fig. 76. In preferred embodiments, the polynucleotides of the invention encode polypeptides having at least about 20%, or at least about 29%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 55%, or at least about 60%, or at least about 65%, or at least about 70%, or at least about 75%, or at least about 80%, or at least about 85%, or at least about 90%, or at least about 92%, or at least about 94%, or at least about 96%, or at least about 98%, or at least about 99%, or at least about 100% amino acid residue sequence identity to a polypeptide encoded by any of the sequences NCU00801, NCU00809, NCU08114, NCU00130, NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, XUT3, NCU07705, NCU05137, NCU01517, NCU09133, or NCU10040.

Polynucleotides of the invention further include polynucleotides that encode conservatively modified variants of polypeptides encoded by the genes listed above. “Conservatively modified variants” as used herein include individual substitutions, deletions or additions to a polypeptide sequence which result in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the disclosure. The following eight groups contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, e.g., Creighton, Proteins (1984)).

Polynucleotides of the invention further include polynucleotides that encode homologs or orthologs of polypeptides encoded by any of the genes listed in Table 10, in Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009; in Tables 14, 15, 16, 29; or in Fig. 76. “Homology” as used herein refers

to sequence similarity between a reference sequence and at least a fragment of a second sequence. Homologs may be identified by any method known in the art, preferably, by using the BLAST tool to compare a reference sequence to a single second sequence or fragment of a sequence or to a database of sequences. As described below, BLAST will compare sequences based upon percent identity and similarity. “Orthology” as used herein refers to genes in different species that derive from a common ancestor gene.

The terms “identical” or percent “identity,” in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same. Two sequences are “substantially identical” if two sequences have a specified percentage of amino acid residues or nucleotides that are the same (i.e., 29% identity, optionally 30%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or 100% identity over a specified region, or, when not specified, over the entire sequence), when compared and aligned for maximum correspondence over a comparison window, or designated region as measured using one of the following sequence comparison algorithms or by manual alignment and visual inspection. Optionally, the identity exists over a region that is at least about 50 nucleotides (or 10 amino acids) in length, or more preferably over a region that is 100 to 500 or 1000 or more nucleotides (or 20, 50, 200, or more amino acids) in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Default program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters. When comparing two sequences for identity, it is not necessary that the sequences be contiguous, but any gap would carry with it a penalty that would reduce the overall percent identity. For blastn, the default parameters are Gap opening penalty=5 and Gap extension penalty=2. For blastp, the default parameters are Gap opening penalty=11 and Gap extension penalty=1.

A “comparison window,” as used herein, includes reference to a segment of any one of the number of contiguous positions including, but not limited to from 20 to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence

of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith and Waterman (1981), by the homology alignment algorithm of Needleman and Wunsch (1970) J Mol Biol 48(3):443-453, by the search for similarity method of Pearson and Lipman (1988) Proc Natl Acad Sci USA 85(8):2444-2448, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection [see, e.g., Brent *et al.*, (2003) Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (Ringbou Ed)].

Two examples of algorithms that are suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.* (1997) Nucleic Acids Res 25(17):3389-3402 and Altschul *et al.* (1990) J. Mol Biol 215(3)-403-410, respectively. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, supra). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) or 10, M=5, N=-4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix [see Henikoff and Henikoff, (1992) Proc Natl Acad Sci USA 89(22):10915-10919] alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

The BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, *e.g.*, Karlin and Altschul, (1993) Proc Natl Acad Sci USA 90(12):5873-5877). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid is considered similar to a reference sequence if the smallest sum probability in a comparison of the test nucleic acid to the reference nucleic acid is less than about 0.2, more preferably less than about 0.01, and most preferably less than about 0.001.

Other than percentage of sequence identity noted above, another indication that two nucleic acid sequences or polypeptides are substantially identical is that the polypeptide encoded by the first nucleic acid is immunologically cross-reactive with the antibodies raised against the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules or their complements hybridize to each other under stringent conditions, as described below. Yet another indication that two nucleic acid sequences are substantially identical is that the same primers can be used to amplify the sequence.

As described herein, polynucleotides of the invention include members of the Major Facilitator Superfamily sugar transporter family, including NCU00988, NCU10021, NCU04963, NCU06138, NCU00801, NCU08114, and NCU05853. Members of the Major Facilitator Superfamily (MFS) (Transporter Classification # 2.A.1) of transporters almost always consist of 12 transmembrane α -helices, with an intracellular N- and C-terminus (S. S. Pao, I. T. Paulsen, M. H. Saier, Jr., *Microbiol Mol Biol Rev* 62, 1 (Mar, 1998)). While the primary sequence of MFS transporters varies widely, all are thought to share the tertiary structure of the *E. coli* lactose permease (LacY) (J. Abramson *et al.*, *Science* 301, 610 (Aug 1, 2003)), and the *E. coli* Pi /glycerol-3-phosphate (GlpT) (Y. Huang, M. J. Lemieux, J. Song, M. Auer, D. N. Wang, *Science* 301, 616 (Aug 1, 2003)). In these examples the six N- and C-terminal helices form two distinct domains connected by a long cytoplasmic loop between helices 6 and 7. This symmetry corresponds to a duplication event thought to have given rise to the MFS. Substrate binds within a hydrophilic cavity formed by helices 1, 2, 4, and 5 of the N-terminal domain, and helices 7, 8, 10, and 11 of the C-terminal domain. This cavity is stabilized by helices 3, 6, 9, and 12.

The Sugar Transporter family of the MFS (Transporter Classification # 2.A.1.1) is defined by motifs found in transmembrane helices 6 and 12 (PESPR (SEQ ID NO: 9)/PETK (SEQ ID NO: 10)), and loops 2 and 8 (GRR/GRK) (M. C. Maiden, E. O. Davis, S. A. Baldwin, D. C. Moore, P. J. Henderson, *Nature* 325, 641 (Feb 12-18, 1987)). The entire Hidden Markov Model (HMM) for this family can be viewed at pfam.janelia.org/family/PF00083#tabview=tab3. PROSITE (N. Hulo *et al.*, *Nucleic Acids Res* 34, D227 (Jan 1, 2006)) uses two motifs to identify members of this family. The first is [LIVMSTAG] - [LIVMFSAG] - {SH} - {RDE} - [LIVMSA] - [DE] - {TD} - [LIVMFYWA] - G - R - [RK] - x(4,6) - [GSTA] (SEQ ID NO: 11). The second is [LIVMF] - x - G - [LIVMFA] - {V} - x - G - {KP} - x(7) - [LIFY] - x(2) - [EQ] - x(6) - [RK] (SEQ ID NO: 12). As an example of how to read a PROSITE motif, the following motif, [AC]-x-V-x(4)-{ED}, is translated as: [Ala or Cys]-any-Val-any-any-any-any-{any but Glu or Asp} (SEQ ID NO: 13).

As described herein, NCU00801, NCU00809, NCU08114, XP_001268541.1, and LAC2 were discovered to encode polypeptides that transport cellodextrins. Further, alanine scanning experiments and sequence analyses were used to determine that a recombinant polypeptide containing 12 transmembrane α -helices, and one or more of the sequences selected from the group consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, and SEQ ID NO: 8 encodes a polypeptide that transports cellodextrin.

Thus, in one aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 comprises SEQ ID NO: 1. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 comprises SEQ ID NO: 2. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 comprises SEQ ID NO: 4. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing

transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 comprises SEQ ID NO: 5. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 comprises SEQ ID NO: 7. In another aspect, the invention provides a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8.

Each of the above described aspects may be combined in any number of combinations. A polynucleotide according to any of these aspects may encode a polypeptide containing 1, 2, 3, 4, 5, 6, or 7 of any of SEQ ID NOs: 1-8, or the polypeptide may contain all of SEQ ID NOs: 1-8. For example, a polynucleotide may encode a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 1 comprises SEQ ID NO: 1, a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, and transmembrane α -helix 7 comprises SEQ ID NO: 7. Or, in another example, a polynucleotide may encode a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 2 comprises SEQ ID NO: 2, transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 6 comprises SEQ ID NO: 5, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8.

In certain embodiments of the above described aspects, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114.

As further described herein, NCU08221 and STL12/XUT6 were discovered to encode polypeptides that transport xylose. XUT1 was discovered to encode a polypeptide that transports arabinose. NCU06138 was discovered to encode a polypeptide that transports arabinose or glucose. SUT2, SUT3, and XUT3 were discovered to encode polypeptides that transport xylose or glucose. NCU04963 was discovered to encode a polypeptide that transports xylose, arabinose, or glucose. In preferred embodiments, polynucleotides of the invention include recombinant polynucleotides encoding a NCU08221 or STL12/XUT6 polypeptide, where the polypeptide transports xylose. In other preferred embodiments, polynucleotides of the invention include recombinant polynucleotides encoding a XUT1 polypeptide, where the polypeptide transports arabinose. In other preferred embodiments, polynucleotides of the invention include recombinant polynucleotides encoding a NCU06138 polypeptide, where the polypeptide transports arabinose or glucose. In other preferred embodiments, polynucleotides of the invention include recombinant polynucleotides encoding a SUT2, SUT3, or XUT3 polypeptide, where the polypeptide transports xylose or glucose. In other preferred embodiments, polynucleotides of the invention include recombinant polynucleotides encoding a NCU04963 polypeptide, where the polypeptide transports xylose, arabinose, or glucose.

The polynucleotides of the invention that encode polypeptides encoded by NCU07705 are predicted by FunCat (Ruepp, 2004; webpage broad.mit.edu/annotation/genome/neurospora/Home.html) to encode an unclassified protein. However, BLAST analysis of the polypeptide encoded by NCU07705 revealed that the polypeptide has high similarity to many C6 zinc finger domain containing transcription factors (see Fig. 1; a list of exemplary homologs can be found in Fig. 23 of related U.S. Appl. No. 61/271,833). Polynucleotides of the invention include polynucleotides that encode these homologs of the polypeptide encoded by NCU07705 or any other homologs identified with any methods known in the art.

In another aspect of the invention, polynucleotides of the invention include those polynucleotides that encode polypeptides encoded by NCU05137. FunCat classifies the polypeptide encoded by NCU05137 to be an unclassified protein. However, NCU05137 is highly conserved in the genomes of a number of filamentous ascomycete fungi (see Fig. 2). Polynucleotides of the invention include polynucleotides that encode these homologs of the polypeptide encoded by NCU05137 or any other homologs identified with any methods known in the art.

In another aspect of the invention, polynucleotides of the invention include those polynucleotides that encode polypeptides encoded by NCU01517, NCU09133, or NCU10040. FunCat classifies the polypeptide encoded by NCU01517 to be a glucoamylase precursor. FunCat classifies the polypeptides encoded by NCU09133 and NCU10040 to be unclassified proteins. Polynucleotides of the invention include polynucleotides that encode these homologs of the polypeptide encoded by NCU01517, NCU09133, or NCU10040 or any other homologs identified with any methods known in the art.

Predicted functions of these polypeptides can be confirmed by performing functional analyses of the polynucleotide and its encoded protein. These analyses may include, for example, phenotypic analysis of strains containing deletions of the polynucleotide, genetic complementation experiments, phenotypic analysis of strains over expressing a wild-type copy of the polynucleotide, expression and purification of a recombinant form of the polypeptide, and subsequent characterization of the biochemical properties and activity of the recombinant polypeptide.

Sequences of the polynucleotides of the invention are prepared by any suitable method known to those of ordinary skill in the art, including, for example, direct chemical synthesis or cloning. For direct chemical synthesis, formation of a polymer of nucleic acids typically involves sequential addition of 3'-blocked and 5'-blocked nucleotide monomers to the terminal 5'-hydroxyl group of a growing nucleotide chain, wherein each addition is effected by nucleophilic attack of the terminal 5'-hydroxyl group of the growing chain on the 3'-position of the added monomer, which is typically a phosphorus derivative, such as a phosphotriester, phosphoramidite, or the like. Such methodology is known to those of ordinary skill in the art and is described in the pertinent texts and literature [e.g., in Matteucci *et al.*, (1980) Tetrahedron Lett 21:719-722; U.S. Pat. Nos. 4,500,707; 5,436,327; and 5,700,637]. In addition, the desired sequences may be isolated from natural sources by splitting DNA using appropriate restriction enzymes, separating the fragments using gel electrophoresis, and thereafter, recovering the desired nucleic acid sequence from the gel via techniques known to those of ordinary skill in the art, such as utilization of polymerase chain reactions (PCR; e.g., U.S. Pat. No. 4,683,195).

Each polynucleotide of the invention can be incorporated into an expression vector. "Expression vector" or "vector" refers to a compound and/or composition that transduces, transforms, or infects a host cell, thereby causing the cell to express nucleic acids and/or proteins other than those native to the cell, or in a manner not native to the cell. An "expression vector" contains a sequence of nucleic

acids (ordinarily RNA or DNA) to be expressed by the host cell. Optionally, the expression vector also comprises materials to aid in achieving entry of the nucleic acid into the host cell, such as a virus, liposome, protein coating, or the like. The expression vectors contemplated for use in the present invention include those into which a nucleic acid sequence can be inserted, along with any preferred or required operational elements. Further, the expression vector must be one that can be transferred into a host cell and replicated therein. Preferred expression vectors are plasmids, particularly those with restriction sites that have been well documented and that contain the operational elements preferred or required for transcription of the nucleic acid sequence. Such plasmids, as well as other expression vectors, are well known to those of ordinary skill in the art.

Incorporation of the individual polynucleotides may be accomplished through known methods that include, for example, the use of restriction enzymes (such as *Bam*HI, *Eco*RI, *Hha*I, *Xho*I, *Xma*I, and so forth) to cleave specific sites in the expression vector, e.g., plasmid. The restriction enzyme produces single stranded ends that may be annealed to a polynucleotide having, or synthesized to have, a terminus with a sequence complementary to the ends of the cleaved expression vector. Annealing is performed using an appropriate enzyme, e.g., DNA ligase. As will be appreciated by those of ordinary skill in the art, both the expression vector and the desired polynucleotide are often cleaved with the same restriction enzyme, thereby assuring that the ends of the expression vector and the ends of the polynucleotide are complementary to each other. In addition, DNA linkers may be used to facilitate linking of nucleic acids sequences into an expression vector.

A series of individual polynucleotides can also be combined by utilizing methods that are known to those having ordinary skill in the art (e.g., U.S. Pat. No. 4,683,195).

For example, each of the desired polynucleotides can be initially generated in a separate PCR. Thereafter, specific primers are designed such that the ends of the PCR products contain complementary sequences. When the PCR products are mixed, denatured, and reannealed, the strands having the matching sequences at their 3' ends overlap and can act as primers for each other. Extension of this overlap by DNA polymerase produces a molecule in which the original sequences are "spliced" together. In this way, a series of individual polynucleotides may be "spliced" together and subsequently transduced into a host cell simultaneously. Thus, expression of each of the plurality of polynucleotides is affected.

Individual polynucleotides, or "spliced" polynucleotides, are then incorporated into an expression vector. The invention is not limited with respect to the process by which the polynucleotide is incorporated into the expression vector. Those of ordinary skill in the art are familiar with the necessary steps for incorporating a polynucleotide into an expression vector. A typical expression vector contains the desired polynucleotide preceded by one or more regulatory regions, along with a ribosome binding site, e.g., a nucleotide sequence that is 3-9 nucleotides in length and located 3-11 nucleotides upstream of the initiation codon in *E. coli*. See Shine and Dalgarno (1975) *Nature* 254(5495):34-38 and Steitz (1979) *Biological Regulation and Development* (ed. Goldberger, R. F.), 1:349-399 (Plenum, New York).

The term "operably linked" as used herein refers to a configuration in which a control sequence is placed at an appropriate position relative to the coding sequence of the DNA sequence or polynucleotide such that the control sequence directs the expression of a polypeptide.

Regulatory regions include, for example, those regions that contain a promoter and an operator. A promoter is operably linked to the desired polynucleotide, thereby initiating transcription of the polynucleotide via an RNA polymerase enzyme. An operator is a sequence of nucleic acids adjacent to the promoter, which contains a protein-binding domain where a repressor protein can bind. In the absence of a repressor protein, transcription initiates through the promoter. When present, the repressor protein specific to the protein-binding domain of the operator binds to the operator, thereby inhibiting transcription. In this way, control of transcription is accomplished, based upon the particular regulatory regions used and the presence or absence of the corresponding repressor protein. Examples include lactose promoters (Lac repressor protein changes conformation when contacted with lactose, thereby preventing the Lac repressor protein from binding to the operator) and tryptophan promoters (when complexed with tryptophan, TrpR repressor protein has a conformation that binds the operator; in the absence of tryptophan, the TrpR repressor protein has a conformation that does not bind to the operator). Another example is the tac promoter (see de Boer *et al.*, (1983) *Proc Natl Acad Sci USA* 80(1):21-25). As will be appreciated by those of ordinary skill in the art, these and other expression vectors may be used in the present invention, and the invention is not limited in this respect.

Although any suitable expression vector may be used to incorporate the desired sequences, readily available expression vectors include, without limitation: plasmids, such as pSC101, pBR322, pBBR1MCS-3, pUR, pEX, pMR100, pCR4, pBAD24, pUC19; bacteriophages, such as M13 phage and λ

phage. Of course, such expression vectors may only be suitable for particular host cells. One of ordinary skill in the art, however, can readily determine through routine experimentation whether any particular expression vector is suited for any given host cell. For example, the expression vector can be introduced into the host cell, which is then monitored for viability and expression of the sequences contained in the vector. In addition, reference may be made to the relevant texts and literature, which describe expression vectors and their suitability to any particular host cell.

Host Cells of the Invention

The invention herein relates to host cells containing recombinant polynucleotides encoding polypeptides where the polypeptides transport cellodextrin or a pentose into the cell. Further described herein are methods of increasing transport of cellodextrin into a host cell, methods of increasing growth of a host cell on a medium containing cellodextrin, methods of co-fermenting cellulose-derived and hemicellulose-derived sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports cellodextrin into the cell. Further described herein are methods of increasing transport of a pentose into a host cell, methods of increasing growth of a host cell on a medium containing pentose sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell.

"Host cell" and "host microorganism" are used interchangeably herein to refer to a living biological cell that can be transformed via insertion of recombinant DNA or RNA. Such recombinant DNA or RNA can be in an expression vector. Thus, a host organism or cell as described herein may be a prokaryotic organism (e.g., an organism of the kingdom Eubacteria) or a eukaryotic cell. As will be appreciated by one of ordinary skill in the art, a prokaryotic cell lacks a membrane-bound nucleus, while a eukaryotic cell has a membrane-bound nucleus.

Any prokaryotic or eukaryotic host cell may be used in the present invention so long as it remains viable after being transformed with a sequence of nucleic acids. Preferably, the host cell is not adversely affected by the transduction of the necessary nucleic acid sequences, the subsequent expression of the proteins (e.g., transporters), or the resulting intermediates. Suitable eukaryotic cells include, but are not limited to, fungal, plant, insect or mammalian cells.

In preferred embodiments, the host is a fungal strain. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, In, Ainsworth and Bisby's Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*).

In particular embodiments, the fungal host is a yeast strain. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner, F. A., Passmore, S. M., and Davenport, R. R., eds, Soc. App. Bacteriol. Symposium Series No. 9, 1980).

In a more preferred embodiment, the yeast host is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* strain.

In certain embodiments, the yeast host is a *Saccharomyces carlsbergensis* (Todkar, 2010), *Saccharomyces cerevisiae* (Duarte *et al.*, 2009), *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, *Saccharomyces monacensis* (GB-Analysts Reports, 2008), *Saccharomyces bayanus* (Kristen Publicover, 2010), *Saccharomyces pastorianus* (Nakao *et al.*, 2007), *Saccharomyces pombe* (Mousdale, 2008), or *Saccharomyces oviformis* strain. In other preferred embodiments, the yeast host is *Kluyveromyces lactis* (O.W. Merten, 2001), *Kluyveromyces fragilis* (Pestal *et al.*, 2006; Siso, 1996), *Kluyveromyces marxianus* (K. Kourkoutas *et al.*, 2008), *Pichia stipitis* (Almeida *et al.*, 2008), *Candida shehatae* (Ayhan Demirbas, 2003), or *Candida tropicalis* (Jamai *et al.*, 2006). In other embodiments, the yeast host may be *Yarrowia lipolytica* (Biryukova E.N., 2009), *Brettanomyces custersii* (Spindler D.D. *et al.*, 1992), or *Zygosaccharomyces roux* (Chaabane *et al.*, 2006).

In another particular embodiment, the fungal host is a filamentous fungal strain. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

In preferred embodiments, the filamentous fungal host is, but not limited to, an *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Scytalidium*, *Thielavia*, *Tolypocladium*, or *Trichoderma* strain.

In certain embodiments, the filamentous fungal host is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, or *Aspergillus oryzae* strain. In other embodiments, the filamentous fungal host is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, or *Fusarium venenatum* strain. In yet other preferred embodiments, the filamentous fungal host is a *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Scytalidium thermophilum*, *Sporotrichum thermophile* (Topakas *et al.*, 2003), or *Thielavia terrestris* strain. In a further embodiment, the filamentous fungal host is a *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* strain.

In other preferred embodiments, the host cell is prokaryotic, and in certain embodiments, the prokaryotes are *E. coli* (Dien, B.S. *et al.*, 2003; Yomano, L. P. *et al.*, 1998; Moniruzzaman *et al.*, 1996), *Bacillus subtilis* (Susana Romero *et al.*, 2007), *Zymomonas mobilis* (B. S. Dien *et al.*, 2003; Weuster Botz, 1993; Alterthum and Ingram, 1989), *Clostridium* sp. (Zeikus, 1980; Lynd *et al.*, 2002; Demain *et al.*, 2005), *Clostridium phytofermentans* (Leschine S., 2010), *Clostridium thermocellum* (Lynd *et al.*, 2002), *Clostridium beijerinckii* (Giles Clark, 2008), *Clostridium acetobutylicum* (*Moorella thermoacetica*) (Huang W.C. *et al.*, 2004; Dominik *et al.*, 2007), *Thermoanaerobacterium saccharolyticum* (Marietta Smith, 2009), or *Klebsiella oxytoca* (Dien, B.S. *et al.*, 2003; Zhou *et al.*, 2001; Brooks and Ingram, 1995). In other embodiments, the prokaryotic host cells are *Carboxydocella* sp. (Dominik *et al.*, 2007), *Corynebacterium glutamicum* (Masayuki Inui, *et al.*, 2004), *Enterobacteriaceae* (Ingram *et al.*, 1995), *Erwinia chrysanthemi* (Zhou and Ingram, 2000; Zhou *et al.*, 2001), *Lactobacillus* sp. (McCaskey, T.A., *et al.*, 1994), *Pediococcus acidilactici* (Zhou, S. *et al.*, 2003), *Rhodopseudomonas capsulata* (X.Y. Shi *et al.*, 2004), *Streptococcus lactis* (J.C. Tang *et al.*, 1988), *Vibrio furnissii* (L.P. Wackett, 2010), *Vibrio furnissii* M1 (Park *et al.*, 2001), *Caldicellulosiruptor saccharolyticus* (Z. Kadar *et al.*, 2004), or *Xanthomonas campestris* (S.T. Yang *et al.*, 1987). In other embodiments, the host cells are cyanobacteria. Additional

examples of bacterial host cells include, without limitation, those species assigned to the *Escherichia*, *Enterobacter*, *Azotobacter*, *Erwinia*, *Bacillus*, *Pseudomonas*, *Klebsiella*, *Proteus*, *Salmonella*, *Serratia*, *Shigella*, *Rhizobia*, *Vitreoscilla*, *Synechococcus*, *Synechocystis*, and *Paracoccus* taxonomical classes.

In especially preferred embodiments of the invention, the host cell is *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, or *Bacillus subtilis*. *Saccharomyces* sp. may include Industrial *Saccharomyces* strains. Argueso *et al.* discuss the genome structure of an Industrial *Saccharomyces* strain commonly used in bioethanol production as well as specific gene polymorphisms that are important for bioethanol production (*Genome Research*, 19: 2258-2270, 2009).

The host cells of the present invention may be genetically modified in that recombinant nucleic acids have been introduced into the host cells, and as such the genetically modified host cells do not occur in nature. The suitable host cell is one capable of expressing one or more nucleic acid constructs encoding one or more proteins for different functions.

"Recombinant nucleic acid" or "heterologous nucleic acid" or "recombinant polynucleotide" as used herein refers to a polymer of nucleic acids wherein at least one of the following is true: (a) the sequence of nucleic acids is foreign to (*i.e.*, not naturally found in) a given host cell; (b) the sequence may be naturally found in a given host cell, but in an unnatural (*e.g.*, greater than expected) amount; or (c) the sequence of nucleic acids comprises two or more subsequences that are not found in the same relationship to each other in nature. For example, regarding instance (c), a recombinant nucleic acid sequence will have two or more sequences from unrelated genes arranged to make a new functional nucleic acid. Specifically, the present invention describes the introduction of an expression vector into a host cell, wherein the expression vector contains a nucleic acid sequence coding for a protein that is not normally found in a host cell or contains a nucleic acid coding for a protein that is normally found in a cell but is

under the control of different regulatory sequences. With reference to the host cell's genome, then, the nucleic acid sequence that codes for the protein is recombinant.

In some embodiments, the host cell naturally produces any of the proteins encoded by the polynucleotides of the invention. The genes encoding the desired proteins may be heterologous to the host cell or these genes may be endogenous to the host cell but are operatively linked to heterologous promoters and/or control regions which result in the higher expression of the gene(s) in the host cell. In other embodiments, the host cell does not naturally produce the desired proteins, and comprises heterologous nucleic acid constructs capable of expressing one or more genes necessary for producing those molecules.

“Endogenous” as used herein with reference to a nucleic acid molecule or polypeptide and a particular cell or microorganism refers to a nucleic acid sequence or peptide that is in the cell and was not introduced into the cell using recombinant engineering techniques; for example, a gene that was present in the cell when the cell was originally isolated from nature.

“Genetically engineered” or “genetically modified” refer to any recombinant DNA or RNA method used to create a prokaryotic or eukaryotic host cell that expresses a protein at elevated levels, at lowered levels, or in a mutated form. In other words, the host cell has been transfected, transformed, or transduced with a recombinant polynucleotide molecule, and thereby been altered so as to cause the cell to alter expression of a desired protein. Methods and vectors for genetically engineering host cells are well known in the art; for example various techniques are illustrated in *Current Protocols in Molecular Biology*, Ausubel *et al.*, eds. (Wiley & Sons, New York, 1988, and quarterly updates). Genetically engineering techniques include but are not limited to expression vectors, targeted homologous recombination and gene activation (see, for example, U.S. Pat. No. 5,272,071 to Chappel) and trans-activation by engineered transcription factors (see, for example, Segal *et al.*, (1999) *Proc Natl Acad Sci USA* 96(6):2758-2763).

Genetic modifications that result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, or up-regulation of a gene. More specifically, reference to increasing the action (or activity) of enzymes or other proteins discussed herein generally refers to any genetic modification of the host cell in question which results in increased expression and/or functionality (biological activity) of the enzymes or proteins and includes

higher activity or action of the proteins (e.g., specific activity or *in vivo* enzymatic activity), reduced inhibition or degradation of the proteins, and overexpression of the proteins. For example, gene copy number can be increased, expression levels can be increased by use of a promoter that gives higher levels of expression than that of the native promoter, or a gene can be altered by genetic engineering or classical mutagenesis to increase the biological activity of an enzyme or action of a protein. Combinations of some of these modifications are also possible.

Genetic modifications which result in a decrease in gene expression, in the function of the gene, or in the function of the gene product (i.e., the protein encoded by the gene) can be referred to as inactivation (complete or partial), deletion, interruption, blockage, silencing, or down-regulation, or attenuation of expression of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such gene, can be the result of a complete deletion of the gene (i.e., the gene does not exist, and therefore the protein does not exist), a mutation in the gene which results in incomplete or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no enzymatic activity or action). More specifically, reference to decreasing the action of proteins discussed herein generally refers to any genetic modification in the host cell in question, which results in decreased expression and/or functionality (biological activity) of the proteins and includes decreased activity of the proteins (e.g., decreased transport), increased inhibition or degradation of the proteins as well as a reduction or elimination of expression of the proteins. For example, the action or activity of a protein of the present invention can be decreased by blocking or reducing the production of the protein, reducing protein action, or inhibiting the action of the protein. Combinations of some of these modifications are also possible. Blocking or reducing the production of a protein can include placing the gene encoding the protein under the control of a promoter that requires the presence of an inducing compound in the growth medium. By establishing conditions such that the inducer becomes depleted from the medium, the expression of the gene encoding the protein (and therefore, of protein synthesis) could be turned off. Blocking or reducing the action of a protein could also include using an excision technology approach similar to that described in U.S. Pat. No. 4,743,546, incorporated herein by reference. To use this approach, the gene encoding the protein of interest is cloned between specific genetic sequences that allow specific, controlled excision of the gene from the genome. Excision could be prompted by, for example, a shift in the cultivation temperature of the culture, as in U.S. Pat. No. 4,743,546, or by some other physical or nutritional signal.

In general, according to the present invention, an increase or a decrease in a given characteristic of a mutant or modified protein (e.g., enzyme activity, ability to transport compounds) is made with reference to the same characteristic of a wild-type (i.e., normal, not modified) protein that is derived from the same organism (from the same source or parent sequence), which is measured or established under the same or equivalent conditions. Similarly, an increase or decrease in a characteristic of a genetically modified host cell (e.g., expression and/or biological activity of a protein, or production of a product) is made with reference to the same characteristic of a wild-type host cell of the same species, and preferably the same strain, under the same or equivalent conditions. Such conditions include the assay or culture conditions (e.g., medium components, temperature, pH, etc.) under which the activity of the protein (e.g., expression or biological activity) or other characteristic of the host cell is measured, as well as the type of assay used, the host cell that is evaluated, etc. As discussed above, equivalent conditions are conditions (e.g., culture conditions) which are similar, but not necessarily identical (e.g., some conservative changes in conditions can be tolerated), and which do not substantially change the effect on cell growth or enzyme expression or biological activity as compared to a comparison made under the same conditions.

Preferably, a genetically modified host cell that has a genetic modification that increases or decreases the activity of a given protein (e.g., a transporter, an enzyme) has an increase or decrease, respectively, in the activity or action (e.g., expression, production and/or biological activity) of the protein, as compared to the activity of the wild-type protein in a wild-type host cell, of at least about 5%, and more preferably at least about 10%, and more preferably at least about 15%, and more preferably at least about 20%, and more preferably at least about 25%, and more preferably at least about 30%, and more preferably at least about 35%, and more preferably at least about 40%, and more preferably at least about 45%, and more preferably at least about 50%, and more preferably at least about 55%, and more preferably at least about 60%, and more preferably at least about 65%, and more preferably at least about 70%, and more preferably at least about 75%, and more preferably at least about 80%, and more preferably at least about 85%, and more preferably at least about 90%, and more preferably at least about 95%, or any percentage, in whole integers between 5% and 100% (e.g., 6%, 7%, 8%, etc.). The same differences are preferred when comparing an isolated modified nucleic acid molecule or protein directly to the isolated wild-type nucleic acid molecule or protein (e.g., if the comparison is done *in vitro* as compared to *in vivo*).

In another aspect of the invention, a genetically modified host cell that has a genetic modification that increases or decreases the activity of a given protein (e.g., a transporter, an enzyme) has an increase or decrease, respectively, in the activity or action (e.g., expression, production and/or biological activity) of the protein, as compared to the activity of the wild-type protein in a wild-type host cell, of at least about 2-fold, and more preferably at least about 5-fold, and more preferably at least about 10-fold, and more preferably about 20-fold, and more preferably at least about 30-fold, and more preferably at least about 40-fold, and more preferably at least about 50-fold, and more preferably at least about 75-fold, and more preferably at least about 100-fold, and more preferably at least about 125-fold, and more preferably at least about 150-fold, or any whole integer increment starting from at least about 2-fold (e.g., 3-fold, 4-fold, 5-fold, 6-fold, etc.).

Host Cell Components

In one aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 1 comprises SEQ ID NO: 1. In another aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 2 comprises SEQ ID NO: 2. In another aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where the loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3. In another aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 5 comprises SEQ ID NO: 4. In another aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 6 comprises SEQ ID NO: 5. In another aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6. In another

aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 7 comprises SEQ ID NO: 7. In another aspect, host cells of the invention contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8.

Each of the above described aspects may be combined in any number of combinations. A host cell may contain a polynucleotide encoding a polypeptide containing 1, 2, 3, 4, 5, 6, or 7 of any of SEQ ID NOs: 1-8, or the polypeptide may contain all of SEQ ID NOs: 1-8. For example, a host cell may contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 1 comprises SEQ ID NO: 1, a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, and transmembrane α -helix 7 comprises SEQ ID NO: 7. Or, in another example, a host cell may contain a polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, where transmembrane α -helix 2 comprises SEQ ID NO: 2, transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 6 comprises SEQ ID NO: 5, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8.

In certain embodiments of the above described aspects, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114.

In preferred embodiments, the host cells further contain a polynucleotide, where the polynucleotide encodes a catalytic domain of a β -glucosidase. As used herein, β -glucosidase refers to a β -D-glucoside glucohydrolase (E.C. 3.2.1.21), which catalyzes the hydrolysis of terminal non-reducing β -D-glucose residues with the release of β -D-glucose. A catalytic domain of β -glucosidase has β -glucosidase activity as determined, for example, according to the basic procedure described by Venturi *et al.*, 2002. A

catalytic domain of a β -glucosidase is any domain that catalyzes the hydrolysis of terminal non-reducing residues in β -D-glucosides with release of glucose. In preferred embodiments, the β -glucosidase is a glycosyl hydrolase family 1 member. Members of this group can be identified by the motif, [LIVMFSTC] - [LIVFYS] - [LIV] - [LIVMST] - E - N - G - [LIVMFAR] - [CSAGN] (SEQ ID NO: 14). Here, E is the catalytic glutamate (webpage expasy.org/cgi-bin/prosite-search-ac?PDOC00495). In certain embodiments, the polynucleotide encoding a catalytic domain of β -glucosidase is heterologous to the host cell. In preferred embodiments, the catalytic domain of β -glucosidase is located intracellularly in the host cell. In preferred embodiments, the β -glucosidase is from *N. crassa*, and in particularly preferred embodiments, the β -glucosidase is NCU00130. In certain embodiments, the β -glucosidase may be an ortholog of NCU00130. Examples of orthologs of NCU00130 include, without limitation, *T. melanosporum*, CAZ82985.1; *A. oryzae*, BAE57671.1; *P. placenta*, EED81359.1; *P. chrysosporium*, BAE87009.1; *Kluyveromyces lactis*, CAG99696.1; *Laccaria bicolor*, EDR09330; *Clavispora lusitaniae*, EEQ37997.1; and *Pichia stipitis*, ABN67130.1. Other β -glucosidases could be used include those from the glycosyl hydrolase family 3. These β -glucosidases can be identified by the following motif according to PROSITE: [LIVM](2) - [KR] - x - [EQKRD] - x(4) - G - [LIVMFTC] - [LIVT] - [LIVMF] - [ST] - D - x(2) - [SGADNIT] (SEQ ID NO: 15). Here D is the catalytic aspartate. Typically, any β -glucosidase may be used that contains the conserved domain of β -glucosidase/6-phospho- β -glucosidase/ β -galactosidase found in NCBI sequence COG2723. Catalytic domains from specific β -glucosidases may be preferred depending on the cellodextrin transporter contained in the host cell.

In certain embodiments, the host cell contains one or more polynucleotides, where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. The one or more polynucleotides may be endogenous or heterologous to the host cell. Pentose, as used herein, refers to any monosaccharide with five carbon atoms. Examples of pentoses include, without limitation, xylose, arabinose, mannose, galactose, and rhamnose. The one or more enzymes involved in pentose utilization may include, for example, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase in any combination. These enzymes may come from any organism that naturally metabolizes pentose sugars. Examples of such organisms include, for example, *Kluyveromyces* sp., *Zymomonas* sp., *E. coli*, *Clostridium* sp., and *Pichia* sp.

Examples 12-15 describe ways in which the pentose utilization pathway in the host cell may be improved or made to be more efficient. Strain background of a host cell can affect the efficiency of its pentose utilization pathway. In embodiments of the invention where the host cell is a *Saccharomyces* sp., preferred pentose utilizing strains include DA24-16 (see Example 13) and L2612 (see Example 16). Other host cells containing polynucleotides encoding enzymes involved in pentose utilization include a DuPont *Zymomonas* strain (WO 2009/058927) and a *Saccharomyces* strain (US 5,789,210).

In certain embodiments of the invention, the host cell contains a recombinant polynucleotide encoding a pentose transporter. In certain embodiments, pentose transporters include those transporters discovered and described herein, including NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, and XUT3 (see Example 11). In other embodiments, pentose transporters may include Gxs1 from *C. intermedia*, Aut1 from *P. stipitis*, Xylhp from *D. hansenii* (Nobre *et al.*, 1999), xylose transporter from *K. marxianus* (Stambuk *et al.*, 2003), LAT1 and LAT2 from *Ambrosiozyma monospora* (EMBL AY923868 and AY923869, respectively, R. Verho *et al.*), ART1 from *C. arabinofementans* (Fonseca *et al.*, 2007), KmLAT1 from *K. marxianus* (Knoshaug *et al.*, 2007), PgLAT2 from *P. guilliermondii* (Knoshaug *et al.*, 2007), and araT from *P. stipitis* (Boles & Keller, 2008).

Methods of Producing and Culturing Host Cells of the Invention

The invention herein relates to host cells containing recombinant polynucleotides encoding polypeptides where the polypeptide transports cellodextrin or a pentose into the cell. Further described herein are methods of increasing transport of cellodextrin into a host cell, methods of increasing growth of a host cell on a medium containing cellodextrin, methods of co-fermenting cellulose-derived and hemicellulose-derived sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports cellodextrin into the cell. Further described herein are methods of increasing transport of a pentose into a host cell, methods of increasing growth of a host cell on a medium containing pentose sugars, and methods of making hydrocarbons or hydrocarbon derivatives by providing a host cell containing a recombinant polynucleotide encoding a polypeptide where the polypeptide transports a pentose into the cell.

Methods of producing and culturing host cells of the invention may include the introduction or transfer of expression vectors containing the recombinant polynucleotides of the invention into the host

cell. Such methods for transferring expression vectors into host cells are well known to those of ordinary skill in the art. For example, one method for transforming *E. coli* with an expression vector involves a calcium chloride treatment wherein the expression vector is introduced via a calcium precipitate. Other salts, e.g., calcium phosphate, may also be used following a similar procedure. In addition, electroporation (i.e., the application of current to increase the permeability of cells to nucleic acid sequences) may be used to transfect the host cell. Also, microinjection of the nucleic acid sequences provides the ability to transfect host cells. Other means, such as lipid complexes, liposomes, and dendrimers, may also be employed. Those of ordinary skill in the art can transfect a host cell with a desired sequence using these or other methods.

The vector may be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or plasmids which together contain the total DNA to be introduced into the genome of the host, or a transposon may be used.

The vectors preferably contain one or more selectable markers which permit easy selection of transformed hosts. A selectable marker is a gene the product of which provides, for example, biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Selection of bacterial cells may be based upon antimicrobial resistance that has been conferred by genes such as the *amp*, *gpt*, *neo*, and *hyg* genes.

Suitable markers for yeast hosts are, for example, ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenylyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in *Aspergillus* are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*. Preferred for use in *Trichoderma* are *bar* and *amdS*.

The vectors preferably contain an element(s) that permits integration of the vector into the host's genome or autonomous replication of the vector in the cell independent of the genome.

For integration into the host genome, the vector may rely on the gene's sequence or any other element of the vector for integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleotide sequences for directing integration by homologous recombination into the genome of the host. The additional nucleotide sequences enable the vector to be integrated into the host genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host. Furthermore, the integrational elements may be non-encoding or encoding nucleotide sequences. On the other hand, the vector may be integrated into the genome of the host by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host in question. The origin of replication may be any plasmid replicator mediating autonomous replication which functions in a cell. The term "origin of replication" or "plasmid replicator" is defined herein as a sequence that enables a plasmid or vector to replicate *in vivo*. Examples of origins of replication for use in a yeast host are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6. Examples of origins of replication useful in a filamentous fungal cell are AMA1 and ANS1 (Gems *et al.*, 1991; Cullen *et al.*, 1987; WO 00/24883). Isolation of the AMA1 gene and construction of plasmids or vectors comprising the gene can be accomplished according to the methods disclosed in WO 00/24883.

For other hosts, transformation procedures may be found, for example, in Jeremiah D. Read, *et al.*, *Applied and Environmental Microbiology*, Aug. 2007, p. 5088–5096, for *Kluyveromyces*, in Osvaldo Delgado, *et al.*, *FEMS Microbiology Letters* 132, 1995, 23-26, for *Zymomonas*, in US 7,501,275 for *Pichia stipitis*, and in WO 2008/040387 for *Clostridium*.

More than one copy of a gene may be inserted into the host to increase production of the gene product. An increase in the copy number of the gene can be obtained by integrating at least one additional copy of the gene into the host genome or by including an amplifiable selectable marker gene with the nucleotide sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the gene, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, e.g., Sambrook *et al.*, 1989, *supra*).

The host cell is transformed with at least one expression vector. When only a single expression vector is used (without the addition of an intermediate), the vector will contain all of the nucleic acid sequences necessary.

Once the host cell has been transformed with the expression vector, the host cell is allowed to grow. Methods of the invention may include culturing the host cell such that recombinant nucleic acids in the cell are expressed. For microbial hosts, this process entails culturing the cells in a suitable medium. Typically cells are grown at 35°C in appropriate media. Preferred growth media in the present invention include, for example, common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular host cell will be known by someone skilled in the art of microbiology or fermentation science. Temperature ranges and other conditions suitable for growth are known in the art (see, e.g., Bailey and Ollis 1986).

According to some aspects of the invention, the culture media contains a carbon source for the host cell. Such a “carbon source” generally refers to a substrate or compound suitable to be used as a source of carbon for prokaryotic or simple eukaryotic cell growth. Carbon sources can be in various forms, including, but not limited to polymers, carbohydrates, acids, alcohols, aldehydes, ketones, amino acids, peptides, etc. These include, for example, various monosaccharides such as glucose, oligosaccharides, polysaccharides, a biomass polymer such as cellulose or hemicellulose, xylose, arabinose, disaccharides, such as sucrose, saturated or unsaturated fatty acids, succinate, lactate, acetate,

ethanol, etc., or mixtures thereof. The carbon source can additionally be a product of photosynthesis, including, but not limited to glucose.

In preferred embodiments, the carbon source is a biomass polymer such as cellulose or hemicellulose. "A biomass polymer" as described herein is any polymer contained in biological material. The biological material may be living or dead. A biomass polymer includes, for example, cellulose, xylan, xylose, hemicellulose, lignin, mannan, and other materials commonly found in biomass. Non-limiting examples of sources of a biomass polymer include grasses (e.g., switchgrass, *Miscanthus*), rice hulls, bagasse, cotton, jute, hemp, flax, bamboo, sisal, abaca, straw, leaves, grass clippings, corn stover, corn cobs, distillers grains, legume plants, sorghum, sugar cane, sugar beet pulp, wood chips, sawdust, and biomass crops (e.g., *Crambe*).

In addition to an appropriate carbon source, media must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures and promotion of the enzymatic pathways necessary for the fermentation of various sugars and the production of hydrocarbons and hydrocarbon derivatives. Reactions may be performed under aerobic or anaerobic conditions where aerobic, anoxic, or anaerobic conditions are preferred based on the requirements of the microorganism. As the host cell grows and/or multiplies, expression of the enzymes, transporters, or other proteins necessary for growth on various sugars or biomass polymers, sugar fermentation, or synthesis of hydrocarbons or hydrocarbon derivatives is affected.

Methods of Increasing Transport of a Sugar into a Cell

The present invention provides methods of increasing transport of a sugar into a cell. In one aspect, the invention provides a method of transporting cellodextrin into a cell, including a first step of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, and α -helix 12, where one or more of the following is true: transmembrane α -helix 1 comprises SEQ ID NO: 1, transmembrane α -helix 2 comprises SEQ ID NO: 2, the loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 5 comprises SEQ ID NO: 4, transmembrane α -helix 6 comprises SEQ ID NO: 5, sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, transmembrane α -helix 7 comprises SEQ ID NO: 7, and transmembrane α -helix 10 and transmembrane α -

helix 11 and the sequence between them comprise SEQ ID NO: 8. The method includes a second step of culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not contain the recombinant polynucleotide. Transport of cellodextrin into a cell may be measured by any method known to one of skill in the art, including those methods described in Example 9 such as measuring uptake of [³H]-cellobiose into cells or measuring the ability of an *S. cerevisiae* host cell to grow when cellobiose is the sole carbon source. Typically, the host cell containing the recombinant polynucleotide and the host cell that does not contain the recombinant polynucleotide will otherwise be identical in genetic background.

In certain embodiments, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments, the host cell also contains a recombinant polynucleotide encoding a catalytic domain of a β -glucosidase. Such embodiments are useful for host cells lacking the endogenous ability to utilize cellodextrins. Preferably, the catalytic domain of the β -glucosidase is intracellular. In preferred embodiments, the β -glucosidase is from *Neurospora crassa*. In particularly preferred embodiments, the β -glucosidase is encoded by NCU00130.

In methods of increasing transport of cellodextrin into a cell, the cell may be cultured in a medium containing a cellulase-containing enzyme mixture from an altered organism, where the mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism. The organism may be altered to reduce the expression of β -glucosidase, such as by mutation of a gene encoding β -glucosidase or by targeted RNA interference or the like.

In another aspect, the invention provides a method of increasing transport of xylose into a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU00821 or STL12/XUT6 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of xylose into the cell compared with a cell that does not contain the recombinant polynucleotide. In another aspect, the invention provides a method of increasing transport of arabinose into a cell, including the steps of providing a host cell, where the host cell contains a recombinant

polynucleotide encoding a XUT1 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of arabinose into the cell compared with a cell that does not contain the recombinant polynucleotide. In yet another aspect, the invention provides a method of increasing transport of arabinose or glucose into a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU06138 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of arabinose or glucose into the cell compared with a cell that does not contain the recombinant polynucleotide. In yet another aspect the invention provides a method of increasing transport of xylose or glucose into a cell, including the steps of providing a host cell, where the host cell comprises a recombinant polynucleotide encoding a SUT2, SUT3, or XUT3 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of xylose or glucose into the cell compared with a cell that does not contain the recombinant polynucleotide. In another aspect, the invention provides a method of increasing transport of xylose, arabinose, or glucose into a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU04963 polypeptide, and culturing the cell such that the recombinant polynucleotide is expressed, where expression of the recombinant polynucleotide results in increased transport of xylose, arabinose, or glucose into the cell compared with a cell that does not contain the recombinant polynucleotide.

Transport of xylose, arabinose, or glucose into a cell may be measured by any method known to one of skill in the art, including those methods described in Example 11. These methods include, for example, measuring D-xylose or L-arabinose transport by extracting accumulated D-xylose and xylitol or L-arabinose and arabinitol from the host cell using osmosis and analyzing it using high performance liquid chromatography and measuring glucose transport by using host cells lacking the ability to grow on glucose as a sole carbon source. Typically, the host cell containing the recombinant polynucleotide and the host cell that does not contain the recombinant polynucleotide will otherwise be identical in genetic background.

In certain embodiments, the host cell also contains one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. The one or more enzymes may be, for example, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4

epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase, xylitol dehydrogenase, or any other pentose utilization enzymes known in the art.

Methods of Increasing Growth of a Cell

The present invention further provides methods of increasing the growth of a cell. In one aspect the invention provides methods of increasing growth of a cell, including a first step of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, and α -helix 12, where one or more of the following is true: transmembrane α -helix 1 comprises SEQ ID NO: 1, transmembrane α -helix 2 comprises SEQ ID NO: 2, the loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 5 comprises SEQ ID NO: 4, transmembrane α -helix 6 comprises SEQ ID NO: 5, sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, transmembrane α -helix 7 comprises SEQ ID NO: 7, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8, and the polypeptide transports cellodextrin. The method includes a second step of culturing the host cell in a medium containing cellodextrin, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. The growth rate of a host cell may be measured by any method known to one of skill in the art. Typically, growth rate of a cell will be measured by evaluating cell concentration in suspension by optical density. Preferably, the host cell containing the recombinant polynucleotide and the host cell that does not contain the recombinant polynucleotide will otherwise be identical in genetic background. Media containing cellodextrins may have resulted from enzymatic treatment of biomass polymers such as cellulose.

In certain embodiments, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments, the host cell also contains a recombinant polynucleotide encoding a catalytic domain of a β -glucosidase. Such embodiments are useful for host cells lacking the endogenous ability to utilize cellodextrins. Preferably, the catalytic domain of the β -

glucosidase is intracellular. In preferred embodiments, the β -glucosidase is from *Neurospora crassa*. In particularly preferred embodiments, the β -glucosidase is encoded by NCU00130.

In methods of increasing growth of a cell, the culturing medium may contain a cellulase-containing enzyme mixture from an altered organism, where the mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism. The organism may be altered to reduce the expression of β -glucosidase, such as by mutation of a gene encoding β -glucosidase or by targeted RNA interference or the like.

In another aspect, the invention provides a method of increasing growth of a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a NCU00821 or STL12/XUT6 polypeptide, and the polypeptide transports xylose, and culturing the host cell in a medium containing xylose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. In another aspect the invention provides a method of increasing growth of a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a XUT1 polypeptide, and the polypeptide transports arabinose, and culturing the host cell in a medium containing arabinose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. In yet another aspect, the invention provides a method of increasing growth of a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a NCU06138 polypeptide, and the polypeptide transports arabinose and glucose, and culturing the host cell in a medium containing arabinose or glucose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. In another aspect, the invention provides a method of increasing growth of a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a SUT2, SUT3, or XUT3 polypeptide, and the polypeptide transports xylose and glucose, and culturing the host cell in a medium containing xylose or glucose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide. In yet another aspect, the invention provides a method of increasing growth of a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a NCU04963 polypeptide, and the polypeptide transports xylose, arabinose, and glucose, and culturing the

host cell in a medium containing xylose, arabinose, or glucose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

The growth rate of a host cell may be measured by any method known to one of skill in the art. Typically, growth rate of a cell will be measured by evaluating cell concentration in suspension by optical density. Preferably, the host cell containing the recombinant polynucleotide and the host cell that does not contain the recombinant polynucleotide will otherwise be identical in genetic background. Media containing xylose or arabinose may have resulted from acid treatment of biomass polymers such as hemicellulose. Media containing glucose may have resulted from enzymatic treatment of biomass polymers such as cellulose.

In certain embodiments, the host cell also contains one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. The one or more enzymes may be, for example, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase, xylitol dehydrogenase, or any other pentose utilization enzymes known in the art.

In one aspect, the invention provides methods of increasing growth of a cell on a biomass polymer. In preferred embodiments, the biomass polymer is cellulose. In other preferred embodiments, the biomass polymer is hemicellulose. According to one aspect of the invention, the method includes providing a host cell comprising a recombinant polynucleotide that encodes a NCU07705 polypeptide. According to another aspect of the invention, the method includes culturing the cell in a medium comprising the biomass polymer wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

In another aspect of the invention, the invention provides a method of increasing growth of a cell, including the steps of providing a host cell, where the host cell contains a recombinant polynucleotide where the polynucleotide encodes a NCU01517, NCU09133, or NCU10040 polypeptide, and culturing the cell in a medium containing hemicellulose, where the host cell grows at a faster rate in the medium than a cell that does not contain the recombinant polynucleotide.

According to another aspect of the invention, the method includes providing a host cell comprising an endogenous polynucleotide that encodes a NCU05137 polypeptide. According to another

aspect of the invention, the method includes inhibiting expression of the endogenous polynucleotide and culturing the cell in a medium comprising a biomass polymer wherein the host cell grows at a faster rate in the medium than a cell in which expression of the endogenous polynucleotide is not inhibited.

Methods of the invention may include culturing the host cell such that recombinant nucleic acids in the cell are expressed. For microbial hosts, this process entails culturing the cells in a suitable medium. Typically cells are grown at 35°C in appropriate media. Preferred growth media in the present invention include, for example, common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth, or Yeast medium (YM) broth. Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular host cell will be known by someone skilled in the art of microbiology or fermentation science. Temperature ranges and other conditions suitable for growth are known in the art (see, e.g., Bailey and Ollis 1986).

The source of the biomass polymer in the medium may include, for example, grasses (e.g., switchgrass, *Miscanthus*), rice hulls, bagasse, cotton, jute, hemp, flax, bamboo, sisal, abaca, straw, leaves, grass clippings, corn stover, corn cobs, distillers grains, legume plants, sorghum, sugar cane, sugar beet pulp, wood chips, sawdust, and biomass crops (e.g., *Crambe*). In addition to a biomass polymer, the medium must contain suitable minerals, salts, cofactors, buffers and other components, known to those skilled in the art, suitable for the growth of the cultures. The rate of growth of the host cell may be measured by any methods known to one of skill in the art.

In certain embodiments of the invention, the expression of cellulases is increased in the host cell upon expression of a recombinant polynucleotide. "Cellulase" as used herein refers to a category of enzymes capable of hydrolyzing cellulose polymers to shorter cello-oligosaccharide oligomers, cellobiose, and/or glucose. Cellulases include, without limitation, exoglucanases, exocellobiohydrolases, endoglucanases, and glucosidases. Expression of cellulases may be measured by RT-PCR or other methods known in the art.

In certain embodiments of the invention, the expression of hemicellulases is increased in the host cell upon expression of a recombinant polynucleotide. "Hemicellulase" as used herein refers to a category of enzymes capable of hydrolyzing hemicellulose polymers. Hemicellulases include, without limitation, xylanases, mannanases, arabinases (both endo and exo kinds) and their corresponding

glycosidases. Expression of hemicellulases may be measured by RT-PCR or other methods known in the art.

Inhibition of expression of the endogenous polynucleotide may be achieved, for example, by genetic modifications which result in a decrease in gene expression, in the function of the gene, or in the function of the gene product (i.e., the protein encoded by the gene) and can be referred to as inactivation (complete or partial), deletion, interruption, blockage, silencing, or down-regulation, or attenuation of expression of a gene. For example, a genetic modification in a gene which results in a decrease in the function of the protein encoded by such a gene can be the result of a complete deletion of the gene (i.e., the gene does not exist, and therefore the protein does not exist), a mutation in the gene which results in incomplete or no translation of the protein (e.g., the protein is not expressed), or a mutation in the gene which decreases or abolishes the natural function of the protein (e.g., a protein is expressed which has decreased or no enzymatic activity or action). More specifically, reference to decreasing the action of proteins discussed herein generally refers to any genetic modification in the host cell in question which results in decreased expression and/or functionality (biological activity) of the proteins and includes decreased activity of the proteins (e.g., decreased transport), increased inhibition or degradation of the proteins, as well as a reduction or elimination of expression of the proteins. For example, the action or activity of a protein of the present invention can be decreased by blocking or reducing the production of the protein, reducing protein action, or inhibiting the action of the protein. Combinations of some of these modifications are also possible. Blocking or reducing the production of a protein can include placing the gene encoding the protein under the control of a promoter that requires the presence of an inducing compound in the growth medium. By establishing conditions such that the inducer becomes depleted from the medium, the expression of the gene encoding the protein (and therefore, of protein synthesis) could be turned off. Blocking or reducing the action of a protein could also include using an excision technology approach similar to that described in U.S. Pat. No. 4,743,546. To use this approach, the gene encoding the protein of interest is cloned between specific genetic sequences that allow specific, controlled excision of the gene from the genome. Excision could be prompted by, for example, a shift in the cultivation temperature of the culture, as in U.S. Pat. No. 4,743,546, or by some other physical or nutritional signal.

In certain embodiments of the invention, cellulase activity of the host cell is increased upon inhibiting expression of an endogenous polynucleotide. Cellulase activity may be measured as described in Example 5 and by any other methods known in the art.

In certain embodiments of the invention, hemicellulase activity of the host cell is increased upon inhibiting expression of an endogenous polynucleotide. Hemicellulase activity may be measured as described in Example 17 and by any other methods known in the art.

Methods of Co-Fermentation

One aspect of the present invention provides methods of co-fermenting cellulose-derived and hemicellulose-derived sugars. As used herein, co-fermentation refers to simultaneous utilization by a host cell of more than one sugar in the same vessel. The method includes the steps of providing a host cell, where the host cell contains a first recombinant polynucleotide encoding a cellodextrin transporter and a second recombinant polynucleotide encoding a catalytic domain of a β -glucosidase, and culturing the host cell in a medium containing a cellulose-derived sugar and a hemicellulose-derived sugar, where expression of the recombinant polynucleotides enables co-fermentation of the cellulose-derived sugar and the hemicellulose-derived sugar.

The first recombinant polynucleotide may encode any polypeptide that is capable of transporting cellodextrin into a cell. Cellodextrin transport may be measured by any method known to one of skill in the art, including the methods discussed in Example 9. In preferred embodiments, the first recombinant polynucleotide encodes a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, and α -helix 12, where one or more of the following is true: transmembrane α -helix 1 comprises SEQ ID NO: 1, transmembrane α -helix 2 comprises SEQ ID NO: 2, the loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 5 comprises SEQ ID NO: 4, transmembrane α -helix 6 comprises SEQ ID NO: 5, sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, transmembrane α -helix 7 comprises SEQ ID NO: 7, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8. Examples of such polypeptides include NCU00801, NCU00809, NCU08114, XP_001268541.1, and LAC2. In preferred embodiments, the first recombinant polypeptide encodes NCU00801.

The second recombinant polynucleotide may encode any catalytic domain capable of catalyzing the hydrolysis of terminal non-reducing residues in β -D-glucosides with release of glucose. Preferably, the β -glucosidase catalytic domain is located intracellularly in the host cell. In certain embodiments the source of the β -glucosidase domain is a *N. crassa* β -glucosidase. In preferred embodiments the source of the β -glucosidase domain is NCU00130. Catalytic domains from different sources may work best with different cellodextrin transporters.

In certain embodiments, the host cell also contains one or more recombinant polynucleotides where the one or more polynucleotides encode one or more enzymes involved in pentose utilization. Alternatively, one or more polynucleotides encoding one or more enzymes involved in pentose utilization may be endogenous to the host cell. The one or more enzymes may include, for example, L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabitol 4-dehydrogenase, L-xylulose reductase, xylitol dehydrogenase, or any other pentose-utilizing enzymes known to one of skill in the art.

In certain embodiments, the host cell contains a third recombinant polynucleotide where the polynucleotide encodes a pentose transporter. Alternatively, the host cell may contain an endogenous polynucleotide encoding a pentose transporter. In preferred embodiments, the pentose transporter transports xylose and/or arabinose into the cell. In certain embodiments, the third recombinant polynucleotide encodes a polypeptide such as NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, or XUT3. The expression of a pentose transporter in the host cell may enhance the efficiency of co-fermentation if glucose is present along with a pentose sugar in the growth medium.

In methods of co-fermentation as described herein, cellulose-derived sugars preferably include cellobiose, cellotriose, and celltetraose, and hemicellulose-derived sugars preferably include xylose and arabinose. Typically, in order to prepare the cellulose-derived sugars and hemicellulose-derived sugars for co-fermentation by a host cell, lignocellulosic biomass is first pretreated to alter its structure and allow for better enzymatic hydrolysis of cellulose. Pretreatment may include physical or chemical methods, including, for example, ammonia fiber/freeze explosion, the lime method based on calcium or sodium hydroxide, and steam explosion with or without an acid catalyst. Acid treatment will release xylose and arabinose from the hemicellulose component of the lignocellulosic biomass. Next, preferably, the cellulose component of the pretreated biomass is hydrolyzed by a mixture of cellulases. Examples of

commercially available cellulase mixtures include Celluclast 1.5L[®] (Novozymes), Spezyme CP[®] (Genencor) (Scott W. Pryor, 2010, *Appl Biochem Biotechnol*), and Cellulyve 50L (Lyven).

Cellulase mixtures typically contain endoglucanases, exoglucanases, and β -glucosidases. In methods of co-fermentation as described herein, the amount of β -glucosidase activity in the cellulase mixture should be minimized as much as possible. For example, the culturing medium may contain a cellulase-containing enzyme mixture from an altered organism, where the mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism. The organism may be altered to reduce the expression of β -glucosidase, such as by mutation of a gene encoding β -glucosidase or by targeted RNA interference or the like.

Surprisingly, as described in Example 17, co-fermentation of cellobiose and xylose by the methods of the invention resulted in a synergistic effect on sugar consumption and ethanol production by the host cell.

Methods of Synthesis of Hydrocarbons or Hydrocarbon Derivatives

One aspect of the present invention provides methods for increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell.

“Hydrocarbons” as used herein are organic compounds consisting entirely of hydrogen and carbon. Hydrocarbons include, without limitation, methane, ethane, ethene, ethyne, propane, propene, propyne, cyclopropane, allene, butane, isobutene, butene, butyne, cyclobutane, methylcyclopropane, butadiene, pentane, isopentane, neopentane, pentene, pentyne, cyclopentane, methylcyclobutane, ethylcyclopropane, pentadiene, isoprene, hexane, hexene, hexyne, cyclohexane, methylcyclopentane, ethylcyclobutane, propylcyclopropane, hexadiene, heptane, heptene, heptyne, cycloheptane, methylcyclohexane, heptadiene, octane, octene, octyne, cyclooctane, octadiene, nonane, nonene, nonyne, cyclononane, nonadiene, decane, decene, decyne, cyclodecane, and decadiene.

“Hydrocarbon derivatives” as used herein are organic compounds of carbon and at least one other element that is not hydrogen. Hydrocarbon derivatives include, without limitation, alcohols (e.g., arabinitol, butanol, ethanol, glycerol, methanol, 1,3-propanediol, sorbitol, and xylitol); organic acids (e.g., acetic acid, adipic acid, ascorbic acid, citric acid, 2,5-diketo-D-gluconic acid, formic acid, fumaric acid, glucaric acid, gluconic acid, glucuronic acid, glutaric acid, 3-hydroxypropionic acid, itaconic acid, lactic

acid, malic acid, malonic acid, oxalic acid, propionic acid, succinic acid, and xylonic acid); esters; ketones (e.g., acetone); aldehydes (e.g., furfural); amino acids (e.g., aspartic acid, glutamic acid, glycine, lysine, serine, and threonine); and gases (e.g., carbon dioxide and carbon monoxide).

In preferred embodiments, the hydrocarbon or hydrocarbon derivative can be used as fuel. In particularly preferred embodiments, the hydrocarbon or hydrocarbon derivative is ethanol or butanol.

According to one aspect of the invention, a method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell includes a first step of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a polypeptide containing transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, and α -helix 12, where one or more of the following is true: transmembrane α -helix 1 comprises SEQ ID NO: 1, transmembrane α -helix 2 comprises SEQ ID NO: 2, the loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, transmembrane α -helix 5 comprises SEQ ID NO: 4, transmembrane α -helix 6 comprises SEQ ID NO: 5, sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, transmembrane α -helix 7 comprises SEQ ID NO: 7, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8, and where the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives. The method includes a second step of culturing the host cell in a medium containing cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide. In certain embodiments, the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114. In certain embodiments, the host cell also contains a recombinant polynucleotide encoding a catalytic domain of a β -glucosidase. Such embodiments are useful for host cells lacking the endogenous ability to utilize cellodextrins. Preferably, the catalytic domain of the β -glucosidase is intracellular. In preferred embodiments, the β -glucosidase is from *Neurospora crassa*. In particularly preferred embodiments, the β -glucosidase is encoded by NCU00130. Transport of cellodextrin into the cell may be measured by any methods known to one of skill in the art, including the methods described in Example 9. Typically, the source of the cellodextrin is cellulose.

The culturing medium may contain a cellulase-containing enzyme mixture from an altered organism, where the mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism. The organism may be altered to reduce the expression of β -glucosidase, such as by mutation of a gene encoding β -glucosidase or by targeted RNA interference or the like.

According to another aspect of the invention, a method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell includes the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU00821 or STL12/XUT6 polypeptide, where the polypeptide transports xylose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing xylose or a source of xylose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of xylose into the cell is increased upon expression of the recombinant polynucleotide.

According to another aspect, a method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell includes the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a XUT1 polypeptide, where the polypeptide transports arabinose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing arabinose or a source of arabinose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of arabinose into the cell is increased upon expression of the recombinant polynucleotide.

According to yet another aspect, a method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell includes the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU06138 polypeptide, where the polypeptide transports arabinose or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium comprising arabinose or glucose or a source of arabinose or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of arabinose or glucose into the cell is increased upon expression of the recombinant polynucleotide.

According to yet another aspect, a method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell includes the steps of providing a host cell, where the host cell

contains a recombinant polynucleotide encoding a SUT2, SUT3, or XUT3 polypeptide, where the polypeptide transports xylose or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium containing xylose or glucose or a source of xylose or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of xylose or glucose into the cell is increased upon expression of the recombinant polynucleotide.

According to another aspect, a method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell includes the steps of providing a host cell, where the host cell contains a recombinant polynucleotide encoding a NCU04963 polypeptide, where the polypeptide transports xylose, arabinose, or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives, and culturing the host cell in a medium comprising xylose, arabinose, or glucose or a source of xylose, arabinose, or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, where transport of xylose, arabinose, or glucose into the cell is increased upon expression of the recombinant polynucleotide.

Transport of xylose, arabinose, or glucose into the cell may be measured by any methods known to one of skill in the art, including the methods described in Example 11. Typically, the source of glucose is cellulose, and the source of xylose and arabinose is hemicellulose.

Methods of Degrading Cellulose

One aspect of the present invention provides methods of degrading cellulose. The methods include a first step of providing a composition comprising cellulose. The cellulose is preferably from plant material, such as switchgrass, *Miscanthus*, rice hulls, bagasse, flax, bamboo, sisal, abaca, straw, leaves, grass clippings, corn stover, corn cobs, distillers grains, legume plants, sorghum, sugar cane, sugar beet pulp, wood chips, sawdust, and biomass crops.

The methods include a second step of contacting the composition with a cellulase-containing enzyme mixture from an altered organism, where the cellulase-containing mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism. The cellulose is degraded by the cellulase-containing mixture. The organism may be altered by mutation of a gene encoding a β -glucosidase or by reducing the expression of a β -glucosidase with a technique such as

RNA interference. The organism may be a fungus or a bacterium. In preferred embodiments, the organism is a filamentous fungus such as *T. reesei*.

Alternatively, the methods include a second step of contacting the composition with a cellulase-containing enzyme mixture that has been altered to reduce its β -glucosidase activity. For example, the cellulase-containing enzyme mixture may be altered by affinity chromatography where β -glucosidase enzymes are captured during the chromatography, and thus removed from the mixture. In another example, the cellulase-containing enzyme mixture is altered by inactivation of β -glucosidase enzymes in the mixtures with an inhibitor. Examples of commercially available cellulase mixtures include Celluclast 1.5L[®] (Novozymes), Spezyme CP[®] (Genencor) (Scott W. Pryor, 2010, *Appl Biochem Biotechnol*), and Cellulite 50L (Lyven).

It is to be understood that, while the invention has been described in conjunction with the preferred specific embodiments thereof, the foregoing description is intended to illustrate and not limit the scope of the invention. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

The invention having been described, the following examples are offered to illustrate the subject invention by way of illustration, not by way of limitation.

EXAMPLES

Example 1 – Transcriptome Analysis of *N. crassa* grown on *Miscanthus* and Avicel

In this example, the expression profile of the *N. crassa* genome was examined during growth on *Miscanthus* or Avicel. Growth and cellulase activity of *N. crassa* (FGSC 2489) cultured on Vogel's minimal media with crystalline cellulose (Avicel) as the sole carbon source was similar to that of *T. reesei* (QM9414) (Fig. 3); *N. crassa* completely degraded Avicel in approximately 4 days. *N. crassa* also grew rapidly on ground *Miscanthus* stems, suggesting functional cellulase and hemicellulase degradative capacity. To determine the transcriptome associated with plant cell wall deconstruction in *N. crassa*, we used full genome microarrays (Kasuga and Glass 2008; Tian *et al.*, 2007; Kasuga *et al.*, 2005) to monitor gene expression profiles during growth of *N. crassa* on ground *Miscanthus* stems. RNA was sampled after 16 hrs of growth on sucrose and compared to RNA isolated from *N. crassa* grown on *Miscanthus* medium at 16 and 40 hrs, 5 days and 10 days (Fig. 4; also see Supplemental Data, Dataset S1, page 1 in

Tian *et al.*, 2009; data can also be found at bioinfo.townsend.yale.edu/browse.jsp, Experiment IDs 52 and 53).

A total of 769 *N. crassa* genes showed a statistically significant difference in relative expression level among the four *Miscanthus* samples as compared to the sucrose sample (see Supplemental Data, Dataset S1, page 3 in Tian *et al.*, 2009). Hierarchical clustering showed that these genes fell into three main clusters (Fig. 4A). The first cluster of genes (C1; 300 genes) showed the highest expression levels in minimal medium with sucrose as a carbon source. Functional category (FunCat) analysis (Ruepp 2004) of these genes showed an enrichment for ribosomal proteins and other functional categories associated with primary metabolism, such as respiration, electron transport and amino acid metabolism (see Supplemental Data, Dataset S1, page 4 in Tian *et al.*, 2009). The second cluster (C2) included 327 genes that showed the highest expression levels in *Miscanthus* cultures at later time points (40 hrs to 10 days; Fig. 4A). Within this group were 89 genes that showed a high relative expression level in *Miscanthus* cultures at all time points. For further analyses, these 89 genes were added to the cluster of genes that showed the highest expression levels from the 16 hr *Miscanthus* cultures (C3 cluster, see below). FunCat analysis (Ruepp 2004) of the remaining 238 genes showed one functional category (C-compound and carbohydrate metabolism) was slightly enriched (see Supplemental Data, Dataset S1, page 5 in Tian *et al.*, 2009).

A third cluster of 142 genes showed the highest relative expression level after 16 hrs of growth of *N. crassa* on *Miscanthus* (C3, Fig. 4A). FunCat analysis (Ruepp 2004) of these 142 genes plus the 89 genes that showed high expression levels in *Miscanthus* cultures at all time points (C3+ cluster; total 231 genes) showed an enrichment for proteins involved with carbon metabolism, including predicted cellulases and hemicellulases (Fig. 4C; also see Supplemental Data, Dataset S1, page 6 in Tian *et al.*, 2009). Of the 23 predicted cellulase genes in the *N. crassa* genome, 18 showed significant increases in expression levels during growth on *Miscanthus* (see Table 1 in Tian *et al.*, 2009), particularly at the 16 hr time point (Fig. 5). Five genes showed an increase in expression level over 200-fold (*cbh-1* (CBH(I); NCU07340, *gh6-2* (CBH(II)-like gene; NCU09680), *gh6-3* (NCU07190) and two GH61 genes (*gh61-4*; NCU01050 and NCU07898)).

Plant cell walls are complex structures composed of cellulose microfibrils, hemicellulose, lignin, pectin, cutin, and protein. Thus, we compared expression profiles of *N. crassa* grown on

Miscanthus to expression profiles of *N. crassa* grown on Avicel, a pure form of crystalline cellulose (see Tian *et al.*, 2009, Supplemental Data, Dataset S1, page 2; data can also be found at bioinfo.townsend.yale.edu/browse.jsp, Experiment IDs 52 and 53). Over 187 genes showed a significant increase in relative expression level during growth of *N. crassa* on Avicel. Of these genes, 114 overlapped with the 231 genes in the C3+ cluster (Fig. 4B). FunCat analysis of the 114-overlap gene set showed a clear enrichment for genes predicted to be involved in carbon metabolism (see Supplemental Data, Dataset S1, page 6 in Tian *et al.*, *PNAS*, 2009). Within this gene set, there was a further enrichment for secreted proteins; 53 of the 114 gene products were predicted to be secreted. Of these 53 genes, 32 encode predicted proteins that have annotation suggesting a role in plant cell wall degradation, while 16 encode putative or hypothetical proteins that lack any functional prediction. The remaining 61 genes encode predicted intracellular proteins, including ten predicted major facilitator superfamily transporters (NCU00801, NCU00988, NCU01231, NCU04963, NCU05519, NCU05853, NCU05897, NCU06138, NCU08114 and NCU10021) and 23 putative or hypothetical proteins.

Of the 117 genes within the *Miscanthus*-specific cluster (Fig. 4B), 37 encode proteins predicted to be secreted. Nine predicted hemicellulases or enzymes related to the degradation of hemicellulose were identified (NCU00710, NCU04265, NCU04870, NCU05751, NCU05965, NCU09170, NCU09775, NCU09923 and NCU09976) (Tian *et al.*, 2009-Table 2). The remaining 80 *Miscanthus*-specific genes encode predicted intracellular proteins, including genes involved in the metabolism of pentose sugars (for example, NCU00891, xylitol dehydrogenase and NCU00643, a predicted arabinitol dehydrogenase), a predicted sugar transporter (NCU01132), and 48 proteins of unknown function.

Example 2 – Secretome Analysis of N. crassa Grown on Miscanthus and Avicel

Lignocellulose degradation by fungi takes place extracellularly and requires the secretion of proteins associated with depolymerization of cell wall constituents (Lynd *et al.*, 2002). To compare with transcriptional profiling data, which showed that genes encoding predicted cellulases, hemicellulases, and other secreted proteins increased in expression levels when *N. crassa* was grown on *Miscanthus* or Avicel, we analyzed the secretome of *N. crassa* using a shotgun proteomics approach (Fig. 4B). Supernatants from seven day old *Miscanthus* and Avicel cultures were digested with trypsin and analyzed by liquid chromatography nano-electrospray ionization tandem mass spectrometry (MS; see Example 5).

Secreted proteins that bound to phosphoric acid swollen cellulose (PASC) were enriched and also analyzed by MS.

A total of 50 proteins were identified with confidence by tandem MS (Tables 2 and 3). There were 34 proteins detected in the *Miscanthus* grown *N. crassa* cultures, while 38 proteins were identified from Avicel grown cultures; twenty-two proteins were detected in both samples. Of these 22 proteins, 21 were predicted to be secreted based on computational analyses and 19 showed increased expression levels in both the *Miscanthus* and Avicel grown cultures (Table 2). The overlap dataset included eight of the 23 predicted cellulases in *N. crassa* (Table 3). There were also five predicted hemicellulases, a predicted β -glucosidase (*gh3-4*; NCU04952), five proteins with predicted activity on carbohydrates, and two proteins with unknown function (NCU07143 and NCU05137) (Table 4-5).

For Table 2, the annotation was generated by the Broad Institute at webpage broad.mit.edu/annotation/genome/neurospora/Home.html. The “sample detected” was the sample in which peptides were detected for a particular protein. Peptides were validated by manual inspection. A protein was determined to be present if at least 1 peptide was detected in each biological repeat. “TOTAL” refers to peptides detected from a tryptic digest of all extracellular proteins. “PASC BOUND” refers to peptides detected after enrichment for proteins that bind to phosphoric acid swollen cellulose. “UNBOUND” refers to proteins remaining in solution after removal of PASC bound proteins.

Table 2
Proteins identified by LC-MS/MS In the culture filtrates of Avicel grown *Neurospora crassa*

GENE ID	ANNOTATION	SAMPLE DETECTED
NCU00206	Neurospora crassa hypothetical protein similar to cellobiose dehydrogenase 830 nt	TOTAL
NCU00762	Neurospora crassa endoglucanase 3 precursor 391 nt	TOTAL
NCU01050	Neurospora crassa hypothetical protein similar to endoglucanase II 239 nt	TOTAL
NCU02343	Neurospora crassa hypothetical protein similar to alpha L arabinofuranosidase A 668 nt	TOTAL
NCU04870	Neurospora crassa hypothetical protein similar to acetyl xylan esterase 313 nt	TOTAL
NCU04952	Neurospora crassa hypothetical protein similar to beta D glucoside glucohydrolase 736 nt	TOTAL
NCU05137	Neurospora crassa conserved hypothetical protein 692 nt	TOTAL
NCU05159	Neurospora crassa acetylxyln esterase precursor 301 nt	TOTAL
NCU05924	Neurospora crassa endo 1 4 beta xylanase 330 nt	TOTAL
NCU07143	Neurospora crassa predicted protein 391 nt	TOTAL

NCU07190	Neurospora crassa conserved hypothetical protein 384 nt	TOTAL
NCU07225	Neurospora crassa endo 1 4 beta xylanase 2 precursor 255 nt	TOTAL
NCU07326	Neurospora crassa predicted protein 327 nt	TOTAL
NCU07340	Neurospora crassa exoglucanase 1 precursor 522 nt	TOTAL
NCU07898	Neurospora crassa predicted protein 242 nt	TOTAL
NCU08189	Neurospora crassa hypothetical protein similar to endo 1 4 beta xylanase 385 nt	TOTAL
NCU08398	Neurospora crassa conserved hypothetical protein 391 nt	TOTAL
NCU08760	Neurospora crassa predicted protein 343 nt	TOTAL
NCU08785	Neurospora crassa conserved hypothetical protein 291 nt	TOTAL
NCU09491	Neurospora crassa feruloyl esterase B precursor 293 nt	TOTAL
NCU09680	Neurospora crassa exoglucanase 2 precursor 485 nt	TOTAL
NCU09923	Neurospora crassa hypothetical protein similar to beta xylosidase 775 nt	TOTAL
NCU00206	Neurospora crassa hypothetical protein similar to cellobiose dehydrogenase 830 nt	PASC BOUND
NCU00762	Neurospora crassa endoglucanase 3 precursor 391 nt	PASC BOUND
NCU05159	Neurospora crassa acetylxyln esterase precursor 301 nt	PASC BOUND
NCU05955	Neurospora crassa hypothetical protein similar to Cel74a 862 nt	PASC BOUND
NCU07225	Neurospora crassa endo 1 4 beta xylanase 2 precursor 255 nt	PASC BOUND
NCU07340	Neurospora crassa exoglucanase 1 precursor 522 nt	PASC BOUND
NCU08760	Neurospora crassa predicted protein 343 nt	PASC BOUND
NCU09680	Neurospora crassa exoglucanase 2 precursor 485 nt	PASC BOUND
NCU09708	Neurospora crassa conserved hypothetical protein 465 nt	PASC BOUND
NCU00762	Neurospora crassa endoglucanase 3 precursor 391 nt	UNBOUND
NCU01651	Neurospora crassa conserved hypothetical protein 783 nt	UNBOUND
NCU02343	Neurospora crassa hypothetical protein similar to alpha L arabinofuranosidase A 668 nt	UNBOUND
NCU04202	Neurospora crassa nucleoside diphosphate kinase 1 153 nt	UNBOUND
NCU04870	Neurospora crassa hypothetical protein similar to acetyl xylan esterase 313 nt	UNBOUND
NCU04952	Neurospora crassa hypothetical protein similar to beta D glucoside glucohydrolase 736 nt	UNBOUND
NCU05057	Neurospora crassa endoglucanase EG 1 precursor 439 nt	UNBOUND
NCU05137	Neurospora crassa conserved hypothetical protein 692 nt	UNBOUND
NCU05751	Neurospora crassa conserved hypothetical protein 242 nt	UNBOUND
NCU05924	Neurospora crassa endo 1 4 beta xylanase 330 nt	UNBOUND
NCU06239	Neurospora crassa conserved hypothetical protein 514 nt	UNBOUND

NCU07143	Neurospora crassa predicted protein 391 nt	UNBOUND
NCU07190	Neurospora crassa conserved hypothetical protein 384 nt	UNBOUND
NCU07225	Neurospora crassa endo 1 4 beta xylanase 2 precursor 255 nt	UNBOUND
NCU07326	Neurospora crassa predicted protein 327 nt	UNBOUND
NCU07898	Neurospora crassa predicted protein 242 nt	UNBOUND
NCU08189	Neurospora crassa hypothetical protein similar to endo 1 4 beta xylanase 385 nt	UNBOUND
NCU08398	Neurospora crassa conserved hypothetical protein 391 nt	UNBOUND
NCU08412	Neurospora crassa conserved hypothetical protein 401 nt	UNBOUND
NCU08760	Neurospora crassa predicted protein 343 nt	UNBOUND
NCU08785	Neurospora crassa conserved hypothetical protein 291 nt	UNBOUND
NCU09024	Neurospora crassa conserved hypothetical protein 625 nt	UNBOUND
NCU09175	Neurospora crassa conserved hypothetical protein 411 nt	UNBOUND
NCU09267	Neurospora crassa conserved hypothetical protein 1048 nt	UNBOUND
NCU09491	Neurospora crassa feruloyl esterase B precursor 293 nt	UNBOUND
NCU09775	Neurospora crassa hypothetical protein similar to alpha L arabinofuranosidase 343 nt	UNBOUND
NCU09923	Neurospora crassa hypothetical protein similar to beta xylosidase 775 nt	UNBOUND

Table 3: 22 secreted proteins detected in both *Miscanthus* and Avicel cultures

Gene name	Gene annotation	Profilin g	kos	CBM1	Signal P
NCU00206.2	CBDH	both	heter	yes	yes
NCU00762.2	probable cellulase precursor	both	16747	yes	yes
NCU01050.2	related to cel1 protein precursor	both	16543	no	yes
NCU04952.2	probable beta-D-glucoside glucohydrolase	both	13732	no	yes
NCU05057.2	probable endo-1,4-beta-glucanase	both	13343	no	yes
NCU05137.2	conserved hypothetical protein	both	11682	no	yes
NCU05924.2	probable endo-beta-1,4-D-xylanase	both	15626	no	yes
NCU05955.2	probable endoglucanase C	both	13535	yes	yes
NCU07143.2	hypothetical	both	No	no	yes
NCU07190.2	CBHII homolog	both	19315	no	yes
NCU07225.2	probable endo-1,4-beta-xylanase B precursor	both	heter	yes	yes
NCU07326.2	related to putative arabinase	both	19534	no	yes
NCU07340.2	CBHI	both	15630	yes	yes
NCU07898.2	related to cel1 protein precursor	both	19600	no	yes
NCU08189.2	similar to endo-1,4-beta xylanase	both	19861	no	yes
NCU08398.2	related to aldose 1-epimerase	both	20310	no	yes
NCU08412.2	hypothetical protein 401 nt	none	No	no	no

Gene name	Gene annotation	Profilin g	kos	CBM1	Signal P
NCU08760.2	related to family 61 endoglucanase	both	15664	yes	yes
NCU09024.2	hypothetical protein 625 nt	none	No	no	yes
NCU09175.2	glucan 1,3-beta-glucosidase precursor	both	11750	no	yes
NCU09491.2	feruloyl esterase B precursor	mis	No	no	yes
NCU09680.2	CBHII	both	15633	yes	yes

Table 4 shows predicted cellulase genes in *Neurospora crassa*

Gene	GH ¹ Family	CBM1 ²	SP ³	MS ⁴	EL ⁵ <i>Miscanthus</i>	EL ⁵ Avicel
NCU00762	5	yes	yes	both	29.6	31.5
NCU03996	6	no	no	ND6	ND	ND
NCU07190	6	no	yes	both	526.0	119
NCU09680	6	yes	yes	both	230.9	251.3
NCU04854	7	no	yes	ND	32.9	10.8
NCU05057	7	no	yes	both	8.7	7.4
NCU05104	7	no	yes	ND	11.6	NC7
NCU07340	7	yes	yes	both	426.4	382.2
NCU05121	45	yes	yes	avi	8.6	17.2
NCU00836	61	yes	yes	ND	91.2	31
NCU01050	61	no	yes	both	206.7	382.1
NCU01867	61	yes	yes	ND	2.2	NC
NCU02240	61	yes	yes	avi	193.5	84
NCU02344	61	no	yes	ND	8.1	4.1
NCU02916	61	yes	yes	ND	85.2	17.7
NCU03000	61	no	yes	ND	NC	ND

NCU03328	61	no	yes	ND	26.4	23.8
NCU05969	61	no	yes	ND	ND	12.7
NCU07520	61	no	yes	ND	ND	ND
NCU07760	61	yes	yes	ND	3.7	NC
NCU07898	61	no	yes	both	376.3	230
NCU07974	61	no	yes	ND	NC	NC
NCU08760	61	yes	yes	both	107.5	44.7

¹Glucoside Hydrolase; ²CBM1, carbohydrate binding module; ³Signal peptide prediction (signalP = webpage cbs.dtu.dk/services/SignalP/); ⁴MS, mass spectrometry analysis; ⁵EL, relative expression level; ⁶ND, not detected; ⁷NC, no change in expression level versus minimal media.

Table 5
Cellulases and Hemicellulases identified by LC-MS/MS

Predicted cellulases in the genome of *Neurospora crassa*

Gene ID	GH Family	AV MS	MIS MS	AV ARRAY	MIS ARRAY
NCU00762	5	+	+	31.5	29.6
NCU00836	61	-	-	31	91.2
NCU01050	61	+	+	382.1	206.7
NCU01867	61	-	-	1	1
NCU02240	61	+	-	84	193.5
NCU02344	61	-	-	4.1	8.1
NCU02916	61	-	-	17.7	85.2
NCU03000	61	-	-	1	1
NCU03328	61	-	-	23.8	26.4
NCU03996	6	-	-	2.5	6.3
NCU04854	7	-	-	10.8	32.9
NCU05057	7	+	+	7.4	8.7
NCU05104	7	-	-	1	1
NCU05121	45	+	-	17.2	8.6
NCU05969	61	-	-	12.7	12.3
NCU07190	6	+	+	119	526
NCU07340	7	+	+	382.2	426.4

NCU07520	61	-	-	1	1
NCU07760	61	-	-	1	3.4
NCU07898	61	+	+	230.5	376.3
NCU07974	61	-	-	1	1
NCU08760	61	+	+	44.7	107.5
NCU09680	6	+	+	251.3	230.9

Predicted hemicellulases in the genome of *Neurospora crassa*

Gene ID	GH Family	AV MS	MIS MS	AV Array	MIS Array
NCU00852	43	-	-	1	1
NCU00972	53	-	-	9.03	15.6
NCU01900	43	-	-	10.03	26
NCU02343	51	-	+	6.63	174.6
NCU02855	11	+	-	10.2	364
NCU04997	10	-	-	1	25.6
NCU05924	10	+	+	55.9	149.3
NCU05955	74	+	+	19.9	50.5
NCU05965	43	-	-	1	5.4
NCU06861	43	-	-	1	1
NCU07130	10	-	-	1	1
NCU07225	11	+	+	11.43	33.5
NCU07326	43	+	+	104.5	426.6
NCU07351	67	-	-	1	1
NCU08087	26	-	-	1	1
NCU08189	10	+	+	39.8	94.4
NCU09170	43	-	-	1	16.7
NCU09652	43	-	-	12.2	95.4
NCU09775	54	-	+	1	48.3

GH Family - Glycosyl Hydrolase Family; AV MS - Protein detected by LC-MS/MS in the culture filtrates of Avicel grown *Neurospora crassa*. (+) detected, (-) not detected; MIS MS - Protein detected by LC-MS/MS in the culture filtrates of Miscanthus grown *Neurospora crassa*. (+) detected, (-) not detected; AV ARRAY - Fold upregulation after 30 hours of growth on Avicel relative to 16 hours of growth on sucrose from profiling data; MIS ARRAY - Fold upregulation after 16 hours of growth on Miscanthus relative to 16 hours of growth on sucrose from profiling data, peptides detected only in Miscanthus culture filtrates.

There were 16 proteins identified with confidence only in the Avicel culture and 14 of these were predicted to be secreted (Table 6) including two predicted cellulases (*gh61-1*; NCU02240 and

gh45-1; NCU05121), one xylanase (*gh11-1*; NCU02855), one predicted protease (NCU04205), three other proteins with predicted activity on carbohydrates (NCU08909, NCU05974 and *gh30-1* (NCU04395)), three *Neurospora*-specific proteins of unknown function, and four conserved hypothetical proteins, including one protein with a cellulose binding domain (NCU09764). Twelve proteins were specific for culture filtrates of *Miscanthus* cultures and seven of these were predicted to be secreted (Table 3). Three of the five predicted intracellular proteins were conserved hypothetical proteins. The remaining two included a predicted glyoxal oxidase (NCU09267, identified from the *N. crassa* *Miscanthus* transcriptome) and a nucleoside diphosphate kinase (*ndk-1*; NCU04202, not identified in the *N. crassa* transcriptome). The seven proteins predicted to be secreted included three predicted esterases (NCU04870, NCU05159, and NCU08785), two predicted xylanases (GH51; NCU02343 and GH54; NCU09775), a predicted β -xylosidase (*gh3-7*; NCU09923) and a conserved hypothetical protein (NCU05751).

Table 6
Proteins identified by LC-MS/MS in the culture filtrates of Avicel grown *Neurospora crassa*

GENE ID	ANNOTATION	SAMPLE DETECTED
NCU00206	<i>Neurospora crassa</i> hypothetical protein similar to cellobiose dehydrogenase 830 nt	TOTAL
NCU00762	<i>Neurospora crassa</i> endoglucanase 3 precursor 391 nt	TOTAL
NCU00798	<i>Neurospora crassa</i> predicted protein 313 nt	TOTAL
NCU01050	<i>Neurospora crassa</i> hypothetical protein similar to endoglucanase II 239 nt	TOTAL
NCU01595	<i>Neurospora crassa</i> protein SOF1 446 nt	TOTAL
NCU02240	<i>Neurospora crassa</i> hypothetical protein similar to endoglucanase II 323 nt	TOTAL
NCU02696	<i>Neurospora crassa</i> hypothetical protein similar to DEAD DEAH box RNA helicase 1195 nt	TOTAL
NCU02855	<i>Neurospora crassa</i> endo 1 4 beta xylanase A precursor 221 nt	TOTAL
NCU04952	<i>Neurospora crassa</i> hypothetical protein similar to beta D glucoside glucohydrolase 736 nt	TOTAL
NCU05057	<i>Neurospora crassa</i> endoglucanase EG 1 precursor 439 nt	TOTAL
NCU05137	<i>Neurospora crassa</i> conserved hypothetical protein 692 nt	TOTAL
NCU05924	<i>Neurospora crassa</i> endo 1 4 beta xylanase 330 nt	TOTAL
NCU05955	<i>Neurospora crassa</i> hypothetical protein similar to Cel74a 862 nt	TOTAL
NCU07143	<i>Neurospora crassa</i> predicted protein 391 nt	TOTAL
NCU07190	<i>Neurospora crassa</i> conserved hypothetical protein 384 nt	TOTAL
NCU07225	<i>Neurospora crassa</i> endo 1 4 beta xylanase 2 precursor 255 nt	TOTAL
NCU07326	<i>Neurospora crassa</i> predicted protein 327 nt	TOTAL
NCU07340	<i>Neurospora crassa</i> exoglucanase 1 precursor 522 nt	TOTAL

NCU07898	Neurospora crassa predicted protein 242 nt	TOTAL
NCU08171	Neurospora crassa predicted protein 382 nt	TOTAL
NCU08412	Neurospora crassa conserved hypothetical protein 401 nt	TOTAL
NCU08760	Neurospora crassa predicted protein 343 nt	TOTAL
NCU09491	Neurospora crassa feruloyl esterase B precursor 293 nt	TOTAL
NCU09680	Neurospora crassa exoglucanase 2 precursor 485 nt	TOTAL
NCU09764	Neurospora crassa conserved hypothetical protein 406 nt	TOTAL
NCU00206	Neurospora crassa hypothetical protein similar to cellobiose dehydrogenase 830 nt	PASC BOUND
NCU00762	Neurospora crassa endoglucanase 3 precursor 391 nt	PASC BOUND
NCU05121	Neurospora crassa endoglucanase V 294 nt	PASC BOUND
NCU05955	Neurospora crassa hypothetical protein similar to Cel74a 862 nt	PASC BOUND
NCU07225	Neurospora crassa endo 1 4 beta xylanase 2 precursor 255 nt	PASC BOUND
NCU07340	Neurospora crassa exoglucanase 1 precursor 522 nt	PASC BOUND
NCU08760	Neurospora crassa predicted protein 343 nt	PASC BOUND
NCU09680	Neurospora crassa exoglucanase 2 precursor 485 nt	PASC BOUND
NCU00206	Neurospora crassa hypothetical protein similar to cellobiose dehydrogenase 830 nt	UNBOUND
NCU00762	Neurospora crassa endoglucanase 3 precursor 391 nt	UNBOUND
NCU00798	Neurospora crassa predicted protein 313 nt	UNBOUND
NCU01050	Neurospora crassa hypothetical protein similar to endoglucanase II 239 nt	UNBOUND
NCU04205	Neurospora crassa predicted protein 346 nt	UNBOUND
NCU04395	Neurospora crassa endo 1 6 beta D glucanase precursor 481 nt	UNBOUND
NCU04952	Neurospora crassa hypothetical protein similar to beta D glucoside glucohydrolase 736 nt	UNBOUND
NCU05057	Neurospora crassa endoglucanase EG 1 precursor 439 nt	UNBOUND
NCU05134	Neurospora crassa hypothetical protein 124 nt	UNBOUND
NCU05137	Neurospora crassa conserved hypothetical protein 692 nt	UNBOUND
NCU05852	Neurospora crassa conserved hypothetical protein 254 nt	UNBOUND
NCU05924	Neurospora crassa endo 1 4 beta xylanase 330 nt	UNBOUND
NCU05974	Neurospora crassa hypothetical protein similar to cell wall glucanosyltransferase Mwg1 365 nt	UNBOUND
NCU07143	Neurospora crassa predicted protein 391 nt	UNBOUND
NCU07190	Neurospora crassa conserved hypothetical protein 384 nt	UNBOUND
NCU07225	Neurospora crassa endo 1 4 beta xylanase 2 precursor 255 nt	UNBOUND
NCU07326	Neurospora crassa predicted protein 327 nt	UNBOUND
NCU07340	Neurospora crassa exoglucanase 1 precursor 522 nt	UNBOUND

NCU07898	Neurospora crassa predicted protein 242 nt	UNBOUND
NCU08171	Neurospora crassa predicted protein 382 nt	UNBOUND
NCU08189	Neurospora crassa hypothetical protein similar to endo 1 4 beta xylanase 385 nt	UNBOUND
NCU08398	Neurospora crassa conserved hypothetical protein 391 nt	UNBOUND
NCU08412	Neurospora crassa conserved hypothetical protein 401 nt	UNBOUND
NCU08760	Neurospora crassa predicted protein 343 nt	UNBOUND
NCU08909	Neurospora crassa hypothetical protein similar to beta 1 3 glucanosyltransferase 543 nt	UNBOUND
NCU08936	Neurospora crassa clock controlled gene 15 412 nt	UNBOUND
NCU09024	Neurospora crassa conserved hypothetical protein 625 nt	UNBOUND
NCU09046	Neurospora crassa predicted protein 187 nt	UNBOUND
NCU09175	Neurospora crassa conserved hypothetical protein 411 nt	UNBOUND
NCU09491	Neurospora crassa feruloyl esterase B precursor 293 nt	UNBOUND

ANNOTATION - Generated by the Broad Institute (webpage at broad.mit.edu/annotation/genome/neurospora/Home.html);
SAMPLE DETECTED - Sample in which peptides were detected for a particular protein. Peptides were validated by manual inspection. A protein was determined to be present if at least 1 peptide was detected in each biological repeat. TOTAL, peptides detected from a tryptic digest of all extracellular proteins; PASC BOUND, peptides detected after enrichment for proteins that bind to phosphoric acid swollen cellulose; UNBOUND, proteins remaining in solution after removal of PASC bound proteins.

Many plant cell wall degrading enzymes contain a cellulose-binding module (CBM), which aids in attachment of the enzyme to the substrate (Linder and Teeri 1996). Within the *N. crassa* genome, proteins encoded by 19 genes are predicted to contain a CBM1 domain (Cantarel *et al.*, 2009). Of these 19 genes, 16 showed an increase in relative gene expression in *Miscanthus*-grown cultures (Table 7).

Table 7: Effect of *Miscanthus* and Avicel on *N. crassa* gene expression

Gene name	CBM prediction	Annotation	Mis Array	Avicel array	MS
NCU00206	cazy and mips	probable cellobiose dehydrogenase	164	12	both
NCU00710	cazy and mips	acetylxy lan esterase	30	no detect	none
NCU00762	cazy and mips	EG2	29	31	both
NCU00836	cazy and mips	EG, GH61	91	30	none
NCU01867	cazy and mips	EG, GH61	2.2-d10	no difference	none
NCU02240	cazy and mips	EG, GH61	193	84	avi
NCU02916	cazy and mips	EG, GH61	85	17	none
NCU04500	cazy and mips	similar to chitinase 4	no detect	no detect	none
NCU04997	cazy and mips	similar to xylanase	no detect	no detect	none

NCU05121	cazy and mips	EG, GH45	8.5	17	avi
NCU05159	cazy and mips	acetylxyln esterase precursor	34	10	mis
NCU05955	cazy and mips	GH74	50	19	both
NCU07225	cazy and mips	xylanase	33	11	both
NCU07340	cazy and mips	CBH1	426	382	both
NCU07760	cazy and mips	EG,GH61	3.7	no difference	none
NCU08760	cazy and mips	EG,GH61	107	44	both
NCU09416	cazy and mips	hypothetical	no detect	27	none
NCU09680	cazy and mips	CBH2	230	251	both
NCU09764	cazy and mips	hypothetical	18	16.6	avi

From the 50 proteins identified by MS, 11 contained a CBM1 domain. PASC was used to enrich for proteins that bind to cellulose (see Example 4 for methods). Nine proteins were identified by MS that bound to PASC from the supernatant of *Miscanthus*-grown *N. crassa* cultures, while eight proteins from the Avicel supernatants were identified; seven cellulose binding proteins were identified in both (Tables 2, 3, 8). These included NCU00206, a predicted cellobiose dehydrogenase; *gh5-1* (NCU00762), a predicted endoglucanase; NCU05955, a predicted GH74 xyloglucanase; *gh11-2* (NCU07225), a predicted endoxylanase; *cbh-1* (NCU07340); *gh61-5* (NCU08760), a predicted endoglucanase; and *gh6-2* (NCU09680), a predicted cellobiohydrolase 2 precursor.

Table 8: Proteins identified by LC-MS/MS in the culture filtrates of Avicel-grown *Neurospora crassa*

GENE ID	ANNOTATION	CULTURE
NCU00206	<i>Neurospora crassa</i> hypothetical protein similar to cellobiose dehydrogenase 830 nt	BOTH
NCU00762	<i>Neurospora crassa</i> endoglucanase 3 precursor 391 nt	BOTH
NCU01050	<i>Neurospora crassa</i> hypothetical protein similar to endoglucanase II 239 nt	BOTH
NCU04952	<i>Neurospora crassa</i> hypothetical protein similar to beta D glucoside glucohydrolase 736 nt	BOTH
NCU05057	<i>Neurospora crassa</i> endoglucanase EG 1 precursor 439 nt	BOTH
NCU05137	<i>Neurospora crassa</i> conserved hypothetical protein 692 nt	BOTH
NCU05924	<i>Neurospora crassa</i> endo 1 4 beta xylanase 330 nt	BOTH
NCU05955	<i>Neurospora crassa</i> hypothetical protein similar to Cel74a 862 nt	BOTH
NCU07143	<i>Neurospora crassa</i> predicted protein 391 nt	BOTH

NCU07190	Neurospora crassa conserved hypothetical protein 384 nt	BOTH
NCU07225	Neurospora crassa endo 1 4 beta xylanase 2 precursor 255 nt	BOTH
NCU07326	Neurospora crassa predicted protein 327 nt	BOTH
NCU07340	Neurospora crassa exoglucanase 1 precursor 522 nt	BOTH
NCU07898	Neurospora crassa predicted protein 242 nt	BOTH
NCU08189	Neurospora crassa hypothetical protein similar to endo 1 4 beta xylanase 385 nt	BOTH
NCU08398	Neurospora crassa conserved hypothetical protein 391 nt	BOTH
NCU08412	Neurospora crassa conserved hypothetical protein 401 nt	BOTH
NCU08760	Neurospora crassa predicted protein 343 nt	BOTH
NCU09024	Neurospora crassa conserved hypothetical protein 625 nt	BOTH
NCU09175	Neurospora crassa conserved hypothetical protein 411 nt	BOTH
NCU09491	Neurospora crassa feruloyl esterase B precursor 293 nt	BOTH
NCU09680	Neurospora crassa exoglucanase 2 precursor 485 nt	BOTH
NCU00798	Neurospora crassa predicted protein 313 nt	AV
NCU01595	Neurospora crassa protein SOF1 446 nt	AV
NCU02240	Neurospora crassa hypothetical protein similar to endoglucanase II 323 nt	AV
NCU02696	Neurospora crassa hypothetical protein similar to DEAD DEAH box RNA helicase 1195 nt	AV
NCU02855	Neurospora crassa endo 1 4 beta xylanase A precursor 221 nt	AV
NCU04205	Neurospora crassa predicted protein 346 nt	AV
NCU04395	Neurospora crassa endo 1 6 beta D glucanase precursor 481 nt	AV
NCU05121	Neurospora crassa endoglucanase V 294 nt	AV
NCU05134	Neurospora crassa hypothetical protein 124 nt	AV
NCU05852	Neurospora crassa conserved hypothetical protein 254 nt	AV
NCU05974	Neurospora crassa hypothetical protein similar to cell wall glucanosyltransferase Mwg1 365 nt	AV
NCU08171	Neurospora crassa predicted protein 382 nt	AV
NCU08909	Neurospora crassa hypothetical protein similar to beta 1 3 glucanosyltransferase 543 nt	AV
NCU08936	Neurospora crassa clock controlled gene 15 412 nt	AV
NCU09046	Neurospora crassa predicted protein 187 nt	AV
NCU09764	Neurospora crassa conserved hypothetical protein 406 nt	AV
NCU01651	Neurospora crassa conserved hypothetical protein 783 nt	MIS
NCU02343	Neurospora crassa hypothetical protein similar to alpha L arabinofuranosidase A 668 nt	MIS

NCU04202	Neurospora crassa nucleoside diphosphate kinase 1 153 nt	MIS
NCU04870	Neurospora crassa hypothetical protein similar to acetyl xylan esterase 313 nt	MIS
NCU05159	Neurospora crassa acetylxylan esterase precursor 301 nt	MIS
NCU05751	Neurospora crassa conserved hypothetical protein 242 nt	MIS
NCU06239	Neurospora crassa conserved hypothetical protein 514 nt	MIS
NCU08785	Neurospora crassa conserved hypothetical protein 291 nt	MIS
NCU09267	Neurospora crassa conserved hypothetical protein 1048 nt	MIS
NCU09708	Neurospora crassa conserved hypothetical protein 465 nt	MIS
NCU09775	Neurospora crassa hypothetical protein similar to alpha L arabinofuranosidase 343 nt	MIS
NCU09923	Neurospora crassa hypothetical protein similar to beta xylosidase 775 nt	MIS

ANNOTATION - Generated by the Broad Institute (webpage broad.mit.edu/annotation/genome/neurospora/Home.html); CULTURE- Culture in which peptides were detected for a particular protein. BOTH, peptides detected in both Avicel and Miscanthus culture filtrates; AV, peptides detected only in Avicel culture filtrates; MIS, peptides detected only in Miscanthus culture filtrates.

Example 3 – Characterization of Extracellular Proteins and Cellulase Activity in Strains Containing Deletions in Genes Identified in the Overlap of the Transcriptome/Secretome Datasets

Of the 22 extracellular proteins detected in both the *Miscanthus* and Avicel grown cultures, homokaryotic strains containing deletions in genes encoding 16 of these extracellular proteins were available to the public (Dunlap *et al.*, 2007). None of these 16 deletion strains had been previously characterized with respect to their influence on plant cell wall or cellulose degradation in *N. crassa*. The 16 deletion strains were grown both on media containing sucrose or Avicel as a preferred carbon source. All strains showed a wild type growth phenotype on sucrose. On medium containing Avicel, the bulk growth of the 16 deletion strains was monitored for a 7-day period. After seven days, the total secreted protein, endoglucanase activity, β -glucosidase activity, and aggregate Avicelase activity of the culture filtrates was measured and compared with the wild-type strain from which all the mutants were derived (Fig. 6). SDS-PAGE was also done on unconcentrated culture supernatants to investigate the relative abundance of secreted proteins.

There were growth deficiencies on Avicel for strains containing deletions of two predicted exoglucanases (*cbh-1*; NCU07340 and *gh6-2*; NCU09680) and a predicted β -glucosidase (*gh3-4*; NCU04952). The *cbh-1* mutant was the most severe; after seven days much of the Avicel remained, while in the wild-type strain all of the Avicel was degraded by this time. For 10 of the 16 deletion strains,

SDS-PAGE analysis of the secreted proteins showed an altered extracellular protein profile where a single band disappeared, thus allowing assignment of a particular protein band to a predicted gene (Fig. 6A, boxes; Fig. 7). These included NCU00762 (*gh5-1*), NCU04952 (*gh3-4*), NCU05057 (*gh7-1*), NCU05137, NCU05924 (*gh10-1*), NCU05955, NCU07190 (*gh6-3*), NCU07326, NCU07340 (*cbh-1*), and NCU09680 (*gh6-2*).

For the majority of the deletion strains, the total secreted protein, endoglucanase, β -glucosidase, and Avicelase activities of the culture supernatants were similar to wild type (Fig. 6B, C and Table 9).

Table 9: Enzyme Activity of Deletion Strains

Gene Name	Growth on Avicel	[Secreted Protein] (% of WT)	Azo-CMCase (% of WT)	Bgl (% of WT)	[CB] (mM)	[GLC] (mM)
NCU00762	***	113 $\pm$ 8	33 $\pm$ 2	102 $\pm$ 2	0.9 $\pm$ 0.0	2.6 $\pm$ 0.1
NCU01050	***	98 $\pm$ 12	92 $\pm$ 8	88 $\pm$ 5	0.8 $\pm$ 0.2	2.9 $\pm$ 0.3
NCU04952	***	146 $\pm$ 6	124 $\pm$ 5	1 $\pm$ 0.3	2.24 $\pm$ 0.2	0.6 $\pm$ 0.0
NCU05057	***	143 $\pm$ 10	98 $\pm$ 3	100 $\pm$ 10	1.7 $\pm$ 0.1	3.6 $\pm$ 0.1
NCU05137	***	154 $\pm$ 12	156 $\pm$ 10	178 $\pm$ 3	1.0 $\pm$ 0.0	3.8 $\pm$ 0.1
NCU05924	***	108 $\pm$ 3	108 $\pm$ 5	101 $\pm$ 4	1.1 $\pm$ 0.1	2.6 $\pm$ 0.2
NCU05955	***	92 $\pm$ 10	94 $\pm$ 8	98 $\pm$ 7	0.9 $\pm$ 0.1	2.3 $\pm$ 0.1
NCU07190	***	111 $\pm$ 7	136 $\pm$ 6	92 $\pm$ 1	1.1 $\pm$ 0.0	2.6 $\pm$ 0.0
NCU07326	***	105 $\pm$ 4	114 $\pm$ 17	85 $\pm$ 11	1.0 $\pm$ 0.0	2.3 $\pm$ 0.0
NCU07340	*	41 $\pm$ 2.2	43 $\pm$ 9	56 $\pm$ 9	0.1 $\pm$ 0.0	0.5 $\pm$ 0.1
NCU07898	***	84 $\pm$ 7	86 $\pm$ 1.5	59 $\pm$ 15	0.5 $\pm$ 0.3	2.3 $\pm$ 0.5
NCU08189	***	83 $\pm$ 12	80 $\pm$ 8	69 $\pm$ 15	0.5 $\pm$ 0.1	2.3 $\pm$ 0.4
NCU08398	***	95 $\pm$ 11	107 $\pm$ 7	97 $\pm$ 3	0.6 $\pm$ 0.1	1.8 $\pm$ 0.0
NCU08760	***	115 $\pm$ 3	126 $\pm$ 6	115 $\pm$ 8	0.9 $\pm$ 0.1	2.6 $\pm$ 0.1

NCU09175	* * *	96 ± 7	115 ± 0	101 ± 8	0.7 ± 0.0	1.9 ± 0.1
NCU09680	* *	118 ± 7	165 ± 7	150 ± 1	0.23 ± 0.1	1.7 ± 0.1
WT	* * *	100 ± 7	100 ± 12	100 ± 6	0.97 ± 0.0	2.4 ± 0.1

Deviations from this trend were seen with the $\Delta gh5-1$ (NCU00762), $\Delta gh3-4$ (NCU04952), $\Delta NCU05137$, $\Delta cbh-1$ (NCU07340), and $\Delta gh6-2$ (NCU09680) mutants. In $\Delta gh5-1$ (NCU00762), $\Delta gh3-4$ (NCU04952), and $\Delta cbh-1$ (NCU07340), Avicelase, endoglucanase or Δ -glucosidase activities were lower than the corresponding wild-type activities. In particular, the deletion of NCU04952 eliminated all β -glucosidase activity from the culture supernatant, as evidenced by PNPGase activity and by higher levels of cellobiose and lower levels of glucose in the Avicelase enzyme assays (Fig. 6B, C). Despite lowering endoglucanase activity, the culture filtrate from $\Delta gh5-1$ (NCU00762) showed no significant deficiency in Avicelase activity relative to the wild-type strain (Fig. 6C). As expected, mutations in *cbh-1* (NCU07340) resulted in lower endoglucanase and Avicelase activity, due to poor growth. A strain containing a deletion of NCU09680, encoding a CBH(II)-like protein (*gh6-2*), also showed reduced cellobiose accumulation, as observed with $\Delta cbh-1$ mutant (Fig. 6C).

Mutations in three strains resulted in an increased level of secreted proteins, especially CBH(I) (Fig. 6A); *gh3-4* (NCU04952), *gh7-1* (NCU05057) and a hypothetical protein gene (NCU05137). In addition to increased levels of secreted proteins, the $\Delta NCU05137$ mutant showed increased endoglucanase, β -glucosidase, and Avicelase activity (Fig. 6B, C). NCU05137 is highly conserved in the genomes of a number of filamentous ascomycete fungi, including other cellulolytic fungi, but notably does not have an ortholog in *T. reesei* (Fig. 2). It is possible that the increase in CBH(I) levels observed in $\Delta gh3-4$, $\Delta gh7-1$, and $\Delta NCU05137$ could be due to either increased secretion, protein stability or, alternatively, feedback that results in an increase in expression of *cbh-1*. To differentiate these possibilities, the profile of extracellular proteins produced by $\Delta NCU05137$ and $\Delta gh3-4$ (NCU04952) was compared with gene expression levels of *cbh-1* (NCU07340) and *gh6-2* (CBH(II); NCU09680) as assayed by quantitative RT-PCR (Fig. 8). The strains $\Delta NCU05137$ and $\Delta gh3-4$ showed a higher level of CBH(I) protein as early as two days in an Avicel-grown culture. Quantitative RT-PCR of *cbh-1* and *gh6-2* from Avicel-grown cultures showed that both genes exhibited high expression levels in wild type and the $\Delta NCU05137$ and $\Delta gh3-4$ mutants after two days of growth. However, although expression of both of

these genes decreased significantly on day three in the wild-type strain, both *cbh-1* and *gh6-2* expression levels increased in the Δ NCU05137 mutant, and decreased less than wild type in Δ *gh3-4* (Fig. 8). Sustained expression of *cbh-1* and *gh6-2* genes in the Δ NCU05137 and Δ *gh3-4* mutants could be responsible for the observed increase in CBH(I) and CBH(II) protein levels.

Example 4 – Materials and Methods for Transcriptome and Secretome Studies

Strains

All *Neurospora crassa* strains were obtained from the Fungal Genetics Stock Center (FGSC; webpage fgsc.net) (Supplemental Data, Dataset S1, page 1 in Tian *et al.*, 2009). Gene deletion strains were from the *N. crassa* functional genomics project (Dunlap *et al.*, 2007). *Trichoderma reesei* QM9414 was a gift from Dr. Monika Schmoll (Vienna University of Technology). Strains were grown on Vogel's salts (Vogel 1956) with 2% (w/v) carbon source (*Miscanthus*, sucrose or Avicel (Sigma)). *Miscanthus x giganteus* (milled stem to ~0.1 mm) was a gift from the University of Illinois.

Enzyme Activity Measurements

Total extracellular protein content was determined using a Bio-Rad DC Protein Assay kit (Bio-Rad). Endoglucanase activity in culture supernatants was measured with an azo-CMC kit (Megazyme SCMCL). β -glucosidase activity was measured by mixing 10-fold diluted culture supernatant with 500 μ M 4-nitrophenyl β -D-glucopyranoside in 50 mM sodium acetate buffer, pH 5.0, for 10 minutes at 40 °C. The reaction was quenched with 5% w/v sodium carbonate, and the absorbance at 400 nm was measured. Avicelase activity was measured by mixing 2-fold diluted culture supernatant with 50 mM sodium acetate, pH 5.0, and 5 mg/mL Avicel at 40 °C. Supernatants were analyzed for glucose content using a coupled enzyme assay with glucose oxidase/peroxidase. Fifty μ L of the avicelase reaction was transferred to 150 μ L of glucose detection reagent containing 100 mM sodium acetate pH 5.0, 10 U/mL horseradish peroxidase, 10 U/mL glucose oxidase, and 1 mM o-dianisidine. After 30 minutes absorption was measured at 540 nm. Cellobiose concentrations were determined using a coupled enzyme assay with cellobiose dehydrogenase (CDH) from *Sporotrichum thermophile*. CDH was isolated from *S. thermophile* similar to previous reports (Canevascini 1988). Fifty μ L of the avicelase reaction was transferred to 250 μ L of cellobiose detection reagent containing 125 mM sodium acetate pH 5.0, 250 μ M

dichlorophenol indophenol, and 0.03 mg/mL CDH. After 10 minutes absorption was measured at 530 nm.

RNA Isolation, Microarray Analysis, and Signal Peptide Predictions

Mycelia were harvested by filtration and flash frozen in liquid nitrogen. Total RNA was isolated using trizol (Tian *et al.*, 2007; Kasuga *et al.*, 2005). Microarray hybridization and data analysis were as previously described (Tian *et al.*, 2007). Normalized expression values were analyzed using BAGEL (Bayesian analysis of gene expression levels) (Townsend 2004; Townsend and Hartl 2002), which infers relative gene expression levels and credible intervals for each gene at each experimental time point. Signal peptides were predicted using the N-terminal 70 amino acid region of each predicted protein with the signalP3 program (webpage cbs.dtu.dk/services/SignalP-3.0/). Original profiling data is obtainable at (webpage yale.edu/townsend/Links/ffdatabase/).

Protein Gel Electrophoresis

Except where otherwise noted, unconcentrated culture supernatants were treated with 5x SDS loading dye and boiled for 5 minutes before loading onto Criterion 4-15% Tris-HCl polyacrylamide gels. Coomassie dye was used for staining.

Preparation of Tryptic Peptides for Secretome Analysis

Culture supernatants were concentrated with 10 kDa MWCO PES spin concentrators. Cellulose binding proteins were isolated from the culture supernatant by addition of phosphoric acid swollen cellulose (PASC). Five mL of a suspension of 10 mg/mL PASC was added to 10 mL of culture supernatant. After incubation at 4 °C for 5 minutes, the mixture was centrifuged and the pelleted PASC was then washed with 20 pellet volumes of 100 mM sodium acetate pH 5.0. The supernatant after treatment with PASC was saved as the unbound fraction and concentrated. 36 mg of urea, 5 µL of 1M Tris PH 8.5, and 5 µL of 100 mM DTT were then added to 100 µL of concentrated culture supernatant or protein-bound PASC and the mixture was heated at 60 °C for 1 hour. After heating 700 µL of 25 mM ammonium bicarbonate and 140 µL of methanol were added to the solution followed by treatment with 50 µL of 100 µg/mL trypsin in 50 mM sodium acetate pH 5.0. For the PASC bound proteins, the PASC was removed by centrifugation after heating, and the supernatant was then treated with trypsin. The trypsin was left to react overnight at 37 °C. After digestion the volume was reduced by speedvac and washed

with MilliQ water three times. Residual salts in the sample were removed by using OMIX microextraction pipette tips according to the manufacturer's instructions.

Liquid Chromatography of Tryptic Peptides

Trypsin-digested proteins were analyzed using a tandem mass spectrometer that was connected in-line with ultraperformance liquid chromatography (UPLC). Peptides were separated using a nanoAcquity UPLC (Waters, Milford, MA) equipped with C18 trapping (180 $\mu\text{m} \times 20 \text{ mm}$) and analytical (100 $\mu\text{m} \times 100 \text{ mm}$) columns and a 10 μL sample loop. Solvent A was 0.1% formic acid/99.9% water and solvent B was 0.1% formic acid/99.9% acetonitrile (v/v). Sample solutions contained in 0.3 mL polypropylene snap-top vials sealed with septa caps (Wheaton Science, Millville, NJ) were loaded into the nanoAcquity autosampler prior to analysis. Following sample injection (2 μL , partial loop), trapping was performed for 5 min with 100% A at a flow rate of 3 $\mu\text{L}/\text{min}$. The injection needle was washed with 750 μL each of solvents A and B after injection to avoid cross-contamination between samples. The elution program consisted of a linear gradient from 25% to 30% B over 55 min, a linear gradient to 40% B over 20 min, a linear gradient to 95% B over 0.33 min, isocratic conditions at 95% B for 11.67 min, a linear gradient to 1% B over 0.33 min, and isocratic conditions at 1% B for 11.67 min, at a flow rate of 500 nL/min. The analytical column and sample compartment were maintained at 35 $^{\circ}\text{C}$ and 8 $^{\circ}\text{C}$, respectively.

Mass Spectrometry

The column was connected to a NanoEase nanoelectrospray ionization (nanoESI) emitter mounted in the nanoflow ion source of a quadrupole time-of-flight mass spectrometer (Q-ToF Premier, Waters). The nanoESI source parameters were as follows: nanoESI capillary voltage 2.3 kV, nebulizing gas (nitrogen) pressure 0.15 mbar, sample cone voltage 30 V, extraction cone voltage 5 V, ion guide voltage 3 V, and source block temperature 80 $^{\circ}\text{C}$. No cone gas was used. The collision cell contained argon gas at a pressure of 8×10^{-3} mbar. The ToF analyzer was operated in "V" mode. Under these conditions, a mass resolving power¹ of 1.0×10^4 (measured at $m/z = 771$) was routinely achieved, which is sufficient to resolve the isotopic distributions of the singly and multiply charged peptide ions measured in this study. Thus, an ion's mass and charge could be determined independently, i.e., the ion charge was determined from the reciprocal of the spacing between adjacent isotope peaks in the m/z spectrum. External mass calibration was performed immediately prior to analysis, using solutions of sodium

formate. Survey scans were acquired in the positive ion mode over the range $m/z = 450-1800$ using a 0.95 s scan integration and a 0.05 s interscan delay. In the data-dependent mode, up to five precursor ions exceeding an intensity threshold of 35 counts/second (cps) were selected from each survey scan for tandem mass spectrometry (MS/MS) analysis. Real-time deisotoping and charge state recognition were used to select 2+, 3+, 4+, 5+, and 6+ charge state precursor ions for MS/MS. Collision energies for collisionally activated dissociation (CAD) were automatically selected based on the mass and charge state of a given precursor ion. MS/MS spectra were acquired over the range $m/z = 50-2500$ using a 0.95 s scan integration and a 0.05 s interscan delay. Ions were fragmented to achieve a minimum total ion current (TIC) of 30,000 cps in the cumulative MS/MS spectrum for a maximum of 3 s. To avoid the occurrence of redundant MS/MS measurements, real time exclusion was used to preclude re-selection of previously analyzed precursor ions over an exclusion width of ± 0.25 m/z unit for a period of 180 s.

Mass Spectrometry Data Analysis

The data resulting from LC-MS/MS analysis of trypsin-digested proteins were processed using ProteinLynx Global Server software (version 2.3, Waters), which performed background subtraction (threshold 35% and fifth order polynomial), smoothing (Savitzky-Golay2 10 times, over three channels), and centroiding (top 80% of each peak and minimum peak width at half height four channels) of the mass spectra and MS/MS spectra. The processed data were searched against the *N. crassa* database (Broad Institute) using the following criteria: tryptic fragments with up to five missed cleavages, precursor ion mass tolerance 50 ppm, fragment ion mass tolerance 0.1 Da, and the following variable post-translational modifications: carbamylation of N-terminus and Lys side chains, Met oxidation, and Ser/Thr dehydration. The identification of at least three consecutive fragment ions from the same series, i.e., b or y-type fragment ions, was required for assignment of a peptide to an MS/MS spectrum. The MS/MS spectra were manually inspected to verify the presence of the fragment ions that uniquely identify the peptides.

Quantitative RT-PCR

The RT-PCR was performed in an ABI7300 with reagents from Qiagen (SYBR-green RT-PCR kit (Cat No. 204243)). The primers for CBHI (NCU07340) were: forward 5'-ATCTGGGAAGCGAACAAAG-3' (SEQ ID NO: 16) and reverse 5'-TAGCGGTCGTCGGAATAG-3' (SEQ ID NO: 17). The primers for CBHII (NCU09680) were: forward 5'-CCCATCACC ACTACTACC-

3' (SEQ ID NO: 18) and reverse 5'-CCAGCCCTGAACACCAAG-3' (SEQ ID NO: 19). Actin was used as a control for normalization. The primers for actin were: forward 5'-TGA TCT TAC CGA CTA CCT - 3' (SEQ ID NO: 20) and reverse 5'-CAG AGC TTC TCC TTG ATG -3' (SEQ ID NO: 21). Quantitative RT-PCR was performed according to Dementhon *et al.*, (2006).

Example 5 – Discussion of Transcriptome and Secretome Studies

Degradation of plant biomass requires the production of many different enzymatic activities, which are regulated by the type and complexity of the available plant material (Fig. 9) (Bouws *et al.*, 2008). The first systematic analyses of plant cell wall degradation by a cellulolytic fungus are described here, which include transcriptome, secretome, and mutant analyses. Profiling data showed that *N. crassa* coordinately expresses a host of extracellular and intracellular proteins when challenged by growth on *Miscanthus* or Avicel (Fig. 9). Many of the most highly expressed genes during growth on cellulosic substrates encode proteins predicted to be involved in the metabolism of plant cell wall polysaccharides, many of which were identified by MS analyses. Genome comparisons of filamentous fungi show a large number of glycosyl hydrolases (~200) with varying numbers of predicted cellulases, from 10 in *T. reesei* (Martinez *et al.*, 2008) to 60 in *Podospora anserina* (Espagne *et al.*, 2008), a dung-degrading species closely related to *N. crassa*. A comparison between these results and a recent transcriptome/secretome study on the white rot basidiomycete fungus, *Phanerochaete chrysosporium*, (Wymelenberg *et al.*, 2009) showed little overlap in regulated genes (18 genes) and secreted proteins (2 proteins) when both species were grown on pure cellulose. These data suggest that different fungi may utilize different gene sets for plant cell wall degradation. However, one aspect that both studies had in common was the high number of uncharacterized genes/proteins associated with cellulose degradation. Other cellulolytic fungi, including *P. chrysosporium*, do not have the genetic and molecular tools that are readily available with *N. crassa*. Using the functional genomic tools available with *N. crassa*, both the function and redundancy of plant cell wall degrading enzyme systems can be addressed to create optimal enzyme mixtures for industrial production of liquid fuels from lignocellulose biomass.

In this study, it was found that cellobiohydrolase(I) (CBHI) in *N. crassa* is the most highly produced extracellular protein during growth on Avicel or *Miscanthus*, and deletion of this gene caused the most severe growth deficiencies on cellulosic substrates. These results are similar to those reported in *T. reesei* (Suominen *et al.*, 1993, Seiboth *et al.*, 1997). Deletion of cellobiohydrolase(II) also caused

growth deficiencies on cellulosic substrates, but to a much lesser extent than CBH(I), suggesting that exoglucanase activity in *N. crassa* is predominantly from CBH(I) and that cellulases and other CBHs do not compensate for the loss of CBH(I). Here, it was shown that the three most highly produced endoglucanases during growth on cellulosic substrates are the proteins encoded by NCU05057, NCU00762, and NCU07190. These proteins have homology to endoglucanases EG1, EG2, and EG6, respectively. Deletion of these genes did not affect growth on Avicel, although differences in the secreted protein levels and endoglucanase activity were observed. Unexpectedly, in the Δ NCU05057 strain, extracellular protein levels were much higher, especially CBH(I), suggesting that to maintain the wild-type growth phenotype on crystalline cellulose the mutant was forced to increase production of other cellulases or that the products of NCU05057 catalysis may repress cellulase production. It was concluded that no one endoglucanase in *N. crassa* is required for growth on crystalline cellulose and that the different endoglucanases have overlapping substrate specificities.

The glycoside hydrolase family 61 enzymes are greatly expanded in *N. crassa* compared to *T. reesei* (Martinez *et al.*, 2008). These enzymes have poorly defined biological function, but their general conservation and abundance in cellulolytic fungi suggests an important role in plant cell wall metabolism. Here, genes for 10 of the 14 GH61 enzymes were identified in the *N. crassa* transcriptome, suggesting that these enzymes are utilized during growth on cellulosic biomass. The four GH61 deletion strains tested showed only small differences compared to wild type in the secreted protein levels, endoglucanase, and total cellulase activities. However, analyses of additional GH61 mutants and the capacity to create strains containing multiple mutations in *N. crassa* via sexual crosses will address redundancy and expedite functional analysis of this family.

In addition to predicted cellulase genes, genes encoding hemicellulases, carbohydrate esterases, β -glucosidases, β -xylosidases, and other proteins predicted to have activity on carbohydrates were identified in the *N. crassa* transcriptome from both *Miscanthus* and Avicel. The fact that Avicel contains no hemicellulose components suggests that cellulose is probably the primary inducer of genes encoding plant cell wall degrading enzymes in *N. crassa*. However, genes encoding some hemicellulases and carbohydrate esterases were only expressed during growth on *Miscanthus*. Similarly, in other cellulolytic fungi such as *T. reesei* and *Aspergillus niger*, genes encoding some cellulases and hemicellulases are coordinately regulated, while others are differentially regulated (Stricker *et al.*, 2008). As expected, deletions of non-cellulase genes had little effect on growth on Avicel or cellulase activity,

with the exception of NCU05137 and *gh3-4*. The Δ NCU05137 strain secreted more protein, had higher cellulase activity, and showed higher expression of *cbh-1* (CBH(I)) and *gh6-2* (CBH(II)) than wild type. NCU05137 encodes a secreted hypothetical protein that has no homology to proteins of known function, but is highly conserved in other cellulolytic fungi (Fig. 2; E value 0.0). NCU05137 also has more distant homologs, but also of unknown function, in a number of bacterial species. The protein product of NCU05137 may interfere with signaling processes associated with induction of cellulase gene expression in *N. crassa* (Fig. 9). Similarly, mutations in *gh3-4* (NCU04952) also increased CBH(I) activity. Deletion of this gene completely removed PNPGase activity and cellobiose accumulated in *in vitro* cellulase assays using Δ *gh3-4* culture filtrates. All the data together suggested that NCU04952 encodes the primary extracellular β -glucosidase in *N. crassa*. These data were consistent with catabolite repression of cellulase production by glucose.

Extracellular degradation of cellulose and hemicellulose results in the formation of soluble carbohydrates that are subsequently transported into the cell (Fig. 9). In this study, 10 genes encoding permeases/transporters were identified which showed significantly increased expression when *N. crassa* was grown on *Miscanthus* or Avicel, suggesting their involvement in transport of plant cell wall degradation products into the cell. The major degradation products by cellulases and hemicellulases *in vitro* are cellobiose, glucose, xylobiose, and xylose. Some of these transporters may be functionally redundant or capable of transporting oligosaccharides. The function of these putative transporters was further explored (see Examples 7-9). Construction of downstream processing strains capable of transporting oligosaccharides by heterologous expression of *N. crassa* transporters may improve industrial fermentation of biomass hydrolysis products. None of these transporters or what they may transport has been characterized at the molecular or functional level in any filamentous fungi.

Many genes that showed increased expression levels during growth on *Miscanthus* and Avicel encode proteins of unknown function that are conserved in other cellulolytic fungi. By assessing the phenotype of only 16 strains, a mutant in a gene encoding a protein of unknown function that significantly affects cellulase activity was identified. The well-understood genetics and availability of functional genomic resources in *N. crassa* make it an ideal model organism to determine the biological function of these proteins, as well as regulatory aspects of cellulase and hemicellulase production, and to dissect redundancies and synergies between extracellular enzymes involved in the degradation of plant cell walls.

Example 6 – Screening of Mutants of Genes Upregulated during Growth on *Miscanthus*

In order to analyze additional genes identified in the transcriptional profiling experiment, the phenotypes of mutants of 188 genes that were upregulated in *Neurospora* grown on *Miscanthus* for 16 hours were analyzed (see Example 1). A knockout mutant of each gene was grown on minimal Vogel's medium for 10-14 days. Conidia were harvested with 2 mL ddH₂O and inoculated into 100 mL media in 250 mL flasks at a concentration of 10⁶ conidia per mL. One of three different carbon sources was added to each flask: 2% sucrose, 2% Avicel, or 2% *Miscanthus* (1 mm particles from Calvin Laboratory, University of California, Berkeley, CA). Cultures were grown at 25 °C with 220 rpm of shaking for 4 days.

Table 10 lists the phenotypes of the mutants that showed a significant difference in cellulase activity and growth on Avicel or *Miscanthus* compared to wild-type. Growth on Avicel or *Miscanthus* was evaluated by eye with a “+” scoring system. Wild-type growth was set at “++”. Total protein in the culture supernatant was measured by Bradford assay (100 µl supernatant to 900 µl Bradford dye). Endoglucanase activity was measured with the Azo-CMC kit from Megazyme and indicated in Table 10 as the percentage of endoglucanase activity in the mutant compared to wild type. Total cellulase activity was measured by detecting cellobiose levels in the supernatant as described in Example 4. Results are indicated in Table 10 as a percentage of wild-type.

Table 10 shows mutant screening data

NCU#	FGSC #	Broad Annotation (Domains)	Pfam*	Up-Regulation	Growth (Avi, Mis)	%WT Bradford (Avi)	%WT Bradford (Mis)	% WT endo (Avi)	% WT endo (Mis)	%WT cellobiose (Avi)	%WT cellobiose (Mis)
NCU0013 0.2	FGSC 11823	beta-glucosidase (GH1)	Glycosyl Hydrolase 1 (2.5e-196)	394.6	++,++	203.24779 47	118.39879 72	152.2858578	129.35474 94	n/a	n/a
NCU0024 8.2	FGSC 12214	Predicted Protein	no significant hit	9.74	+,++	86.960132 89	86.044718 58	30.39187506	156.50501 44	93.05143 946	89.2769821 9
NCU0032 6.2	FGSC 15868	Conserved Hypothetical (SMP-30/glucanase)	SMP-30/Gluconolactonase /LRE-like region (3.5e-82)	7.7	+,++	33.028792 91	144.12104 86	39.91568458	227.03668 09	89.76872 415	79.0515463 9

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NCU0076 2.2	FGSC 16747	Endoglu canase- 3 precurs or (GH5, CBD1)	Cellulase (1.4e-69), Fungal cellulose binding domain (9.2e-14)	29.6	++,++	104.35044 11	84.570569 44	26.92790756	39.268900 58	n/a	n/a
NCU0081 0.2	FGSC 11285	Similar to Glycosyl Hydrola se (GH2, beta- galactos idase)	Glycosyl hydrolase s family 2 (1.7e-145)	5.3	++,++	163.80504 7	123.55647 57	161.2908993	159.49837 44	102.2745 211	91.7334566 4
NCU0089 0.2	FGSC 16749	Similar to beta- manosid ase (GH2)	Glycosyl hydrolase s family 2 (4.1e-06)	20.45	+,+	47.574178 03	101.59744 41	43.25546345	164.08197 18	n/a	n/a
NCU0332 8.2	FGSC 16589	Conserv ed Hypothe tical (GH61)	Glycosyl hydrolase family 61 (2.3e-10)	26.4	++,++	100.17528 48	109.96672 48	142.6962073	167.00754 81	n/a	n/a
NCU0341 5.2	FGSC 12922	Aldehyd e Dehydro genase	Aldehyde dehydroge nase family (2.5e-267)	9.8	++,++	104.22782 04	96.614353 73	96.4633125	63.455233 29	76.96643 943	103.127398 3
NCU0373 1.2	FGSC 18653	Similar to HAD Superfa mily Hydrola se	haloacid dehalogen ase-like hydrolase (9.2e-21)	2.7	++,++	131.36911 28	110.58014 46	145.0235135	134.62799 5	230.1450 412	100.417237 5
NCU0375 3.2	FGSC 16379	ccg-1 (clock controlle d gene)	no significant hit	10.5	++,++	107.67928 92	111.34810 86	74.42402278	129.21967 77	n/a	n/a
NCU0419 7.2	FGSC 17499	Conserv ed Hypothe tical	no significant hit	5.04	++,++	103.06681 27	99.083054 14	108.9737808	89.861286 25	75.17285 531	96.0507505 4
NCU0424 9.2	FGSC 18628	Hypothe tical Protein	no significant hit	5.3	++,++	93.296823 66	106.10121 67	79.0053469	84.161412 36	64.07989 522	100.124185
NCU0428 7.2	FGSC 14573	Predict ed Protein	no significant hit	4.7	++,++	115.51578 59	102.23610 65	125.5086234	127.92825 77	202.5161 29	183.867924 5
NCU0434 9.2	FGSC 18634	Similar to mitocho ndrial pyruvate	BCDHK_A dom3 (4.7e-78), HistidineA TPase_c	2.9	++,++	87.877764 65	89.362051 96	71.41381803	145.24158 13	208.4329 349	101.299376 3

		dehydro genase kinase	(6.9e-14)								
NCU0447 5.2	FGSC 15386	Predicted Protein	no significant hit	76.7	+++,,+	98.102053 52	122.20348 51	156.3643221	127.06766 92	n/a	n/a
NCU0499 7.2	FGSC 15623	Similar to xylanase (GH10, CBD1)	Glycosyl hydrolase family 10 (3.3e- 148), Fungal cellulose binding domain (2.1e-16)	25.6	++,++	105.35201 76	114.58401 84	123.3295466	231.69838 95	136.4189 483	102.540398 3
NCU0505 7.2	FGSC 13342	Endoglu canase EG-1 precursor (GH7)	Glycosyl hydrolase family 7 (3.3e-189)	8.7	++,++	137.53165 63	95.692206 51	133.5226686	174.26793 56	182.0237 75	97.8133065 7
NCU0515 9.2	FGSC 13439	acetylxy lan esterase precursor (Cutinase, CBD1)	Cutinase (3.4e- 110), Fungal cellulose binding domain (7.4e-14)	34.8	+++,,+	86.185438 71	39.516582 35	92.2873845	67.117794 49	n/a	n/a
NCU0549 3.2	FGSC 14625	Predicted Protein	no significant hit	4.5	+,++	73.252660 13	104.41025 64	102.3841739	116.89545 93	70.37185 126	99.4283792 9
NCU0551 9.2	FGSC 19924	Similar to Tna1 (MFS transporter)	Major Facilitator Superfamily (3.7e- 40)	2.8	++,++	85.311913 21	101.06666 67	118.8447721	87.777191 13	51.68869 31	97.8750165 5
NCU0575 1.2	FGSC 15757	Conserved Hypothetical (GDSL-like lipase)	GDSL-like Lipase/Ac ylhydrolase (1.3e- 11)	3.9	+,++	97.016482 37	111.40512 82	114.7202911	136.37803 59	87.71492 649	105.592058 3
NCU0577 0.2	FGSC 11532	Peroxidase/Cat alase 2	Peroxidase (9.4e- 195)	11.9	++,++	109.86309 89	86.734120 29	69.1872525	146.21553 88	n/a	n/a
NCU0585 3	FGSC 13771	Sugar Transporter	Sugar Transporter	130.7	+	40.279246 87	24.412597 90	n/a	n/a	n/a	n/a
NCU0589 7.2	FGSC 13717	Similar to l-fucose permease (MFS transporter)	Major Facilitator Superfamily (3.8e- 16)	20.9	+,++	33.784641 42	34.727545 41	26.3266891	86.259541 98	n/a	n/a

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		ter)									
NCU0593 2.2	FGSC 19952	Predicted Protein	no significant hit	38.2	++,++	70.898264 28	76.871320 44	80.78910753	117.95968 23	58.07431 478	96.7108463
NCU0600 9.2	FGSC 14922	Similar to aldo/ket o reductas e	Aldo/keto reductase family (4.8e-63)	6.9	+,++	148.66337 26	74.067844 13	120.602266	99.480757 48	70.89513 625	97.0057324 1
NCU0649 0.2	FGSC 15539	Conserv ed Hypothe tical	no significant hit	13.8	+,++	77.461041 43	80.263526 77	76.95289207	79.383017 72	59.91109 168	99.9937138 5
NCU0734 0.2	FGSC 15630	Exogluc anase-1 precurs or, CBH1 (GH7)	Glycosyl hydrolase family 7 (1e-999), Fungal cellulose binding domain (4.9e-18)	426.4	+,++	21.096345 51	95.219737 86	35.54661301	96.991344 96	93.62619 808	78.4490255 3
NCU0785 3.2	FGSC 19036	Uricase	Uricase (1.7e-119)	4.3	+++,++	n/a	n/a	120.9286562	168.23406 48	65.75994 56	99.1465917 7
NCU0799 7.2	FGSC 18273	Predicted Protein	no significant hit	4.5	++,++	n/a	n/a	148.127436	98.119122 26	60.65548 063	93.7870427 1
NCU0811 4.2	FGSC 17869	Similar to MFS hexose transport er (MFS transport er)	Sugar (and other) transport er (5.1e- 88), Major Facilitator Superfami ly (3.8e- 24)	6.7	+,++	81.692639 05	79.226240 54	85.18187239	92.974954 18	58.83068 556	93.1432252
NCU0874 4.2	FGSC 11387	Predicted Protein, possible TF (basic region leucine zipper)	no significant hit	2.3	++,++	n/a	n/a	168.8527368	110.76280 04	136.2451 567	97.4413419 7
NCU0874 6.2	FGSC 18358	Conserv ed Hypothe tical (starch binding domain)	Starch binding domain (5.3e-54)	6	++,++	98.695046 24	79.114101 49	111.0713576	120.25045 82	447.2796 518	100.575366 7
NCU0876 0.2	FGSC 15664	Predicted Protein	Fungal cellulose binding	107.5	++,++	158.13953 49	86.179645 34	208.2590783	81.000137 38	97.32646 961	84.3425177 4

		(CBD1)	domain (1.9e-11), Glycosyl hydrolase family 61 (1.3e-9)								
NCU0910 8.2	FGSC 19207	Conserv ed Hypothe tical	no significant hit	4.1	++,++	n/a	n/a	93.22148788	111.80773 25	60.89420 655	97.2451790 6
NCU0949 5.2	FGSC 12411	set-6, histone methyltr ansferas e	SET domain (6.9e-5)	26.2	++,++	109.33001 11	122.53276 79	129.9223915	130.89710 13	152.7495 439	92.2521655 4
NCU0968 0.2	FGSC 15633	Exogluc anase-2 precurs or, CBH2 (GH6, CBD1)	Glycosyl hydrolase s family 6 (1.1e- 152), Fungal cellulose binding domain (1.2e-13)	230.9	+,++	102.71317 83	95.200462 61	89.54680464	102.67893 94	94.61873 756	83.8766134 3
NCU1004 5.2	FGSC 18480	pectines terase precurs or	Pectineste rase (4.4e-22)	10.9	+,++	105.30850 12	101.51387 72	109.8886901	132.52901 65	83.25906 421	103.615164 1

*Note: All sequences were searched against Pfam ls models and hits were accepted with an e-value <.0001

Example 7 – Further Analyses of Transporter Genes

As described in Example 1, ten genes encoding predicted sugar transporter proteins showed increased expression levels when *Neurospora* was grown on *Miscanthus* and Avicel: NCU00801, NCU00988, NCU01231, NCU04963, NCU05519, NCU05853, NCU05897, NCU06138, NCU08114 and NCU10021. Deletion strains for nine of these genes were available from the Fungal Genetics Stock Center. A deletion strain of NCU10021 was not available.

Deletion mutations of NCU05853, NCU05897, or NCU08114 resulted in strains that showed a growth defect on *Miscanthus* or Avicel and/or had a cellulase enzyme defect (see Example 6; Table 10). ΔNCU05853 showed reduced growth on Avicel and reduced endoglucanase activity compared to wild-type. ΔNCU05897 showed reduced growth on Avicel and reduced endoglucanase activity compared to wild-type, and ΔNCU08114 showed reduced growth on Avicel and reduced cellobiose levels compared to

wild-type. Notably, in a comparison with expression analysis of *Sporotrichum thermophile*, another filamentous fungus, the homologs of NCU05853 (ST8454) and NCU08114 (ST5194) were also upregulated when *S. thermophile* was grown on Avicel compared to glucose (see Example 8, Table 11), further indicating their importance in cellulose utilization.

Table 11 shows *S. thermophile* expression data

Gene Name	Like NCU#	Gene Length	Glu	Avi	Cot	Glu_norm	Avi_norm	Cot_norm	Avi/Glu	Cot/Glu
jgilSpath1 108890 estExt_fgenes1_pg.C_60848	NCU00988	1937	322	370	293	42.97830583	60.2250594	48.07756207	1.149068	0.9099379
jgilSpath1 48439 e_gw1.3.3367.1	NCU01132	1539	113	59	56	15.08244894	9.60345542	9.188885583	0.522124	0.4955752
jgilSpath1 79030 estExt_Genewise1Plus.C_31624	NCU01231	1776	1171	1206	469	156.2968824	196.30114	76.95691676	1.029889	0.4005124
jgilSpath1 116270 estExt_fgenes1_pm.C_50266	NCU05519	1680	103	78	54	13.74771895	12.6960936	8.860711098	0.757282	0.5242718
jgilSpath1 84164 estExt_Genewise1Plus.C_62100	NCU05853	1706	2703	20760	14284	360.7775176	3379.11414	2343.822173	7.680355	5.2844987
jgilSpath1 102977 fgenes1_pm.5_#_763	NCU05897	1446	1510	546	322	201.5442292	88.8726553	52.8360921	0.361589	0.213245
jgilSpath1 84305 estExt_Genewise1Plus.C_70023	NCU06138	1605	1131	1330	2376	150.9579624	216.484673	389.8712883	1.17595	2.1007958
jgilSpath1 114107 estExt_fgenes1_pg.C_60848	NCU08	1945	2246	22423	10779	299.780356	3649.801	1768.696	9.98352	4.7991986

nesh1_pm.C_20669	114					8	37	388	6	
jgi Spath1 112305 estExt_fgenesh1_kg.C_60263	NCU10021	2026	6204	5287	5619	828.0664888	860.567268	922.006216	0.852192	0.905706
jgi Spath1 43941 le_gw1.2.4209.1	NCU00801	1614	41	71	159	5.472392979	11.5567006	26.08987157	1.731707	3.8780488
jgi Spath1 62521 estExt_Genewise1.C_21757	NCU04963	2204	799	1548	641	106.6449266	251.968627	105.1799225	1.937422	0.8022528

In order to narrow down the identity of each predicted transporter’s substrate, strains containing deletion mutations of NCU05853 or NCU08114 were cultured on glucose, xylose, cellobiose, xylan and Avicel (Table 12). The culturing medium contained Vogel’s medium plus 2% of the carbon source. Both mutants showed greatly reduced growth on Avicel but not on xylan, glucose, xylose, or cellobiose.

Table 12 shows growth of deletion mutants on different sugars

Gene Name	Growth on Sucrose	Growth on Avicel	Growth on Mis	Growth on Xylan	Growth on Glucose	Growth on Xylose	Growth on Cellobiose
NCU00801	***	***	***				
NCU00988	***	***	***				
NCU01231	***	***	***				
NCU04963	***	***	***				
NCU05519	***	***	***				
NCU05853	***	*	**	***	***	***	***
NCU05897	***	*	**				
NCU06138	***	***	***				
NCU08114	***	*	**	***	***	***	***

NCU10021	No deletion strain						
wt	* * *	* * *	* * *	* * *	* * *	* * *	* * *

To investigate the role of these transporters in utilization of hemicellulose, the expression of the ten transporter genes was examined when *Neurospora* was grown on xylan. Methods were used as described in Example 4, except that strains were grown on Vogel's salts with 2% (w/v) xylan. Expression of all ten transporters was upregulated during growth on xylan (Table 13), suggesting that they can transport sugars derived from hemicellulose degradation (e.g., xylobiose, xylose, arabinose, xylo-oligosaccharides) as well as from cellulose degradation (e.g., cellobiose, glucose, cello-oligosaccharides). The mutant growth results and expression analyses suggested that at least two of the predicted transporters, NCU05853 and NCU08114, can transport disaccharides (cellobiose, xylobiose) and/or oligosaccharides (cellodextrins).

Table 13 shows expression analysis of transporter genes

Gene Name	wt-Xylan 4h	Fold change in St-Avicel-4h/Glucose-4h
NCU00801	~6	10
NCU00988.2	31.1	NO CHANGE
NCU01231.2	732.1	NO CHANGE
NCU04963.2	96.5	NO DETECT
NCU05519.2	3.9	NO CHANGE
NCU05853.2	71.2	8.5
NCU05897.2	122.3	NO CHANGE
NCU06138.2	141.0	NO CHANGE
NCU08114.2	10.0	11
NCU10021.2	44.7	NO CHANGE

Example 8 – Expression Analysis of *Sporotrichum thermophile* Homologs of *N. crassa* Transporters during Growth on Various Carbon Sources

In order to compare the expression of homologous genes from a different filamentous fungus, the expression profile of *Sporotrichum thermophile* was analyzed from cultures grown on glucose, Avicel, or cotton. cDNA was isolated from cultures grown on minimal media with a carbon source of glucose, Avicel, or cotton for 16-30 hours.

First, in order to identify homologs of *Neurospora* transporter proteins in the *S. thermophile* genome, each *Neurospora* sequence was compared against a database of *S. thermophile* proteins with BLAST. The sequences of *S. thermophile* proteins found by this method were then compared to a database of *Neurospora* proteins with BLAST. These results are listed in Figure 10. The amino acid sequences for all of the *S. thermophile* homologs of putative *Neurospora* transporters that were identified can be found in SEQ ID NOs: 22-32.

Next, the expression profile of the *S. thermophile* homologs was examined. The data is presented in Table 11. The first column contains the *S. thermophile* gene name from the Joint Genome Institute *S. thermophile* assembly. The second column contains the NCU number for the most closely related putative transporter in *Neurospora*. The third column contains the gene length of the *S. thermophile* gene in nucleotides. The fourth to sixth columns contain the expression level (number of reads, comparable to absolute expression level) during growth on Vogel's minimal media supplemented with 2% of glucose, Avicel, or cotton balls as the carbon source. The seventh to ninth columns contain the normalized expression data (the # of reads divided by the total reads in the dataset). The final two columns contain the relative expression level data for each gene as a ratio of Avicel/glucose or cotton/glucose. Homologs of NCU5853, NCU8114, and NCU0801 were upregulated when grown on both Avicel and cotton. The homolog of NCU6138 was upregulated when grown on cotton, and the homolog of NCU4963 was upregulated when grown on Avicel. These data provided further support that putative transporters NCU5853, NCU8114, NCU0801, NCU6138, and NCU4963 are important for the utilization of cellulose.

Example 9 - Identification and Analysis of Cellodextrin Transporters

When grown on pure cellulose, *N. crassa* was shown to increase transcription of seven Major Facilitator Superfamily sugar transporters as well as an intracellular β -glucosidase (Ex. 1; also see Supplemental Data, Dataset S1, page 6 in Tian *et al.*, *PNAS*, 2009). Notably, knockout strains lacking individual transporters from this set grew more slowly on crystalline cellulose, suggesting that they may play a direct role in cello-oligosaccharide uptake under cellulolytic conditions (Ex. 7; Tables 10, 12). For example, deletion of NCU08114 resulted in severely retarded *N. crassa* growth (Fig. 11), and reduced *N. crassa* consumption of cellobiose (Figs. 12-13). In this example, transporter genes NCU00801/*cbt1* and NCU08114/*cbt2* were further analyzed and identified to encode transporters of cellodextrin.

To assay the function of each transporter individually, the fact that cellobiose is not catabolized by *S. cerevisiae* and is not accumulated in its cytoplasm was exploited (Fig. 14). It was reasoned that expression of a functional cellobiose transporter in conjunction with an intracellular β -glucosidase would allow *S. cerevisiae* to grow when cellobiose is presented as the sole carbon source. Yeast strains were engineered to express the transporters NCU00801 or NCU08114 fused to Green Fluorescent Protein (GFP), and the putative intracellular β -glucosidase, NCU00130. Both transporters were expressed and localized correctly to the plasma membrane (Fig. 15). The strains expressing NCU00801 or NCU08114 allowed yeast to grow with specific growth rates of 0.0341 hr^{-1} and 0.0131 hr^{-1} , respectively (Fig. 16A). These growth rates correspond to 30% and 12% of the growth rate on glucose, respectively (Fig. 17). Growth could not be explained by the extracellular hydrolysis of cellobiose to glucose followed by transport, as a strain expressing only the putative intracellular β -glucosidase grew at a rate of 0.0026 hr^{-1} (Fig. 16A), and did not grow in large-scale cultures (Fig. 18). Based on these observations, NCU00801 and NCU08114, which were named CBT1 and CBT2, were determined to function as cellobiose transporters.

To directly assay transporter function, the uptake of [^3H]-cellobiose into yeast cells was measured. Both CBT1 and CBT2 were found to be high-affinity cellobiose transporters, with K_m values of $4.0 \pm 0.3 \text{ }\mu\text{M}$ and $3.2 \pm 0.2 \text{ }\mu\text{M}$, respectively (Fig. 19). The expression-normalized V_{max} of CBT1 was 2.2 times that of CBT2, a fact that explained differences seen in the yeast growth assays. Notably, cellodextrin molecules longer than cellobiose supported the growth of yeast expressing *cbt1* and *cbt2* (Fig. 20; Fig. 16B), suggesting that cellodextrin molecules are transported by CBT1 and CBT2. In

agreement, cellobiose transport by CBT1 and CBT2 was inhibited by excess cellotriose, and CBT1 activity was also inhibited by cellotetraose (Fig. 21). Furthermore, upon purification, the β -glucosidase, NCU00130 (Fig. 22), was found to hydrolyze cellobiose, cellotriose, and cellotetraose (Fig. 16C).

Orthologs of *cbt1* and *cbt2* were identified and found to be widely distributed in the fungal kingdom (Fig. 23). Recent expression data shows their importance to various interactions between fungi and plants. For example, when the ascomycete, *Tuber melanosporum*, or the basidiomycete, *Laccaria bicolor*, interacts symbiotically with root tips to form ectomycorrhizas, the ortholog of *cbt1* is upregulated in both (Martin *et al.*, 2010). Likewise, the saprophytes, *Aspergillus oryzae* (Noguchi *et al.*, 2009), *Postia placenta* (Vanden Wymelenberg *et al.*, 2010), and *Phanerochaete chrysosporium* (Vanden Wymelenberg *et al.*, 2010), upregulate orthologs of *cbt2* when in contact with plant wall material. Certain yeasts, such as *Kluyveromyces lactis* and *Pichia stipitis* grow on cellobiose (Freer, 1991; Preez *et al.*, 1986), and cellobiose transport has been reported in *Clavispora lusitaniae* (Freer and Greene 1990). It was determined in this study that all of these yeasts contain orthologs of *cbt1*, *cbt2*, or both (see below for methods). Cellobiose transport has been observed in *Hypocrea jecorina* (*Trichoderma reesei*), but since the transporter was not identified, it is not clear if this activity can be ascribed to orthologs of *cbt1* or *cbt2* (Kubicek *et al.*, 1993).

The use of cellobiose transporters by cellulolytic fungi suggests that they are essential for their optimal growth on cellulose. To test whether cellobiose catabolism could improve yeast ethanol production, the yeast strains constructed above were grown under fermentation conditions. With little optimization, yeast with a complete cellobiose catabolism pathway ported from *N. crassa* were shown to ferment cellobiose to ethanol efficiently (Fig. 24A), with an ethanol yield of 0.47, 86% of the theoretical value (Bai *et al.*, 2008). This was comparable to industrial yields from glucose of 90 – 93% (Basso *et al.*, 2008). The high affinity of CBT1 and CBT2 for cellobiose compared to the hexose transporters of *S. cerevisiae* (Reifenberger *et al.*, 1997), and reported extracellular β -glucosidases (Chauve *et al.*, 2010), suggested that a cellobiose/cellodextrin transport system would be particularly useful during SSF. For example, cellobiose/cellodextrin transport would lower the requirement for full hydrolysis of cellulose to glucose, decrease cellobiose-mediated inhibition of cellulolytic enzymes, and reduce the risk of contamination by glucose-dependent organisms. Indeed, yeasts expressing a cellobiose/cellodextrin transport system markedly improved the efficiency of SSF reactions by reducing the steady state concentration of both cellobiose and glucose, and increasing the ethanol production rate (Fig. 24B, C).

Biofuel production from cellulose requires efficient and economical depolymerization of plant biomass to sugars coordinated with fuel production by improved host strains (Kumar *et al.*, 2008). Here it was shown that cellulolytic fungi use cello-oligosaccharide transport pathways for optimal growth on plant biomass. Furthermore, reconstitution of these pathways in yeast revealed that they can be ported in a modular fashion to improve cellobiose catabolism, with a minimal pathway composed of a transporter and an intracellular cello-oligosaccharide hydrolase (Fig. 25). The use of cellodextrin transport in biofuel-producing strains of yeast and other organisms is critical for making cellulosic biofuel processes more economically viable.

Transporter and β -glucosidase orthologs

GenBank accession numbers or Joint Genome Institute (JGI) protein ID (PID) numbers for cellodextrin transporters are as follows: *Tuber melanosporum*, CAZ81962.1; *Pichia stipitis*, ABN65648.2; *Laccaria bicolor*, EDR07962; *Aspergillus oryzae*, BAE58341.1; *Phanerochaete chrysosporium*, PID 136620 (JGI) (Martinez *et al.*, 2004); *Postia placenta*, PID 115604 (JGI) (Martinez *et al.*, 2009). The GenBank accession number for *Saccharomyces cerevisiae* HXT1 and *Kluyveromyces lactis* LACP are DAA06789.1 and CAA30053.1, respectively. The *P. chrysosporium* and *P. placenta* genomes can be accessed at genome.jgi-psf.org/Phchr1/Phchr1.home.html and genome.jgi-psf.org/Pospl1/Pospl1.home.html, respectively.

GenBank accession numbers for cellodextrin hydrolases that are orthologs of NCU00130 are as follows: *T. melanosporum*, CAZ82985.1; *A. oryzae*, BAE57671.1; *P. placenta*, EED81359.1; and *P. chrysosporium*, BAE87009.1. The other organisms that contain cellodextrin transporter orthologs contain genes in the GH3 family predicted to be intracellular β -glucosidases (Bendtsen *et al.*, 2004; Cantarel *et al.*, 2009), as follows: *Kluyveromyces lactis*, CAG99696.1; *Laccaria bicolor*, EDR09330; *Clavispora lusitaniae*, EEQ37997.1; and *Pichia stipitis*, ABN67130.1.

Strains and Media

The yeast strain used in this study was YPH499 (Sikorski *et al.*, 1989), which has the genotype: *MATa ura3-52 lys2-801_amber ade2-101_ochre trp1- Δ 63 his3- Δ 200 leu2- Δ 1*. It was grown in YPD media supplemented to 100 mg/L adenine hemisulfate. Transformed strains (Becker *et al.*, 2001) were grown in the appropriate complete minimal dropout media, supplemented to 100 mg/L adenine

hemisulfate. *Neurospora crassa* stains used in this study were obtained from the Fungal Genetics Stock Center (McCluskey 2004) and include WT (FGSC 2489) and two cellobiose transporter deletion strains (FGSC 16575, Δ NCU00801.2 and FGSC 17868, Δ NCU08114.2 (Colot *et al.*, 2006)).

Plasmids and Cloning

Transporters were cloned into the 2 μ plasmid, pRS426, which was modified to include the *S. cerevisiae* PGK1 promoter inserted between *SacI* and *SpeI* using the primers, ATATATGAGCTCGTGAGTAAGGAAAGAGTGAGGAACTATC (SEQ ID NO: 53) and ATATATACTAGTTGTTTTATATTTGTTGTAAAAAGTAGATAATTACTTCC (SEQ ID NO: 54). (In all primers above and below, restriction sites are underlined). NCU00801 with a C-terminal Myc-tag and optimized Kozak sequence (Miyasaka 1999) was then inserted between *Bam*HI and *Eco*RI using the primers, ATGGATCCAAAAATGTCGTCTCACGGCTCC (SEQ ID NO: 55) and ATGAATTCCTACAAATCTTCTTCAGAAATCAATTTTTGTTTCAGCAACGATAGCTTCGGAC (SEQ ID NO: 56), and NCU08114 with a C-terminal Myc-tag and optimized Kozak sequence was inserted between *SpeI* and *ClaI* using the primers, ATACTAGTAAAAATGGGCATCTTCAACAAGAAGC (SEQ ID NO: 57) and GCATATCGATCTACAAATCTTCTTCAGAAATCAATTTTTGTTTCAGCAACAGACTTGCCCTCATG (SEQ ID NO: 58). To make GFP fusions, superfolder GFP (Pedelacq *et al.*, 2006) with an N-terminal linker of Gly-Ser-Gly-Ser was first inserted between the *ClaI* and *SalI* site of the PGK1 promoter-containing pRS426 plasmid with the primers, TATTAAATCGATGGTAGTGGTAGTGTGAGCAAGGGCGAGGAG (SEQ ID NO: 59) and TATTAAAGTCGACCTACTTGTACAGCTCGTCCATGCC (SEQ ID NO: 60). Transporters were then fused to GFP as follows: NCU00801 was inserted between *Bam*HI and *Eco*RI using the primers, GCATGGATCCATGTCGTCTCACGGCTCC (SEQ ID NO: 61) and TATAATGAATTCAGCAACGATAGCTTCGGAC (SEQ ID NO: 62), and NCU08114 was inserted between *SpeI* and *Eco*RI using the primers, TATTAACTAGTATGGGCATCTTCAACAAGAAGC (SEQ ID NO: 63) and TTATAAGAATTCAGCAACAGACTTGCCCTCATG (SEQ ID NO: 64).

The β -glucosidase, NCU00130, was cloned into the 2 μ plasmid, pRS425, modified to include the PGK1 promoter described above. NCU00130 with an optimized Kozak sequence and a C-terminal 6xHis tag was inserted between *SpeI* and *PstI* using the primers,

GCATACTAGTAAAAATGTCTCTTCCTAAGGATTTCCTCT (SEQ ID NO: 65) and ATACTGCAGTTAATGATGATGATGATGATGGTCCTTCTTGATCAAAGAGTCAAAG (SEQ ID NO: 66). All constructs included the Cyc transcriptional terminator between *Xho*I and *Kpn*I. All *N. crassa* genes were amplified by PCR from cDNA synthesized from mRNA isolated from *N. crassa* (FGSC 2489) cultured on minimal media with pure cellulose (Avicel) as the sole carbon source.

Yeast Growth Assays

To monitor growth on cello-oligosaccharides, engineered strains were grown in 5 mL of complete minimal media with appropriate dropouts overnight. These starter cultures were washed three times with 25 mL of ddH₂O, and resuspended to an OD (at 600 nm) of 0.1 in Yeast Nitrogen Base (YNB) plus the appropriate Complete Supplemental Media (CSM) and 1% (w/v) of cellobiose, or 0.5% (w/v) of either cellotriose or cellotetraose. Assays were performed in a Bioscreen C™ with constant shaking at maximum amplitude at 30 °C and a final assay volume of 0.4 mL. The change in OD was measured either at 600 nm or using a wideband filter from 450 – 580 nm. Growth rates were taken from the linear portion of each growth curve, and are reported as the mean of three independent experiments ± the standard deviation between these experiments. Cellotriose and cellotetraose were obtained from Seikagaku Biobusiness Corporation (Tokyo, Japan).

Purification of NCU00130 and Assay of its activity

A 1 L culture of *S. cerevisiae* expressing *cbt1* and NCU00130 was grown to an OD of 2.0 in complete minimal media. Cells were harvested by centrifugation and resuspended in 30 mL of lysis buffer (50 mM NaH₂PO₄ [pH 8.0], 300 mM NaCl, 10 mM imidazole, 2 mM β-ME, Complete™ Mini, EDTA free protease inhibitor cocktail). Cells were lysed by sonication, and the lysate was cleared by centrifugation at 15,000 g for 30 minutes. The lysate was bound to 1 mL of nickel-NTA resin by gravity flow, and washed three times with 25 mL wash buffer (identical to lysis buffer but with 20 mM imidazole). NCU00130 was eluted with 5 mL of elution buffer (identical to lysis buffer but with 250 mM imidazole), and the appropriate fractions were pooled, exchanged into storage buffer (Phosphate Buffered Saline (PBS), 2 mM DTT, 10% glycerol), aliquoted, frozen in liquid nitrogen, and stored at -80 °C. Purity was determined by SDS-PAGE (Fig. 22), and protein concentration was determined from the absorbance at 280 nm, using an extinction coefficient of 108,750 M⁻¹cm⁻¹.

Purified NCU00130 was assayed for hydrolysis activity with different cellodextrin substrates. Activity was measured by incubating 5 pmol of enzyme with 500 μ M of each sugar in 150 μ L PBS plus 3 mM DTT. Reactions proceeded for 40 minutes at 30 °C before 100 μ L was removed and quenched in 400 μ L of 0.1 M NaOH. The results were analyzed by ion chromatography with a Dionex ICS-3000, with CarboPac PA200 column. Peaks were detected with an electrochemical detector.

Phylogenetic Analysis of Transporter Orthologs

Amino acid sequences of orthologs of CBT1 and CBT2 were obtained from online databases. Multiple sequence alignments were performed using T-Coffee (Notredame *et al.*, 2000). A maximum likelihood phylogeny was determined using PhyML version 3.0 (Guindon and Gascuel 2003) with 100 Bootstraps. Both programs were accessed through Phylogeny.fr (webpage phylogeny.fr/). The resulting tree was visualized with FigTree v.1.2.1 (webpage tree.bio.ed.ac.uk/).

Fermentation and SSF

In fermentation and SSF experiments, comparisons were made between yeast expressing NCU00130 and either Myc-tagged *cbt1*, or no transporter. These strains were grown aerobically overnight in complete minimal media, washed three times with 25 mL water, and resuspended to a final OD of 2.0 in 50 mL YNB plus the appropriate CSM, and either 2% (w/v) cellobiose or 3% (w/v) pure cellulose (Avicel), in sealed serum flasks. The SSF reactions also included 50 Filter Paper Units/g cellulose of filter-sterilized Celluclast (Sigma C2730), without β -glucosidase supplementation. Reactions were carried out anaerobically at 30 °C with shaking. At indicated time points, 1 mL samples were removed and filtered through a 0.2 μ m syringe filter. The ethanol, glucose, and cellobiose concentration in the filtrate was determined by HPLC with an Aminex HPX-87H column and refractive index detection.

N. crassa Growth and Alamar Blue[®] Assays

WT *N. crassa* (FGSC 2489), and the homokaryotic NCU08114 (FGSC 17868) (Colot *et al.*, 2006) were acquired from the Fungal Genetics Research Center (McCluskey 2003), and grown at 25 °C in 50 mL of Vogel's salts plus 2% of either sucrose or pure cellulose (Avicel) in a 250 mL unbaffled flask. After 16 or 28 hours, respectively, 100 μ L of Alamar Blue[®] was added, and cultures were incubated at room temperature for 20 minutes. At this time, 1 mL samples were removed, debris pelleted,

and the fluorescence of 100 μ L of the supernatant determined with excitation/emission wavelengths of 535/595 nm in a Beckman Coulter Paradigm plate reader.

N. crassa Cellobiose Transport Assays

WT *N. crassa* (FGSC 2489), and homokaryotic deletion lines (Colot *et al.*, 2006) of NCU00801 (FGSC 16575) and NCU08114 (FGSC 17868) were acquired from the Fungal Genetics Stock Center (McCluskey 2003), and grown for 16 hours in 50 mL of Vogel's salts plus 2% (w/v) sucrose at 25 °C, starting with an inoculum of 10^6 conidia/mL. Mycelia were harvested by centrifugation, washed three times with Vogel's salts, and transferred to Vogel's salts plus 0.5% (w/v) pure cellulose (Avicel) for 4 hours to induce the transporter expression. Ten mL of the culture was harvested by centrifugation, washed three times with Vogel's salts, and resuspended in 1 mL ddH₂O plus cycloheximide (100 μ g/mL) and 90 μ M of the respective cellodextrin (cellobiose, cellotriose, or cellotetraose). To measure cellodextrin consumption, 100 μ L was removed after 15 minutes, clarified by centrifugation, and transferred into 900 μ L of 0.1 M NaOH. The amount of sugar remaining in the supernatant was determined by HPLC with a Dionex ICS-3000, using a CarboPac PA200 column. Peaks were detected with an electrochemical detector.

GFP Fluorescence and Confocal Fluorescence Microscopy

Bulk-cell GFP fluorescence measurements were made in a Beckman Coulter Paradigm plate reader with excitation/emission wavelengths of 485/535 nm. Confocal fluorescence microscopy was performed with cells at an OD (at 600 nm) of 0.8 – 1.2, using a 100x 1.4 NA oil immersion objective on a Leica SD6000 microscope attached to a Yokogawa CSU-X1 spinning disc head with a 488 nm laser and controlled by Metamorph software. Z series were recorded with a 200 nm step size and analyzed using ImageJ.

[³H] Cellobiose Transport Assays and Kinetic Parameters

Transport assays were performed using a modification of the oil-stop method (Arendt *et al.*, 2007). Yeast strains expressing either *cbt1* or *cbt2* fused to GFP were grown to an OD (at 600 nm) of 1.5 – 3.0 in selective media, washed three times with ice cold assay buffer (30 mM MES-NaOH [pH 5.6] and 50 mM ethanol), and resuspended to an OD of 20. To start transport reactions, 50 μ L of cells were

added to 50 μ L of [^{3} H] cellobiose layered over 100 μ L of silicone oil (Sigma 85419). Reactions were stopped by spinning cells through oil for 1 minute at 17,000 g, tubes were frozen in ethanol/dry ice, and tube-bottoms containing the cell-pellets were clipped off into 1 mL of 0.5 M NaOH. The pellets were solubilized overnight, 5 mL of Ultima Gold scintillation fluid added, and CPM determined in a Tri-Carb 2900TR scintillation counter. [^{3} H] cellobiose was purchased from Moravsek Biochemicals, Inc. and had a specific activity of 4 Ci/mmol and a purity of >99%. Kinetic parameters were determined by measuring the linear rate of [^{3} H] cellobiose uptake over 3 minutes for a range of cellobiose concentrations. V_{max} and K_m values were determined by fitting a single rectangular, 2-parameter hyperbolic function to a plot of rates vs. cellobiose concentration by non-linear regression in SigmaPlot[®]. V_{max} values were normalized for differences in transporter abundance by measuring the GFP fluorescence from 100 μ L of cells at OD 20 immediately before beginning transport assays. Kinetic parameters reported in the text are mean $\pm$ the standard deviation from three separate experiments. Competition assays were performed by measuring transport of 50 μ M [^{3} H]-cellobiose over 20 seconds in the percent of 250 μ M of the respective competitors.

Large Scale Yeast Growth

To monitor growth on different carbon sources, engineered strains were grown in 5 mL of complete minimal media with appropriate dropouts overnight. These starter cultures were washed three times with 25 mL of ddH₂O and resuspended to an OD (at 600 nm) of 0.1 in 50 mL Yeast Nitrogen Base (YNB) plus the appropriate Complete Supplemental Media (CSM) and 2% (w/v) cellobiose. Cultures were grown in 250 mL unbaffled flasks at 30 °C, with shaking at 200 rpm. The change in OD (at 600 nm) was monitored by periodically removing samples.

Example 10 – Identification of Critical Residues for Cellodextrin Transporter Function

In this example, sequence analysis and mutagenesis studies were used to identify conserved and functionally important residues in the cellodextrin transporters. In addition, additional cellodextrin transporters were identified.

The growth rates of yeast strains expressing various mutants of the cellodextrin transporter NCU00801 (*cbt1*) or NCU08114 (*cbt2*) and the wild-type β -glucosidase NCU00130 were grown with cellobiose as the sole carbon source. Amino acid residues at 96 positions of NCU00801 and at 96

positions of NCU08114 were individually mutated to alanine using QuickChange[®] II Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) as per the manufacturer's instructions. Strains were grown in synthetic defined media -ura -leu 100 mg/L adenine with 2% cellobiose. Cultures were started from two independent colonies.

As the results shown in Fig. 26 (a, b) indicate, mutant strains that expressed NCU00801 with substitutions at W66, L73, Y74, N87, Y89, D90, Q 104, F107, G113, F120, Y123, D139, G142, K144, M147, G150, Q169, F170, G173, R174, G178, G180, P189, Y191, E194, P198, R201, Y208, W235, R236, Q242, ²⁵⁷PESPRF²⁶² (SEQ ID NO: 67), Y279, G283, E296, D307, K308, W310, D312, R325, G336, Y345, N369, D385, F462, P468, E476, T480, or G486 showed at least a 25% growth defect compared to wild-type strain.

The alanine scanning experiment on NCU08114 indicated the following residues as being functionally important: L38, Y39, G54, D56, F73, G91, P100, D104, G107, R108, M118, R139, F144, Q150, P154, E159, P163, H165, R166, Y173, N174, W199, Q214, ²²²PESP²²⁵ (SEQ ID NO: 68), Y244, H245, D249, E258, E268, Q302, W303, S304, N306, Y312, F359, L360, F402, Y403, S404, Y414, E417, P420, Y421, K426, N442, N446, P447, W459, K460, E482, T483, L488, E489, E490, D496, and G497 (Fig. 26b).

In particular, the motifs ⁷³LYF⁷⁵, ²⁵⁷PESP²⁶⁰ (SEQ ID NO: 69), and ²⁷⁸KYH²⁸⁰ (residue numbering of NCU00801) appeared to be functionally important in both transporters (residues ²⁵⁷PESP²⁶⁰ (SEQ ID NO: 69) of NCU00801 and residues ²²²PESP²²⁵ (SEQ ID NO: 68) of NCU08114), which have an amino acid sequence identity of 29% (Fig. 26b, c). Several residues that are conserved in transporters in general (italicized in Fig. 26b, c), or in β -linked transporters in particular (double-underlined), were experimentally shown to be important for transporter function (underlined), e.g., D90 (NCU00801) and D56 (NCU08114), and L73 (NCU00801) and L38 (NCU08114). Results of the mutagenesis experiment also implicated residues conserved in the NCU00801/NCU08114 clade (capped) as being functionally important, e.g., Q168 (NCU00801) and Q214 (NCU08114). Moreover, multiple residues determined to be functionally important in this experiment were previously shown to be conserved in the *S. cerevisiae* sugar transporters (Hxt1/Hxt3), e.g., L73 (NCU00801) and L38 (NCU08114).

Orthologs of *N. crassa* cellodextrin transporters from different organisms were also studied (Fig. 27). Representative orthologs were synthesized by Genescript and cloned into the expression vector,

pRS426 containing the Cup1 promoter using the sites *Bam*HI and *Hind*III. These constructs were transformed into the yeast strain, YPH499 along with the intracellular β -glucosidase, NCU00130. Transporter activity was determined by measuring the growth rates of these strains when cellobiose was present as the sole carbon source.

Alternatively, different fungal strains containing putative orthologs were cultivated in rich media supplemented with cellobiose. Total RNA was isolated and reverse transcribed into cDNA. Polymerase chain reaction (PCR) was used to amplify the putative transporter genes directly from cDNA. However, because the regulation mechanism and expression pattern were unknown for cellodextrin transporters in fungal species, cDNAs encoding the putative transporters were not always obtainable despite alteration of cultivation condition. In this case, primers were designed according to the corresponding cDNA sequences from GenBank and used to amplify the exons using genomic DNA as a template. Overlap-extension PCR was then used to assemble the exons into the full-length genes. The resulting PCR products were cloned into the pRS424 shuttle vector containing a HXT7 promoter and a HXT7 terminator using the DNA assembler method. Yeast plasmids isolated from transformants were retransformed into *E. coli* DH5 α , and isolated *E. coli* plasmids were first checked by diagnostic PCR using the primers used to amplify the original transporter genes. The entire open reading frames were submitted for sequencing to confirm the correct construction of the plasmids. In the orthologs LAC2, LAC3, HXT2.1, and HXT2.6 from *P. stipitis*, one or more alternative codons (CUG) substitute Ser for Leu. Most of the cloning work was carried out using the yeast homologous recombination mediated DNA assembler method. pRS424-HXT7-GFP plasmid was used for cloning of putative cellodextrin transporters. In this plasmid, the HXT7 promoter, the GFP gene flanked with the EcoRI sites at both ends, and the HXT7 terminator were assembled into the pRS424 shuttle vector (New England Biolabs) linearized by ClaI and BamHI. PCR products of the putative transporters flanked with DNA fragments sharing sequence identity to the HXT7 promoter and terminator were co-transferred into CEN.PK2-1C with EcoRI digested pRS424-HXT7-GFP using the standard lithium acetate method. The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose to recover transformants. Yeast expressing putative cellodextrin transporter orthologs and NCU00130 were tested for growth on cellobiose as the sole carbon source.

A listing of the putative cellodextrin transporter orthologs and results obtained from the study are shown in Table 14.

Table 14: Listing of putative cellodextrin transporter orthologs and summary of results.

<i>N. crassa</i> ortholog	Species	NCBI Reference Sequence/ NCBI GI Number/JGI number ¥	Aver. Growth Rate	Growth Rate error	Seq results *
NCU00809	<i>Chaetomium globusom</i> CBS148.51	XP_001220480	-	-	OK
NCU00809	<i>Podospora anserina</i>	XP_001912722	-	-	-
NCU00809	<i>Nectria haematococca</i> mpVI77-13-4	EEU41662	-	-	-
NCU00809	<i>Aspergillus nidulans</i> FGSC A4	XP_660803	-	-	1 intron and 50 bp insertion
NCU00809	<i>Aspergillus terreus</i> NIH2624	XP_001218592	-	-	-
NCU00809	<i>Talaromyces stipitatus</i> ATCC 10500	XP_002341594	-	-	-
NCU00809	<i>Aspergillus niger</i>	XP_001395979	-	-	Ala>Val
NCU00809	<i>Aspergillus fumigatus</i> Af293	XP_747891	-	-	-
NCU00809	<i>Aspergillus terreus</i> NIH2624	XP_00120996	-	-	-
NCU00809	<i>Aspergillus oryzae</i> RIB40	XP_001817400	-	-	OK
NCU08114	<i>Podospora anserina</i>	XP_001908539	-	-	N/A
NCU08114	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	XP_002568019	-	-	N/A
NCU08114	<i>Aspergillus terreus</i> NIH2624	XP_001209810	-	-	Wrong
NCU08114	<i>Aspergillus oryzae</i> RIB40	XP_001820343	-	-	OK
NCU08114	<i>Aspergillus terreus</i> NIH2624	XP_001210859	-	-	N/A
NCU08114	<i>Neurospora crassa</i> OR74A	XP_001728155	-	-	N/A
NCU08114	<i>Aspergillus oryzae</i> RIB40	XP_001826848	-	-	N/A
NCU08114	<i>Aspergillus nidulans</i> FGSC A4	XP_657617	-	-	OK
NCU08114	<i>Talaromyces stipitatus</i> ATCC 10500	XP_002487579	-	-	N/A
NCU08114	<i>Chaetomium globosum</i> CBS 148.51	XP_001227497	-	-	Wrong
NCU08114	<i>Trichoderma atroviridae</i>	215408	0.000836 364	0.0006487 1	I, D
NCU08114	<i>Chaetomium globosum</i>	XP_001220290	0.004036	0.0004716	OK

		.1	364	8	
NCU08114	<i>Aspergillus nidulans</i>	ANID_08347	0.011109 091	0.0000727 27	Other
NCU08114	<i>Pleurotus ostreatus</i>	51322	0.003903 03	0.0001821 2	-
NCU08114	<i>Sporotrichum thermophile</i>	114107	0.009569 697	0.0021636 6	-
NCU00801	<i>Aspergillus nidulans</i>	XP_660418.1	0.000860 606	0.000438	P
NCU00801	<i>Magnaporthe grisea</i>	XP_364883.1	00509090 9	0.0013831 3	OK
NCU00801	<i>Aspergillus fumigatus</i>	XP_753099.1	0.003975 758	0.0021195 1	OK
NCU00801	<i>Trichoderma atroviridae</i>	211304	0.002678 788	0.0003119 3	D
NCU00801	<i>Chaetomium globosum</i>	XP_001220469 .1	0.005890 909	0.0001028 5	OK
NCU00801	<i>Tremella mesenterica</i>	63529	0.004381 818	0.0011575 1	D
NCU00801	<i>Heterobasidion. annosum</i>	105952	0.002751 515	0.0006876 3	D
NCU00801	<i>Cryphonectria parasitica</i>	252427	0.022503 03	0.0002169 2	D
NCU00801	<i>Trichoderma reesei</i>	67752	0.003672 727	0.0006623 3	D
NCU00801	<i>Aspergillus clavatus</i>	XP_001268541 .1	0.014381 818	0.0005961 3	OK
NCU00801	<i>Neurospora discreta</i>	77429	0.007060 606	0.0011056 6	D
NCU00801	<i>Trichoderma reesei</i>	3405	0.003264 646	0.0010339 98	D
NCU00801	<i>Sporotrichum thermophile</i>	43941	0.013654 545	0.0043153 4	-
NCU00801	<i>Neurospora crassa</i>	XP_963801.1	0.048754 872	0.0035401 7	-
NCU05853	<i>Chaetomium globosum</i>	XP_001226269 .1	0.003593 939	0.0006230 6	OK
NCU05853	<i>Trichoderma reesei</i>	46819	0.002042 424	0.0000859 24	D
NCU05853	<i>Mycosphaerella graminicola</i>	68287	0.002901 01	0.0006012 3	D
NCU05853	<i>Aspergillus flavus</i>	AFLA_000820 A	0.003078 788	0.0020913 2	-
-	None	-	0.0026	0.0001	-
NCU00809	<i>Pichia stipitis</i> CBS6054 (LAC1)	XP_001383110 .1/GI:12613317	See Fig. 27	-	-

		0			
NCU00809	<i>Pichia stipitis</i> CBS6054 (LAC2)	XP_001387231 .1/GI:12627633 7	See Fig. 27	-	-
NCU00809	<i>Pichia stipitis</i> CBS6054 (LAC3)	XP_001383677 .2/GI:15086472 7	See Fig. 27	-	-
NCU08114	<i>Pichia stipitis</i> CBS6054 (HXT2.1)	XP_001386873 .1/GI:12627557 1	See Fig. 27	-	-
NCU05853	<i>Pichia stipitis</i> CBS6054 (HXT2.3)	XP_001382754 .1/GI:12613245 8	See Fig. 27	-	-
NCU08114	<i>Pichia stipitis</i> CBS6054 (HXT2.4)	XP_001387757 .1/GI:12627393 9	See Fig. 27	-	-
NCU08114	<i>Pichia stipitis</i> CBS6054 (HXT2.5)	XP_001385684 .1/GI:12613832 2	See Fig. 27	-	-
NCU08114	<i>Pichia stipitis</i> CBS6054 (HXT2.6)	XP_001384653 .2/GI:15086543	See Fig. 27	-	-

* Wrong = difference between tested sequence and sequence in NCBI or JGI databases; I = insertion in tested sequence; D = deletion in tested sequence; P = point mutation in tested sequence; OK = no difference between tested sequence and sequence deposited in NCBI or JGI databases; Other = other problems in sequencing, excluding insertion, deletion, and point mutations in tested sequence; “-” = results not yet available (study in progress).

¥ When accession numbers were not available, the JGI number was used. The JGI number allows access to the gene sequence via the JGI genome portal for this organism (accessible from the following page: genome.jgi-psf.org/programs/fungi/index.jsf). The *A. flavus* and *A. nidulans* identifiers allow access to the genes through their genome portals at webpage cadre-genomes.org.uk/ and webpage broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html, respectively.

In certain cases, the sequences of the cloned orthologs were determined to be correct, and the yeast expressing those clones were able to utilize cellobiose. Thus, these clones, LAC2 from *Pichia stipitis* and XP_001268541.1 from *Aspergillus clavatus* were confirmed to be functional cellobiose transporters. Testing of the cellobiose transporting function of other clones is still in progress. Cloned orthologs with sequences different from the published sequences in databases (e.g., ones with insertions, deletions, etc.) (Table 14) will be re-cloned, re-sequences, and similarly tested for cellobiose transport activity by expressing them in *S. cerevisiae* and monitoring growth rates.

An alignment of NCU00801, NUC08114, and functional orthologs of these transporters is shown in Fig. 28. The alignment in Fig. 28a includes both putative and confirmed cellodextrin transporters, whereas the alignment in Fig. 28b includes only confirmed cellodextrin transporters. In addition, Fig. 28c shows an alignment of NCU00801 and NCU08114. The two transporters share 29% amino acid sequence identity.

Motifs critical for cellodextrin transporter function were identified by visual inspection of multiple sequence alignments between sugar transporters. Specifically, motifs common to cellodextrin transporters were identified from multiple sequence alignments produced in T-COFFEE between putative cellodextrin transporter orthologs and confirmed cellodextrin transporters. To ensure that these motifs were largely unique to cellodextrin transporters, their absence was confirmed from a multiple sequence alignment between the hexose transporters of *S. cerevisiae*, the human glucose transporter, Glut1, and two *N. crassa* monosaccharide transporters produced in T-COFFEE.

The identified motifs are described below. In the motifs, residues that were found to be critical to the function of NCU00801 are underlined. The residues that were critical for the function of NCU08114 are marked with the superscript “†”. The residues that were critical to the function of both transporters are marked with the superscript “*”. All motifs were defined using PROSITE notation. As an example of how to read a PROSITE motif, the following motif, [AC]-x-V-x(4)-{ED}, is translated as: [Ala or Cys]-any-Val-any-any-any-any-{any but Glu or Asp} (SEQ ID NO: 13)

Cellodextrin transporters, like all sugar transporters, have 12 transmembrane α -helices. The N- and C-terminus of cellodextrin transporters are both intracellular.

The sequence before transmembrane helix 1 had no distinguishing features.

Transmembrane helix 1 contained the motif, [L^{*}IVM]-Y^{*}-[FL]-x(13)-[YF]-D^{*} (SEQ ID NO: 1).

Transmembrane helix 2 contained the motif, [YF]-x(2)-G[†]-x(5)-[PVF]-x(6)-[DQ]^{*} (SEQ ID NO: 2).

The loop connecting transmembrane helix 2 and transmembrane helix 3 contained the motif, G^{*}-R[†]-[RK]^{*} (SEQ ID NO: 3).

Transmembrane helix 3 had no distinguishing features.

Transmembrane helix 4 had no distinguishing features.

Transmembrane helix 5 contained the motif, $R^*-x(6)-[YF]^*-N^\dagger$ (SEQ ID NO: 4).

Transmembrane helix 6 contained the motif, $W^*\underline{R}-[IVLA]-P-x(3)-\underline{Q}$ (SEQ ID NO: 5).

The sequence between transmembrane helix 6 and transmembrane helix 7 contained the motif, $P^*-E^*-S^*-P^*\underline{R}-x-L-x(8)-A-x(3)-L-x(2)-Y^*-H^\dagger$ (SEQ ID NO: 6).

Transmembrane helix 7 contained the motif, $F^\dagger-[GST]-Q^*-x-S^\dagger-G-N^\dagger-x-[LIV]$ (SEQ ID NO: 7).

Transmembrane helix 8 had no distinguishing features.

Transmembrane helix 9 had no distinguishing features.

Transmembrane helix 10 and transmembrane helix 11 and the sequence between them contained the motif, $L-x(3)-[YIV]^\dagger-x(2)-E^*-x-L-x(4)-R-[GA]-K^\dagger-\underline{G}$ (SEQ ID NO: 8).

Transmembrane helix 12 had no distinguishing features.

The sequence after transmembrane helix 12 had no distinguishing features.

Homology models of NCU00801 and NCU08114 were produced from the primary amino acid sequences of NCU00801 and NCU08114 using the I-TASSER server at: zhanglab.ccmb.med.umich.edu/I-TASSER/ (Roy *et al.*, 2010). The top structural models produced by I-TASSER were visualized in PYMOL ([webpage pymol.org/](http://webpage.pymol.org/)). Mapping of the motifs was also performed in PYMOL. The homology models of NCU00801 and NCU08114 with the cellodextrin transporter motifs marked are shown in Fig. 29 (a, b). Fig. 29 (c) shows the predicted secondary structures of NCU00801 and NCU08114.

Example 11 – Characterization of Novel Pentose-specific Transporters from Neurospora crassa and Pichia stipitis in Saccharomyces cerevisiae

In this example, a bioinformatics approach was taken to identify novel pentose-specific transporters in *N. crassa* and *P. stipitis*.

Genome Mining of Pentose-Specific Transporters

Bioinformatics study

To discover novel D-xylose-specific transporters, the genes encoding the D-glucose/D-xylose symporter Gxsl from *C. intermedia* (Leandro *et al.*, 2006) and the uncharacterized putative L-arabinose-proton symporter Aut1 from *P. stipitis* (locus tag PICST_87108) were used as probes in BLAST searches (webpage ncbi.nlm.nih.gov/) against the sequenced genomes of two efficient xylose-utilizing species, *N. crassa* and *P. stipitis* (Galagan *et al.*, 2003; Jeffries *et al.*, 2007). Any proteins with known D-glucose transport activity or activity other than sugar transport were eliminated from the analyses. Using a cut-off of 25% minimal sequence identity, 17 putative pentose transporter genes were identified (Table 15), in addition to *AUT1* from *P. stipitis*. These putative pentose transporter genes shared 25-50% identity with either *GXS1* from *C. intermedia* or *AUT1* from *P. stipitis*. All 17 putative pentose transporters were annotated as either sugar-transport proteins or hypothetical proteins with unknown activity. The D-glucose transporter genes *SUT1* and *SUT2* from *P. stipitis* were also cloned for comparison.

Table 15 shows the putative pentose transporters obtained from BLAST using (a) *AUT1* from *P. stipitis* as a probe and (b) *GSX1* from *C. intermedia* as a probe.

a. BLAST search results using *AUT1* as a probe.

Name	Origin	% identity with <i>AUT1</i>	Annotation from NCBI	Length (cDNA)	Locus Tag
Ap31/SUT2	<i>P. stipitis</i>	31	sugar uptake (tentative)	1653	ABN66266
Ap26/XP_001387242	<i>P. stipitis</i>	26	sugar transporter	1404	XP_001387242
AN49/NCU01494	<i>N. crassa</i>	49	hypothetical protein NCU01494, similar to MFS sugar transporter	2025	EAA2669I
AN41/NCU09287	<i>N. crassa</i>	41	hypothetical protein NCU09287, similar to galactose-proton symporter	1968	EAA28903
AN29-2/NCU04963	<i>N. crassa</i>	29	hypothetical protein NCU04963, similar to MFS monosaccharide transporter	1584	EAA30175
AN28-3/NCU02188	<i>N. crassa</i>	28	hypothetical protein NCU02188, conserved hypothetical protein	1458	EAA30346

AN25/NCU 00821	<i>N. crassa</i>	25	sugar transporter	1689	EAA35128

b. BLAST search results using *GSX1* as a probe.

Name	Origin	% identity with <i>GSX1</i>	Annotation from NCBI	Length (cDNA)	Locus Tag
Xy50/NCU 04537	<i>N. crassa</i>	50	hypothetical protein NCU04537 similar to monosaccharide transporter	1626	EAA26741
Xy31/NCU 06138	<i>N. crassa</i>	31	hypothetical protein NCU06138, similar to MFS monosaccharide transporter	1757	EAA30764
Xy33/NCU 00988	<i>N. crassa</i>	33	hypothetical protein NCU00988, similar to MFS quinate transporter	1614	EAA34662
Xyp37/SUT 3	<i>P. stipitis</i>	37	sugar uptake (tentative)	1653	ABN6799 0
Xyp33/XUT 3	<i>P. stipitis</i>	33	sugar transporter, putative xylose uptake (tentative); predicted transporter (major facilitator superfamily)	1656	EAZ63115
Xyp32/XUT 1	<i>P. stipitis</i>	32	sugar transporter, high affinity, putative; xylose uptake (tentative)	1701	ABN6755 4
Xyp30/STL1	<i>P. stipitis</i>	30	sugar transporter, strongly conserved	1590	ABN6574 5
Xyp31/XUT 2	<i>P. stipitis</i>	31	sugar transporter, xylose transporter (tentative) similarly to GXSI (STL1)	1407	AAVQOI OOOO02
Xyp29/STL1 2/XUT6	<i>P. stipitis</i>	29	sugar transporter, putative (STL12); .xylose uptake (tentative)	1641	ABN6856 0
Xyp30- 1/HGT3	<i>P. stipitis</i>	30	high affinity xylose transporter (putative), xylose uptake (tentative)	1587	ABN6868 6
Xyp28/XUT 7	<i>P. stipitis</i>	28	xylose transporter, high affinity, putative similarity to STL13, high affinity sugar transporters	1257	EAZ63044

Cloning of putative pentose transporters

N. crassa and *P. stipitis* were cultivated in rich media supplemented with either D-xylose or L-arabinose as carbon sources. Total RNA was isolated and reverse transcribed into cDNA. Polymerase chain reaction (PCR) was used to amplify the putative transporter genes directly from cDNA. However, because the regulatory mechanism and expression patterns of pentose transporters in fungal species were unknown, cDNAs encoding the putative pentose transporters were not always obtainable despite alteration of cultivation conditions. In those cases, primers were designed according to the corresponding cDNA sequences from GenBank and used to amplify the exons with genomic DNA as templates. Overlap-extension PCR was then used to assemble the exons into full length genes. The resulting PCR products were cloned into the pRS424-HXT7-GFP shuttle vector using the yeast homologous recombination-mediated DNA assembler method (Shao *et al.*, 2009). In this plasmid, an HXT7 promoter, a GFP gene flanked with the *EcoRI* sites at both ends, and an HXT7 terminator were assembled into the pRS424 shuttle vector (New England Biolabs) linearized by *ClaI* and *BamHI*. PCR products of the putative pentose transporters flanked with DNA fragments, sharing sequence identity with the HXT7 promoter and terminator (Fig. 30a) were co-transferred into *S. cerevisiae* CEN.PK2-1C strain (MAT α *leu2-3,112 ura3-52, trp1-289, his3- Δ 1 MAL2-8c*) purchased from Euroscarf (Frankfurt, Germany) with *EcoRI* digested pRS424-HXT7-GFP using the standard lithium acetate method. The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose.

Yeast plasmids isolated from transformants using Zymoprep Yeast Plasmid Miniprep II (Zymo Research, Orange, CA) were re-transferred into *Escherichia coli* DH5 α cells (Cell Media Facility, University of Illinois at Urbana-Champaign, Urbana, IL). The plasmids were isolated using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and then checked by diagnostic PCR with the primers used to amplify the original transporter genes. The entire open reading frames were also submitted for DNA sequencing to confirm correct construction (Core Sequencing Facility, University of Illinois at Urbana-Champaign, Urbana, IL). The DNA sequencing results were compared to gene sequences in databases using Sequencher 4.7 (Gene Codes Corporation, Ann Arbor, MI). All sequences of cloned putative transporters are listed in SEQ ID NOs: 33-52.

Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid

drop out mix). YPA media supplemented with 2% of sugar was used to grow yeast strains harboring no plasmids (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate). *S. cerevisiae* strains were cultured at 30 °C and 250 rpm for aerobic growth and at 30 °C and 100 rpm for oxygen-limited conditions. Yeast strains were grown under aerobic conditions for cell manipulation unless specified otherwise. *E. coli* strains were cultured at 37 °C and 250 rpm in Luria broth (LB) (Fisher Scientific, Pittsburg, PA). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific.

Transporter Activity Assay for Cloned Putative Transporters

Intracellular accumulation of pentose sugars

The cloned putative pentose transporters were over-expressed in an *S. cerevisiae* sugar transporter deletion strain, and uptake of pentose sugars was measured. The D-xylose-uptake ability of putative pentose transporters was determined by summation of intracellular D-xylose and xylitol concentrations. D-xylose accumulated within *S. cerevisiae* cells can be partially converted to xylitol due to the presence of endogenous aldose reductase. Both D-xylose and xylitol were extracted using osmosis and analyzed using high performance liquid chromatography (HPLC).

The sugar transporter knock-out *S. cerevisiae* strain EBY.VW4000 (CEN.PK2-1c $\Delta hxt1-17$, $\Delta stl1$, $\Delta agt1$, $\Delta ydl247w$, $\Delta yjr160c$, $\Delta gal2$), which was a gift from Professor E. Boles' laboratory (Institut für Mikrobiologie, Heinrich-Heine-Universität, Universitätsstr. 1, Geb. 26.12.01, D-40225 Düsseldorf, Germany), had concurrent knock-outs of more than 20 sugar transporters and sensors including *HXT1-17* and *GAL2*. Growth on D-glucose as the sole carbon source was completely abolished in this strain, whereas uptake of maltose through a different sugar transport system was retained. The EBY.VW4000 strain also exhibited minimal pentose-uptake under HPLC assay conditions, which made it a suitable host for testing recombinant D-xylose uptake. Plasmids over-expressing the cloned putative pentose transporter genes were transferred into the EBY.VW4000 strain using the standard lithium acetate method, and single colonies were used for measuring sugar uptake activity.

Cells were first cultured in 2 mL SC-Trp medium supplemented with 2% maltose. Seed culture was then used to inoculate a 50 mL culture in a 250 mL flask. The cells were harvested by centrifugation after 24 hours of growth and re-suspended in YPA medium supplemented with 2% D-xylose or L-

arabinose to a final OD₆₀₀ of 10. At 30 min, 60 min, 120 min, and 24 hours, 5 mL cultures were taken for measuring intracellular sugar concentrations. Culture samples were washed twice with ice-cold water and re-suspended in 3 mL of deionized water. Cell suspensions were incubated at 37 °C with 250 rpm agitation for 2 days to extract intracellular sugars. The resulting cell suspension was filtered through a 0.22 µm PES filter (Corning, Lowell, MA) before HPLC analysis. The concentrations of sugar and corresponding sugar alcohol (discussed below) were determined using Shimadzu HPLC equipped with a BioRad HPX-87C column (BioRad Laboratories, Hercules, CA) and Shimadzu ELSD-LTII low temperature-evaporative light scattering detector (Shimadzu) following the manufacturer's protocol. The sugar-uptake activity was calculated as mg of sugar extracted through osmosis per mL of cell culture at OD ~ 10.

Several putative pentose transporters were identified to be active in uptake of D-glucose or D-xylose or both. Since D-glucose can be metabolized once inside yeast, the D-glucose transport activity could not be determined by measuring intracellular D-glucose concentration. However, because the EBY.VW4000 strain normally cannot grow on media containing D-glucose as the sole carbon source, growth of the strain transformed with a putative pentose transporter on D-glucose indicated that the putative transporter has D-glucose transport activity.

Introduction of SUT3 (Xyp37), XUT3 (Xyp33), SUT2 (Ap31), NCU04963 (An29-2), and NCU06138 (Xy31) restored growth of the EBY.VW4000 strain on D-glucose and, thus, enabled glucose transport activity. SUT3, XUT3, SUT2, and NCU04963 also had xylose transport activity, whereas NCU04963 and NCU06138 showed arabinose transport activity (Fig. 31). The rest of the putative transporters failed to enable growth on D-glucose, and most of them also did not show any pentose transport activity. However, NCU00821 and STL12/XUT6 showed xylose transport activity, and XUT1 exhibited arabinose transport activity, indicating they may be sugar transporters specific for pentoses (Fig. 32).

To further confirm that STL12/XUT6 and XUT1 from *P. stipitis* and NCU00821 from *N. crassa* were actually pentose-specific transporters with no D-glucose-uptake activity, the sugar-uptake assay was performed using ¹⁴C-labeled D-glucose, D-xylose, and L-arabinose as substrates. It was found that D-glucose- and L-arabinose-uptake activities of the EBY.VW4000 strain over-expressing only STL12/XUT6

and NCU00821 were too low to be measured under assay conditions used to determine D-xylose-uptake kinetics of both transporters.

^{14}C -labeled D-glucose, L-arabinose, and D-xylose were purchased from American Radiolabeled Chemicals (St. Louis, MO) as solutions in 90% ethanol. Radiolabeled sugars were first dried in a chemical hood and then re-suspended in water. Sugar solutions at concentrations of 1.33 M and 1 M with specific radioactivity of approximately 40,000 dpm/ μL , and at concentrations of 500 mM, 350 mM, 250 mM, 100 mM, and 50 mM with specific radioactivity of about 20,000 dpm/ μL were used for the sugar-uptake assay. Cell culture at the exponential phase was harvested and washed twice with ice-cold water and re-suspended to about 60 mg dry cell weight (DCW) per mL in 100 mM Tris-citrate buffer at pH 5. Three aliquots of 160 μL cell suspension were dried at 65 °C for 24 hours to determine the DCW. The rest of the cell suspension was kept on ice before use. For the sugar-uptake assay, cell suspension was equilibrated at 30°C for 5 min before the assay. In a 50 mL conical tube, 160 μL of cell suspension was mixed with 40 μL of radio-labeled sugar solution for 40 or 60 seconds (accurately timed). The reaction was stopped by adding 10 mL of ice-cold water delivered by a syringe. The zero-time-point sample was obtained by adding ice-cold water and cell suspension simultaneously in a culture tube containing the radio-labeled solution. The mixture was then filtered immediately through a Whatman GF/C filter (Whatman, Florham Park, NJ) pre-soaked in 40% sugar solution and washed with 15 mL of ice-cold water. The filter was placed in 3 mL of Econo I scintillation cocktail (Fisher Scientific) and counted using a Beckman LS6500 scintillation counter (Beckman Coulter, Brea, CA) for 1 min. All data points were measured in three independent experiments. The sugar-uptake rate was calculated as mmol sugar transported per hour per gram of dry cell weight.

Intracellular accumulation of both D-xylose and L-arabinose in EBY.VW4000 strains over-expressing STL12/XUT6, NCU00821, or XUT1 was also measured using HPLC. Cell cultures incubated with pentose sugars for 30 min, 60 min, 120 min, and 24 hours were analyzed by HPLC. The EBY.VW4000 strains over-expressing STL12/XUT6 or NCU00821 exhibited D-xylose uptake activity, whereas the strain over-expressing XUT1 exhibited L-arabinose-uptake activity after a 24-hour incubation (Fig. 33).

The ^{14}C -labeled sugar uptake assay together with HPLC analysis of intracellular sugar accumulations confirmed that among the three most abundant monosaccharides in lignocellulosic

hydrolysates, D-glucose, D-xylose, and L-arabinose, STL12/XUT6 and NCU00821 were responsible for D-xylose uptake and XUT1 was responsible for L-arabinose uptake. Of note, most sugar transporters studied in yeast for D-xylose uptake have higher uptake activity towards D-glucose than towards D-xylose. Only Trxlt1 from *Trichoderma reesei* after adaptive evolution exhibited D-xylose-specific uptake activity (Saloheimo *et al.*, 2007). This data indicated that STL12/XUT6 from *P. stipitis*, NCU00821 from *N. crassa* are the first two experimentally confirmed naturally-occurring D-xylose-specific transporters introduced into *S. cerevisiae*. Similarly, XUT1 from *P. stipitis* is the first experimentally confirmed naturally-occurring L-arabinose-specific transporter introduced into *S. cerevisiae*.

Kinetic parameters

Using the ^{14}C -labeled sugar-uptake assay, kinetic parameters of D-xylose transport through NCU00921, STL12/XUT6, and XUT1 were determined. It was observed that under the assay conditions, sugar uptake was within a linear range for the first 60 seconds (Fig. 34). The EBY.VW4000 strains over-expressing NCU00821, STL12/XUT6, or XUT1 were incubated with labeled D-xylose or L-arabinose for 40 or 60 seconds followed by addition of ice-cold water to stop further sugar uptake. The reaction mixture was then filtered and washed before measurement using a liquid scintillation counter. The sugar-uptake rates and substrate concentrations were fitted into a Michaelis-Menten equation by non-linear regression using the Origin software (OriginLab Corporation, Northampton, MA). The K_m values for D-xylose uptake by the EBY.VW4000 strain harboring only NCU00821 or STL12/XUT6 were 175.7 ± 21.4 mM and 56.0 ± 9.4 mM, respectively. The corresponding V_{max} values were 36.7 ± 2.9 and 41.5 ± 2.3 $\mu\text{mol/h/gram DCW}$, respectively. Similarly, the K_m and V_{max} values for L-arabinose uptake by the EBY.VW4000 strain harboring XUT1 were 48.0 ± 13.2 mM and 5.6 ± 1.6 $\mu\text{mol/h/gram DCW}$ respectively.

In naturally-occurring D-xylose-assimilating fungal species, both the high affinity D-xylose-proton symport system and the low affinity D-xylose facilitated diffusion system are present. The K_m values of these two systems were determined to be 0.4-4 mM for the symport system and around 140 mM for the facilitated diffusion system (Leandro *et al.*, 2006; Stambuk *et al.*, 2003). These values are close to the affinity of the D-glucose-uptake system in *S. cerevisiae*, which has a K_m of 1.5 mM for the high affinity system and 20 mM for the low affinity system (Lang and Cirillo 1987; Ramos *et al.*, 1988). Unfortunately, the D-xylose uptake affinity of wild-type *S. cerevisiae* is two orders of magnitude lower

than its affinity for D-glucose. The K_m values for D-xylose uptake in *S. cerevisiae* are only 190 mM for the high affinity system and 1.5 M for the low affinity system (Kötter and Ciriacy, 1993). The affinities of the newly discovered D-xylose-specific transporters were lower when compared to the high affinity D-xylose-uptake system in naturally occurring D-xylose-assimilating yeasts. However, compared to the D-xylose-uptake system in wild-type *S. cerevisiae*, NCU00821 and STL12/XUT6 showed higher affinity towards D-xylose. In particular, the K_m of D-xylose uptake by STL12/XUT6 and XUT1 were only one-fourth of the K_m of xylose uptake by the transporter in wild-type *S. cerevisiae*. The K_m values of the D-xylose-specific transporters were also close to those of Gxfl (K_m 88 mM) and Sut1 (K_m 145 mM), which have been shown to improve D-xylose fermentation in recombinant *S. cerevisiae* (Runquist *et al.*, 2009; Katahira *et al.*, 2008). Thus, D-xylose fermentation may be improved by introducing these newly discovered D-xylose-specific transporters into *S. cerevisiae*.

Cellular Localization of Sugar Transporters

Sugar transporters are transmembrane proteins, and correct folding and localization in the cell membrane is required for them to be functional. Since no signal peptide was specifically added when the putative pentose transporters were cloned, it was important to ensure that the D-xylose-specific transporters were correctly localized to the cell membrane. This was particularly true for putative pentose transporters like NCU00821 cloned from the filamentous fungi *N. crassa*, which exhibits a very different physiology compared to *S. cerevisiae*. To study the cellular localization of D-xylose-specific transporters in *S. cerevisiae*, NCU00821, STL12/XUT6, and XUT1 were fused with Green Fluorescent Protein (GFP) at the C-termini via linkers, and their localization was monitored by fluorescent imaging.

The fusion proteins of the pentose-specific transporters with the GFP at the C-terminus were constructed for the transporter localization study. A GS-linker (Gly-Gly-Gly-Gly-Ser-Gly-Gly-Gly-Gly-Ser (SEQ ID NO: 70)) was introduced between the transporter and the GFP. The GS-linker was added to the N-terminus of the GFP open reading frame by a PCR primer, resulting in a PCR product of GS-linker-GFP flanked with nucleotide sequence homologous to the transporters at the 5'-end and the HXT7 terminator at the 3'-end. Transporter genes were amplified from the original pRS424-HXT7-transporter constructs to generate DNA fragments of the transporters flanked with nucleotide sequence identical to the HXT7 promoter at the 5'-end and GS-linker-GFP at the 3'-end. These two fragments were then co-transferred into the *S. cerevisiae* strain CEN.PK2-1C with pRS424-HXT7-GFP digested with *EcoRI* (Fig.

30b). The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose.

Single colonies were inoculated into 2 mL of SC-Trp liquid medium supplemented with 2% maltose. Cell culture was harvested at the exponential phase. In a centrifuge tube, 250 μ L of cell culture was stained with 10 μ L Hoechst 33342 nuclei dye (Invitrogen, Carlsbad, CA) for 10 minutes at room temperature. A small droplet of cell culture was then transferred onto a piece of cover glass and fluorescent images were taken using an Andor Technology Revolution System Spinning Disk Confocal Microscope (Core facilities, Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana, IL). Images were processed using Imaris image analysis and visualization software (Bitplane, Saint Paul, MN).

Yeast strains over-expressing pentose-specific transporters showed a distinctive fluorescent halo at the cell periphery (Fig. 35). For NCU00821 and XUT1, almost all the GFP fluorescence appeared in the cell membrane, while a large portion of fluorescence in STL12/XUT6-over-expressing cells remained in the cytoplasm. This could indicate inefficient export of the STL12/XUT6 transporter due to elevated expression of the membrane protein. It was also noticed that not all the cells showed fluorescence, indicating that expression of the transporter was not optimal. Further improvements of transporter expression can be achieved through altering the expression level and/or integrating the transporter genes into the genome of recombinant *S. cerevisiae*.

Determination of the Type of Pentose Transporters

There are two types of sugar transporters in *S. cerevisiae*, symporters and facilitators. For symporters, sugar uptake is coupled to proton uptake. Sugar symporters usually exhibit high affinity towards sugar. Meanwhile, sugar uptake through facilitators is not coupled to proton transport, and facilitators usually exhibit low sugar-uptake affinities (Leandro *et al.*, 2006). Symporter assays were performed for NCU00821, STL12/XUT6, and XUT1 expressed in the EBY.VW4000 strain.

To determine the type of transporters, pH change of the EBY.VW4000 over-expressing pentose-specific transporters was measured in un-buffered cell suspension containing D-xylose, L-arabinose, or maltose using a Seven Multi pH meter equipped with an USB communication module and Direct pH software (Mettler Toledo, Columbus, OH). Plasmids encoding pentose-specific transporters were

transferred into EBY.VW4000 strain followed by plating on the SC-Trp plates supplemented with 2% maltose. Single colonies were inoculated in 2 mL SC-Trp medium supplemented with 2% maltose. Seed culture was then used to inoculate a 400 mL culture in 2 L flasks. The culture was harvested at OD ~ 1 and washed twice with ice-cold water. Cell pellets were re-suspended in 4 mL of water and kept on ice before use. For the symporter assay, the pH electrode was immersed in a water-jacketed beaker of 50 mL capacity kept at 25 °C and provided with magnetic stirring. To the beaker, 23 mL of deionized water and 1 mL of cell suspension equilibrated at 25 °C was added. The pH was adjusted to 5, and a base line was obtained. The pH change was recorded with addition of 1 mL of 50% sugar solution at pH 5.

Fig. 36 shows pH changes in un-buffered cell suspension after the addition of maltose. As was reported, pH in un-buffered *S. cerevisiae* cell suspension went up with the addition of maltose. One mL of 50% maltose solution was added to the un-buffered cell suspension to ensure that the pH recording system was functional. The pH elevations observed in all samples indicated that the pH recording system could monitor transient pH changes in the experimental setting.

No elevation of pH in un-buffered cell suspensions was observed for any of the pentose-specific transporters, indicating that pentose uptake through these transporters is not coupled with proton transport (Fig. 37). Thus, NCU00821, STL12/XUT6, and XUT1 were determined to be pentose facilitators.

This result was consistent with the fact that the kinetic parameters of NCU00821 and STL12/XUT6 were similar to those of the low affinity D-xylose facilitated diffusion system in naturally-occurring D-xylose-assimilating yeasts. Despite the fact that symporters have higher affinities towards D-xylose, over-expression of symporters may not always facilitate sugar utilization by D-xylose-assimilating strains due to the ATP requirement to create the proton gradient. In fact, most of the transporters shown to be beneficial for D-xylose fermentation are facilitators (Runquist *et al.*, 2009; Katahira *et al.*, 2008).

Heterologous Over-Expression of D-Xylose-Specific Transporters

The over-expression of active heterologous D-xylose-specific transporters in *S. cerevisiae* strains containing the D-xylose utilization pathway was also investigated to determine whether their over-expression could improve xylose utilization. Xylose utilization was studied using a shake-flask under aerobic conditions. Plasmids expressing the xylose transporters NCU00821, NCU04963, XUT1, STL12/XUT6, and Hxt7 were introduced into strain HZE63 (CEN.PK2 *ura3::xylose utilization pathway*).

This strain had a xylose utilization pathway integrated into the URA3 site onto the chromosome. It was constructed using a plasmid from previous work that contained xylulose reductase (XR) and xylitol dehydrogenase (XDH) from *N. crassa* and xylulokinase (XKS) from *P. stipitis*. This plasmid was digested with *ApaI* and transformed into yeast strain CEN.PK2 to yield the strain HZE63.

The HZE63 strain transformed with the xylose transporter-encoding plasmids was selected by plating on SC-Ura plates supplemented with 2% glucose. The transformed strain was pre-cultured in SC-Trp-Ura with 2% glucose and then inoculated into SC-Trp-Ura supplemented with 0.5% or 5% of xylose to an initial OD₆₀₀ = 1.0. Cell cultures were grown in a 125 mL shake-flask containing 50 mL of culture at 30 °C and 250 rpm (Fig. 38).

Yeast plasmids of transformants were transformed into *E. coli* DH5α cells. The plasmids were then isolated and checked by diagnostic PCR and submitted for sequencing to confirm correct construction. Plasmid maps can be found in Fig. 39.

Unfortunately, the advantage of pentose-specific transporter over-expression could not be observed despite alteration of expression strategies, cultivation conditions, and choice of the D-xylose utilization pathway. There are several possible reasons. Firstly, the over-expression of membrane proteins, such as sugar transporters, could affect the integrity of the cell membrane and consequently hamper cell growth (Wagner *et al.*, 2006). It was observed that transporter over-expression strains displayed a slower growth rate even when D-glucose was used as a carbon source. The final OD of 2-day cultures of strains carrying transporters grown in glucose-containing SC-ura media was only 4, whereas the OD of the negative control was around 6. Secondly, the D-xylose-uptake activity of the wild-type *S. cerevisiae* through hexose transporters is much higher than the D-xylose-uptake activity of a certain D-xylose transporter over-expressed in a hexose transporter knockout strain. The low sugar transport activity of newly discovered D-xylose-specific transporters may make it hard to observe the improvement of sugar uptake ability. Thirdly, even if the introduction of new D-xylose-specific transporters could improve the uptake of D-xylose into *S. cerevisiae* cells, the benefit of D-xylose utilization can only be observed when the D-xylose utilization pathway is efficient enough to make sugar-uptake the limiting step. It was shown that the effect of over-expression of sugar transporters depends on the strain background and cultivation conditions (Runquist *et al.*, 2010). Examples 12-15 below describe the optimization of the xylose utilization pathway in yeast.

Cloning of Additional Pentose-Specific Transporters

Orthologs of NCU00821, STL12/XUT6, and XUT1 were cloned and tested for pentose uptake. Different fungal strains were cultivated in rich media supplemented with glucose or pentoses. Total RNA was isolated and reverse transcribed into cDNA. Polymerase chain reaction (PCR) was used to amplify the putative transporter genes directly from cDNA. However, because the regulation mechanism and expression pattern were unknown for pentose transporters in fungal species, cDNAs encoding the putative pentose transporters were not always obtainable despite alteration of cultivation condition. In this case, primers were designed according to the corresponding cDNA sequences from GenBank and used to amplify the exons using genomic DNA as a template. Overlap-extension PCR was then used to assemble the exons into the full-length genes. The resulting PCR products were cloned into the pRS424 shuttle vector containing a HXT7 promoter and a HXT7 terminator using the DNA assembler method. Yeast plasmids isolated from transformants were retransformed into *E. coli* DH5 α , and isolated *E. coli* plasmids were first checked by diagnostic PCR using the primers used to amplify the original transporter genes. The entire open reading frames were submitted for sequencing to confirm the correct construction of the plasmids.

Most of the cloning work was carried out using the yeast homologous recombination mediated DNA assembler method. pRS424-HXT7-GFP plasmid was used for cloning of putative pentose transporters. In this plasmid, the HXT7 promoter, the GFP gene flanked with the EcoRI sites at both ends, and the HXT7 terminator were assembled into the pRS424 shuttle vector (New England Biolabs) linearized by ClaI and BamHI. PCR products of the putative pentose transporters flanked with DNA fragments sharing sequence identity to the HXT7 promoter and terminator were co-transferred into CEN.PK2-1C with EcoRI digested pRS424-HXT7-GFP using the standard lithium acetate method. The resulting transformation mixture was plated on SC-Trp plates supplemented with 2% D-glucose. Transformants were then tested for pentose transport activity.

The results are shown below in Fig. 40 and Table 16. Among the eight putative pentose specific transporters [XP_960000 (NC52), CAG88709 (DH48), XP_457508 (DH61), XP_681669 (32-10), XP_001487429 (29-6), XP_001727326 (29-9), XP_657854 (32-8), XP_720384 (29-4)], only NC52 enabled cell growth on a glucose plate, which suggested that the other seven transporters may be pentose-specific or inactive. Using the HPLC-based pentose uptake assay, four xylose-specific transporters were

found, including XP_457508 (DH61), XP_001727326 (29-9), XP_720384 (29-4), and XP_681669 (32-10). In addition, one arabinose-specific transporter, XP_657854 (32-8) was identified (Fig. 40; Top). Five additional putative pentose specific transporters (XP_002488227, AB070824.1, XP_001389300, XP_002488227, EEQ43601.1) were also tested, none of which enabled cell growth in a glucose plate. Further pentose uptake assays indicated that XP_002488227 and AB070824.1 were xylose specific transporters (Fig. 40; Bottom). The summary of these results are shown in Table 16D.

Table 16A: Cloning of xylose-specific transporter NCU00821 orthologs

NCBI Reference Sequence	Origin	Sequence Results*	Uptake Assay	Status
XP_002488227	<i>Talaromyces stipitatus</i>	Correct	Yes	Cloned
XP_001400900	<i>Aspergillus niger</i>	Correct	Yes	Cloned
XP_001220481	<i>Chaetomium globosum</i> CBS 148.51	No	No	Sequenced, one intron
XP_001912725	<i>Podospora anserina</i>	No	No	OE-PCR, no PCR product
XP_660079	<i>Aspergillus nidulans</i> FGSC A4	Correct	Yes	Cloned
AAL89823	<i>Aspergillus niger</i>	Correct	Yes	Cloned
XP_002382573	<i>Aspergillus flavus</i> NRRL3357	Wrong	Yes	Cloned
XP_459386	<i>Debaryomyces hansenii</i> CBS767	No	No	Genomic DNA, no PCR product
XP_001825132	<i>Aspergillus oryzae</i> RIB40	Correct	Yes	Cloned
XP_001389300	<i>Aspergillus niger</i>	Correct	Yes	Cloned

* “Correct” = Sequence of clone matched sequence in database(s); “Wrong” = Sequence of clone did not match sequence in database(s); “No” = Results not available (work in progress)

Table 16B: Cloning of xylose-specific transporter STL12/XUT6 orthologs

NCBI Reference Sequence	Origin	Sequence Results*	Uptake Assay	Status
XP_457508 (DH61)	<i>Debaryomyces hansenii</i> CBS767	Correct	No	Cloned
XP_002551364	<i>Candida tropicalis</i> MYA-3404	Wrong	No	No
XP_001523322	<i>Lodderomyces elongisporus</i> NRRL	Wrong	No	No
XP_720384 (29-4)	<i>Candida albicans</i> SC5314	Correct	No	Cloned
XP_456868	<i>Debaryomyces hansenii</i> CBS767	Wrong	No	No
XP_001487429 (29-6)	<i>Pichia guilliermondii</i> ATCC 6260	Wrong	No	Cloned
XP_961039	<i>Neurospora crassa</i>	Wrong	No	No
CAG88709 (DH48)	<i>Debaryomyces hansenii</i> CBS767	Correct	No	Cloned
XP_001727326 (29-9)	<i>Aspergillus oryzae</i>	Correct	No	Cloned
XP_001816757	<i>Aspergillus oryzae</i>	Correct	No	Cloned

* “Correct” = Sequence of clone matched sequence in database(s); “Wrong” = Sequence of clone did not match sequence in database(s); “No” = Results not available (work in progress)

Table 16C: Cloning of arabinose-specific transporter XUT1 orthologs

NCBI Reference Sequence	Origin	Sequence Results *	Uptake Assay	Status
XP_002545773	<i>Candida tropicalis</i> MYA-3404	Correct	Yes	Cloned
EEQ43601	<i>Candida albicans</i> WO-1	Correct	Yes	Cloned
XP_001818631	<i>Aspergillus oryzae</i> RIB40	No	No	No PCR product
XP_002558275	<i>Penicillium chrysogenum</i> Wisconsin 54-1255	Wrong	Yes	Cloned
XP_001390883	<i>Aspergillus niger</i>	No	No	No PCR product
XP_750103	<i>Aspergillus fumigatus</i> Af293	Wrong	No	No
XP_960000 (NC52)	<i>Neurospora crassa</i> OR74A	Wrong	No	Cloned
XP_657854 (32-8)	<i>Aspergillus nidulans</i> FGSC A4	Correct	No	Cloned
XP_001825068	<i>Aspergillus oryzae</i> RIB40	Correct	No	Cloned
XP_681669 (32-10)	<i>Aspergillus nidulans</i> FGSC	Correct	No	Cloned

* “Correct” = Sequence of clone matched sequence in database(s); “Wrong” = Sequence of clone did not match sequence in database(s) (e.g., because of mutation in clone) “No” = Results not available (work in progress)

Table 16D: Listing of new xylose-specific transporters and one arabinose-specific transporter.

NCBI Reference Sequence	Origin	Xylose-specific	Arabinose-specific
XP_457508 (DH61)	<i>Debaryomyces hansenii</i> CBS767	Yes	
XP_001727326 (29-9)	<i>Aspergillus oryzae</i>	Yes	
XP_720384 (29-4)	<i>Candida albicans</i> SC5314	Yes	
XP_681669 (32-10)	<i>Aspergillus nidulans</i> FGSC A4	Yes	
XP_657854 (32-8)	<i>Aspergillus nidulans</i> FGSC A4		Yes
XP_002488227	<i>Talaromyces stipitatus</i>	Yes	
AB070824.1	<i>Aspergillus oryzae</i>	Yes	

The orthologs with sequences inconsistent with the sequences in databases (e.g., ones with mutations) will be re-cloned, sequenced, expressed in yeast strains, and tested for sugar uptake function.

Similarly, the orthologs for which there is no sequencing results will also be tested for transporter function.

Sequence alignments of the pentose transporter orthologs were analyzed to identify conserved residues, which could have potential roles in transporter function. Alignments of a sample of xylose transporters (NCU0821, STL12/XUT6, XP_002488227.1, and XP_002382573.1) and arabinose transporters (XUT1 and EEQ43601.1) are shown in Fig. 41 (a, b) respectively. Several residues are specifically conserved in xylose transporters whereas others are specifically conserved in the arabinose transporters. These residues may have critical roles in transporting the specific pentose. An overall comparison of the sequences of the xylose and arabinose transporters (Fig. 41c) shows that there are also residues that are conserved in both types of pentose transporters, indicating functional roles in uptake of pentoses in general.

Examples 12 - 15 relate to optimization of the xylose utilization pathway in yeast.

Example 12 - Engineering Pentose-Utilizing *S. cerevisiae* Strain

An efficient xylose metabolic pathway was reconstituted by exploiting the concept of isoenzymes. Isoenzymes catalyze the same chemical reaction with different kinetic or regulatory properties, and are known to confer fine-tuned control of metabolic fluxes in response to dynamic changes in the cytosolic environment. However, no prior metabolic engineering approaches had employed isoenzymes to increase fluxes of interest. This study demonstrated that simultaneous expression of both wild-type and mutant xylulose reductase (XR) isozymes could decrease xylitol accumulation and increase the overall xylose fermentation rate.

Inspired by the prevalence of isoenzymes in living systems, wild type XR and mutant XR (R276H) were co-expressed in *S. cerevisiae* along with xylitol dehydrogenase (XDH) and xylulokinase (XK) in order to construct a functional xylose metabolic pathway in *S. cerevisiae*. The XR mutant had been reported to exhibit much lower preference for NADPH over NADH whereas wild type XR showed 116 two-fold higher preference for NADPH over NADH (Watanabe *et al.*, 2007).

The xylose-metabolizing genes (wild-type *XYL1*, 2, and 3 and mutant *XYL1*) from *P. stipitis* were PCR-amplified and placed under the control of constitutive promoters (PGK1 and TDH3) to construct expression cassettes. These integration cassettes were integrated into the genome of the D452-2 strain.

Transformation of expression cassettes for constructing xylose metabolic pathways was performed using the yeast EZ-Transformation kit (BIO 101, Vista, CA). To select transformants using an amino acid auxotrophic marker, yeast synthetic complete (YSC) medium was used, which contained 6.7 g/liter yeast nitrogen base plus 20 g/liter glucose, 20 g/liter agar, and CSM-Leu-Trp-Ura (BIO 101), which supplied appropriate nucleotides and amino acids. Yeast strains were routinely cultivated at 30 °C in YP medium 234 (10 g/liter yeast extract, 20 g/liter Bacto peptone) with 20 g/liter glucose.

The effect of *S. cerevisiae* strain background on xylose-metabolizing efficiency was also tested by expressing identical constructs containing optimized xylose utilization pathway enzymes in several different yeast strains. The three laboratory strains used were D452-2 (*MATa, leu2, his3, ura3, can1*), L2612 (*MATa, leu2-3, leu2-112, ura3-52, trp1-298, can1, cyn1, gal+*), and CEN.PK. Production of xylitol, acetate, and ethanol was monitored together with use of xylose and OD₆₀₀. The results indicated that the D452-2 strain was the best amongst the three tested strains (Fig. 42-44). *S. cerevisiae* D452-2 was used for engineering of the xylose-metabolizing enzymes in yeast. Strains and plasmids used in this study are described in Table 17.

Table 17: Strain and plasmids used in study

Strain or plasmid	Description	Reference
Strain		
D452-2	<i>MATa, leu2, his3, ura3, can1</i>	Hosaka <i>et al.</i> , (1992)
D801-130	D452-2 expressing β -glucosidase (NCU00130) and <i>cbt1</i> (NCU00801)	In this study
D809-130	D452-2 expressing β -glucosidase (NCU00130) and NCU00809	In this study
D8114-130	D452-2 expressing β -glucosidase (NCU00130) and <i>cbt2</i> (NCU08114)	In this study
DA24	D452-2 expressing <i>XYL1, mXYL1, XYL2</i> , and <i>XKS1</i> (Isogenic of D452-2 except for <i>leu2::TDH3P-XYL1-TDH3T, ura3::URA3-PGKP-mXYL1-PGKT-PGKP-XYL2-PGKT, Ty3::neo-TDHP-XKS1-TDHT</i>)	In this study
DA24-16	Evolved strain of DA24 in xylose containing media	In this study
DA24-16BT3	DA24-16 expressing β -glucosidase (NCU00130) in a multi-copy plasmid and <i>cbt1</i> (NCU00801) though single-copy integration	In this study

DA24-16BT-M	DA24-16 expressing β -glucosidase (NCU00130) and <i>cbt1</i> (NCU00801) in multi-copy plasmids	In this study
Plasmid		
pRS425	<i>LEU2</i> , a multi copy plasmid	Christianson <i>et al.</i> , (1992)
pRS426	<i>URA3</i> , a multi copy plasmid	Christianson <i>et al.</i> , (1992)
pRS403	<i>HIS3</i> , an integrative plasmid	Sikorski <i>et al.</i> , (1989)
pRS405	<i>URA3</i> , an integrative plasmid	Sikorski <i>et al.</i> , (1989)
pRS425- β -glucosidase	β -glucosidase (NCU00130) under the control of PGK promoter in pRS425	Submitted
pRS426- <i>cbt1</i>	<i>cbt1</i> under the control of PGK promoter in pRS426	Submitted
pRS426- <i>cbt2</i>	<i>cbt2</i> under the control of PGK promoter in pRS426	Submitted
pRS426-NCU00809	NCU00809 under the control of PGK promoter in pRS426	Submitted
pRS403- <i>cbt1</i>	<i>cbt1</i> under the control of PGK promoter in pRS403	In this study

The engineered xylose-fermenting *S. cerevisiae* strain (DA24) consumed xylose and produced ethanol with negligible amounts of xylitol accumulation. When 40 and 80 g/L of xylose were used as a sole carbon source, the DA24 strain produced ethanol with consistent yields ($Y_{\text{Ethanol/Xylose}} = 0.31 \sim 0.32$ g/g) in both shaker-flask and bioreactor fermentation experiments (Fig. 45). However, the DA24 strain consumed xylose slower than the naturally existing xylose-fermenting yeast, *P. stipitis*. Xylose fermentation capability of DA24 was further improved using an evolutionary engineering approach (Sauer 2001). One of the strains (DA24-16) isolated after repeated sub-cultures of the DA24 on xylose-containing medium showed much faster xylose fermentation rates as compared to the parental strain under various culture conditions (Table 18).

Table 18 shows the comparison of fermentation parameters of the two *S. cerevisiae* strains DA24 and DA24-16 under different sugar conditions.

Carbon source	Strains	Produced Ethanol (g/L)	Sugar consumption rate (g/L/h)	Yield (g/g)	Productivity (g/L·h)
Xylose (80 g/L)	DA24	24	1.16	0.34	0.40
	DA24-16	28	1.32	0.35	0.47
Glucose (70 g/L) and xylose (40 g/L)	DA24	34	1.45	0.39	0.74
	DA24-16	45	1.78	0.42	0.96

Interestingly, the DA24-16 strain consumed xylose as fast as *P. stipitis*, the fastest xylose-fermenting yeast known. However, ethanol yield by DA24-16 was slightly lower than that by *P. stipitis* (Fig. 46).

A screen was set up using *S. cerevisiae* strain L2612 expressing the xylose-utilizing enzymes (strain YSX3) transformed with a genomic library. Transformation was followed by serial culture transfer in 40 g/L xylose under oxygen-limiting conditions to enrich for strains that are efficient in utilizing xylose. Fermentations were performed in 50 mL YPX media under oxygen-limited conditions and 0.1% (50 μ L) of a fully grown cell culture was transferred to the next serial culture when OD₆₀₀=10 was reached. After 10 serial cultures, cells were spread with serial dilution on YPX (40 g/L) agar media. Through fermentation experiments using 5 mL of YPX media, colonies were screened for low xylitol and high ethanol formation. DNA sequencing revealed that the two most efficient strains contained integrated copies of *XYL2*, which was then cloned into a multi-copy plasmid through homologous recombination and transformed into YSX3 cells.

The *XYL2* gene was placed in integration vectors under the control of promoters of different strength, e.g., TDHp or PGKp, and transformed into YSX3 cells (Fig. 47). Studies were conducted to monitor the effect of these plasmids on xylitol and ethanol formation in the transformed yeast cells. The results indicated that the YSX3 cells expressing higher levels of *XYL2* (under the PGKp) were more efficient at ethanol production and in addition, produced lower amounts of xylitol (Fig. 48). When additional *XYL3* was expressed in these cells (termed SR1 strain), the amount of xylitol produced was further decreased in the resulting strain SRu-23 (Fig. 49). Therefore, it appeared that *XYL2* expression

level in engineered *S. cerevisiae* strains is a key factor for implementing xylose fermentation, and when expression is under a strong promoter, the strain has less xylitol accumulation as well as high ethanol yield. Simultaneous over-expression of *XYL2* and *XYL3* can further decrease the amount of xylitol accumulation. However, when *XYL1* was further over-expressed in a strain over-expressing *XYL2* and *XYL3*, there was considerable xylitol accumulation and consequently decreased xylose fermentation (Figs. 50-51). Therefore, it appeared that there was an optimal level of *XYL1* for efficient xylose fermentation.

Experiments were also carried out to test if over-expression of endogenous *GRE3* in *S. cerevisiae* expressing *XYL2* and *XYL3* could facilitate xylose fermentation. For the construction of pRS403-GRE3, *GRE3* gene was amplified from *S. cerevisiae* D452-2 and inserted into pR403 vector with TDH3 promoter and CYC terminator. After linearization of pRS403-GRE3, it was integrated into the genome of D452-2. The xylose-utilizing genes were introduced into the yeast strain D452-2 (Fig. 52), and xylose fermentation parameters were monitored. The results indicated that over-expression of *GRE3* was as effective as the over-expression of *XYL1* in ethanol production and xylitol accumulation, particularly when cells were grown in 80g/L of xylose at high OD inoculations (Figs. 53-54).

Example 13 – Engineering LAD and XDH

L-arabinitol and xylitol accumulation, thought to be caused by cofactor imbalance between NADPH-dependent XR and NAD⁺-dependent XDH and LAD, has been regarded as a major bottleneck during xylose fermentation in engineered *S. cerevisiae* expressing the pentose-utilizing enzymes. While the imbalance between XR and XDH has been corrected by engineering enzymes with reversed cofactor preferences (Watanabe *et al.*, 2007; Matsushika *et al.*, 2008; Bengtsson *et al.*, 2009), this approach resulted in reduced flux, as the modified enzymes had reduced specific activities. The *P. stipitis* XR mutant had been reported to exhibit much lower preference for NADPH over NADH whereas wild type *psXR* showed two-fold higher preference for NADPH (Watanabe *et al.*, 2007).

In this study, similar studies were done on L-arabinitol 4-dehydrogenase (LAD) and XDH from *N. crassa* to alter cofactor specificity and hence improve xylose fermentation in engineered *S. cerevisiae*.

Identification of Putative LAD-Encoding Genes

Methods of identifying putative LAD-encoding genes and of cloning LAD-encoding and putative LAD-encoding genes are described.

Identification of putative LAD-encoding genes

From a protein BLAST search using ncLAD (EAA36547.1) as a probe, two putative genes were identified in *P. chrysogenum* (XP_002569286.1) and *P. guilliermondii* (EDK37120.2), respectively. The amino acid sequence identities of these two proteins with ncLAD were 71% and 46%, respectively.

Cloning LAD-encoding and putative LAD-encoding genes

A. niger (NRRL 326), *P. guilliermondii* (NRRL Y2075), and *P. chrysogenum* (NRRL 807) were obtained from the United States Department of Agriculture Agricultural Research Service Culture Collection (Peoria, IL). *T. longibrachiatum* (*T. reesei*, YSM 768) was obtained from the German Resource Centre for Biological Material (DSMZ).

A. niger, *T. longibrachiatum*, *P. chrysogenum*, and *P. guilliermondii* were grown in liquid media or on agar plates containing 1% yeast extract, 2% peptone, and 2% L-arabinose. Cells were frozen in liquid nitrogen for the isolation of total RNA or genomic DNA. Reverse transcription-PCR (RT-PCR) was performed on mRNAs isolated from *T. longibrachiatum*, *P. chrysogenum*, and *P. guilliermondii* to obtain cDNA, and PCR was used to obtain the genes encoding (putative) LADs. For *A. niger*, the putative LAD gene could not be amplified from cDNA due to unknown reasons. Thus, overlap extension-PCR (OE-PCR) was used to clone this intron-containing gene from the isolated genomic DNA. Note that all primer sequences used to clone these genes are listed in Table 19.

Table 19: Primers used for the cloning of wild type LADs. Restriction enzyme sites are in bold and italicized.

	Restriction Enzyme	Primer	Sequence
anLAD	<i>NdeI</i>	Fwd-fragment1 ^a	5'-GACATCGATGAC <i>ATATG</i> ^c GCTACCGCAAC-3'
		Rev-fragment1	5'-GTGCACGTCGGACCCGCAGATTCC-3'
	<i>BamHI</i>	Fwd-fragment2 ^b	5'-GGAATCTGCGGGTCCGACGTGCAC-3'
		Rev-fragment2	5'-CAGAAGATTTAAG <i>GATCCT</i> GAAACGTAGA-3'
tlLAD	<i>NdeI</i>	Fwd	5'-GACATCAGTGAC <i>ATATG</i> TGCGCCTTCC-3'
	<i>BamHI</i>	Rev	5'-CCTGGATTGAG <i>GATCCT</i> GAAACGTATA-3'

			SEQ ID NO: 76
pcLAD	<i>NdeI</i>	For	5'-GACATCGATGAC <i>ATAT</i> GGCTTCCGCAAC-3'
	<i>EcoRI</i>	Rev	5'-CCAGAAGTATTGAG <i>AATT</i> CTGAACGTAGA-3'
pgLAD	<i>NdeI</i>	Fwd	5'-GACATCGATGAC <i>ATAT</i> GGCGACTCTGC-3'
	<i>BamHI</i>	Rev	5'-GGATACAGAATGAG <i>GATC</i> CTGAACGTAGA-3'

^{a,b} Fragment 1 and 2 indicate the upstream and downstream exons flanking the intron.

^c Sequences in bold (italicized) indicate restriction enzyme sites.

PCR products were subcloned into pET-28a vector and the constructs were used to transform into two *E. coli* strains, DH5 α and BL21 (DE3), by electroporation for cloning and expression, respectively. *NdeI/BamHI* restriction sites were used for the subcloning of the predicted genes from *A. niger*, *T. longibrachiatum*, and *P. guilliermondii*, and *NdeI/EcoRI* sites were used for *P. chrysogenum*. The constructs encoded (putative) LADs as N-terminal His₆-tagged fusions. Plasmids were sequenced using BIGDYE (TM) Terminator sequencing method and analyzed with 3730xL Genetic Analyzer (Applied Biosystems, Foster City, CA) at the Biotechnology Center at the University of Illinois at Urbana-Champaign (Urbana, IL).

Protein expression and purification

Genes encoding pcLAD (XP_002569286.1), pgLAD (EDK37120.2), anLAD (CAH69383.1), and tlLAD (AAL08944.1) were cloned into the pET-28a vector and expressed in *E. coli* BL21 (DE3). *E. coli* BL21 (DE3) containing the LAD genes were grown overnight at 30 °C on a rotary shaker at 250 rpm. Overnight culture (50 μ L) was used to inoculate a fresh culture (5 mL), which was grown at 30 °C with shaking at 250 rpm until the optical density at 600 nm (OD₆₀₀) reached 0.6-1.0. The cultures were then induced with 0.3 mM IPTG at 30 °C for 3-4 hrs or at 18 °C for 20 hrs.

The induced cells (1 mL) were lysed by re-suspending them in 1 mL of 50 mM potassium phosphate buffer (pH 7.0) with 1 mg/mL lysozyme and shaking at 30 °C and 250 rpm for 30 min. Cells were kept at -80 °C overnight and thawed at room temperature. The resulting cell lysates were centrifuged at 13,200 rpm for 15 min, and the supernatant and precipitate were analyzed for protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

For protein purification, the induced cells (400 mL) were treated with 15 mL of Buffer A (20 mM Tris, 0.5 M NaCl, 20% glycerol, pH 7.6) with 1 mg/mL lysozyme and shaken at 30 °C and 250 rpm for 30 min. After a freeze-thaw cycle, the resulting product was further lysed by sonication followed by centrifugation for 20 min at 12,000 rpm to remove cell debris. The supernatants were applied to a column packed with Co^{2+} -immobilized metal affinity chromatography resin to purify His₆-tagged proteins following the manufacturer's instructions. The purified proteins were desalted by ultrafiltration (Amicon Ultra, Millipore, Billerica, MA) and washed with HEPES buffer (pH 7.0) containing 150 mM NaCl and 15% glycerol and kept at -20 °C. Protein concentrations were determined by the Bradford method (Bradford 1976) according to the manufacturer's protocol.

Characterization of LAD Proteins

The steady-state kinetics, molecular weight, quaternary structure, temperature dependence, pH dependence, L-arabinitol dehydrogenase activity, and metal content of LAD enzymes were analyzed.

L-Arabinitol Dehydrogenase Activity

Lysates were prepared from host cells expressing LAD from *P. chrysogenum*, *P. guilliermondii*, *A. niger*, and *T. longibrachiatum*. Ten microliters of cell lysate were used for an activity assay with 200 mM L-arabinitol and 2 mM NAD^+ as the substrates in 50 mM potassium phosphate buffer (pH 7.0). NADH production was monitored by measuring absorbance at 340 nm ($\epsilon = 6.22 \text{ mM}^{-1}\text{cm}^{-1}$) using a Cary 300 Bio UV-vis spectrophotometer (Varian, Cary, NC).

Steady-State Kinetics

Kinetic parameters of different LAD enzymes were determined. Initial rates were determined by measuring the absorbance change at 340 nm using a UV-vis spectrophotometer at room temperature in 50 mM potassium phosphate buffer (pH 7.0). Initial rates were measured at various concentrations of the substrate (L-arabinitol) and cofactors ($\text{NAD}^+/\text{NADP}^+$) (5 to 320 mM for L-arabinitol, 0.5 to 3.2 mM for cofactors). Enzyme kinetics for the substrate and cofactors were analyzed using Michaelis-Menten kinetics, and kinetic parameters were determined by fitting data to the Lineweaver-Burk plot. The parameters for substrate were determined by measuring initial rates at saturated cofactor concentrations (3.2 mM) and those for cofactors were determined at saturated substrate concentrations (320 mM). Assays were performed in triplicate.

The cloned LADs showed different binding affinities and catalytic activities for L-arabinitol: K_m differed by two fold and k_{cat} by about three fold amongst the LADs. For L-arabinitol, the K_m values of anLAD, tlLAD, and pcLAD were 25 ± 1 , 18 ± 1 , and 37 ± 2 mM, and the k_{cat} values were 507 ± 22 , 346 ± 41 , and 1085 ± 71 min⁻¹, respectively (Table 20). The tlLAD enzyme had the lowest K_m while pcLAD showed the highest catalytic activity (k_{cat}) and efficiency (k_{cat}/K_m) despite having the highest K_m (Table 20). For cofactor NAD⁺ kinetics, the cloned LADs showed K_m values in the range of 0.2-0.3 mM and catalytic efficiencies in the range of 2526 to 3460 mM⁻¹·min⁻¹ (Table 21). All cloned LADs showed minimal activities toward NADP⁺ (Tables 20, 21). The initial rates were not saturated at highest substrate and cofactor concentration (320 mM for L-arabinitol and 3.2 mM for NADP⁺) due to the large K_m . Therefore, only the catalytic efficiency of the enzyme was determined using 0.1 or 0.2 mM for NADP⁺ and 10 or 20 mM for L-arabinitol ($K_m \gg [S]$) (Tables 20, 21).

Table 20: Kinetic parameters of LADs for L-arabinitol at saturated cofactor concentrations.

		Specific activity (U/mg protein)	K_m (mM)	k_{cat} (min⁻¹)	k_{cat}/K_m (mM⁻¹·min⁻¹)
anLAD	NAD ⁺	11.7 ± 0.3^a	25 ± 1	507 ± 22	20.0 ± 0.8
	NADP ⁺	- ^b	-	-	0.04 ± 0.01
tlLAD	NAD ⁺	8.7 ± 0.1	18 ± 1	346 ± 41	19.0 ± 0.8
	NADP ⁺	-	-	-	0.13 ± 0.02
pcLAD	NAD ⁺	25.3 ± 1.4	37 ± 2	1085 ± 71	29 ± 1
	NADP ⁺	-	-	-	0.04 ± 0.02

^a Error indicates standard deviation from the mean, n=3

^b Dash indicates not determined due to high K_m for indicated cofactor

Table 21: Kinetic parameters of LADs for NAD⁺ and NADP⁺ at saturated L-arabinitol concentration.

		K_m (mM)	k_{cat} (min⁻¹)	k_{cat}/K_m (mM⁻¹·min⁻¹)
anLAD	NAD ⁺	0.20 ± 0.01^a	494 ± 11	2526 ± 83
	NADP ⁺	- ^b	-	20 ± 9
tlLAD	NAD ⁺	0.2 ± 0.1	436 ± 96	2689 ± 646
	NADP ⁺	-	-	17 ± 9
pcLAD	NAD ⁺	0.3 ± 0.1	1039 ± 165	3460 ± 505
	NADP ⁺	-	-	15 ± 4

^a Error indicates standard deviation from the mean, n = 3

^b Dash indicates not determined due to high K_m for indicated cofactor

Molecular weight and quaternary structure

Calculated molecular weights of the subunits of the four proteins were 43 kDa (anLAD), 41 kDa (tlLAD), 42 kDa (pcLAD), and 42 kDa (pgLAD). The molecular weights of the proteins were determined using a Bio-Sil SEC-250 column (300×7.8 mm, Bio-Rad, Hercules, CA) on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan). The mobile phase consisted of 50 mM Na₂HPO₄, 50 mM NaH₂PO₄, 150 mM NaCl, and 10 mM NaN₃ (pH 6.8) and the flow rate was 1.0 mL/min. The molecular weights were calculated by comparing the retention times with those of protein molecular weight standard.

The quaternary structures were determined based on the molecular weights observed by HPLC and the molecular weights of monomeric subunits which were determined by SDS-PAGE analysis. Molecular weights of an-, tl-, and pcLAD were determined to be 178, 194, and 173 kDa, respectively. Comparing to the molecular weights of the subunits determined by SDS-PAGE, results suggested that the LADs were non-covalently linked tetramers in their native forms.

Temperature and pH dependence

The optimal temperatures of the proteins were determined by assaying enzyme activities at temperatures ranging from 10 to 70 °C. Thermal inactivation was determined by measuring enzyme activity after various incubation times at 50 °C in phosphate buffer. Enzyme activity was measured with 2 mM NAD⁺ and 200 mM L-arabinitol. Half-life of enzyme activity was determined using a first-order exponential decay function. Temperature was controlled by a Cary temperature controller connected to the UV-vis spectrophotometer (Varian, Cary, NC). pH-dependent enzyme activity was determined by measuring activity at pH between 5.0 and 11.0 at saturated concentrations of NAD⁺ (2 mM) and L-arabinitol (200 mM) in a universal buffer (50 mM morpholineethanesulfonic acid/50 mM Tris/50 mM glycine) (Ellis and Morrison 1982).

The optimal temperatures of anLAD and pcLAD were between 40 and 50 °C, whereas tlLAD showed higher optimal temperature between 55 and 65 °C (Fig. 55a). Catalytic activities of the LADs exponentially decreased with the length of incubation time at 50 °C and were almost completely deactivated after 100 min (Fig. 55b). tlLAD was the most thermally stable with a half-life of 20 min at 50

°C, and anLAD was least stable with a half-life of less than 5 min at 50 °C. All characterized LADs showed activity in the pH range of 7 to 11 with maximum activity around pH 9.4 (Fig. 55c). In the pH range outside of 9 to 10, activity was significantly reduced and approximately 20% of activity remained at pH 7.0 (Fig. 55c). No activity was detected at or below pH 5.0.

Metal analysis

Duplicate samples for metal analysis were prepared in phosphate buffered saline (PBS) by buffer exchange and lyophilization. Each sample contained 1-2 mg of protein in 1 mL buffer solution. The identity and content of the metal were analyzed by inductively coupled plasma atomic emission spectrometry (OES Optima 2000 DV, Perkin Elmer, Boston, MA) in the Microanalytical Laboratory at the University of Illinois at Urbana-Champaign (Urbana, IL).

Measured weight percentages of Zn^{2+} were close to those calculated based on the 1:1 molar ratio (Table 22).

Table 22: Calculated and measured Zn^{2+} contents.

	Calculated Weight^a (%)	Measured weight (%)
anLAD	0.027	0.027 ± 0.003^b
tlLAD	0.047	0.048 ± 0.003
pcLAD	0.048	0.061 ± 0.013

^a Calculated molecular weights were determined based on the buffer composition, protein concentration, and 1:1 molar ratio of LAD monomer subunit and Zn^{2+} . Buffer solution (1 L) contained NaCl (8 g), KCl (0.2 g), Na_2HPO_4 (1.44 g), and KH_2PO_4 (0.24 g).

^b All samples were analyzed in duplicate and errors were standard deviations.

Engineering of LAD Enzymes with Altered Cofactor Specificity

Methods of altering the cofactor specificity of LADs were determined, and mutated LADs were analyzed for altered cofactor specificity and other characteristics.

Development of LADs with Altered Cofactor Specificity

Site-directed mutagenesis was performed to alter the cofactor specificity of anLAD, tlLAD, and pcLAD from NAD^+ to NADP^+ . Amino acid numbers 224, 225, and 362 of naturally occurring tlLAD were substituted with serine, arginine, and threonine, respectively, to generate the tlLAD with altered cofactor specificity. The amino acid sequences of cloned anLAD and pcLAD were aligned with the *T.*

longibrachiatum LAD (tlLAD) sequence, and the amino acids that correspond to tlLAD amino acid numbers 224, 225, and 362 were mutated. For all of the LADs with altered cofactor specificity, two amino acid residues within the β - α - β motif of the coenzyme binding domain were replaced with serine and arginine, respectively: D213 and I214 for anLAD, D224 and I225 for tlLAD, and D212 and I213 for pcLAD (Korkhin *et al.*, 1998; Pauly *et al.*, 2003; Watanabe *et al.*, 2005), and the third mutation was introduced at A359 for anLAD, A362 for tlLAD, and S358 for pcLAD and replaced with threonine (For primer sequences, see Table 23). Megaprimer PCR method was used to introduce site-specific mutations using wild type LAD constructs as the templates (Sarkar and Sommer 1990). Correct mutations were confirmed by DNA sequence analysis.

Table 23: Primers used for site directed mutagenesis by the megaprimer PCR method.^a

	Fwd-T7-pro Rev-T7-term	5'-TAATACGACTCACTATAGGG-3' SEQ ID NO: 81 5'-GCTAGTTATTGCTCAGCGG-3' SEQ ID NO: 82
anLAD	Fwd-D213S/I214R Rev-D213S/I214R Fwd-A359T	5'-CCTATCGTCATTACCTCAGT ^b GACGAGGGGCGGCTG-3' SEQ ID NO: 83 5'-CAGCCGCCCCCTCGTCACGTGAGGTAATGACGATAGG-3' SEQ ID NO: 84 5'- CCT TCGAAACGGCTACAAACCCCAAGACG -3 SEQ ID NO: 85
tlLAD	Fwd-D214S/I215R Rev-D214S/I215R Fwd-S362T	5'-GCTTGTCATCACATCAGTTCAGAGAGCCGTCTG-3' SEQ ID NO: 86 5'-CAGACGGCTCTCTGAACGTGATGTGATGACAAGC-3' SEQ ID NO: 87 5'-GCATTTGAGACGTCAACAGATCCCAAGAGC-3' SEQ ID NO: 88
pcLAD	Fwd-D212S/I213R Rev-D212S/I213R Fwd-S358T	5'-CCTATTGTCATCACTTCAGTGACGAGGGCCGCTTG-3' SEQ ID NO: 89 5'-CAAGCGGCCCTCGTCACGTGAAGTGATGACAATAGG-3' SEQ ID NO: 90 5'-CCTTTGAGACTGCCACAAACCCTAAGACCGGTG-3' SEQ ID NO: 91

^a To create mutant LADs, fragments 1 and 2 were amplified using Fwd-T7-pro and Rev-D213S/I214R and Fwd-A359T and Rev-T7-term primers, respectively. Fragment 3 was amplified using Fwd-D123S/I214R and fragment 2 (Rev megaprimer). Full mutant genes were amplified by overlap extension of fragment 1 and 3. Template DNA was pET-28a plasmid.

^b Sequences underlined were the mutation sites.

Kinetic Analysis of LADs with Altered Cofactor Specificity

In this example, “tlLAD mutant” is defined as tlLAD with the mutations D224S / I225R / A362T; “anLAD mutant” is defined as anLAD with the mutations D213S / I214R / A359T; and “pcLAD mutant”

is defined as pcLAD with the mutations D212S / I213R / S358T. The tlLAD mutant showed significantly altered cofactor specificity from NAD^+ to NADP^+ . It also demonstrated the highest catalytic activity. The K_m and k_{cat} of the tlLAD mutant for L-arabinitol with NADP^+ were 46 ± 4 mM and 170 ± 9 min^{-1} , respectively (Table 24). In all assays including the tlLAD mutant with saturated NAD^+ , a plateau of reaction rate was not observed in the tested concentration range, so catalytic efficiencies were determined at 0.8 mM for NAD^+ and 80 mM for L-arabinitol (Tables 24, 25). For cofactors, anLAD and tlLAD mutants showed significantly higher preference for NADP^+ over NAD^+ (Table 25). The K_m values of the anLAD and tlLAD mutants were 0.46 ± 0.09 and 0.10 ± 0.01 mM, and the k_{cat} values were 55.7 ± 6.4 and 90.5 ± 9.2 min^{-1} , respectively (Table 25). The catalytic efficiencies of anLAD and tlLAD mutants were 130 ± 32 and 934 ± 72 $\text{mM}^{-1} \cdot \text{min}^{-1}$, and the ratios of the catalytic efficiencies with NADP^+ to NAD^+ were 100 and 161, respectively. For the tlLAD mutant, the ratio of catalytic efficiency for NADP^+ to NAD^+ was increased by 2.5×10^4 fold (Tables 21, 25). The pcLAD mutant showed no activity with NAD^+ .

Table 24 shows kinetic parameters of LAD mutants for L-arabinitol at saturated cofactor concentrations.

		Specific activity (U/mg protein)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{min}^{-1}$)
anLAD mutant	NAD^+	- ^a	-	-	0.010 ± 0.002^b
	NADP^+	-	-	-	0.45 ± 0.20
tlLAD mutant	NAD^+	-	-	-	0.050 ± 0.007
	NADP^+	3.9 ± 0.2	46 ± 4	170 ± 9	3.7 ± 0.2
pcLAD mutant	NAD^+	-	-	-	-
	NADP^+	-	-	-	0.02 ± 0.02

^a Dash indicates not determined due to high K_m for indicated cofactor

^b Error indicates standard deviation from the mean, n=3

Table 25 shows kinetic parameters of LAD mutants for NAD^+ and NADP^+ at saturated L-arabinitol concentration.

		K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{mM}^{-1} \cdot \text{min}^{-1}$)
anLAD mutant	NAD^+	- ^a	-	1.3 ± 0.3^b
	NADP^+	0.46 ± 0.09	55.7 ± 6.4	130 ± 32
tlLAD mutant	NAD^+	-	-	5.8 ± 0.8

	NADP ⁺	0.097 ± 0.011	90.5 ± 9.2	934 ± 72
pcLAD mutant	NAD ⁺	-	-	-
	NADP ⁺	-	-	3.6 ± 1.0

^a Dash indicates not determined due to high K_m for indicated cofactor

^b Error indicates standard deviation from the mean, n = 3

Engineering of *N. crassa* XDH (ncXDH) with altered cofactor specificity

Cloning and characterization of putative ncXDH

A putative *N. crassa* xylitol dehydrogenase (ncXDH) sequence was found using a protein BLAST search on the National Center for Biotechnology Information website (webpage ncbi.nlm.nih.gov) using the *P. stipitis* xylitol dehydrogenase (psXDH) enzyme as a query sequence. The two enzymes were aligned fully using a ClustalW algorithm and found to share 44% identity and 60% similarity (Fig. 56). The whole-genome sequence of *Neurospora crassa* has been published (Galagan *et al.*, 2003) and it was utilized to design primers for cloning of the putative xylitol dehydrogenase (XDH) gene.

RT-PCR performed on total RNA isolated from D-xylose-induced *N. crassa* 10333 showed the expected size of gene product (~1.1 kb). The RT-PCR product was cloned into the pET-28a vector using *Nde*I and *Sac*I restriction sites and was transformed into *E. coli* BL21 (DE3). This construct (pET-28a ncXDH) expressed ncXDH as an N-terminal His₆-tagged fusion with a thrombin cleavage site. Cell lysates of IPTG-induced cultures of these cells were prepared, analyzed by SDS-PAGE, and assayed for XDH activities. The XDH was then purified by immobilized metal ion affinity chromatography (IMAC) using Talon[®] Co²⁺ Superflow resin (Clontech, Mountain View, CA) according to manufacturer's protocol. The purified protein was desalted by ultrafiltration with several washes of 50 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.25) + 15% glycerol and stored frozen at -80 °C. Protein concentrations were determined by the Bradford method (Bradford 1976).

ncXDH is a strictly NAD⁺-preferring enzyme. ncXDH also displays high stability (half-life of ~200 min at 50 °C) and expression. Previous work by Watanabe *et al.* (2005b) was aimed at reversing the cofactor specificity of psXDH.

Development of ncXDH with Altered Cofactor Specificity

Through sequence alignment, residues D204, I205, and V206 of ncXDH were targeted for site-directed mutagenesis to alanine, arginine, and serine, respectively, to create ncXDH-ARS. Table 26 shows that ncXDH-ARS has completely reversed cofactor specificity, now preferring NADP⁺. The affinity for substrate xylitol did not suffer very much from the affinity-change for the co-factor.

Table 26 shows kinetic parameters for *N. crassa* and *P. stipitis* XDH and XDH-ARS with nicotinamide cofactors NAD⁺ and NADP⁺ at saturated xylitol concentrations.

NAD ⁺				NADP ⁺			
Enzyme	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	Source
ncXDH-wt	2160	0.127	17000	-a	~5.6	~68	This work
ncXDH-ARS	-a	~3.5	~165	2080	0.325	6400	This work
psXDH	1050	0.381	2760	110	170	0.65	Watanabe <i>et al.</i> (2005b)
psXDH-ARS	240	1.3	181	2500	0.897	2790	Watanabe <i>et al.</i> (2005b)

a Not determined, cofactor saturation not reached.

All assays were performed at 25 °C in 50 mM Tris, pH 8.0.

Kinetic Analysis of ncXDH Mutant

The mutant ncXDH had a dramatic reversal of cofactor specificity. The K_m of the mutant ncXDH for NADP⁺ was only about 2.5-fold higher than the K_m of wild-type ncXDH for NAD⁺ whereas the k_{cat} values were similar (Table 27).

Table 27 shows kinetic parameters of ncXDH mutants for substrate xylitol.

Enzyme	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m ($\text{mM}^{-1} \text{min}^{-1}$)
ncXDH-wt	2170 ± 135	6.6 ± 2.0	330
ncXDH-ARS	2090 ± 35	4.3 ± 0.3	490

a Not determined, cofactor saturation not reached.

All assays were performed at 25 °C in 50 mM Tris, pH 8.0.

All enzymes were purified and characterized with N-His₆-tag

As shown in Fig. 57, XDH activity exhibits a higher tolerance to more acidic conditions with activity extending down to pH 4.0, whereas LAD activity is abolished at pH 5.0 in the *in vitro* activity assay.

Example 14 - Expression of xylose isomerase from Bacteroides stercoris in S. cerevisiae

Bacterial xylose isomerase (XI) is involved in converting xylose into xylulose. Recently, three successful cases of expressing active XI from two species of anaerobic fungi (*Piromyces* sp. and *Orpinomyces* sp.) and from the anaerobic bacteria (*Clostridium phytofermentans*) have been reported. A fungal *XYLA* gene from *Piromyces* sp. E2 was functionally expressed in *S. cerevisiae* and a maximum 1.1 U/mg-protein of XI activity was obtained at 30 °C (Kuyper *et al.*, 2003). The second fungal *XYLA* gene from *Orpinomyces*, which has 94% identity with that from *Piromyces* sp., was also functionally expressed in *S. cerevisiae* (Madhavan *et al.*, 2009). Recently, the first prokaryotic *xylA* gene from *Clostridium phytofermentans* was functionally expressed in *S. cerevisiae* (Brat *et al.*, 2009).

The isomerase gene *xylA* from the anaerobic bacteria *Bacteroides stercoris* (*BtXI*) shares high sequence identity with the isomerase gene from *Piromyces* sp. (82%). *BtXI* was cloned into the pRS424TEF vector and transformed into the *S. cerevisiae* L2612 strain. The gene was also integrated into the *S. cerevisiae* D452-2 strain by using the pRS403TEF vector. Ethanol production was observed in both strains expressing *BtXI* (5g/L in L2612 and 7.8 g/L in D452-2) (Fig. 58-59). However, rates of production were relatively low compared to that of engineered strains expressing the *XYL* genes.

The low ethanol production could be attributed to the inhibitory effect of any accumulated xylitol (formed from xylose by endogenous yeast aldose reductase). To decrease xylitol accumulation, XDH and

XK were expressed in *BTXI*-expressing yeast strain (DBtXI). The resulting strain had slightly improved ethanol yield and decreased xylitol production (Fig. 60). Co-expression of these two *XYL* genes in DBtXI resulted in ethanol production even under aerobic conditions.

Example 15 - Over-Expression of Enzymes in Pentose Phosphate Pathway (PPP)

The PPP enzymes glucose-6-phosphate dehydrogenase (*ZWF1*), 6-phosphogluconate dehydrogenase (*GDN1*), transaldolase (*TAL1*), and transketolase (*TKT1*) from *P. stipitis* were cloned into an integration vector (pRS406) under the control of a strong promoter (P_{GPD}). The plasmid was linearized by the enzyme *StuI* and integrated into the chromosome of *S. cerevisiae*.

However, to get the beneficial effects of over-expressing the PPP enzymes, there also had to be over-expression of *XYL3* (XK) (Fig. 61). Expression of *XYL3* and the PPP enzymes also improved ethanol production in YP-xylulose media.

Example 16 - Expression of Aldose-1-Epimerase

Hydrolysis of cellobiose by β -glucosidase releases β -D-glucose. However, yeast hexokinases prefer (or exclusively use) α -D-glucose, and the rate of mutarotation of β -D-glucose to α -D-glucose could effectively slow down metabolic rate. One way of enhancing the conversion was to over-express the predicted aldose-1-epimerase NCU09705. This hypothesis was tested by over-expressing NCU09705 homologs: *galM* in *E. coli*; *GAL10*, *YHR210C*, and *YNR071C* in *S. cerevisiae*; and *GAL 10* in *P. stipitis*. The strains were then tested for cellobiose consumption and ethanol production (Fig. 62). The results indicated that over-expression of the homologs in *S. cerevisiae* caused a slight increase in cellobiose consumption and ethanol production.

Example 17 - Co-Fermentation of Xylose and Cellobiose

In this example a new strategy was used to overcome glucose repression in which a dimer of glucose, cellobiose, was co-fermented with xylose (a pentose). Cellobiose is an intermediate product from enzymatic hydrolysis of cellulose, which is further converted to glucose by β -glucosidases in the cocktail of cellulases including exocellulases, endocellulases, and β -glucosidases, whereas pentose sugars are the products of dilute acid hydrolysis of hemicellulose. Wild type *S. cerevisiae* cannot assimilate cellobiose because it lacks both a cellobiose transporter and a β -glucosidase capable of hydrolyzing

cellobiose into glucose. Hence, the newly discovered cellodextrin transporter genes described in Example 9 and a β -glucosidase gene from *N. crassa* were co-expressed in *S. cerevisiae* and a mixture of xylose and cellobiose was used as carbon source (Fig. 63). Similar approaches have employed either secretion, or cell surface display, of β -glucosidases to allow cellobiose fermentation by *S. cerevisiae* (van Rooyen *et al.*, 2005; Skory *et al.*, 1996; Kotaka *et al.*, 2008; Katahira *et al.*, 2006). In those cases, cellobiose was hydrolyzed into glucose extracellularly before being transported by the endogenous hexose transport system of *S. cerevisiae*. In contrast, in this strategy, cellobiose was hydrolyzed intracellularly following transport.

In the conventional methods for mixed sugar fermentation in *S. cerevisiae*, a mixture of glucose and pentose sugars derived from lignocellulose is used. However, in this new strategy, a mixture of cellobiose and pentose sugars was used. The cellobiose was transported inside yeast cells via the heterologous cellodextrin transporters while pentose sugars were transported inside yeast cells by endogenous hexose transporters, thus removing the direct competition between glucose and pentose sugars for the same transporters, a phenomenon that is partly responsible for glucose repression. Once inside yeast cells, cellobiose was converted to glucose by β -glucosidase and immediately consumed by yeast cells, which resulted in low intracellular glucose concentration, thereby further alleviating glucose repression.

The engineered xylose-utilizing yeast strain L2612 was used as a host to co-express cellodextrin transporter and β -glucosidase genes. In this strain, the D-xylose utilization pathway consisting of xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis* was integrated into the chromosome. The cellodextrin transporters from *Neurospora crassa* including NCU008011, NCU08114, and, NCU00809, and two β -glucosidase genes, one from *Neurospora crassa* and the other from *Aspergillus aculeatus*, were evaluated.

S. cerevisiae L2612 (*MAT α* , *leu2-3*, *leu2-112*, *ura3-52*, *trp1-298*, *can1*, *cyn1*, *gal+*) was cultivated in synthetic dropout media to maintain plasmids (0.17% of Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% of ammonium sulfate, 0.05% of amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% of sugar was used to grow yeast strains.

To integrate the D-xylose utilization pathway consisting of D-xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis*, the corresponding genes were PCR-amplified and cloned into the pRS416 plasmid using the DNA assembler method (Shao *et al.*, 2009). *Bam*HI and *Hind*III were used to remove the DNA fragment encoding the D-xylose utilization pathway and then ligated into the pRS406 plasmid digested by the same two restriction enzymes. The resulting plasmid was then linearized by *Apa*I and integrated into the *URA3* locus on the chromosome of L2612.

The pRS425 plasmid (New England Biolabs, Ipswich, MA) was used to co-express a cellodextrin transporter gene and a β -glucosidase gene. As shown in Fig. 64, the pRS425 plasmid was digested by *Bam*HI and *Apa*I. The *PYK1* promoter and the *ADH1* terminator were added to N-terminus and C-terminus of the cellodextrin transporter, respectively, while the *TEF1* promoter and the *PGK1* terminator were added to the N-terminus and C-terminus of the β -glucosidase, respectively. These DNA fragments were assembled into the linearized pRS425 shuttle vector using the DNA assembler method (Shao *et al.*, 2009). Three cellodextrin transporter genes NCU00801 (XM_958708), NCU08114 (XM_958780), and NCU00809 (XM_959259) from *Neurospora crassa* and two β -glucosidase genes NCU00130 (XM_951090) from *Neurospora crassa* and BGL1 (D64088) from *Aspergillus aculeatus* were used. There were six combinations in total, each with one cellodextrin transporter gene and one β -glucosidase gene.

Yeast plasmids were then transferred into *E. coli* DH5 α , which were used for recombinant DNA manipulation. The transformants were plated on Luria broth plates containing 00 mg/L ampicillin. Single colonies of *E. coli* transformants were then inoculated into the liquid Luria broth media (Fisher Scientific, Pittsburgh, PA) and grown at 37 °C and 250 rpm. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN). These plasmids were transformed into the L2612 strain individually to yield the following strains: SL01 (contained the plasmid harboring the NCU00801 cellodextrin transporter gene and the NCU00130 β -glucosidase gene from *Neurospora crassa*), SL02 (contained the plasmid harboring the NCU00809 cellodextrin transporter gene and the NCU00130 β -glucosidase gene from *Neurospora crassa*), SL03 (contained the plasmid harboring the NCU08114 cellodextrin transporter gene and the NCU00130 β -glucosidase gene from *Neurospora crassa*), SL04 (contained the plasmid harboring the NCU00801 cellodextrin transporter gene and the BGL1 gene from *Aspergillus aculeatus*), SL05 (contained the plasmid harboring the NCU00809 cellodextrin transporter gene and the BGL1 gene from *Aspergillus aculeatus*), and SL06 (contained the plasmid harboring the NCU08114 cellodextrin

transporter gene and the BGL1 gene from *Aspergillus aculeatus*). The empty pRS425 plasmid was transformed into the L2612 strain to yield the SL00 strain, which was used as a negative control. Yeast transformation was carried out using the standard lithium acetate method (Gietz *et al.*, 1995). The resulting transformation mixtures were plated on SC-Ura-Leu medium supplemented with 2% D-glucose.

To confirm the proper construction of plasmids using the DNA assembler method, plasmids were isolated from yeast cells using the Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research, Orange, CA) and then transferred into *E. coli* DH5 α cells. The resulting cells were spread on LB plates containing 100 mg/L ampicillin. Single *E. coli* colonies were inoculated into the LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion using *Cla*I and *Hind*III. All restriction enzymes were obtained from New England Biolabs (Ipswich, MA). All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

For each yeast strain, single colony was first grown up in 2 mL SC-Ura-Leu medium plus 2% glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, cells were spun down and inoculated into 50 mL of YPA medium supplemented with 4% cellobiose and 5% D-xylose, or 4% cellobiose, 5% xylose, and 0.5% glucose, or 4% cellobiose, 5% xylose, and 1% glucose in a 250 mL unbaffled shake-flask. Starting from an initial OD₆₀₀ ~ 1, cell culture was grown at 30 °C at 100 rpm for fermentation under oxygen limited condition. OD₆₀₀ reading and cell culture sample were taken at various time points. Sugar concentrations were analyzed using HPLC, while ethanol formation was analyzed using the Ethanol Kit (R-biopharm, Darmstadt, Germany). For each data point, triplicate samples were taken. The mixed sugar fermentation results for the strains ranging from SL00 to SL06 are shown in Fig. 65. The best strain SL01 was selected for further characterization.

A total of six different strains, ranging from SL01 to SL06, were constructed by introducing a pRS425 plasmid harboring one of the cellodextrin transporter genes and one of the β -glucosidase genes into the L2612 strain. In each plasmid, the cellodextrin transporter gene and the β -glucosidase gene were added with a yeast promoter and terminator, respectively, and assembled into the pRS425 multi-copy plasmid by the DNA 10 assembler method (Shao *et al.*, 2009) (Fig. 64). The empty pRS425 plasmid was introduced into the L2612 strain to yield the SL00 strain, which was used as a negative control. All

strains were cultivated with a mixture of 40 g/L cellobiose and 50 g/L D-xylose in shake-flasks, and their sugar consumption rates, cell growth rates, and ethanol titers were determined (Fig. 65). Amongst all strains, the SL01 strain containing the β -glucosidase from *Neurospora crassa* and the cellodextrin transporter NCU00801 showed the highest sugar consumption rate and ethanol productivity. Thus, this strain was selected for further characterization.

Both SL01 and SL00 were cultivated using a mixture of 40 g/L cellobiose and 50 g /L D-xylose in both shake-flasks and bioreactors (Fig. 66). In the shake-flask cultivation (Fig. 66a-b), 83% cellobiose was consumed in 96 hours by SL01, with 41.2% higher average D-xylose consumption rate compared to SL100 (from 0.33 g/L/h to 0.46 g/L/h). Consistent with the enhanced sugar consumption rate, 1.32-fold increased average biomass growth rate was observed (from 0.031 g dry cell weight/L/h to 0.072 g dry cell weight/L/h). The ethanol productivity was increased by more than 2.1-fold, from 0.07 g/L/h to 0.23 g/L/h. The highest ethanol yield of 0.31 g per g sugar was reached in 48 hours, and the average ethanol yield was 0.28 g per g sugar, representing a 23% increase compared to the SL00 strain. In the SL01 cultivation, a faster D-xylose consumption rate was observed, without the lag phase that is the hallmark of glucose repression in co-fermentation of glucose and D-xylose. Moreover, enhanced biomass growth and ethanol production were also observed.

The Multifors system (Infors-HT, Bottmingen, Switzerland) was used for mixed sugar fermentation in bioreactors. Each vessel had a total capacity volume of 750 mL. For each vessel, there was one individual set of pO₂ sensor, air sparger, exit gas cooler, temperature sensor, inoculation port, spare port, dip tube, antifoam sensor, pH sensor, drive shaft, heater block, rotameter, and peristaltic pumps system. The whole bioreactor system was equipped with a cooling system, ThermoFlex900 (Thermo Scientific, Waltham, MA).

Single colonies of yeast strains were first grown up in 2 mL SC-Ura-Leu medium plus 2% glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, 10 mL saturated culture were inoculated in 400 mL YPA medium supplemented with 4% cellobiose and 5% D-xylose, or 4% cellobiose, 5% xylose, and 0.5% glucose, or 4% cellobiose, 5% xylose, and 1% glucose. The temperature was maintained at 30 °C and the pH was maintained at 5.5, adjusted by addition of either 2 N H₂SO₄ or 4 N NaOH. In the first 48 hours, the air flow rate was maintained at 0.5 L/min, with the impeller speed at

250 rpm. Afterwards, the air flow rate was adjusted to 0.2 L/min to achieve high ethanol production under oxygen limited condition. Triplicate samples were taken at various time points and the OD₆₀₀, sugar concentration, and ethanol concentration were determined as described above.

In the bioreactor cultivation (Fig. 66c-d), almost all cellobiose and 66% D-xylose were consumed in 48 hours, representing 44% increased D-xylose consumption rate (from 0.47 g/L/h to 0.68 g/L/h) and 1.1-fold increased biomass growth rate (from 0.08 g dry cell weight/L/h to 0.17 g dry cell weight/L/h). The ethanol productivity was increased by more than 4.3-fold (from 0.09 g/L/h to 0.50 g/L/h), and the ethanol yield was 0.39 g per g sugar. Compared to shake-flask cultivations, sugar consumption rates in the first 24 hours were lower, which was due to the low cell density used in the beginning of batch cultivation.

Unexpectedly, a small amount of glucose was detected even though there was no glucose added in fermentation (Fig. 66a-b). The maximum glucose concentration was reached in approximately 24 hours in both shake-flasks (12.1 g/L) and bioreactors (17.5 g/L) and then dropped to a very low level. However, no obvious glucose repression was observed even in the presence of such glucose. Because no glucose was detected in the SL00 strain, the extracellular glucose may result from the slow conversion of β -glucose to its epimer α -glucose, the main form of glucose used in glycolysis. Typically, β -glucose can be efficiently converted to α -glucose either enzymatically or chemically because of its relatively low concentration in glucose (Bouffard *et al.*, 1994). However, in the engineered SL01 strain, catalyzed by β -glucosidase, an excess amount of β -glucose is produced from cellobiose intracellularly and a small fraction may be secreted outside cells, similar to what was observed with β -galactose (Bouffard *et al.*, 1994).

Because a small amount of glucose (less than 10% of total sugars) is typically present in lignocellulosic hydrolysates in industrial settings, the fermentation performance of the engineered SL01 strain was also investigated using a mixture of cellobiose, D-xylose, and glucose. Two concentrations of glucose, 5 g/L or 10 g/L, were combined with 40 g/L cellobiose and 50 g/L D-xylose as mixed carbon source in bioreactors. With 5 g/L glucose (Fig. 67a-b), 81.7% cellobiose was consumed by SL01, with 67.8% D-xylose consumed at 48 hours in batch cultivation. The D-xylose consumption rate was increased by 1.19-fold, from 0.32 g/L/h to 0.69 g/L/h. The ethanol productivity was increased by 3.3-fold (from 0.11 g/L/h to 0.46 g/L/h) while the ethanol yield was increased from 0.26 g per g sugar to 0.33 g per g

sugar. With 10 g/L glucose (Fig. 67c-d), 83.8% cellobiose was consumed by SL01, with 74.7% D-xylose consumed at 48 hour in batch cultivation. The D-xylose consumption rate was increased by 68%, from 0.45 g/L/h to 0.76 g/L/h. The ethanol productivity was increased by 2.1-fold (from 0.16 g/L/h to 0.50 g/L/h) and the ethanol yield was increased from 0.30 g per g sugar to 0.33 g per g sugar. As expected, the engineered SL01 strain showed both a higher efficiency of sugar consumption and a higher rate of ethanol production than the SL00 wild type strain. More importantly, there was no significant glucose repression in the co-fermentation of three sugars even with glucose up to 10% of total sugars (Fig. 67c-d) suggesting that this approach may be viable for industrial applications.

A similar study was carried out in the *S. cerevisiae* strain D452-2, where the three *N. crassa* cellodextrin transporters NCU00801, NCU08114, and NCU00809 were introduced together with the β -glucosidase NCU00130. The transformants were selected on YSC medium containing 20 g/liter cellobiose expressing an intracellular β -glucosidase (NCU00130). Strains and plasmids used in this work are described in Table 17 (Ex. 12). The primers used are listed in Table 28.

Table 28 shows the synthetic oligonucleotides used in the study.

Name	Sequences
NCU00801-F	ATGGATCCAAAAATGTCGTCTCACGGCTCC SEQ ID NO: 92
NCU00801-R	ATGAATTCCTACAAATCTTCTTCAGAAATCAATTTTTGTTCA GCAACGATAGCTTCGGAC SEQ ID NO: 93
NCU08114-F	ATACTAGTAAAAAATGGGCATCTTCAACAAGAAGC SEQ ID NO: 94
NCU08114-R	GCATATCGATCTACAAATCTTCTTCAGAAATCAATTTTTGTT CAGCAACAGACTTGCCCTCATG SEQ ID NO: 95
NCU00130-F	GCATACTAGTAAAAAATGTCTCTTCTTAAGGATTTCCTCT SEQ ID NO: 96
NCU00130-R	ATACTGCAGTTAATGATGATGATGATGGTCCTTCTTGA TCAAAGAGTCA AAG SEQ ID NO: 97

Yeast were grown in YP medium containing 20 g/L of glucose or 20 g/L of cellobiose to prepare inoculums for xylose or cellobiose fermentation experiments, respectively. Cells at mid-exponential phase from YP media containing 20 g/L of glucose or cellobiose were harvested and inoculated after washing twice with sterilized water. All of the flask fermentation experiments were performed using 50

mL of YP medium containing 40 g/L or 80 g/L of xylose in 250 mL flask at 30 °C with initial OD₆₀₀ of 1.0 under oxygen limited conditions. Bioreactor fermentations were performed in 400 mL of YP medium containing appropriate amounts of sugars using Sixfors Bioreactors (Appropriate Technical Resources, Inc) at 30 °C with an agitation speed of 200 rpm under oxygen limited 250 conditions. Initial cell densities were adjusted to OD₆₀₀ = 1.0.

Cell growth was monitored by optical density (OD) at 600 nm using UV-visible Spectrophotometer (Biomate 5, Thermo, NY). Glucose, xylose, xylitol, glycerol, acetate, and ethanol concentrations were determined by high performance liquid chromatography 264 (HPLC, Agilent Technologies 1200 Series) equipped with a refractive index detector using 265 a Rezex ROA-Organic Acid H+ (8%) column (Phenomenex Inc., Torrance, CA). The column was eluted with 0.005 N of H₂SO₄ at a flow rate of 0.6 mL/min at 50 °C.

All three transformants were able to grow and produce ethanol when cellobiose was the sole carbon source (Fig. 68), but the three transformants exhibited different cellobiose fermentation rates (NCU00801 > NCU08114 > NCU00809). The fastest cellulose-fermenting transformant (D801-130), expressing both NCU00801 and NCU00130, consumed 40 g/L of cellobiose within 4 hours, producing 16.8 g/L of ethanol. The volumetric productivity of cellobiose fermentation ($P_{\text{Ethanol/Cellobiose}} = 0.7 \text{ g/L/h}$) was lower than that of glucose fermentation ($P_{\text{Ethanol/Glucose}} = 1.2 \text{ g/L/h}$), and ethanol yield from cellobiose ($Y_{\text{Ethanol/Cellobiose}} = 0.42 \text{ g/g}$) was about the same as ethanol yield from glucose ($Y_{\text{Ethanol/Glucose}} = 0.43 \text{ g/g}$) under the same culture conditions. However, the observed cellobiose consumption rate and ethanol yield by D801-130 were an improvement over *S. cerevisiae* strains engineered to ferment cellobiose through surface display of β -glucosidase (Kotaka *et al.*, 2008; Nakamura *et al.*, 2008). These results suggest that simultaneous expression of NCU00801 and NCU00130 in *S. cerevisiae* can result in efficient cellobiose fermentation.

After developing the efficient xylose fermenting strain DA24-16 (described in Example 13), genes coding cellodextrin transporter and β -glucosidase (NCU00801 and NCU00130) enzyme were introduced into the strain enabling it to consume cellobiose and xylose simultaneously. It was hypothesized that glucose repression of xylose utilization may be alleviated in this strain, due to the intracellular hydrolysis of cellobiose. The NCU00801 gene was integrated into the genome of DA24-16, and NCU00130 was

expressed from a multi-copy plasmid. The resulting transformant, DA24-16-BT3, was selected on an agar plate containing cellobiose as the sole carbon source.

The DA24-16- BT3 strain grown in media containing various amounts of cellobiose and xylose co-consumed cellobiose and xylose, and produced ethanol with yields of 0.38-0.39 g/g in all conditions tested (Fig. 69). The potential synergistic effects of co-fermentation were tested by culturing DA2416-BT3 under three different conditions: 40 g/L of cellobiose, 40 g/L of xylose, and 40 g/L of both sugars (total 80 g/L of sugars). Surprisingly, DA24-16BT3 was able to co-consume 80 g/L of a cellobiose/xylose mixture within the same period that was required to consume 40 g/L of cellobiose or 40 g/L xylose separately (Fig. 70). Moreover, DA24-16BT3 produced ethanol with a higher yield (0.39 g/g) from a mixture of cellobiose and xylose as compared to ethanol yields (0.31 ~ 0.33 g/g) from single sugar fermentations (cellobiose or xylose). Ethanol productivity also drastically increased from 0.27 g/L/h to 0.65 g/L/h during co-fermentation. These results demonstrated that co-fermentation of cellobiose and xylose can enhance overall ethanol yield and productivity. Fermentation experiments were also done to compare this engineered *S. cerevisiae* strain (DA24-16BT3) to *P. stipitis*, which is capable of co-fermenting cellobiose and xylose efficiently.

A simulated hydrolysate (10 g/L of glucose, 80 g/L of cellobiose, 40 g/L of xylose) based on the composition of energycane was used. The composition of different lignocellulosic plants varies in a broad range. For instance, the US Department of Energy biomass database lists the composition of more than 150 biomass samples (webpage eere.energy.gov/biomass/m/feedstock_databases.html). The cellulose-to-hemicellulose ratios of these samples are between 1.4 and 19, and the average is 2.3. Energy crops typically have higher hemicellulose content than woody biomass. The average cellulose to hemicellulose ratios of sugarcane bagasse, corn stover, sorghum are 2.0, 1.85 and 2.14, respectively. We therefore used a glucan/xylan ratio of 2 in our simulated sugar experiment design. The engineered yeast will likely be used in conjunction with traditional cellulase cocktails that are deficient in β -glucosidase activities for the biofuels production. The biomass hydrolysis process may result in small amounts of glucose in the lignocellulosic hydrolysates as 6-30% glucan-to-glucose conversions with incomplete cellulase cocktails were reported (Medve *et al.*, 1998). Considering all the above factors, a sugar combination of 10 g/L glucose, 80 g/L cellobiose, and 40 g/L xylose was chosen in the simulated sugar experiments.

The DA24-16BT3 consumed glucose first before co-consuming cellobiose and xylose rapidly. A total of 130 g/L of sugars was consumed within 60 hours even though small inoculums were used ($OD_{600} = 1$). In contrast, *P. stipitis* could not finish fermenting the sugar mixture within the same period under identical culture conditions (Fig. 71). DA24-16BT3 produced 48 g/L of ethanol within 60 hours ($Y_{\text{Ethanol/Sugars}} = 0.37 \text{ g/g}$ and $P_{\text{Ethanol/Sugars}} = 0.79 \text{ g/L/h}$).

A transient accumulation of cellodextrins in the medium during cellobiose consumption was observed (Fig. 72-73). The accumulated cellotriose and cellotetraose were again consumed after depletion of cellobiose. It is likely that the accumulated cellodextrins were generated by the trans-glycosylation activity (Christakopoulos *et al.*, 1994) of β -glucosidase (NCU00130), and secreted by the cellodextrin transporter (NCU00801), which might facilitate the transport of cellodextrins in both directions (intracellular $\leftrightarrow$ extracellular). This transient cellodextrin accumulation would probably not reduce product yields since the accumulated cellodextrins would eventually be consumed by the engineered yeast. However, it might decrease productivity because the transport rates of cellotriose and cellotetraose might be slower than that of cellobiose.

Small amounts of glucose were constantly detected in the medium during co-fermentation. Since even low amounts of glucose accumulation can repress xylose fermentation, glucose levels have to be kept at a minimum. It can be hypothesized that the relative expression levels of the cellodextrin transporter and β -glucosidase are likely to affect glucose accumulation. In support of this, it was observed that more glucose was accumulated in the medium when NCU00801 was introduced on a multi-copy plasmid than when NCU00801 was integrated into the yeast genome. The strain (DA24-16-BT), containing both NCU00801 and NCU00130 on multi-copy plasmids, had relatively slower xylose utilization rates than those observed in DA24-16-BT3, a potential reason being glucose repression (Fig. 74). Further adjustments of the cellodextrin transporter and β -glucosidase expression levels, or the identification of β -glucosidases with reduced trans-glycosylation activities, may be able to reduce the accumulation of glucose and cellodextrin during co-fermentation.

Co-fermentation of xylose and cellobiose could also be achieved by mixed cultivation of two different yeast strains: the xylose-fermenting DA24-16 strain and the cellobiose-fermenting DA452BT (Fig: 75). As explained above, the yeast strain DA24-16 expressed the xylose-utilizing enzymes wild type xylose reductase (*XYL1*), mutant xylose reductase R276H (*mXYL1*), xylitol dehydrogenase (*XYL2*),

and xylulokinase (*XKS1*) (Ex. 12; Table 17). D452BT was formed by engineering D452 to express the cellodextrin transporter NCU00801 and the β -glucosidase NCU00130. In the mixed culture, the DA24-16 strain took up xylose (xylose molecule shown as a green pentagon in Fig: 75a) and metabolized it using the enzymes *XYL1* (wild type and mutant), *XYL2*, and *XYL3*, whereas the other strain D452BT was able to take up cellobiose (cellobiose molecule shown as two red hexagons in Fig: 75a) using the transporter NCU00801 and convert the cellobiose into glucose using the enzyme NCU00130. Hence, the mixed culture was able to co-ferment both xylose and cellobiose to produce ethanol (Fig: 75b).

This study demonstrated a novel strategy to allow co-fermentation of hexose and pentose sugars by *S. cerevisiae*. By combining an efficient xylose utilization pathway with a cellodextrin transport system, the problem caused by glucose repression was over-come. As a result, the engineered yeast co-fermented two non-metabolizable sugars in cellulosic hydrolysates synergistically into ethanol. The new co-fermentation method described herein advances lignocellulosic technologies on both the saccharification and fermentation fronts. Most traditional fungal cellulase cocktails are deficient in β -glucosidase and end the cellulose hydrolysis with cellobiose that is not fermented efficiently by yeast. As a result, extra β -glucosidase enzyme must be added to convert cellobiose into glucose. The cellobiose/xylose co-fermentation yeast makes it possible to use these cellulase cocktails with limited β -glucosidase activities, lowering enzyme usage and cost associated with the cellulose saccharification process. Further, the synergy between cellobiose and xylose co-fermentation significantly increases ethanol productivity, thus improving fermentation economics. The presence of a small amount of glucose from the pre-treatment and hydrolysis of lignocellulosic materials does not affect the capacity of the engineered yeast to convert hexose and pentose sugar mixtures into ethanol.

This study involved measuring the capacity of an engineered *S. cerevisiae* strain to ferment various mixtures of sugars meant to mimic hydrolysates from plant biomass. The ability of this strain to co-ferment cellodextrins and xylose is particularly useful during the simultaneous saccharification and co-fermentation (SSCF) of pre-treated plant biomass. During SSCF, hemicellulose would first be hydrolyzed by acid pre-treatment, resulting in formation of xylose and still-crystalline cellulose. Then, fungal cellulases and the yeast strain described herein would be added, allowing the cellulases to co-convert xylose and cellobiose into ethanol. Because of the limited extracellular glucose production in this scheme, there will be reduced repression of xylose utilization and co-fermentation will proceed rapidly and synergistically.

Although the *S. cerevisiae* strain used in this study was a laboratory strain, the fermentation performance of the engineered strain was very impressive when compared to published results. The key fermentation parameters (yield and productivity) may be further improved by the use of industrial yeast strains as a platform. Applications of this co-fermentation strategy would not be limited to ethanol production. Since it is a foundational technology, the strategy presented here can be combined with any other product diversification technologies to produce commodity chemicals and advanced biofuels.

Example 18 - Transcriptome Analysis of N. crassa Grown on Xylan

Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin. Examples 1-3 describe the discovery of genes critical for growth on cellulose through transcriptome and secretome analysis of *N. crassa*. In this example the expression profile of the *N. crassa* genome was examined during growth on xylan to determine which genes are important for utilization of hemicellulose.

Ten day old conidia of WT or $\Delta xlnR$ strains were inoculated at 10^6 conidia/mL on 100mL 1X Vogel's salts minimal medium (2% sucrose), grown for 16 hours at 25 °C with constant light, and washed with 1X Vogel's only medium. Conidia were then transferred into 100mL 1X Vogel's salts with 2% sucrose or 2% Beechwood xylan as the sole carbon source in the medium and allowed to grow for 4 hours. Mycelia were harvested by filtration and immediately flash frozen in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions and treated with DNase (Turbo DNA-free kit; Ambion) (Kasuga, Townsend *et al.*, 2005).

For cDNA synthesis and labeling, the Pronto kit (Catalog No. 40076; Corning) was used according to the manufacturer's specifications except that the total RNA used was 10 µg per sample.

Microarray hybridization and data analysis were performed as previously described (Tian, Kasuga *et al.*, 2007). A GenePix 4000B scanner (Axon Instruments) was used to acquire images, and GenePix Pro6 software was used to quantify hybridization signals and collect the raw data. Normalized expression values were analyzed by using the BAGEL (Bayesian analysis of gene expression levels) software program (Townsend and Hartl 2002; Townsend 2004). 354 genes were found to be induced greater than 2-fold in *N. crassa* grown on xylan. The list is shown in Fig. 76.

Example 19 - Secretome Analysis of *N. crassa* Grown on Xylan

The secretome of *N. crassa* during growth on xylan was analyzed using a shotgun proteomics approach. Supernatants from xylan cultures were digested with trypsin and analyzed by liquid chromatography nano-electrospray ionization tandem mass spectrometry.

Mass spectrometry samples were prepared as follows. *N. crassa* wild type strain was grown on 2% xylan media for 4 or 7 days. Culture supernatants were isolated by centrifugation, filtered through 0.22 μ m filters, and concentrated 10 times with 10 kDa MWCO PES spin concentrators. 3.36 mg of urea, 5 μ L of 1M Tris pH 8.5, and 5 μ L of 100 mM DTT were then added to 100 μ L of concentrated culture supernatant, and the mixture was heated at 60 °C for 1 hour. After heating, 700 μ L of 25 mM ammonium bicarbonate and 140 μ L of methanol were added to the solution followed by treatment with 50 μ L of 100 μ g/mL trypsin in 50 mM sodium acetate pH 5.0. The trypsin was left to react overnight at 37 °C with inverting for about 8-9 hours at basal pH. After digestion the volume was reduced to dryness by speedvac and washed with 300 μ L MilliQ water three times. The final volume was 100 μ L. TFA was added at 0.1 - 0.3% v/v. Residual salts in the sample were removed by using OMIX microextraction pipette tips according to the manufacturer's instructions. The acetonitrile was removed by evaporation. The sample solution was an aqueous solution with 0.1% - 1% TFA, and the final volume was 10 microliters or greater.

Example 20 - Analysis of Xylan-Induced Genes Predicted to Encode Secreted Proteins

The transcriptome and secretome analysis results indicated a total of 71 genes, of which 55 were predicted to be secreted. The list of these genes is in Table 29. Deletion strains were available for 46 out of 69 genes. Out of these 46, six of the strains were heterokaryons, thus the remaining 40 deletion strains were analyzed for total secreted protein, amount of xylose present, and azo-endo-xylanase activity. Results are shown in Fig. 77.

Table 29 shows xylan-induced *N. crassa* genes

Gene Name	Signal P	Data	Annotation
NCU00642	Y	Transcription	probable beta-galactosidase
NCU00695	Y	Transcription	putative protein
NCU00798		MS	hypothetical protein
NCU00937	Y	Transcription	conserved hypothetical protein

NCU01517	Y	Transcription	glucan 1,4-alpha-glucosidase
NCU02136		MS	probable transaldolase
NCU02252		MS	probable phosphoglyceromutase
NCU02343	Y	Transcription	related to alpha-L-arabinofuranosidase A precursor
NCU02455	Y	Transcription	FK506-binding protein 2 precursor (Peptidyl-prolyl cis-trans isomerase)
NCU02583	Y	Transcription	probable Alpha-glucosidase precursor (Maltase)
NCU03013	Y	Transcription	related to cytosolic Cu/Zn superoxide dismutase
NCU03222	Y	Transcription	putative protein
NCU03636	Y	Transcription	
NCU03639	Y	Transcription	probable triacylglycerol lipase precursor
NCU04202		MS	nucleoside-diphosphate kinase
NCU04265	Y	Transcription	related to beta-fructofuranosidase
NCU04388	Y	Transcription	probable phosphatidylglycerol/phosphatidylinositol transfer protein
NCU04395		MS	beta-1,6-glucanase Neg1 NEG-1
NCU04415	Y	Transcription	related to brefeldin A resistance protein
NCU04431	Y	MS	related to endo-1,3-beta-glucanase
NCU04475	Y	Transcription	probable lipase B precursor
NCU04482		MS	hypothetical protein
NCU04623	Y	Transcription	related to beta-galactosidase
NCU04674	Y	Transcription	related to alpha-glucosidase b
NCU04675	Y	Transcription	putative protein
NCU04930	Y	Transcription	related to triacylglycerol lipase
NCU05137	Y	Transcription	conserved hypothetical protein
NCU05143	Y	Transcription	related to Rds1 protein
NCU05159	Y	Transcription	probable acetylxylan esterase precursor
NCU05275		MS	probable ubiquitin fusion protein (ubiquitin / ribosomal protein)
NCU05315	Y	Transcription	hypothetical protein
NCU05395	Y	Transcription	conserved hypothetical protein
NCU05686	Y	MS	probable cell wall protein UTR2
NCU05751	Y	Transcription	related to acetylxylan esterase
NCU05924	Y	Transcription	probable endo-beta-1,4-D-xylanase
NCU05965	Y	Transcription	related to putative arabinase
NCU05974		MS	related to cell wall protein (putative glycosidase)
NCU06364	Y	Transcription	hypothetical protein

NCU06380	Y	Transcription	related to catecholamines up protein
NCU06650	Y	Transcription	conserved hypothetical protein
NCU06781		MS	probable beta (1-3) glucanosyltransferase
NCU06961	Y	Transcription	probable exopolygalacturonase
NCU07067		MS	related to class I alpha-mannosidase 1B
NCU07143	Y	Transcription	
NCU07190	Y	Transcription	related to cellulose 1,4-beta-cellobiosidase II precursor
NCU07200	Y	MS	related to metalloprotease MEP1
NCU07225	Y	Transcription	probable endo-1,4-beta-xylanase B precursor
NCU07281		MS	probable glucose-6-phosphate isomerase
NCU07787	Y	MS	probable SnodProt1 precursor
NCU08131	Y	Transcription	probable alpha-amylase precursor
NCU08171	Y	MS	conserved hypothetical protein
NCU08189	Y	Transcription	related to endo-1,4-beta-xylanase
NCU08384		MS	probable D-xylose reductase
NCU08418		MS	related to tripeptidyl-peptidase I
NCU08457	Y	Transcription	hydrophobin Ccg-2 CCG-2
NCU08516	Y	Transcription	related to aldose 1-epimerase
NCU08750	Y	Transcription	related to isoamyl alcohol oxidase
NCU08752	Y	Transcription	related to esterase
NCU08755	Y	Transcription	hypothetical protein
NCU08909	Y	MS	probable beta (1-3) glucanosyltransferase gel3p
NCU08936		MS	related to sporulation-specific gene SPS2
NCU09024	Y	MS	related to choline dehydrogenase
NCU09133	Y	Transcription	putative protein
NCU09170	Y	MS	probable alpha-N-arabinofuranosidase
NCU09175	Y	Transcription	related to glucan 1,3-beta-glucosidase precursor
NCU09267		MS	related to glyoxal oxidase precursor
NCU09491		MS	feruloyl esterase B precursor (subclass of the carboxylic acid esterases)
NCU09923	Y	Transcription	related to xylan 1,4-beta-xylosidase
NCU09924	Y	Transcription	conserved hypothetical protein
NCU10040	Y	Transcription	
NCU10045	Y	Transcription	

Samples were prepared as follows. 10 day old conidia were grown in 100 mL 2% xylan Vogel's media at 10^6 conidia/mL. Two replicates were prepared for each strain. Cultures were grown at 25 °C with constant light and 220 rpm. Samples were harvested on day 4. Supernatants were isolated by centrifugation and used in assays.

Bradford protein concentrations were measured to determine the total amount of secreted protein. Stocks were prepared with BSA standards: 0 µg/mL, 50 µg/mL, 100 µg/mL, 250 µg/mL, and 500 µg/mL. Bradford solution was diluted 1:4. A multichannel pipette was used to pipette 200 µL of Bradford solution into a 96-well plate. 10 µL of sample and 10 µL of each standard were added. Samples were incubated at room temperature for 10 minutes. The absorbance was read at 595 nm, and the protein concentration was determined.

The assay used to measure xylose was modified from Bailey *et al.*, 1992 (*J Biotech* 23: 257-270). Xylose standards were prepared in H₂O. For concentrated 0.8 M xylose (1.2 g in 10 mL), the standards included 0 mM, 8 mM (1:100 dilution; 990 µl + 10 µl), 20 mM (1:100 dilution; 975 µl + 25 µl), 40 mM (1:100 dilution; 950 µl + 50 µl), 80 mM (1:100 dilution; 900 µl + 100 µl), and 160 mM (1:100 dilution; 800 µl + 200 µl). A multichannel pipette was used to add 900 µL of substrate solution to a deep well 96-well plate. The substrate was allowed to incubate at 50 °C for 10 minutes. One hundred µL of culture supernatant and the standards were added and allowed to incubate at 50 °C for 5 minutes. Samples were centrifuged for 10 minutes at 3,400 rpm. A multichannel pipette was used to pipette 75 µL DNS solution into a 96-well PCR plate. Five µL of solution was removed from the reaction and added to the PCR plate containing DNS solution. The plate was heated at 99 °C in the PCR machine for 5 min. After the samples cooled, they were transferred to clear flat-bottomed plates, and the absorbance was read at 540 nm. Substrate solution (500 mL) contained beechwood xylan (5g; 10 mg/mL), 3M NaOAc, pH 5.0 (8.33 mL; 50 mM), water (491 mL), and was autoclaved for 20 minutes. DNS solution (100 mL) contained 3, 5-dinitrosalicylic acid (707 mg), NaOH (1.32 g), Rochelle salts (Na K tartrate) (20.4 g), Sodium meta-bisulfate (553 mg), phenol (507 µL), and water (94.4 mL).

Azo-endo-xylanase activity was measured with a kit from Megazyme. This assay indirectly measures the amount of endo-xylanase activity in a sample by spectrophotometrically measuring the amount of dye liberated from a xylan chain complexed with the dye. The more enzymes that are present, the more dye will be released. All supernatant samples were diluted 1:10 by adding 50 µL of supernatant

to 450 μ L of Na Acetate buffer (50 mM, pH 4.5) in separate 15mL Falcon tubes. Next, Falcon tubes were pre-warmed about 10 minutes. Substrate solution was added for all samples (500 μ L/sample) to the tubes. Samples and substrate solutions were added into a 40 °C water bath for 10 minutes to pre-equilibrate them. Five hundred μ L substrate solution was added to each 1:10 diluted sample, vortexed for 10 seconds, and incubated at 40 °C for 10 minutes. The reaction was terminated by adding 2.5 mL of precipitant solution (95% ethanol) to each sample and vortexing for 10 seconds. Tubes were allowed to stand at room temperature for 10 minutes. Tubes were vortexed for 10 seconds and then centrifuged at room temperature for 10 minutes at 1,000g. One mL of supernatant solution from each tube was placed directly into a cuvette, and the absorbance was measured at 590 nm. The blank used for this procedure was the supernatant from 500 μ L substrate solution added to 2.5 mL of precipitant solution.

In conclusion, it is anticipated that the modulation of genes identified here that affect the degradation of hemicellulose in *N. crassa* will facilitate engineering strains that have enhanced capacity for plant cell wall breakdown and growth on plant cell wall components such as hemicellulose. Genes of interest include NCU01517, which encodes a predicted glucamylase; NCU02343, which encodes a predicted arabinofuranosidase; NCU05137, which encodes a conserved hypothetical protein; NCU05159, which encodes a predicted acetylxylan esterase precursor; NCU09133, which encodes a conserved hypothetical protein; and NCU10040, which encodes a hypothetical protein.

The growth of a cell on hemicellulose will be increased by providing a host cell that contains a recombinant polynucleotide that encodes a polypeptide encoded by NCU01517, NCU09133, or NCU10040. The host cell will be cultured in a medium that contains hemicellulose such that the recombinant polynucleotide is expressed. The host cell will grow at a faster rate in this medium than a cell that does not contain the recombinant polynucleotide.

Example 21– Further Analysis of the Δ NCU05137 Strain

As described in Examples 1-3 and 18-20, NCU05137 is a predicted secreted protein that was overexpressed during growth of *N. crassa* on any of *Miscanthus*, Avicel, or xylan. A deletion strain of *N. crassa* lacking NCU05137 grown on Avicel showed increased endoglucanase, β -glucosidase, and Avicelase activity. An NCU05137 deletion strain grown on xylan showed increased azo-endo-xylanase activity. As described in this example, the complementation of Δ NCU05137 was performed in order to

verify that the phenotypes observed in the Δ NCU05137 strain were due to the loss of the NCU05137 gene.

A plasmid containing NCU05137 with a C-terminal GFP tag under the control of the *cgl1* promoter was generated. *N. crassa* conidia were transformed with the NCU05137-GFP construct. Experiments were performed according to standard *Neurospora* procedures (webpage fgsc.net/Neurospora/NeurosporaProtocolGuide.htm).

The total secreted protein and carboxymethyl cellulase (CMC) activity of wild-type, Δ NCU05137, and Δ NCU05137-NCU05137-GFP strains was measured. Total secreted protein was measured by taking 100 μ L of supernatant from a culture of each strain, adding it to 900 μ L Bradford Dye, and measuring absorbance at 595 nm. CMC activity was measured with 20X diluted supernatant from each strain culture and an azo-CMC kit (Megazyme SCMCL). Δ NCU05137 knockout strains displayed increased levels of secreted protein and CMC activity. Introduction of the GFP-tagged NCU05137 into Δ NCU05137 strains reduced these levels back to wild-type levels (Fig. 78).

In addition, the localization of NCU05137-GFP in complemented strains was observed. NCU05137-GFP localized to the cell wall of conidia and to the hypha tip (Fig. 79-80). These data indicate that the GFP-tagged NCU05137 protein is fully functional and can be used for purification and experiments addressing the biochemical activity of this protein.

Thus, the normal function of NCU05137 may be to inhibit signaling processes associated with induction of cellulase and hemicellulase gene expression. Reduction of expression of NCU05137 or a homolog of NCU05137 in a cell is likely to increase cellulase and hemicellulase activity in that cell and, consequently, growth of the cell on cellulose or hemicellulose. The growth of a cell on cellulose or hemicellulose will be increased by providing a host cell that contains an endogenous polynucleotide that encodes a polypeptide encoded by NCU05137. The expression of the endogenous polynucleotide will be inhibited, and the cell will be cultured in a medium containing cellulose and/or hemicellulose. The host cell will grow at a faster rate in the medium than a cell in which expression of the endogenous polynucleotide is not inhibited.

Example 22 - Further Analysis of NCU07705

Expression of NCU07705 was found to be upregulated during growth of *N. crassa* on cellulose. BLAST analysis of the polypeptide encoded by NCU07705 revealed that the polypeptide has high similarity to many C6 zinc finger domain containing transcription factors (Fig. 1). To further investigate the role of NCU07705 in the utilization of cellulose, the phenotype of a deletion strain lacking NCU07705 was evaluated.

The Δ NCU07705 strain was unable to grow on 2% cellulose (Avicel), PASC, or CMC as a sole carbon source (Table 30) but grew with similar kinetics to wild-type strain on sucrose, xylan, and xylose. In order to determine whether NCU07705 plays a role in regulating expression of cellulases, the expression of cellulase and hemicellulase genes was examined during growth of Δ NCU07705 on cellulose. Ten-day-old conidia from wild-type (FGSC 2489) and Δ NCU07705 strains were inoculated into Vogel's liquid MM (2% sucrose) (Vogel 1956) and grown for 16 hours. Mycelia were centrifuged, washed with 1x Vogel's salts, and then transferred into either Vogel's media with 2% sucrose or 2% Avicel and grown in constant light for 4 hours. They were harvested by filtration and immediately frozen in liquid nitrogen. Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with DNase (Turbo DNA-free kit, Ambion/Applied Biosystems, Foster City, CA) (Kasuga *et al.*, 2005). ChipShot™ Indirect Labeling/Clean-Up System (Catalog No. Z4000, Promega, Madison, WI) and CyDye Post-Labeling Reactive Dye Pack (Catalog No. RPN5661, GE Healthcare, Piscataway, NJ) were used to synthesize and label cDNA according to the manufacturer's instructions except the amount of RNA used was 10 μ g. The Pronto! Hybridization Kit (Catalog No. 40076, Corning, Lowell, MA) was used for microarray hybridization according to the manufacturer's specifications.

Data analyses were performed as previously described (Tian *et al.*, 2007). A GenePix 4000B scanner (Axon Instruments, Union City, CA) was used to acquire images, and GenePix Pro6 software was used to quantify hybridization signals and collect the raw data. Normalized expression values were analyzed by using BAGEL (Bayesian Analysis of Gene Expression Levels) (Townsend and Hartl, 2002). None of the predicted cellulase genes were induced in the Δ NCU07705 strain, whereas induction of predicted hemicellulase genes was unaffected (see Table 30 below). Thus, NCU07705 has been named *cdr-1*, cellulose degradation regulator 1.

Therefore, the growth of a cell on cellulose will be increased by providing a host cell that contains a recombinant polynucleotide that encodes a polypeptide encoded by NCU07705. The host cell will be cultured in a medium that contains cellulose such that the recombinant polynucleotide is expressed. The host cell will grow at a faster rate in this medium than a cell that does not contain the recombinant polynucleotide.

Table 30 shows expression profile of genes in *N. crassa* Δ NCU07705 strain

7705-switch ²	WT-switch ¹	Gene/locus name	GH Family	Class	up in Avi ³
No	15	NCU00762	5	endo-	31.5
No	No	NCU03996	6	CBHII like	
No	168	NCU07190	6	CBHII like	119
No	26	NCU09680	6	CBHII	251.3
No	18	NCU04854	7	CBHI like	10.8
No	3.8	NCU05057	7	CBHI like	7.4
No	No	NCU05104	7	CBHI like	
No	93	NCU07340	7	CBHI	382.2
No	2	NCU05121	45	endo-	17.2
No	5.8	NCU00836	61	endo-	31
No	3.7	NCU01050	61	endo-	382.1
No	No	NCU01867	61	endo-	
No	49	NCU02240	61	endo-	84
No	No	NCU02344	61	endo-	4.1
No	6.1	NCU02916	61	endo-	17.7
No	No	NCU03000	61	endo-	
No	17	NCU03328	61	endo-	23.8
No	No	NCU05969	61	endo-	12.7
No	No	NCU07520	61	endo-	
No	No	NCU07760	61	endo-	
No	103	NCU07898	61	endo-	230
No	No	NCU07974	61	endo-	
No	25	NCU08760	61	endo-	44.7

¹ Expression levels of predicted cellulase genes from an *N. crassa* (NCU07705) culture grown in Vogel's/sucrose for 16 hours, filtered, and resuspended in Vogel's/Avicel for 4 hours prior to RAN extraction.

² Expression levels of predicted cellulase gene from an *N. crassa* (wild type FGSC 2489) culture grown in Vogel's/sucrose for 16 hours, filtered, and resuspended in Vogel's/sucrose for 4 hours prior to RNA extraction.

³ Expression levels derived from microarray analyses of wild type (FGSC 2489) cells grown for 30 hours in Avicel (Tian *et al.*, 2009).

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CLAIMS

1. A method of increasing transport of cellodextrin into a cell, comprising:
 - providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 comprises SEQ ID NO: 1, and
 - culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.
2. A method of increasing transport of cellodextrin into a cell, comprising:
 - providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 comprises SEQ ID NO: 2, and
 - culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.
3. A method of increasing transport of cellodextrin into a cell, comprising:
 - providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, and

- culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.
4. A method of increasing transport of cellodextrin into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 comprises SEQ ID NO: 4, and
 - culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.
5. A method of increasing transport of cellodextrin into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 comprises SEQ ID NO: 5, and
 - culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.
6. A method of increasing transport of cellodextrin into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12,

and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, and

- culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.

7. A method of increasing transport of cellodextrin into a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 comprises SEQ ID NO: 7, and

- culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.

8. A method of increasing transport of cellodextrin into a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8, and

- culturing the cell in a medium such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of cellodextrin into the cell compared with a cell that does not comprise the recombinant polynucleotide.

9. The method of any of claims 1 - 8 wherein the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least

70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114.

10. The method of any of claims 1 - 9, wherein the host cell further comprises a second recombinant polynucleotide encoding at least a catalytic domain of a β -glucosidase.
11. The method of claim 10 wherein the β -glucosidase is from *Neurospora crassa*.
12. The method of claim 11 wherein the β -glucosidase is encoded by NCU00130.
13. The method of any of claims 1 - 12, wherein the host cell further comprises one or more recombinant polynucleotides wherein the one or more polynucleotides encode one or more enzymes involved in pentose utilization.
14. The method of claim 13, wherein the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.
15. The method of any of claims 1 - 14, wherein the host cell further comprises a third recombinant polynucleotide wherein the third recombinant polynucleotide encodes a pentose transporter.
16. The method of claim 15, wherein the pentose transporter is selected from the group consisting of NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, and XUT3.
17. A method of increasing growth of a cell, comprising:
 - providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 comprises SEQ ID NO: 1, and the polypeptide is a cellodextrin transporter; and

- culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
18. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 comprises SEQ ID NO: 2, and the polypeptide is a cellodextrin transporter; and
 - culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
19. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, and the polypeptide is a cellodextrin transporter; and
 - culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
20. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 comprises SEQ ID NO: 4, and the polypeptide is a cellodextrin transporter; and

- culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

21. A method of increasing growth of a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 comprises SEQ ID NO: 5, and the polypeptide is a cellodextrin transporter; and
- culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

22. A method of increasing growth of a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, and the polypeptide is a cellodextrin transporter; and
- culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

23. A method of increasing growth of a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 comprises SEQ ID NO: 7, and the polypeptide is a cellodextrin transporter; and

- culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
24. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8, and the polypeptide is a cellodextrin transporter; and
 - culturing the host cell in a medium comprising cellodextrin, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
25. The method of any of claims 17 - 24 wherein the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114.
26. The method of any of claims 17 - 25 wherein the host cell further comprises an endogenous or a second recombinant polynucleotide wherein the polynucleotide encodes at least a catalytic domain of a β -glucosidase.
27. The method of claim 26 wherein the β -glucosidase is from *Neurospora crassa*.
28. The method of claim 27 wherein the β -glucosidase is encoded by NCU00130.
29. A method of co-fermenting cellulose-derived and hemicellulose-derived sugars, comprising:
- providing a host cell, wherein the host cell comprises a first recombinant polynucleotide encoding a cellodextrin transporter and a second recombinant polynucleotide encoding a catalytic domain of a β -glucosidase, and

- culturing the host cell in a medium comprising a cellulose-derived sugar and a hemicellulose-derived sugar, wherein expression of the recombinant polynucleotides enables co-fermentation of the cellulose-derived sugar and the hemicellulose-derived sugar.

30. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 comprises SEQ ID NO: 1.
31. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 comprises SEQ ID NO: 2.
32. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3.
33. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 comprises SEQ ID NO: 4.
34. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 comprises SEQ ID NO: 5.
35. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6.

36. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 comprises SEQ ID NO: 7.
37. The method of claim 29 wherein the first recombinant polynucleotide encodes a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8.
38. The method of any of claims 30 - 37 wherein the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114.
39. The method of any of claims 29-38 wherein the β -glucosidase is from *Neurospora crassa*.
40. The method of claim 39 wherein the β -glucosidase is encoded by NCU00130.
41. The method of any of claims 29 - 40 wherein the host cell further comprises one or more recombinant polynucleotides wherein the one or more polynucleotides encode one or more enzymes involved in pentose utilization.
42. The method of claim 41 wherein the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.
43. The method of any of claims 29-42 wherein the host cell further comprises a third recombinant polynucleotide wherein the polynucleotide encodes a pentose transporter.
44. The method of claim 43 wherein the pentose transporter is selected from the group consisting of NCU00821, NCU04963, NCU06138, STL12/XUT6, SUT2, SUT3, XUT1, and XUT3.

45. The method of any of claims 29 - 44 wherein the cellulose-derived sugar is selected from the group consisting of cellobiose, cellotriose, and celltetraose, and the hemicellulose-derived sugar is xylose.
46. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 1 comprises SEQ ID NO: 1, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and
 - culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.
47. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 2 comprises SEQ ID NO: 2, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and
 - culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.
48. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and a loop connecting transmembrane α -helix 2 and transmembrane α -helix 3 comprises SEQ ID NO: 3, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

49. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 5 comprises SEQ ID NO: 4, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

50. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 6 comprises SEQ ID NO: 5, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

51. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and sequence between transmembrane α -helix 6 and transmembrane α -helix 7 comprises SEQ ID NO: 6, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

52. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 7 comprises SEQ ID NO: 7, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.

53. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide comprising transmembrane α -helix 1, α -helix 2, α -helix 3, α -helix 4, α -helix 5, α -helix 6, α -helix 7, α -helix 8, α -helix 9, α -helix 10, α -helix 11, α -helix 12, and transmembrane α -helix 10 and transmembrane α -helix 11 and the sequence between them comprise SEQ ID NO: 8, and the polypeptide transports cellodextrin into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and
 - culturing the host cell in a medium comprising cellodextrin or a source of cellodextrin to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of cellodextrin into the cell is increased upon expression of the recombinant polynucleotide.
54. The method of any of claims 46 - 53, wherein the polypeptide has at least 29%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or at least 100% amino acid identity to NCU00801 or NCU08114.
55. The method of any of claims 46 - 54 wherein the host cell further comprises a second recombinant polynucleotide wherein the polynucleotide encodes at least a catalytic domain of a β -glucosidase.
56. The method of claim 55 wherein the β -glucosidase is from *Neurospora crassa*.
57. The method of claim 56 wherein the β -glucosidase is encoded by NCU00130.
58. The method of claims 46 - 57, wherein the source of the cellodextrin comprises cellulose.
59. The method of any of claims 46 - 58, wherein the hydrocarbons or hydrocarbon derivatives can be used as fuel.
60. The method of claim 59 wherein the hydrocarbons or hydrocarbon derivatives comprise ethanol.

61. The method of claim 59 wherein the hydrocarbon or hydrocarbon derivatives comprise butanol.
62. The method of any of the above claims, wherein the medium comprises a cellulase-containing enzyme mixture from an altered organism, wherein the cellulase-containing mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism.
63. The method of any of the above claims, wherein the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.
64. The method of any of the above claims, wherein the cellodextrin is selected from one or more of the group consisting of cellobiose, cellotriose, and cellotetraose.
65. A host cell comprising a recombinant polynucleotide encoding a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, wherein the polypeptide transports xylose into the cell.
66. A host cell comprising a recombinant polynucleotide encoding a XUT1 polypeptide, wherein the polypeptide transports arabinose into the cell.
67. A host cell comprising a recombinant polynucleotide encoding an NCU06138 polypeptide, wherein the polypeptide transports arabinose and glucose into the cell.
68. A host cell comprising a recombinant polynucleotide encoding a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, wherein the polypeptide transports xylose and glucose into the cell.

69. A host cell comprising a recombinant polynucleotide encoding an NCU04963 polypeptide, wherein the polypeptide transports xylose, arabinose, and glucose into the cell.
70. The host cell of any of claims 65 - 69 further comprising one or more recombinant polynucleotides wherein the one or more polynucleotides encode one or more enzymes involved in pentose utilization.
71. The host cell of claim 70 wherein the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.
72. The host cell of any of claims 65 - 71, wherein the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.
73. A method of increasing transport of xylose into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, and
 - culturing the cell such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of xylose into the cell compared with a cell that does not comprise the recombinant polynucleotide.
74. A method of increasing transport of arabinose into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a XUT1 polypeptide, and

- culturing the cell such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of arabinose into the cell compared with a cell that does not comprise the recombinant polynucleotide.
75. A method of increasing transport of arabinose or glucose into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a NCU06138 polypeptide, and
 - culturing the cell such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of arabinose or glucose into the cell compared with a cell that does not comprise the recombinant polynucleotide.
76. A method of increasing transport of xylose or glucose into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, and
 - culturing the cell such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of xylose or glucose into the cell compared with a cell that does not comprise the recombinant polynucleotide.
77. A method of increasing transport of xylose, arabinose, or glucose into a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a NCU04963 polypeptide, and
 - culturing the cell such that the recombinant polynucleotide is expressed, wherein expression of the recombinant polynucleotide results in increased transport of xylose, arabinose, or glucose into the cell compared with a cell that does not comprise the recombinant polynucleotide.
78. The method of any of claims 73 - 77 further comprising one or more recombinant polynucleotides wherein the one or more polynucleotides encode one or more enzymes involved in pentose utilization.

79. The method of claim 78 wherein the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.
80. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, and the polypeptide transports xylose; and
 - culturing the host cell in a medium comprising xylose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
81. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a XUT1 polypeptide, and the polypeptide transports arabinose; and
 - culturing the host cell in a medium comprising arabinose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
82. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes an NCU06138 polypeptide, and the polypeptide transports arabinose and glucose; and
 - culturing the host cell in a medium comprising arabinose or glucose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
83. A method of increasing growth of a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, and the polypeptide transports xylose and glucose; and

- culturing the host cell in a medium comprising xylose or glucose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

84. A method of increasing growth of a cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a NCU04963 polypeptide, and the polypeptide transports xylose, arabinose, and glucose; and

- culturing the host cell in a medium comprising xylose, arabinose, or glucose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

85. The method of any of claims 80 - 84, wherein the host cell further comprises one or more endogenous or recombinant polynucleotides encoding one or more enzymes involved in pentose utilization.

86. The method of claim 85, wherein the one or more enzymes are selected from one or more of the group consisting of L-arabinose isomerase, L-ribulokinase, L-ribulose-5-P 4 epimerase, xylose isomerase, xylulokinase, aldose reductase, L-arabinitol 4-dehydrogenase, L-xylulose reductase, and xylitol dehydrogenase.

87. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide selected from the group consisting of NCU00821 and STL12/XUT6, wherein the polypeptide transports xylose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising xylose or a source of xylose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of xylose into the cell is increased upon expression of the recombinant polynucleotide.

88. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a XUT1 polypeptide, wherein the polypeptide transports arabinose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising arabinose or a source of arabinose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of arabinose into the cell is increased upon expression of the recombinant polynucleotide.

89. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding an NCU06138 polypeptide, wherein the polypeptide transports arabinose or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising arabinose or glucose or a source of arabinose or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of arabinose or glucose into the cell is increased upon expression of the recombinant polynucleotide.

90. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding a polypeptide selected from the group consisting of SUT2, SUT3, and XUT3, wherein the polypeptide transports xylose or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising xylose or glucose or a source of xylose or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of xylose or glucose into the cell is increased upon expression of the recombinant polynucleotide.

91. A method of increasing the synthesis of hydrocarbons or hydrocarbon derivatives by a host cell, comprising:

- providing a host cell, wherein the host cell comprises a recombinant polynucleotide encoding an NCU04963 polypeptide, wherein the polypeptide transports xylose, arabinose, or glucose into the host cell for the synthesis of hydrocarbons or hydrocarbon derivatives; and

- culturing the host cell in a medium comprising xylose, arabinose, or glucose or a source of xylose, arabinose, or glucose to increase the synthesis of hydrocarbons or hydrocarbon derivatives by the host cell, wherein transport of xylose, arabinose, or glucose into the cell is increased upon expression of the recombinant polynucleotide.

92. The method of any of claims 89 - 91, wherein the source of glucose comprises cellulose.
93. The method any of claims 87 - 92, wherein the source of xylose or arabinose comprises hemicellulose.
94. The method of any of claims 87 - 93, wherein the hydrocarbons or hydrocarbon derivatives can be used as fuel.
95. The method of claim 94 wherein the hydrocarbons or hydrocarbon derivatives comprise ethanol.
96. The method of claim 81 wherein the hydrocarbons or hydrocarbon derivatives comprise butanol.
97. The method of any of claims 73 - 95, wherein the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium*

thermocellum, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.

98. A method of increasing growth of a cell, comprising:
 - providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a NCU07705 polypeptide; and
 - culturing the cell in a medium comprising cellulose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.
99. The method of claim 98, wherein the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.
100. The method of claim 98, wherein the host cell further comprises an inducible promoter operably linked to the recombinant polynucleotide.
101. The method of claim 98, wherein expression of cellulases is increased in the host cell upon expression of the recombinant polynucleotide.
102. A method of increasing growth of a cell on a biomass polymer, comprising:
 - providing a host cell, wherein the host cell comprises an endogenous polynucleotide wherein the polynucleotide encodes an NCU05137 polypeptide;
 - inhibiting expression of the endogenous polynucleotide; and

- culturing the cell in a medium comprising the biomass polymer, wherein the host cell grows at a faster rate in the medium than a cell in which expression of the endogenous polynucleotide is not inhibited.

103. The method of claim 102, wherein the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.
104. The method of claim 102, wherein cellulase activity of the host cell is increased upon inhibiting expression of the endogenous polynucleotide.
105. The method of claim 102, wherein hemicellulase activity of the host cell is increased upon inhibiting expression of the endogenous polynucleotide.
106. The method of claim 102, wherein inhibiting expression of the endogenous polynucleotide comprises mutating or deleting a gene comprising the endogenous polynucleotide.
107. The method of claim 102, wherein the biomass polymer is cellulose.
108. The method of claim 102, wherein the biomass polymer is hemicellulose.
109. A method of increasing growth of a cell, comprising:
- providing a host cell, wherein the host cell comprises a recombinant polynucleotide wherein the polynucleotide encodes a polypeptide selected from the group consisting of NCU01517, NCU09133, and NCU10040; and
 - culturing the cell in a medium comprising hemicellulose, wherein the host cell grows at a faster rate in the medium than a cell that does not comprise the recombinant polynucleotide.

110. The method of claim 109, wherein the host cell is selected from the group consisting of *Saccharomyces* sp., *Saccharomyces cerevisiae*, *Saccharomyces monacensis*, *Saccharomyces bayanus*, *Saccharomyces pastorianus*, *Saccharomyces carlsbergensis*, *Saccharomyces pombe*, *Kluyveromyces* sp., *Kluyveromyces marxianus*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Pichia stipitis*, *Sporotrichum thermophile*, *Candida shehatae*, *Candida tropicalis*, *Neurospora crassa*, *Zymomonas mobilis*, *Clostridium* sp., *Clostridium phytofermentans*, *Clostridium thermocellum*, *Clostridium beijerinckii*, *Clostridium acetobutylicum*, *Moorella thermoacetica*, *Escherichia coli*, *Klebsiella oxytoca*, *Thermoanaerobacterium saccharolyticum*, and *Bacillus subtilis*.
111. The method of claim 109, wherein the host cell further comprises an inducible promoter operably linked to the recombinant polynucleotide.
112. The method of claim 109, wherein hemicellulase activity of the host cell is increased upon expression of the recombinant polynucleotide.
113. A method of degrading cellulose, comprising:
- providing a composition comprising cellulose; and
 - contacting the composition with a cellulase-containing enzyme mixture from an altered organism, wherein the cellulase-containing mixture has reduced β -glucosidase activity compared to a cellulase-containing mixture from an unaltered organism, wherein the cellulose is degraded by the cellulase-containing mixture.
114. The method of claim 113, wherein the organism is altered by mutation of a gene encoding a β -glucosidase.
115. The method of claim 113, wherein the organism is altered by reducing the expression of a β -glucosidase.
116. The method of any of claims 113 - 115, wherein the organism is selected from the group consisting of a fungus and a bacterium.
117. The method of claim 116 wherein the organism is a filamentous fungus.

118. The method of any of claims 113 - 117, wherein the cellulose is from plant material.
119. The method of claim 118, wherein the plant material is selected from the group consisting of switchgrass, *Miscanthus*, rice hulls, bagasse, flax, bamboo, sisal, abaca, straw, leaves, grass clippings, corn stover, corn cobs, distillers grains, legume plants, sorghum, sugar cane, sugar beet pulp, wood chips, sawdust, and biomass crops.

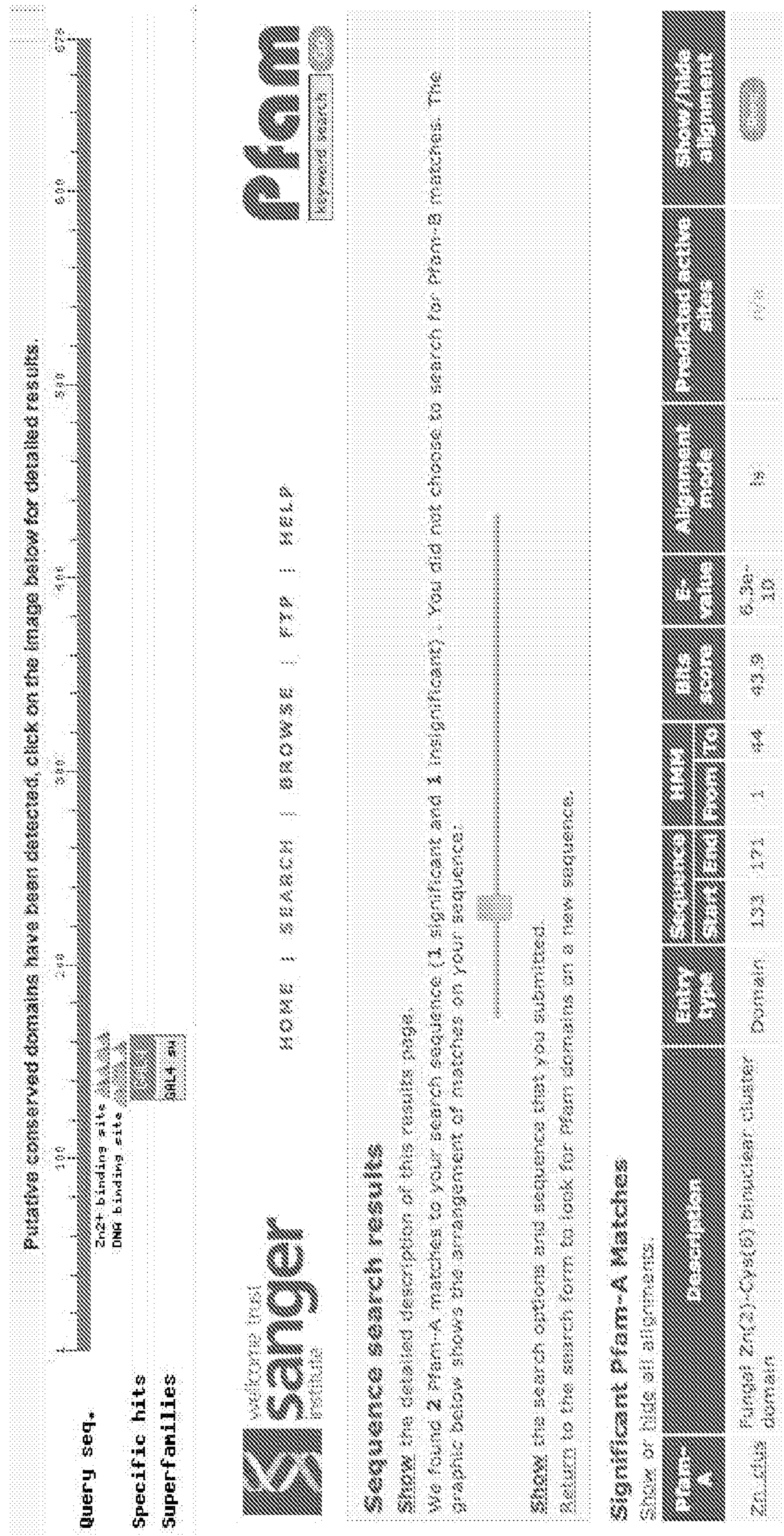


Figure 2

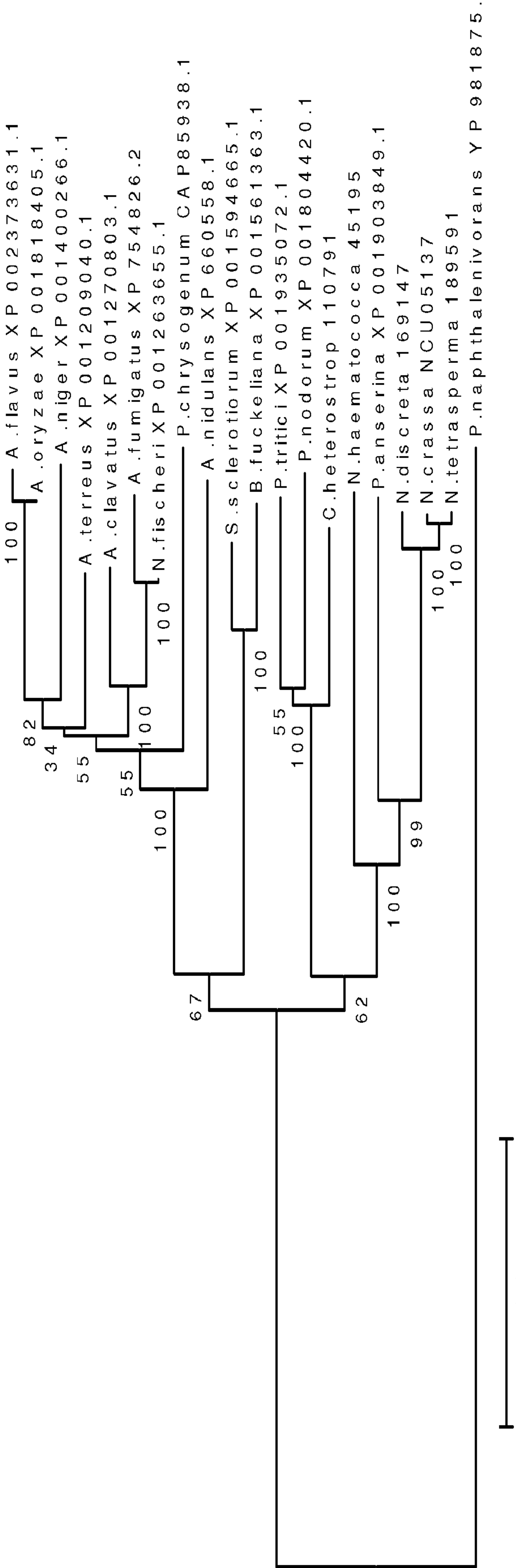


Figure 3

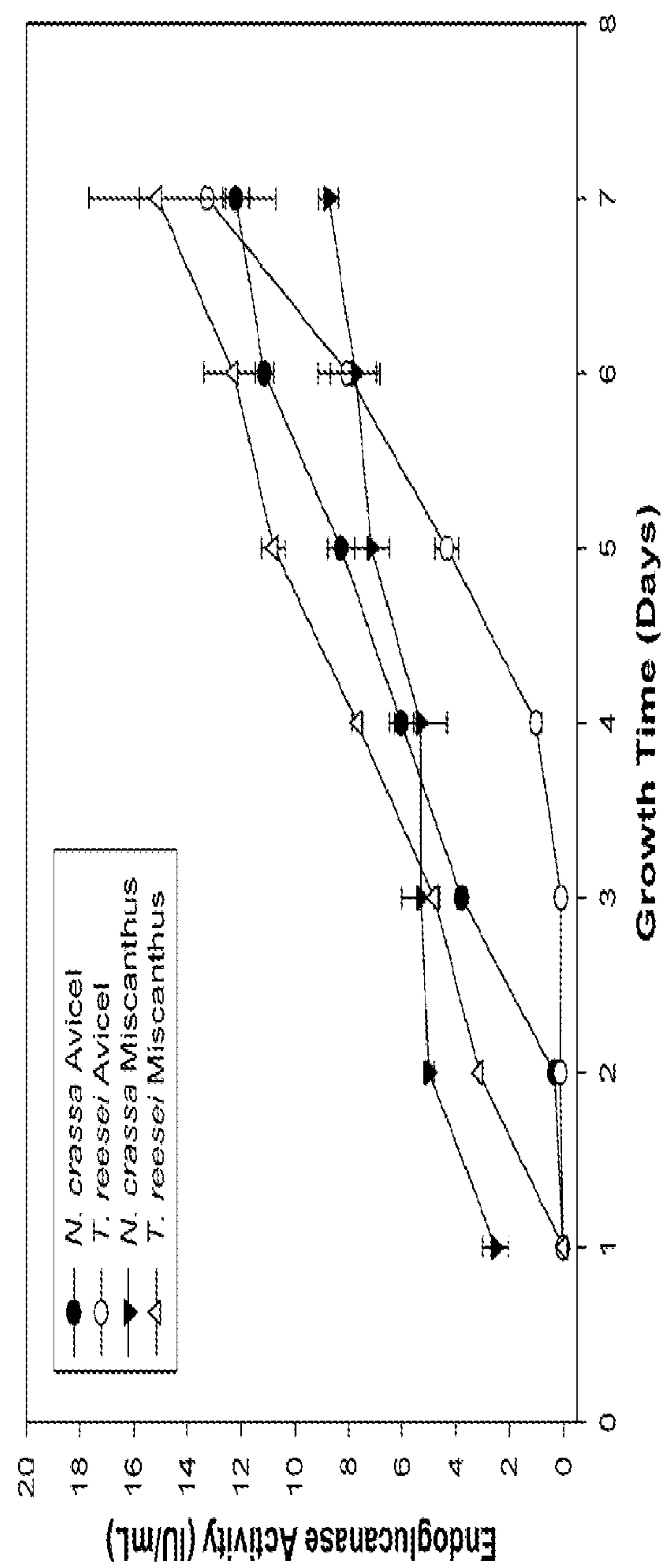


Figure 4

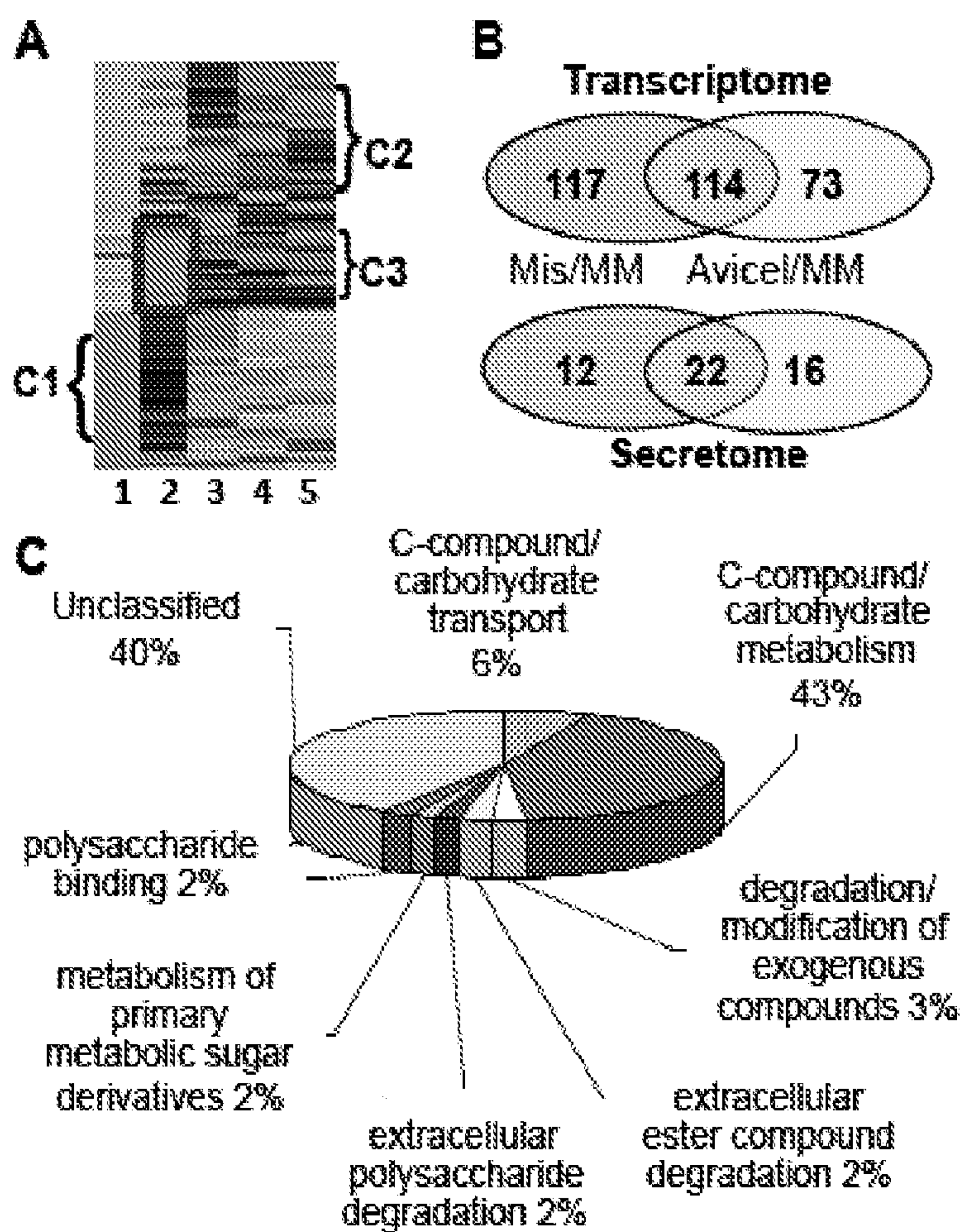


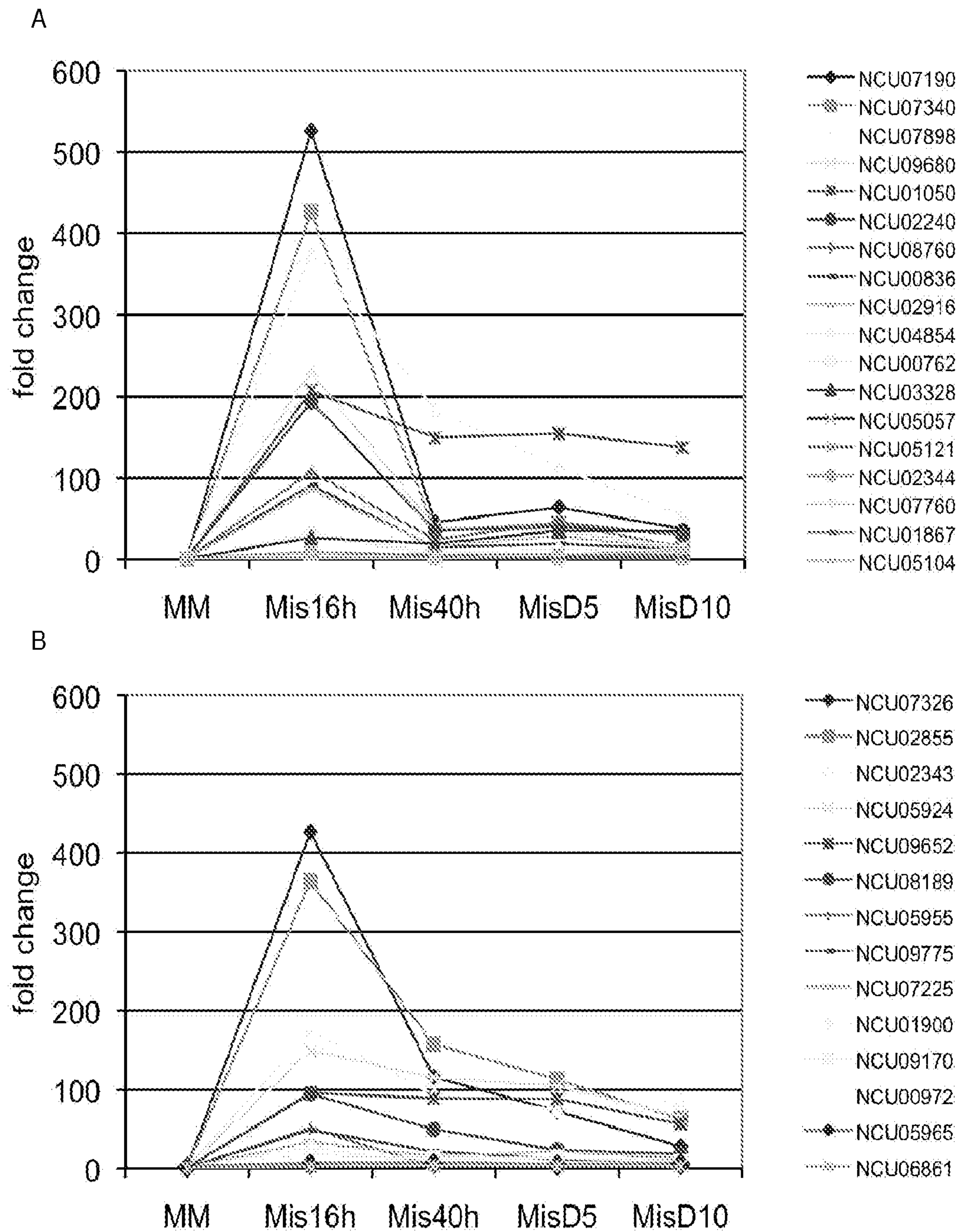
Figure 5

Figure 6

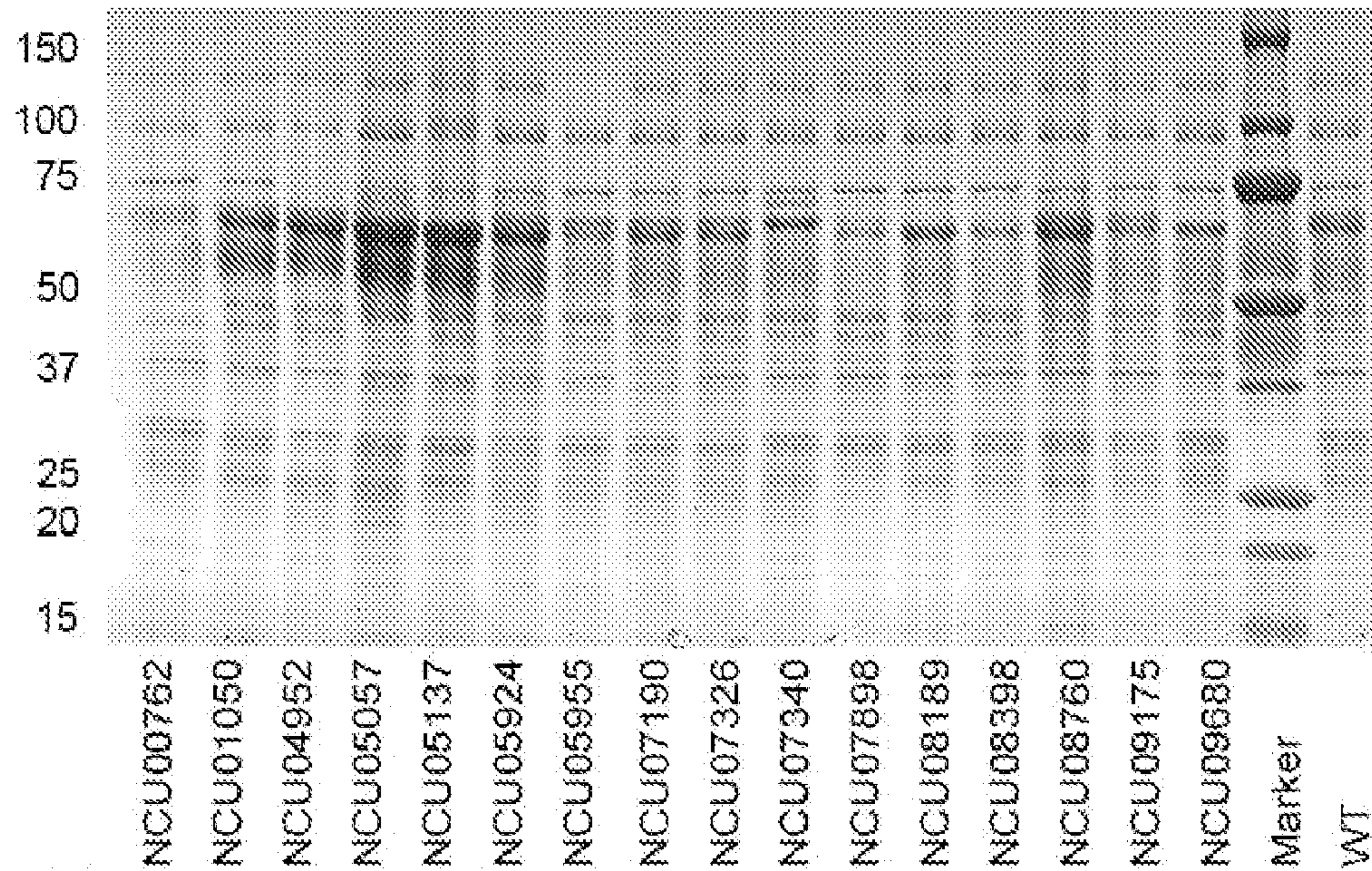
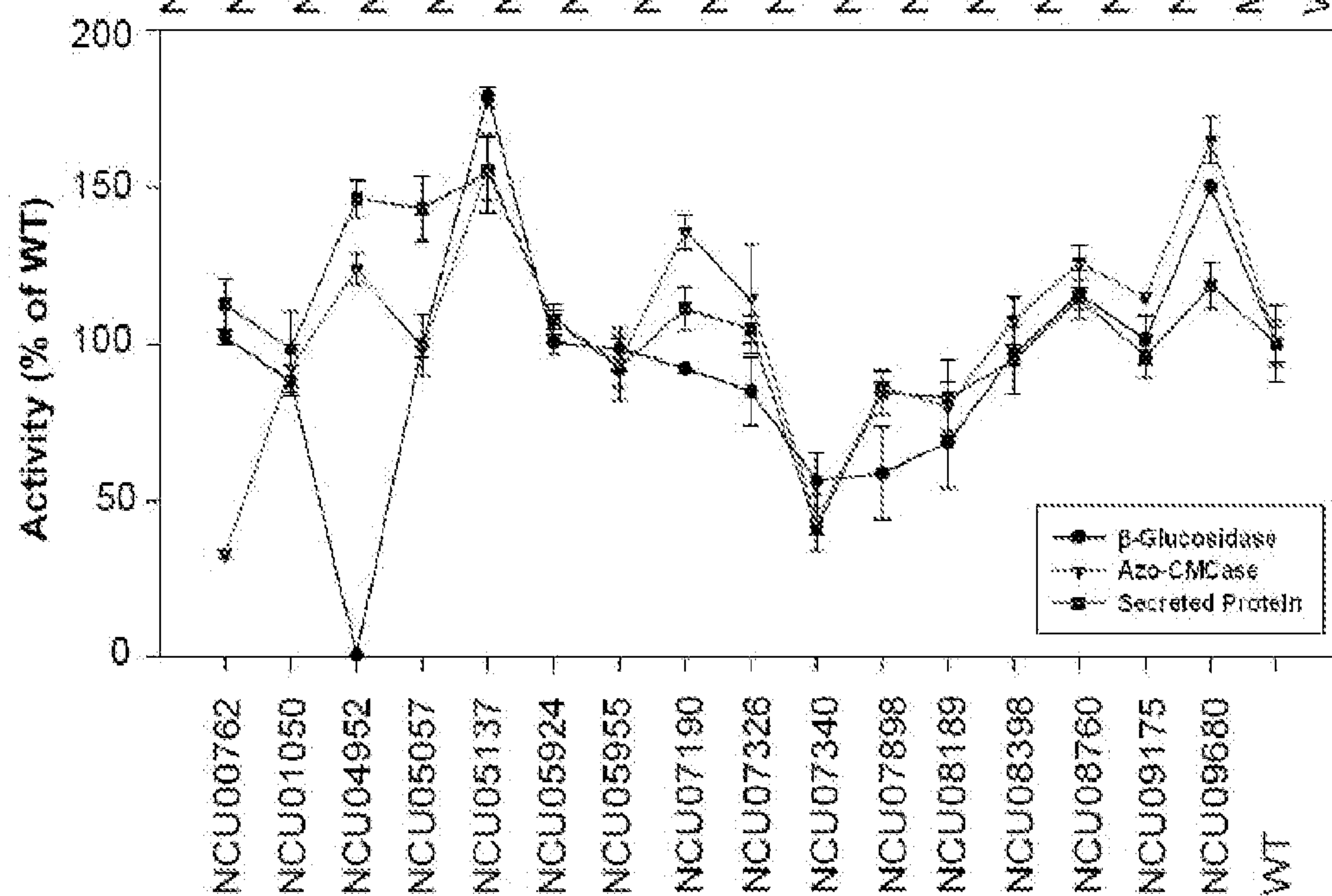
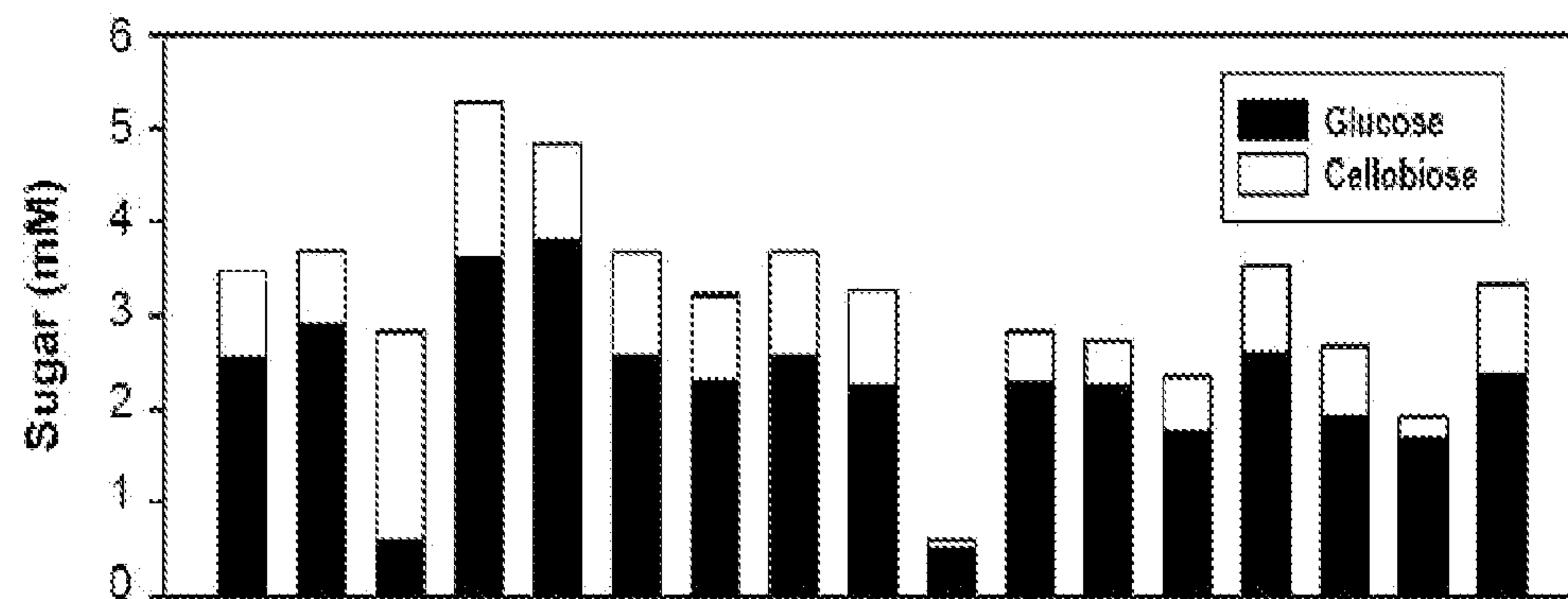
A**B****C**

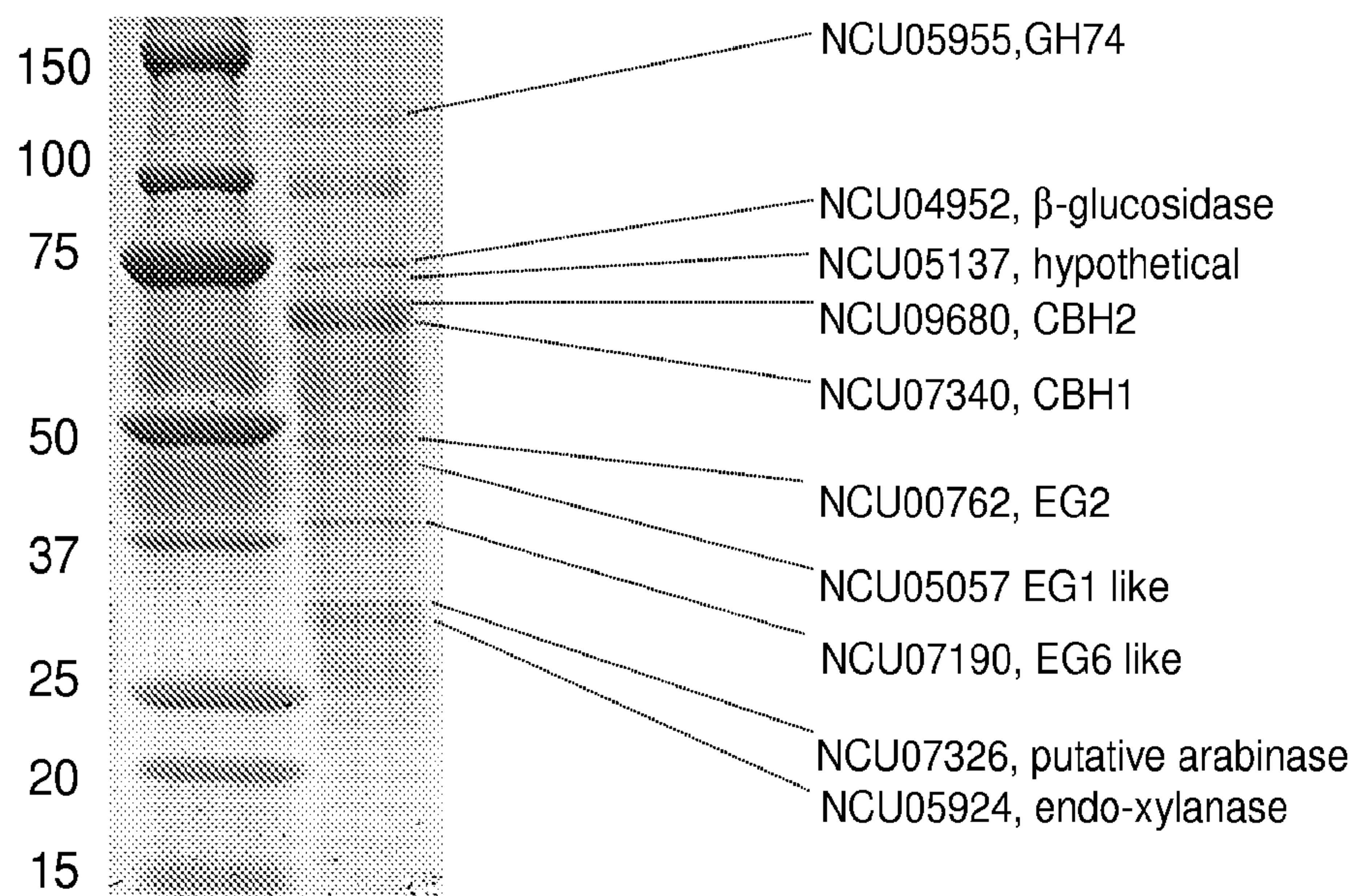
Figure 7

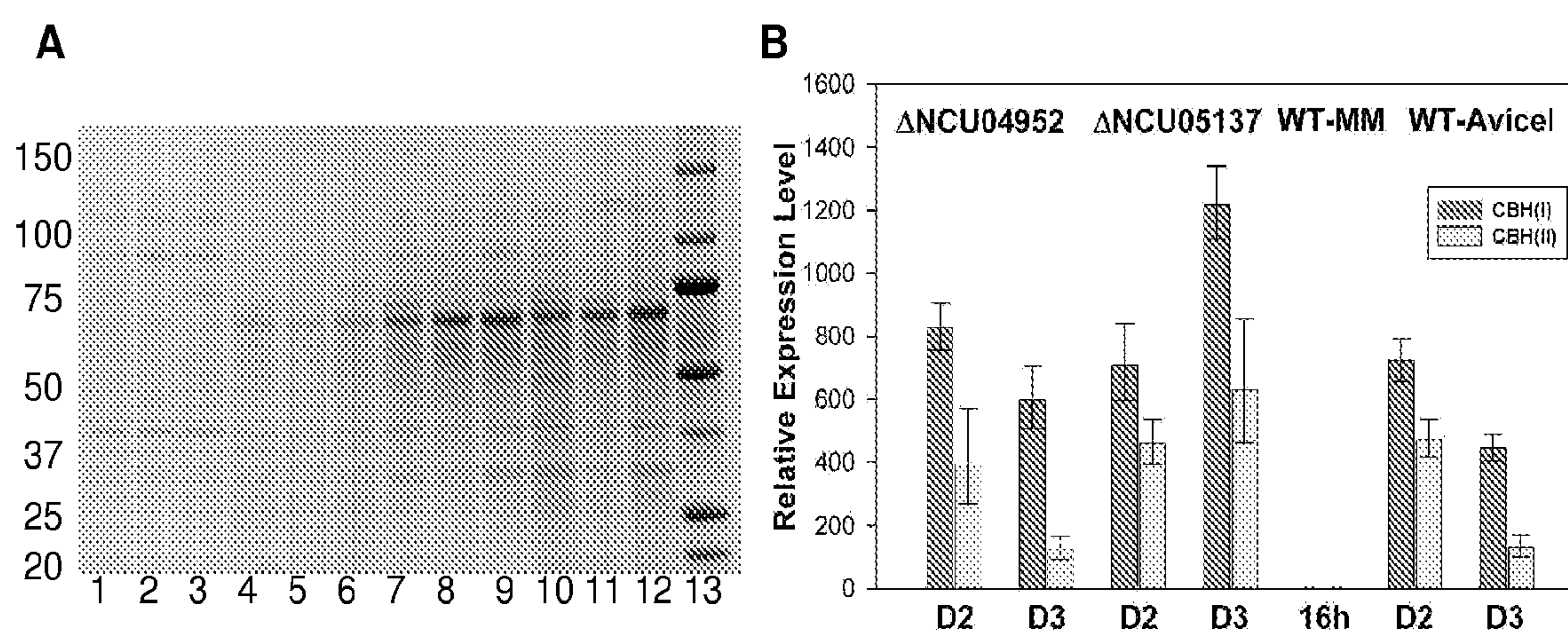
Figure 8

Figure 9

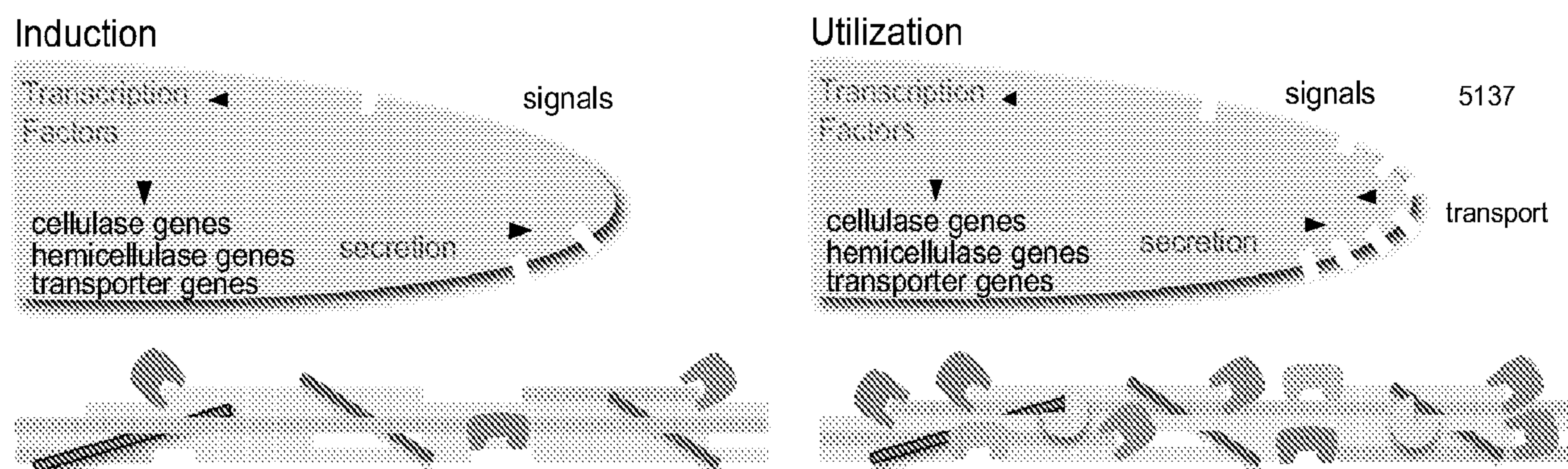


Figure 10-1

Query= NCU00801 | neurospora_crassa hypothetical protein similar to MFS lactose permease (580 nt)
(579 letters)

Database: Spoth1_GeneModels_FilteredModels1_aa.fasta
8806 sequences; 4,205,009 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value
jgi Spath1 43941 e_gw1.2.4209.1	361	e-100

>jgi|Spath1|43941|e_gw1.2.4209.1
Length = 537

Score = 361 bits (926), Expect = e-100, Method: Compositional matrix adjust.
Identities = 199/534 (37%), Positives = 284/534 (53%), Gaps = 29/534 (5%)

Query: 33	KGHGQTATKPGAQEKEVRNAALFAAIKE----SNIKPWSKESIHLYFAIFVAFCCACANG	88
	+ +T A + +R A L + + PWS I LY + +++ C C NG	
Sbjct: 5	RDEKETVVADHADDDALREADLAVQVAHDADGTVYSPWSLRMIRLYIVLSLSYLCGCLNG	64
Query: 89	YDGSLMTGIIAMDKEFQNFHTGDTGPKVSVIFSLYTVGAMVGAPFAAILSDRFGRKKGMF	148
	YDGSLM G+ M +Q FH G +IF++Y +G++ F ++D FGR+ GMF	
Sbjct: 65	YDGSLMGGLNGMTSYQRYFHMSTAGSTTGLIFAMYNIGSVAAVFFTGPVNDYFGRRWGMF	124
Query: 149	IGGIFIIVGSIIVASSSKLAQFVVGRFVLGLGIAIMTVAAPAYSIEIAPPHWRGRCTGFY	208
	+G + +IVG+ + A + QF+ GRFVLG G++ V+AP Y E+A P WRG TG Y	
Sbjct: 125	VGALLVIVGTCVQAPCTTRGQFLAGRFVLGFGVSFCCVSAPCYVSEMAHPKWRGTLTGLY	184
Query: 209	NCGWFGGSIPAACITYGCYFIKSNWSWRIPIILQAFTCLIVMSSVFFLPESPRFLFANGR	268
	NC W+ GSI A+ + YGC +I + +WRIP+ Q T +V VF+LPESPR+L A R	
Sbjct: 185	NCTWYIGSIVASWVVYGC SYIDTLDAWRIPWCQMVTSGLVCLGVFWLPESPRWLMAQDR	244
Query: 269	DAEAVAFLVKYHGNGDPNSKLVLLETEEMRDGIRTDGVDKVWWDYRPLFMTHSGRWRMAQ	328
	+A L YHG G + LV L+ +EM + I T+ DK W-DY L+ THS R R+	
Sbjct: 245	HDDAARVLATYHGEGRADHPLVKLQMDEMNOISTEASDKKWYDYHELWNTHSARRRLIC	304
Query: 329	VLMISIFGQFSGNGL-GYFNTVIFKNIGVTSTSQQLAYNILNSVISAIGALTAVSMTDRM	387
	V+ +++FGQ SGN L Y+ + K+ G+T + LA N +N +S +GA+ MTD +	
Sbjct: 305	VIGMAVFGQISGNSLSSYYLVNMLKSAGITEERRVLALNGVNPALSFLGAILGARMTDVV	364
Query: 388	PRRAVLIIGTFMCAAAALATNSGLSATLDKQTQRTQINLNQGMNEQDAKD NAYLHVDSNY	447
	RR +L+ + A +G S R N	
Sbjct: 365	GRRPLLLYTIVFASVCFVITGTSMATDDPTRTAAAN-----	402
Query: 448	AKGALAAAYFLFNVI FSFTYTP LQGVIPT EALET TIRGKGLALSGFIVNAMGF INQFAGPI	507
	+A F+F ++FSF +TPLQ + E L T R KG A+ F + I Q+A	
Sbjct: 403	--ATIAFIFIFIGIVFSFGWTPLQSMYIAETIPTATRAKGTAVGNFSSSVASTILQYASGP	460
Query: 508	ALHNIGYKYIFVFGWDLIETVAWYFFGVESQGR TLEQLEWVYDQPNPVKASLK	561
	A IGY + VFV WDLIE YF+ E++ RTLE+LE V- PNPVK SL+	
Sbjct: 461	AFEGIGYYFYLVFVFWDLIEGAIMYFYFPETKDRTLEEELEEVFSAPNPVKKSLE	514

Figure 10-2

Query= NCU04963 | neurospora_crassa hypothetical protein similar to
MFS monosaccharide transporter (528 nt)
(527 letters)

Database: Spoth1_GeneModels_FilteredModels1_aa.fasta
8806 sequences; 4,205,009 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value
jgi Spath1 62521 estExt_Genewise1.C_21757	541	e-155
>jgi Spath1 62521 estExt_Genewise1.C_21757		
Length = 566		

Score = 541 bits (1395), Expect = e-155, Method: Compositional matrix adjust.
Identities = 263/508 (51%), Positives = 366/508 (72%), Gaps = 10/508 (1%)

Query: 6 KKPEGVPGKSWPAIVIGLGFVAFGGVLFGYDTGTIGGILAMPYWQDLFSTGYRNPEHHLDV 65
+KP+ V G S PAI++GLFVA GG+LFGYDTG I GILAM -++ F+TGY + + +
Sbjct: 9 QKPDNVAGSSAPAIMVGLFVATGGLLFGYDTGAINGILAMDTFKEDFTTGYTDKQGKPG 68

Query: 66 TASQSATIVSILSAGTFFGALGAAPLADWAGRRRLGLILSSFVFIFGVILQTAAVSIPLFL 125
AS+ + IV+-LSAGT GAL +AP+ D GRRRL LI++ VF G I+Q A ++ + +
Sbjct: 69 YASEVSLIVAMLSAGTATGALLSAPMGDRWGRRRLSLIVAIGVFCVGAI IQVCATNVAMLV 128

Query: 126 AGRFFAGLGVLISATIPLYQSETAPKWIRGVIVGSYQLAITIGLLIASIVNNATHNMQN 185
GR AG+GVG++S +PLYQSE APKWIRG +V +YQL+IT GLL A+ VN T+ +-+
Sbjct: 129 VGRTLAGIGVGVVSVLVPLYQSEMAPKWIRGTLVCAYQLSITAGLLAAATVNILTYKLKS 188

Query: 186 TGCYRIPIAVQFAWAIILIVGMIILPETPRFHIKRDNLPAATRSLAILRRLEQNHPAIE 245
YRIPI +Q WA++L +G++ILPETPR+ +KR AA SL+ LRRL+ HPA+IE
Sbjct: 189 AAAYRIPIGLQLTWALVLALGLVILPETPRYLKRGLEKAAALSLSRLRRLDITHPALIE 248

Query: 246 ELSEIQANHEFEKSLGKATYLDCLKG--NLIKRLLTGCFLQSLQQLTGINFIFYGTQFF 303
EL+EI+ANHE-E +LG TY D + G +L +R LTGC LQ LQQLTG+NFI YYGT FF
Sbjct: 249 ELAEIEANHEYEMALGPDITYKDIIFGEPHLGRRTLTCGLQMLQQLTG VNFIMYYGTTF 308

Query: 304 KNSGFSDSFLISLITNLVNVSTLPGLYAIDKWGRRPVLLWGAVGMCVCQFIVAILGTTT 363
+G ++F -SLI ++N+VST PGL+ ++ WGRR +L+ G-VGM +CQ ++A T +
Sbjct: 309 YGAGIGNAFTVSLIMQVINLVSTFPGLFVVESWGRRKLLIVGSGMAICQLLIASFATAS 368

Query: 364 TSQDASGMIIIVHNLAAQKAAIAFICFYIFFFAASWGPVAVVVTGEIFPLKVRAKSLSITT 423
+ + + I F+ YIFFFAASWGPV WVVT EI+PLKVRAKS+SI-T
Sbjct: 369 GNDNKP-----TQNQILIIFVAIYIFFFAASWGPVWVVTSEIYPLKVRAKSMSIST 420

Query: 424 ASNWLLNWAIAYSTPYLVNYGPGNANLQSKIFFVWGGCCFICIAFVYFMIYETKGLTLEQ 483
ASNW+LN+ IAY TPYLV+ G+ +L S++FFVWG C + IAFV++M+YET ++LEQ
Sbjct: 421 ASNWVLNFGIAYGTPYLVDTSDGSPDLGSRVFFVWGAFCILSIAFVWYMWYETSKISLEQ 480

Query: 484 VDELYEEVSDARKSIGWVPTITFREIRE 511
+DE+YE V+ A S + P+ +F+++R+
Sbjct: 481 IDEMYERVAHAWNSRSFEPWSFSQQMRD 508

Figure 10-3

Query= jgi|Spath1|48439|e_gw1.3.3367.1
(512 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score	E
	(bits)	Value
NCU01132 neurospora_crassa hypothetical protein similar to mo...	727	0.0
>NCU01132 neurospora_crassa hypothetical protein similar to monosaccharide transporter (554 nt) Length = 553		

Score = 727 bits (1876), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 382/548 (69%), Positives = 431/548 (78%), Gaps = 42/548 (7%)

Query: 1	MKKFLGLRGQALNLAVGTIAGCDFLLFGYDQGVMMGGILTLKVFLDAFPMINPEAAGLSHD	60
	MK FLGLRGQ LNLAVG +AGCDFLLFGYDQGVMMGGILTL FL F INP+A GL+	
Sbjct: 1	MKPFLGLRGQPLNLAVGAVAGCDFLLFGYDQGVMMGGILTLPEFLGYFEQINPDAPGLTPH	60
Query: 61	ESSTRSTYQGI+AVASYNLGCFLGAIITIFIGNPLGRKRVIMLGTSVMVIGAILQASSTTL	120
	ESS RSTYQGI+VASYNLGCF+GAIITIFIGNP GRK++I+LGTS+M++GAILQAS+TTL	
Sbjct: 61	ESSMRSTYQGI+SVASYNLGCFIGAIITIFIGNPWGRKKIILLGTSIMIVGAILQASATTL	120
Query: 121	PQFIVGRIITGLGNGGNTSTVPTWQSETSKAHKRGKMIFF-----CAIILAF-----	167
	FI+GRIITG+GNGGNTSTVPTWQSETSKAHKRGKM+ I+L++	
Sbjct: 121	GHFIIGRIITGIGNGGNTSTVPTWQSETSKAHKRGKMVMIEGSLVTAGIMLSYWIDLGLS	180
Query: 168	-----IPFLPESPRWLILKGREDEAREVIAALEDTDTSR	202
	IPFLPESPRWL+ KGR+ EA+E++AAL D + D	
Sbjct: 181	FAPGSVAWRFLAFQIIFCILILIFIPFLPESPRWLVEFKGRDAEAKEILAAALNDVELDDP	240
Query: 203	IVENEFLAIKETVLEMSKGTFRDLFTMDKNRNLHRTLLAYFNQVFQQISGINLITYYAAV	262
	IV+ EF I -TV+EMSKG+F+DLFTMDK+RN HRTLLAY NQVFQQISGINLITYYAAV	
Sbjct: 241	IVDTEFHFIHDTVEMSKGSFKDLFTMDKDRNFHRTLLAYLNQVFQQISGINLITYYAAV	300
Query: 263	IYKGLGMSDFLSRLAALNGTEYFLASWPAVFLVERVGRRLMLFGAVGQAATMAILAGV	322
	IY GLGMSDFL+RLAALNGTEYF+ASWPAVFLVERVGRRLMLFGA+GQAATMAILAGV	
Sbjct: 301	IYSGLGMSDFLARLLAALNGTEYFIASWPAVFLVERVGRRLMLFGAIGQAATMAILAGV	360
Query: 323	NSR-QETGFQIAGIVFLFVFNTFFAVGWLGMTWLYPAEIVPLRIRAPANALSTSANWIFN	381
	NSR + +QIAGIVFLFVFNT FAVGWLGMT+WLYPAEIVPLRIRAPANALSTSANWIFN	
Sbjct: 361	NSRPDDKPYQIAGIVFLFVFNTVFAVGWLGMSWLYPAEIVPLRIRAPANALSTSANWIFN	420
Query: 382	FLVVMITPVAFNNIGYQTYIIFAVINAFMVPCVYFFYPETAYRSLEEMDNIFHKVADGWK	441
	F+VVMITPVAFN I YQTYIIFAVINAF+VP VYFFYPETA RSLEEMD IFHKV DGWK	
Sbjct: 421	FMVVMITPVAFNKIKYQTYIIFAVINAFIVPVVYFFYPETACRSLEEMDMIFHKV-DGWK	479
Query: 442	GVFTVVHQAKVEPRWYGKNGELL--VDYQQTEEHRRHLQQQEGAVTASEKRSVEGAGSGS	499
	G FTVVHQAKVEP+WY K+G+ + D+++T ++ H + K VE +	
Sbjct: 480	GYFTVVHQAKVEPKWYDKDGQRIGGADFEKTAGYQSHSIPESSGFEEKPTKAHVESPRADD	539

Figure 10-4

Query: 500 GSGDVKQD 507

G D

Sbjct: 540 GITSSSSD 547

Query= jgi|Spath1|112305|estExt_fgenesh1_kg.C_60263
(554 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score	E
	(bits)	Value
NCU10021 neurospora_crassa high affinity glucose transporter ...	937	0.0
>NCU10021 neurospora_crassa high affinity glucose transporter		
(554 nt)		
Length = 553		

Score = 937 bits (2421), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 474/542 (87%), Positives = 507/542 (93%), Gaps = 7/542 (1%)

Query: 1 MSSSEKEATGPVAAHVGNLATTQDVEKIEAPVTWKAYLICAFASFSGGIFFGYDSGYINGV 60
MSS+ ++ T P AAH+G LA TQDVE+IEAPVTWKAYLICAFASFSGGIFFGYDSGYINGV
Sbjct: 1 MSSAHEKETAPTAAHIG-LAHTQDVERIEAPVTWKAYLICAFASFSGGIFFGYDSGYINGV 59

Query: 61 LASKLFINAVEGAG--KDAISESHSSLIVSILSCGTFFGALIAGDLADFIGRKYTVILGC 118
L S++FI+AVEG +DA+SESH SL+VSILSCGTFFGALIAGDLAD IGRK+TVILGC
Sbjct: 60 LGSQIFIDAVEGTSPVRDALSESHQSLVVSILSCGTFFGALIAGDLADMIGRKWTVILGC 119

Query: 119 LIYIIGCVIQITGLGNALGAIVAGRLLIAGIGVGFESAIVILYMSEICPRKVRGALVAGY 178
LIY+IGCVIQ-ITGLG+ALGAIVAGRLLIAGIGVGFESA+VILYMSEICPRKVRGALVAGY
Sbjct: 120 LIYLIGCVIQMITGLGDALGAIVAGRLLIAGIGVGFESAVVILYMSEICPRKVRGALVAGY 179

Query: 179 QFCITIGLMLASCVVYGTQNRQDTGQYRIPIGIQFIWALILGGGLLCLPDSPRYFVKRGR 238
QFCITIGLMLASCVVY TQ+R DTG YRIPI IQFIWALIL GGL+CLPDSPRYFVK+G
Sbjct: 180 QFCITIGLMLASCVVYATQDRPDTGAYRIPIAIQFIWALILAGGLMCLPDSPRYFVKKGN 239

Query: 239 LADATSALSRLRGQPEDSEYIQVELAEIVANEEYERQLIPSTTWFGSWANCFKGSLFKAN 298
LA ATS+LSRLRGQ +SEYIQVELAEIVANEEYERQLIPSTTWFGSWANCFKGSL+KAN
Sbjct: 240 LAAATSSLSRLRGQDPNSEYIQVELAEIVANEEYERQLIPSTTWFGSWANCFKGSLWKAN 299

Query: 299 SNLRKTILGTSLQMMQQWTGVNFIFYYSTPFLKSTGAIDDPFLMSMVFTIINVSTPISEF 358
SNLRKTILGTSLQMMQQWTGVNFIFYYSTPFLKSTGAI + FL+SMVFTIINVSTPISEF
Sbjct: 300 SNLRKTILGTSLQMMQQWTGVNFIFYYSTPFLKSTGAISNTFLISMVFTIINVSTPISEF 359

Query: 359 YTVERFGRRTILFWGALGMLICQFLVAIVGVTVGFNHTHPAPTADDPEATLANNISAVNA 418
+TVERFGRRTILFWGALGMLICQFLVAI+GVTGVFN TH P + ++ANN+SAVNA
Sbjct: 360 WTVERFGRRTILFWGALGMLICQFLVAIIGVTGVFNKTHMGPDGE----SMANNVSAVNA 415

Query: 419 QIAFIAIFIFFFASTWGPAGAWIVIGEIFPLPIRSRGVGLSTASNWLWNTIIAVITPYMVG 478
QIAFIAIFIFFFASTWGPAGAWI+IGEIFPLPIRSRGVGLSTASNWLWNTIIAVITPYMVG
Sbjct: 416 QIAFIAIFIFFFASTWGPAGAWILIGEIFPLPIRSRGVGLSTASNWLWNTIIAVITPYMVG 475

Figure 10-5

Query: 479 EDRGNMKSSVFFVWGGCTCAFYTYFLVPETKGLSLEQVDKMMEETTPRTSAKWKP T T 538
 E RGN+KSSVFFVWGGCTCAF+YTYFLVPETKGLSLEQVDKMMEETTPRTSAKWKP T T
 Sbjct: 476 EQRGNLKSSVFFVWGGCTCAFIYTYFLVPETKGLSLEQVDKMMEETTPRTSAKWKPRT 535

Query: 539 FA 540
 FA
 Sbjct: 536 FA 537

Query= jgi|Spath1|114107|estExt_fgenesh1_pm.C_20669
 (522 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
 9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score	E
	(bits)	Value
NCU08114 neurospora_crassa hypothetical protein similar to MF...	832	0.0
>NCU08114 neurospora_crassa hypothetical protein similar to MFS		
hexose transporter (526 nt)		
Length = 525		

Score = 832 bits (2148), Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 397/523 (75%), Positives = 452/523 (86%), Gaps = 6/523 (1%)

Query: 1 MGIFAFNKQKPNAEATAVAQ--EEAPQFERVDWKRDPGLRKLYFYAFVLCIASATTGYDG 58
 MGIF +KP A+A + Q EEAPQFERVDWK+DPGLRKLYFYAF+LCIASATTGYDG
 Sbjct: 1 MGIF---NKKPVAQAVDLNQIQEEAPQFERVDWKKDPGLRKLYFYAFILCIASATTGYDG 57

Query: 59 MFFNSVQNFEETWENYFNHPTGSKLGVLGALYQIGSLASIPLVPIIADRVGRKIPPIAIGCV 118
 MFFNSVQNFEETW YF P GS+LG+LGALYQIGS+ SIP VP++ D GRK PI IGCV
 Sbjct: 58 MFFNSVQNFEETWIKYFGDPRGSELGLLGALYQIGSIGSIPFVPLLTDFGRKTPIIIGCV 117

Query: 119 IMIVGAVLQAACRNIGTFMGGRFLLGFGNSLAQLCSPMLLTELAHPQHRGRLTTVYNCLW 178
 IMIVGAVLQA +NL TFMGGR +LGFGNSLAQ+ SPMLLTELAHPQHR RLTT+YNCLW
 Sbjct: 118 IMIVGAVLQAATAKNLDTFMGGRTMLGFGNSLAQIASPMLLTELAHPQHRARLTTIYNCLW 177

Query: 179 NVGALVVAWVSFGTDYLSKDSWRIPALIQAFPSVIQLLFIFWVPESPRYLMADKHERA 238
 NVGALVV+W+-FGT+Y+ +DWSWRIPAL+QAFPS+IQLL I+WVPESPR+L+AKDKH+ A
 Sbjct: 178 NVGALVVSWLAFGTNYINNDWSWRIPALLQAFPSIIQLLGIWWVPESPRFLIAKDKHDEA 237

Query: 239 LAILAKYHANGDANHPTVQFEYREIKETLRLEFEASKSSSYLDFVTRGNRYRLAVLISL 298
 L ILAKYHANGD NHPTVQFE+REIKET+RIE E++K+SSYLDF ++RGNRYRLA+L+SL
 Sbjct: 238 LHILAKYHANGDPNHPTVQFEFREIKETIRLEMESTKNSSYLDFFKSRGNRYRLAILLSL 297

Query: 299 GIFSQWSGNAIISNYSSKLYDTAGVTGSTQKLGLSAGQTGLSLIISVTMALLVDKFGRRP 358
 G FSQWSGNAIISNYSSKLY+TAGVT ST KLGLSAGQTGL+LI+SVTMALLVDK GRR
 Sbjct: 298 GFFSQWSGNAIISNYSSKLYETAGVTDSTAKLGLSAGQTGLALIVSVTMALLVDKLGRR 357

Query: 359 MFLTSTAGMECTFIFWTLTSGLYEEHNADGARYAMILFIWIHGIFYSISWSGLLVGYAIE 418
 FL ST GM TF+ WTLT+GLY EH GA AMI FIW+ GIFYS++WSGLLVGYAIE
 Sbjct: 358 AFLASTGGMCGTFVIWTLTAGLYGEHRLKGADKAMIFFIWWVFGIFYSLAWSGLLVGYAIE 417

Figure 10-6

Query: 419 VLPYKLRAKGLMIMNLTVQAALTLNTYANPVAFDAF-EGHSWKLYIIYTIWIFLELCFVW 477
 +LPY+LR KGLM+MN++VQ ALTLNTYANPVAFD F HSWKLY+IYT WI E FV+
 Sbjct: 418 ILPYRLRGKGLMVMNMSVQCALTTLNTYANPVAFDYFGPDHSWKLYLIYTCWIAAEFVFVF 477

Query: 478 KMYIETKGPTLEELAKIIDGDEAAVAHVVDIKQVEKETHINEEK 520
 MY+ETKGPTLEELAK+IDGDEA VAH+DI QVEKE I+E -
 Sbjct: 478 FMYVETKGPTLEELAKVIDGDEADVAHIDIHQVEKEVEIHEHE 520

Query= jgi|Spath1|70023|estExt_Genewise1.C_53218
 (504 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
 9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score	E
	(bits)	Value
NCU06138 neurospora_crassa hypothetical protein similar to MF...	263	6e-71
>NCU06138 neurospora_crassa hypothetical protein similar to MFS		
monosaccharide transporter (584 nt)		
Length = 583		

Score = 263 bits (673), Expect = 6e-71, Method: Compositional matrix adjust.
 Identities = 159/488 (32%), Positives = 259/488 (53%), Gaps = 35/488 (7%)

Query: 10 FLVGVFASLGSLLYGYDLGVIAQVIASQSFKSRFSPSD--NEEAA-----VVSVFTGGA 61
 F + FA +G +LYGY+ G+ + V+A +F+ D +E A+ + ++ GA
 Sbjct: 39 FSIACFACIGGVLYGYNQGMFSGVLAMPAFQKHMGEYDPIDENASQTKKGWLTAILLELGA 98

Query: 62 FFGAMAAGPMGDKLGRRWTILCGALVFCLGGALQTGAQALSY--LYAGRSIAGLGVGVLC 119
 + G + +G M + L R++ +L LVF LG +Q + + + + AGR I G+GVG L
 Sbjct: 99 WLGTLISGFMAEVLRSKYGVLVACLVFMLGVVIQATSISGGHETILAGRIFITGMGVGSLA 158

Query: 120 MIVPMYQAE LAHPSIRGRITALQQFMLGIGALAAAWISYGT-YVG---FAPTNDGQWRTS 175
 MI+P+Y +E+A P +RG + ALQQ + G + + WI YGT Y+G +D W
 Sbjct: 159 MIIPINSEVAPPEVRGALVALQQLAICFGIMVSFWIDYGTNYIGGTKLETQSDAAWLVP 218

Query: 176 LGIQVIPAVFLAALILFPESPRWLI DHGRSEEGRLTLAQLHSHGDVDDAWVQAEYQQIR 235
 + +Q+ PA+ L ++ P SPRWLI HGR E + L+ L D V+ E+ + +
 Sbjct: 219 VCLQLAPALILFFGMMFMPFSPRWLIHHGREAEARKILSTLRGLPQ-DHELVELEFLEIK 277

Query: 236 ESVEFVRENEAKSYAEL-----FRDRSCFRRLFLACAIQGSVQMIG 276
 F + + A+ + EL FR ++ FRR+ +A Q -G
 Sbjct: 278 AQSLFEKRSIAELFPELREQTAWNTFKLQFVAIEKLFRTKAMFRRVIVATVTMFFQQWSG 337

Query: 277 VSAIQYYSVTIYGLMGIEGDDT-LKYQAISSIIALVAQALCILLIDRLGRRWTLIGGNLG 335
 ++AI YY+ + +G+ G+ T L + I+ +A +L IDR+GR+ L G LG
 Sbjct: 338 INAILYYAPQIFKQLGLSGNTTSLLATGVVGIVMFIATVPAVLWIDRVGRKPVLTIGALG 397

Query: 336 NCVTFIIATVMLARYPPGTSSNKAAAWGFIVVTWVYNFSEFSAICGPISWIIPAEIFDTKT 395
 II V-+A+ ++KAA W + + W++ F - GP +WII AEI+ T
 Sbjct: 398 MATCHIIIAVIVAKNVDQWETHKAAGWAAVAMVWLFVIHFGYSWGPCAWIIVAEIWPLST 457

Figure 10-7

Query: 396 RSKGVSIATMTSFAINTMIGQVTGPAMKTVGYREFYLLFVVCNFTNALFFWAFLPETAKRP 455
 R GVS+ -++ N ++GQVT +K + Y Y++F + - A F W F+PET +
 Sbjct: 458 RPYGVSLGASSNWMNMFIVGQVTPDMLKAIPYGTYYIIFGLLTVMGAAFIWFFVPETKRLT 517

Query: 456 LEEMNRLF 463
 LEEM+ +F
 Sbjct: 518 LEEMDMIF 525

Query= jgi|Spath1|102977|fgenes1_pm.5_#_763
 (481 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
 9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value
NCU05897 neurospora_crassa hypothetical protein similar to l-...	713	0.0
>NCU05897 neurospora_crassa hypothetical protein similar to l-fucose permease (472 nt) Length = 471		

Score = 713 bits (1841), Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 347/447 (77%), Positives = 385/447 (86%), Gaps = 2/447 (0%)

Query: 1 MLSSGFWKRRSLRVPDNQRTKAAELTLRESLPLSLVTILFFLWGFSGYGLDNLNKHFQN 60
 M S +WKRRSLRV D++ TKAAEL+LRESL PL LVTILFFLWGFSGYGLDNLNKHFQ
 Sbjct: 1 MFSREWWKRRSLRVRDDKVTKAAELSLRESLPLCLVTILFFLWGFSGYGLDNLNKHFQE 60

Query: 61 TLGITKTRSSGLQAAYFGAYPLASLGHAAWILRHYGYRAVFIWGLFLYGLGALLAIPSIM 120
 TL ITK RS+GLQAAYFGAYPLASLGHA+ILR + YRAVFIWGLFLYGLGALLAIP -
 Sbjct: 61 TLHITKARSAGLQAAYFGAYPLASLGHAAYILRRFSYRAVFIWGLFLYGLGALLAIPCIK 120

Query: 121 HHSFAGFCVCIFIIGNGLGSLETAANPYITVCGPPKFSEIRINVAQAFNGIGTVVAPVLG 180
 SFAGFCVCIFIIGNGLGSLETAANPYITVCGPPK+SEIRIN AQAFNGIGTVVAPVLG
 Sbjct: 121 AKSFAGFCVCIFIIGNGLGSLETAANPYITVCGPPKYSEIRINFAQAFNGIGTVVAPVLG 180

Query: 181 SYVFFTFDDQIALRNVQWVYLAIACFVFLLAGVFFLSVIPEITDADMAFQAAETHAGADD 240
 SYVFF FDD AL+NVQWVYLAI FV++LA VFFL +PEITDADM QAAETHAG D
 Sbjct: 181 SYVFFGFDDNLALQNVQWVYLAI AVFVYILAVVFFLIELPEITDADM QHQAETHAGDAD 240

Query: 241 RPFHTQYRLFHAAFAQFCYTGAQVAIAGYFINYATETRPNTDSSLGSKFLAGSQAGFAVG 300
 +PF QYRLFHA+F+QFCYTGAQ+AIAGYFINY TETR NTDS+LG++FLAG+Q FAVG
 Sbjct: 241 QPFRKQYRLFHASFSQFCYTGAQIAIAGYFINYVTETRKNTDSALGAQFLAGAQGTFAVG 300

Query: 301 RFGGAAMMQFIKPRKVFALFMTMCIVFSAPAITQRGNAGLSMLYLVMFFESICFPTIIAL 360
 RF GAA+M F-+PRKVF LF+T CI+F AP ITQR N G+S+LY+ +FFESICFPTI+AL
 Sbjct: 301 RFAGAAIMHFVRPRKVFLFLTACIIFVAPTITQRENTGMSLLYVTLFFESICFPTIMAL 360

Query: 361 GMRGLGRHTKRGSGWIVAGVLGGACVPPLMGAAADARGTGFSMLVPLCFFVAAWTYALAV 420
 GMRGLGRHTKRGSG++VAGV GGA VPPLMGA AD T SM+VPL FF AWTYALAV
 Sbjct: 361 GMRGLGRHTKRGSGFLVAGVFVGAVVPPLMGAVADMHDTAMSMVVPLAFFFAVAWTYALAV 420

Figure 10-8

```
Query: 421 NFAPPYRSVVDAFSTTDVGLREKQRED 447
      NF P YR   DAF+T ++G+R+  RED
Sbjct: 421 NFWPWYRDTCDRAFTTAEIGVRD--RED 445

Query= jgi|Spath1|84164|estExt_Genewise1Plus.C_62100
      (546 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
      9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:
Score      E
(bits) Value

NCU05853 | neurospora_crassa conserved hypothetical protein (54... 586 e-168
>NCU05853 | neurospora_crassa conserved hypothetical protein (542
      nt)
      Length = 541

Score = 586 bits (1511), Expect = e-168, Method: Compositional matrix adjust.
Identities = 276/516 (53%), Positives = 369/516 (71%), Gaps = 11/516 (2%)

Query: 18 ADPIVTRLVQEDKIPWYKKPNLRRMYIFLFICCMGVEMTSGFDSQLINTLQYAETFHKYL 77
      +DP+++RL +ED IPWYKKPNLR +Y LF CMG+E+TSGFDSQ+IN LQ E++ +Y
Sbjct: 11 SDPVISRLAKEDPIPWYKKPNLRFLYFMLFPTCMGIELTSGFDSQMINALQILESWIQYF 70

Query: 78 GNGRKDEDGNYAIEPGLLGFEVNSCYQLGSIFAVPIAPWFAQRFGRRWSIMLGSLIMVGGA 137
      N +          L G +++ Y LG+I ++P+ P RFGRRWSI LGS++M+ GA
Sbjct: 71 DNPQG-----ALKGIISAAYSLGAILSLPLVPIINDRFGRRWSIALGSIVMIVGA 120

Query: 138 IIQGFQHVAMYYIIARMILGMGILFCIISGAALIGELGHPKERAVLTSLFNSSYFIGQIL 197
      IIQGF+QHV MYI+ARMILG GI CI+SG++LIGEL +PKER VLTSLFN SYF+GQI+
Sbjct: 121 IIQGFQHVGMYYIVARMILGFGIPTCIVSGSSLIGELAYPKERPVLTSLFNVSYFVGQIV 180

Query: 198 ASAITIGTTEMKTNWAWRLPSLLQICPSLLQIVTVFFLPESPRFLISKDRDDDAKEVLK 257
      A+AI GT - +NW WR+PSLLQICPSLLQ+ VFF+PESPR+LI+KDR +A ++L K
Sbjct: 181 AAAIVFGTNSIASNWGWRIPLPSLLQICPSLLQLAFVFFIPESPRWLITKDRSQEAHDILKK 240

Query: 258 YHAEGDASSLLVQAEIVQIRETIRTEME-VSNQSWMELVSTYGMRRRLVITLFIGLFTQL 316
      YH E + V AE Q++ IR E E VS SWM+L+ T GMR+RL+I+ +GLFTQ
Sbjct: 241 YHGEMERGEEFVAAEFAQMQAVIRLEYETVSKSSWMDLLRTPGMRKRLISSMLGLFTQW 300

Query: 317 SGNLTSYYSGKLFEMMGYTEASVKTRINVANACWSLLNATTIAFLVPYFKRRHMFMTSA 376
      SGNLTSY+ G L +M+G+T+++ +INV+ ACWSL T-+ LV +RR M+M
Sbjct: 301 SGNLTSYFLGDLKMGFTDSTFIQKINVSIACWSLFCGVTVSLLVTRIRRRIMYMACT 360

Query: 377 LSMCAVFIAITVSLERTQAAQDAGFKNTAAGISGLFWYFAFAPCYNMGNNALTYTYLVEL 436
      +S+ +IA TVS+ER + G N A I+ LF+ + ++PCYNMG NALTYTY+VE+
Sbjct: 361 ISLLLCYIAWTVSMERAMTGKANGTPNNGANIATLFFIYMYSPCYNMGYNALTYTYMVEV 420

Query: 437 WPYSHRSRGIGVQQIFGKLGGFFSTNVNSIALDAIRWKYMAIYCGWIFFEFLIVFFLYPE 496
      WPY+ RSRGI V Q+FG+L GFF+T VN I L + WKY+ YC W+ FE V+F++PE
Sbjct: 421 WPYAERSRGIAVFQFLGRLAGFFTTFVNPIGLKNVGWKYLISYCCWLAFEVCFVYFMFPE 480
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Figure 10-9

Query: 497 TSGRTLEELAFLEFEDASLNEKAAAAVEKQIHYGDEK 532

T GRTLEEL F+FE L +A AA EK +++ +

Sbjct: 481 TMGRTLEELTFMFEGEDLQRQANAAAEKVVNHTEDH 516

Query= jgi|Spath1|116270|estExt_fgenes1_pm.C_50266
(488 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score	E
	(bits)	Value
NCU05519 neurospora_crassa hypothetical protein similar to Tn...	700	0.0

>NCU05519 | neurospora_crassa hypothetical protein similar to Tna1
(520 nt)
Length = 519

Score = 700 bits (1807), Expect = 0.0, Method: Compositional matrix adjust.
Identities = 338/512 (66%), Positives = 400/512 (78%), Gaps = 25/512 (4%)

Query: 1 MADEKRMGSSSDSKAAVQHSETLPGVSSTA AERGFAATDQNGQPIVQFDLKAEARLRRKL 60
M EKR S + E PG + AERG AATD +G P+V+ D AE +LRRK+
Sbjct: 9 MTSEKRRQSLSESDTKEGYFENAPG-AHYRAERGQAATDIHGNPLVELDPVAETKLRRKI 67

Query: 61 DLFIVPTVSLLYLFCFIDRANIGNARIAGLEKDLNLTGYDYNALLSVFYISYIVFEIPSN 120
DL++VPTV++LYLFCFIDRANIGNAR+ LEKDL+L GYDYNALLSVFY+ YIVFEIP-N
Sbjct: 68 DLYVVPTVAILYLFCFIDRANIGNARLDKLEKDLDLHGYDYNALLSVFYVGYIVFEIPAN 127

Query: 121 IACKWIGPGWFIPAIISLGFGVVSLATAFVDNFAQAAGVRFL LGVFEAGMMPGIAYYLSRW 180
I CKW+GPGWF+P SLGFG++S+ AFV+NF+QA GVRFL LGVFEAGM+PGIAYYLSRW
Sbjct: 128 IMCKWMGPGWFLPLTSLGFGIMSVCMFVNNFSQACGVRFL LGVFEAGMLPGIAYYLSRW 187

Query: 181 YRRAELTFRLSLYIVMAPMAGAFGGLLASGILSLDHVGGVTGWRMIFVVEGIITIGLSVI 240
YRR+ELT RLSLYIVM+P+AGAFGGLLASGIL LDH G + GWRMIF +EGIIT+GLS-I
Sbjct: 188 YRRSELT LRLSLYIVMSPLAGAFGGLLASGILKLDHFGSLHGWRMIFGIEGIITVGLSLI 247

Query: 241 SFITLTDRPETARWLTQEEKDLAIARVKSERVATTEVLDRMDTKKLIQGILSPVTLATSF 300
F+TLTD P TA+WLT+QEEKDLAIARVKSER+ TE++D+MD KKL +GI +PV T F
Sbjct: 248 GFLTLDHPATAKWLSTQEEKDLAIARVKSERIGQTEIIDKMDAKKLKRGIFNPVVFETGF 307

Query: 301 MFLNNIT-----QLFTVPPYVVGFFTLALPLLSWYLD RR 336
FLLNNIT QL TVPPY+VVGFFT+ +PL+S +LDRR
Sbjct: 308 AFLNNITVQGLAFFAPTIVVATIYPTKNIIQKQLTVPPYIVVGFFTVLMPLISRWLDRR 367

Query: 337 QIIILLSTPLVIVGYSMFLGTTNPSARYGATFLLSSSLFAVGALSNSQVSANVVS DTARS 396
QIII+ PLV+VGY MFL T N RYGA FL+S+S+FA G L+NSQVSANVVS DTARS
Sbjct: 368 QIIIIACCP LVMVGYIMFLATENAHVRYGAAFLVSTSVFAAGPLTNSQVSANVVS DTARS 427

Query: 397 SAIGLNVMMGNVGGIATWSYLPWDGPNYKIGNGLNLAACCTVLILSAVTLLWMKWDNRR 456
SAI NVM+GNVGGI+ATWS+LP+D P Y IGNGLNLA LI++ L+WMK DN +
Sbjct: 428 SAIAYNVMLGNVGGIVATWSFLPFDA PKYHIGNGINLAASGGTLLIALCLLIWMKRDNNK 487

Figure 10-10

Query: 457 REGRNAEEELAGMSRQEIQDLDWKHPAFRWRP 488
 RE RNAEEEL GMS+QEIQDLD+KHP FRW+P

Sbjct: 488 RELRNAEEELTGMSQQEIQDLDYKHPGFRWKP 519

Query= jgi|Spath1|79030|estExt_Genewise1Plus.C_31624
 (519 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
 9822 sequences; 4,775,003 total letters

Searching.....done

	Score	E
Sequences producing significant alignments:	(bits)	Value
NCU01231 neurospora_crassa hypothetical protein similar to ca...	834	0.0
>NCU01231 neurospora_crassa hypothetical protein similar to carboxylic acid transport protein (520 nt) Length = 519		

Score = 834 bits (2154), Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 395/503 (78%), Positives = 441/503 (87%), Gaps = 1/503 (0%)

Query: 1 MESTHEPADP- AKGVLATAKQSWHDLFIKQRVVVTNELGETSTEWARPVPLRNPISLLA 60
 MESTHEPADP- AKG+L TA+QSW DLFI+KQRVVVTN GET-TEWA+PVPL+NPISLLA
 Sbjct: 1 MESTHEPADPVAKGILPTARQSWKDLFIWKQRVVVTNVYGETATEWAKPVPLKNPISLLA 60

Query: 61 QLSARNWLFF- VGF LAWVADAYDFHALSIQQVKLA E FYNTTKTNISTAITLTLLRSVGA 120
 QLS R+W+ F- VGF AW ADA+DFHALSIQQVKLA +Y +KT++STAITLTLLRS+GA
 Sbjct: 61 QLSGRDWICFLVGFCAWSADAFDFHALSIQQVKLA AYYGVSKTSVSTAITLTLLRSIGA 120

Query: 121 AFFGLAGDKWGRKWPMVANMIVLGVLQIGTIYSVTFSDFLAVRALFGLFMGGVYGNAIAM 180
 A FGLAGD+WGRKWPMV NMIVLG+LQI TIYS T+S FL VRALFGLFMGGVYGNAIAM
 Sbjct: 121 AAFGLAGDRWGRKWPMVNMIVLGILQIATIIYSSTYSQFLGVRALFGLFMGGVYGNAIAM 180

Query: 181 ALENSPPDARGLMSGILQQGYSLGYVIAACANLGVGGDNSWKT VFWIGAGLSIGVGLVR 240
 ALENSP DARGLMSGILQQGY+ GYV AACANLGVGG +SWKT VFWI AGLSIGVG+-R
 Sbjct: 181 ALENSPVDARGLMSGILQQGYAFGYVCAACANLGVGGDTDSWKT VFWIAAGLSIGVGII-R 240

Query: 241 CFFPESQQFLEARAAGKGQASASAFWKETKMMLAQEWKMCVYCIILMTWFNYYSHTSQDS 300
 CFFPES+QFLEAR GK A+ S FW+ETK+ML QEWMKCVYC ILMTWFNYYSHTSQD+
 Sbjct: 241 CFFPESKQFLEARKEGKAHANPSQFWRETKVMLRQEWKMCVYCCILMTWFNYYSHTSQDN 300

Query: 301 YTTFMLTQKELDNDGASRASILMKVGACVGGTIIGYISQWFGRRRTIIVAALISGLIIPA 360
 YTTF+L KE-DN ASRASI+MK GACVGGTIIGY+SQ+FGRRTIIV++LISG +IPA
 Sbjct: 301 YTTFVLRAKEMDNSAASRASIIMKAGACVGGTIIGYLSQYFGRRTIIVSSLISGCMIPA 360

Query: 361 WILPEGERSLSVTGFFMQFFVQGAWGVIP IHLNELSPPAFRSSFPGITYQLGNMISSPSA 420
 WILP ER+LS TGFFMQFFVQGAWGVIP IHLNEL+PPAFRSSFPG+TYQ+GNM+SSPSA
 Sbjct: 361 WILPNSERALSATGFFMQFFVQGAWGVIP IHLNELAPPAFRSSFPGITYQVGNMVSSPSA 420

Query: 421 QIVNAIAESHVTSKSGKSVNAYGPTMG IATAIIATGIAVTTALGPEKRGREFEKTLPAG 480
 QIVNA++E + S +GK V AYGPTMG IATAII GI VTTA GPEKRGREFEK LPAG
 Sbjct: 421 QIVNAVSEKIHIVSHTGKLVEAYGPTMG IATAIIVMGIVVTTAFGPEKRGREFEKALPAG 480

Figure 10-11

Query: 481 MNI-IQGGKAVDDLEKGDSEDEK 502
 MN+ Q GK VDDLE EK
 Sbjct: 481 MNLQKQHGKQVDDLEMETGHMEK 503

Query= jgi|Spath1|108890|estExt_fgenesh1_pg.C_60848
 (533 letters)

Database: neurospora_crassa_7_proteins_no_asterisks.fas
 9822 sequences; 4,775,003 total letters

Searching.....done

Sequences producing significant alignments:	Score (bits)	E Value
NCU00988 neurospora_crassa hypothetical protein similar to MF...	878	0.0
>NCU00988 neurospora_crassa hypothetical protein similar to MFS quinate transporter (538 nt) Length = 537		

Score = 878 bits (2269), Expect = 0.0, Method: Compositional matrix adjust.
 Identities = 429/538 (79%), Positives = 471/538 (87%), Gaps = 10/538 (1%)

Query: 1 MGLS--TKILQKIVRNEAMASDPPEIYGWRVYLLACSACFGAMSFGWDSSVIGGVIVLPP 58
 MGLS +IL-KIV+NEAMA DPPEIYGWRVYLLACSACFGAMSFGWDSSVIGGVI L P
 Sbjct: 1 MGLSIGNRILRKIVKNEAMAEDPPEIYGWRVYLLACSACFGAMSFGWDSSVIGGVIELEP 60

Query: 59 FIRDFN-LGDPKSQASANLSANIVSTLQAGCFLGALVASPMTDRFGRKWCLIGVSLIIII 117
 F DF +G+ K A ANL ANIVSTLQAGCFLGAL+ASP+TDRFGRKWCLI VSL++II
 Sbjct: 61 FKHDFGFIGNDK--AKANLGANIVSTLQAGCFLGALIASPITDRFGRKWCLIAVSLVVI 118

Query: 118 GIIMQAAASGNLGPYAGRFIAGAGVGAASINPIYVSENAPRAIRGLLTGLYQLFIVTG 177
 GIIMQAAASGNL PMY GRF+AG GVGAAS INP++VSENAPR+IRGLLTGLYQLFIVTG
 Sbjct: 119 GIIMQAAASGNLAPMYIGRFVAGVGVAASCINPVFVSENAPRSIRGLLTGLYQLFIVTG 178

Query: 178 GMIAFWINYSVSIHFPETKIMYVFPLAIQALPAALLCLCMLLCQESPRWLARRDRWEDTK 237
 GMIAFWINYSVS+HF + K MY+FPLAIQ LPA LLC+CMLLC ESPRWLARRDRWE+ K
 Sbjct: 179 GMIAFWINYSVSLHF-KGKSMYIFPLAIQGLPAGLLCVCMLLCHESPRWLARRDRWEECK 237

Query: 238 RVLSRIRNLPPDHPYIQDEFQEIVAQLEHERRLIGDASFWNLQREMWTIAGNRRRVLISTI 297
 VL+RIRNLPPDHPYI DEF+EI QLE ERRL GDA++W+L R+MWT+AGNR+R LISI
 Sbjct: 238 SVLARIRNLPPDHPYIVDEFREIQDQLEQERRLQGDATYWDLTRDMWTVAGNRKRALISI 297

Query: 298 ILMICQQMTGTNAINTYAPTIFKNLGLTGTSTSLFSTGVYGVIVKVTSCIIIFLLFMADSLG 357
 LMICQQMTGTNAINTYAPTIFKNLG+TGTSTSLFSTG+YGIVKV SC+IFLLF+ADSLG
 Sbjct: 298 FLMICQQMTGTNAINTYAPTIFKNLGITGTSTSLFSTGIYGVIVKVVSCVIFLLFLADSLG 357

Query: 358 RRRSLLWTSIAQGLAMFYIGLYVRIAPPKEGESVPPAGYFALVCIFLFAAFFQFGWGPAC 417
 RRRSLLWTSIAQGLAMFYIGLYVRI+PP +G+ VPPAGY ALVCIFLFAAFFQFGWGPAC
 Sbjct: 358 RRRSLLWTSIAQGLAMFYIGLYVRISPPIDGQPVPPAGYVALVCIFLFAAFFQFGWGPAC 417

Query: 418 WIYASEIPAARLRSINVAYAAATQWLFNFVVARVPVMIIVTMGEGGYGTYLFGSFCFSM 477
 WIYASEIPAARLRSINV+YAAATQWLFNFVVAR VP M+VT+G GYGTYL+FGSFC SM
 Sbjct: 418 WIYASEIPAARLRSINVSAYAAATQWLFNFVVARAVPTMLVTVGPHGYGTYLIFGSFCFSM 477

Figure 10-12

Query: 478 FVFVWFFVPE Γ KGVSLEAMDKLFGVTD----ESSKSLTVDEDAKEKEKDGP HARQTEV 531
FVFVWFFVPE Γ KG+SLE MD+LFGVTD E S D+ E K ++ EV
Sbjct: 478 FVFVWFFVPE Γ KGISLEHMDELFGVTDGPAAEKSSVHGGDDVGSEMGKGDQKSKHVEV 535

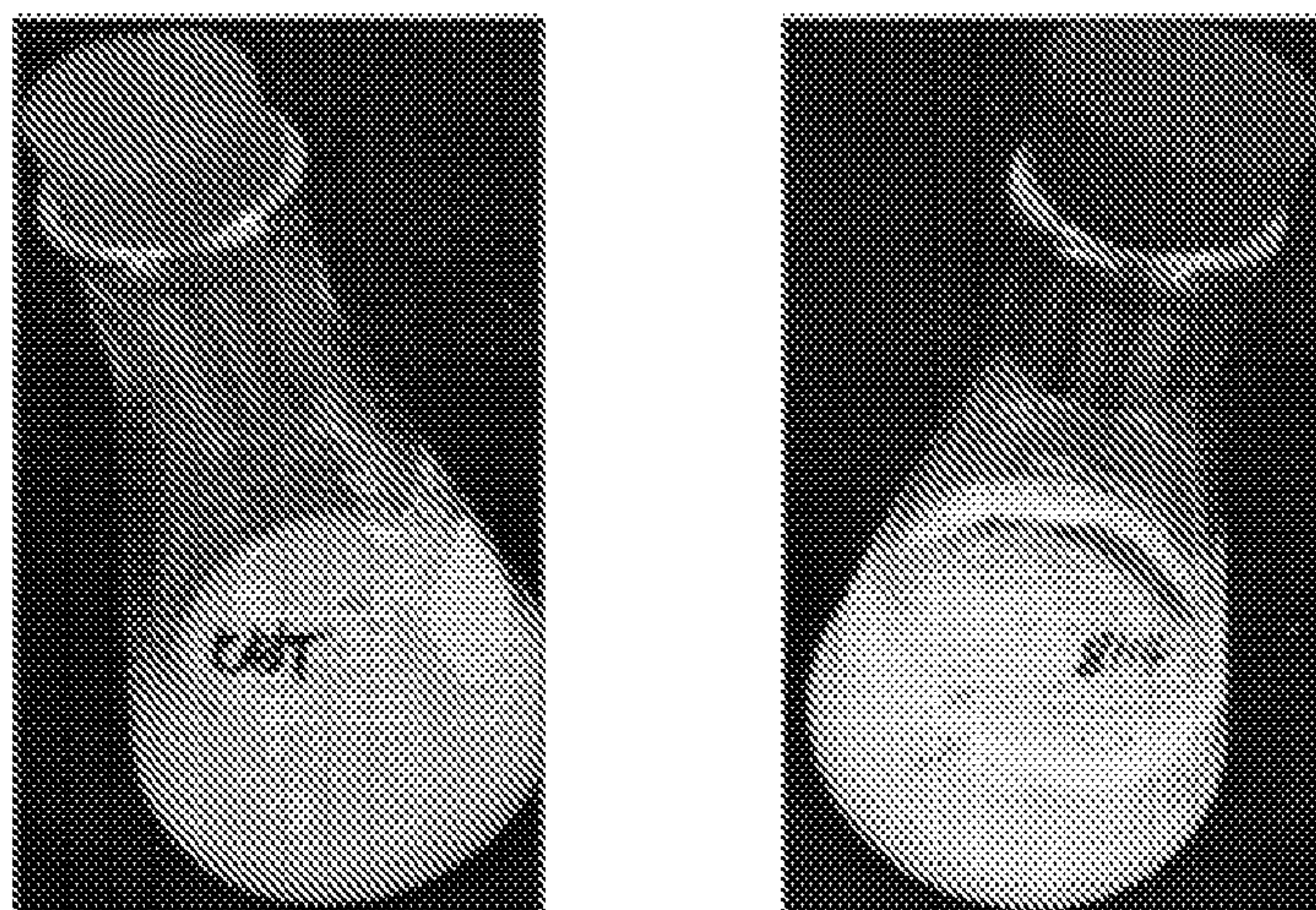
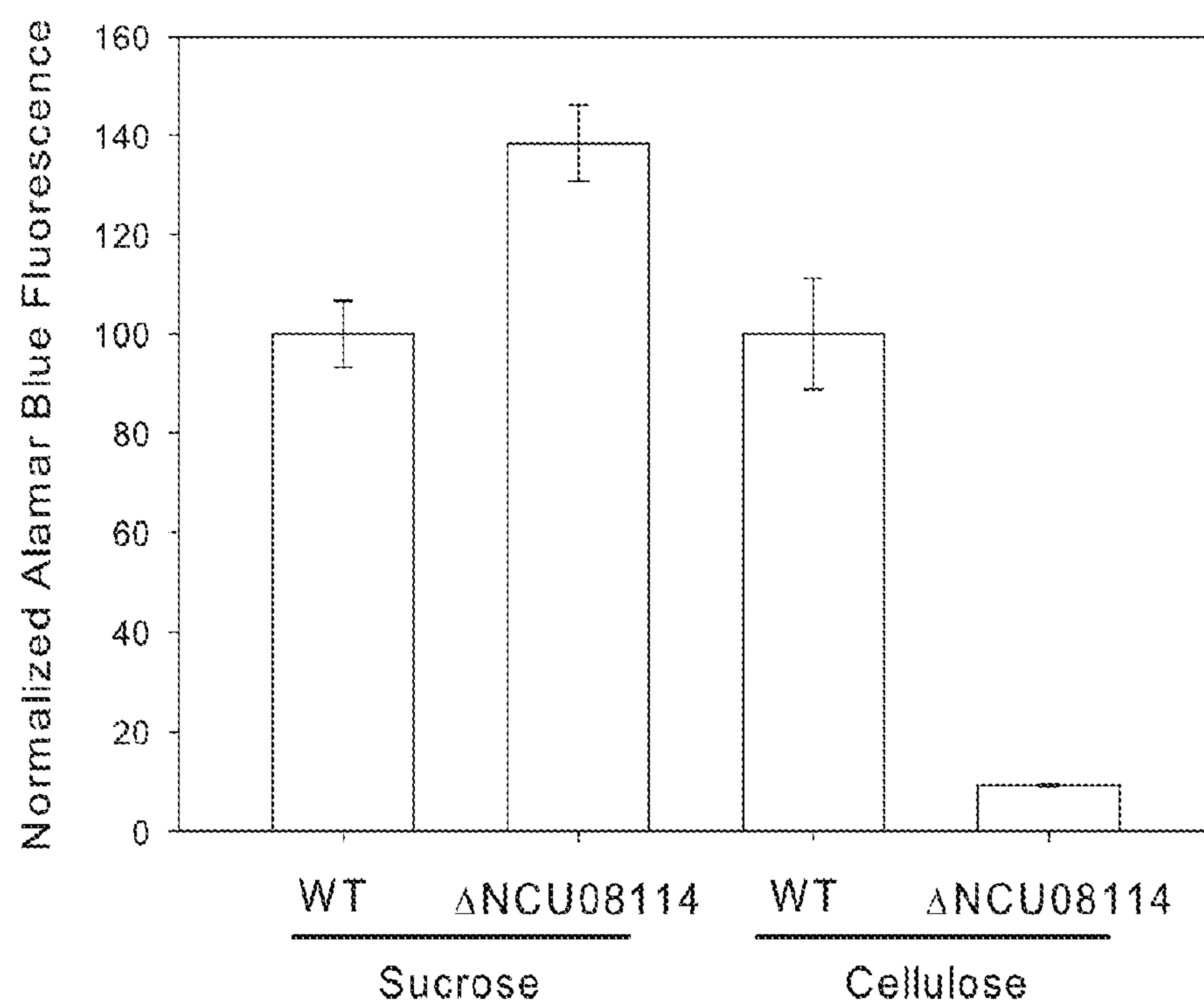
Fig. 11**A****B**

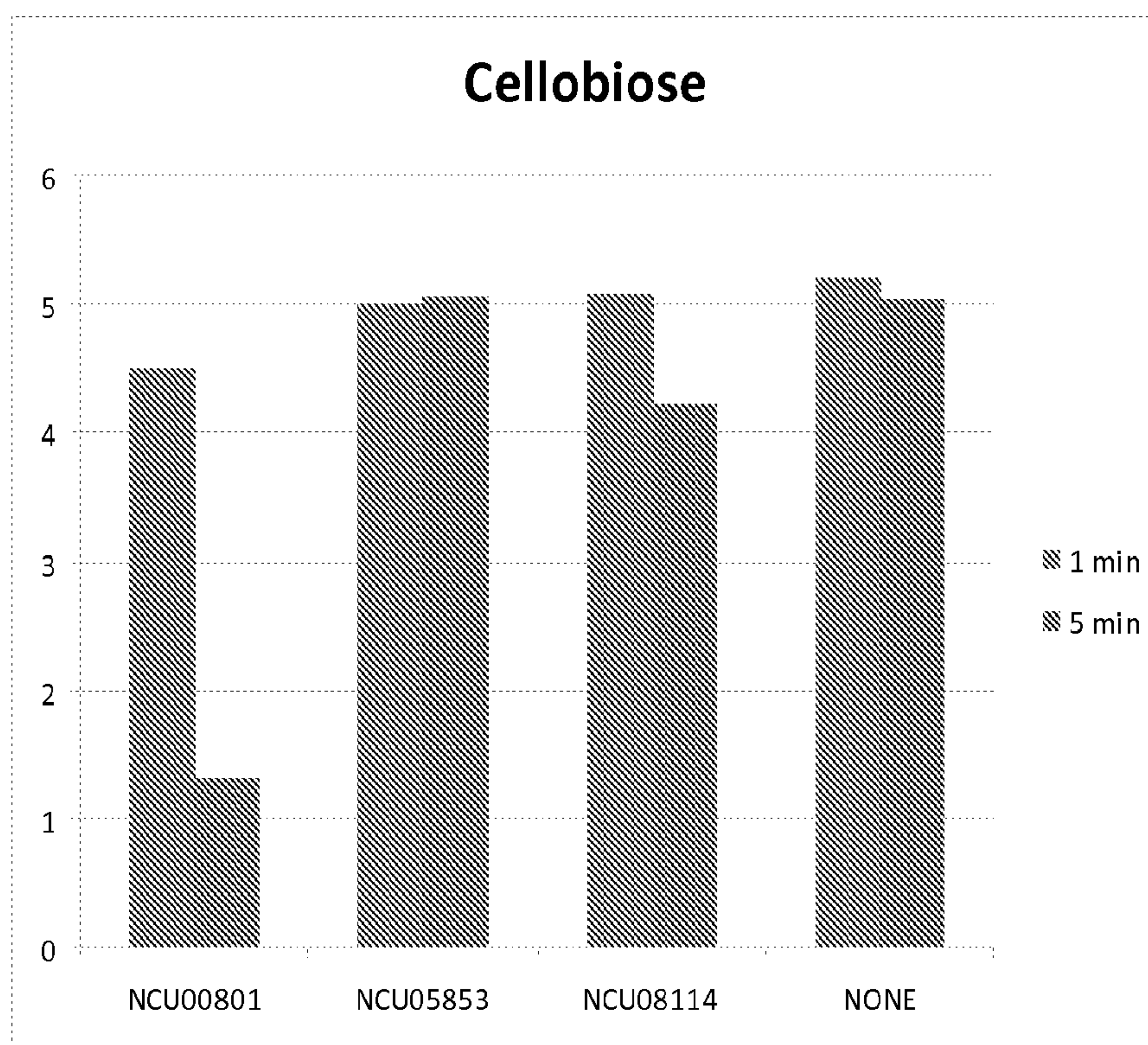
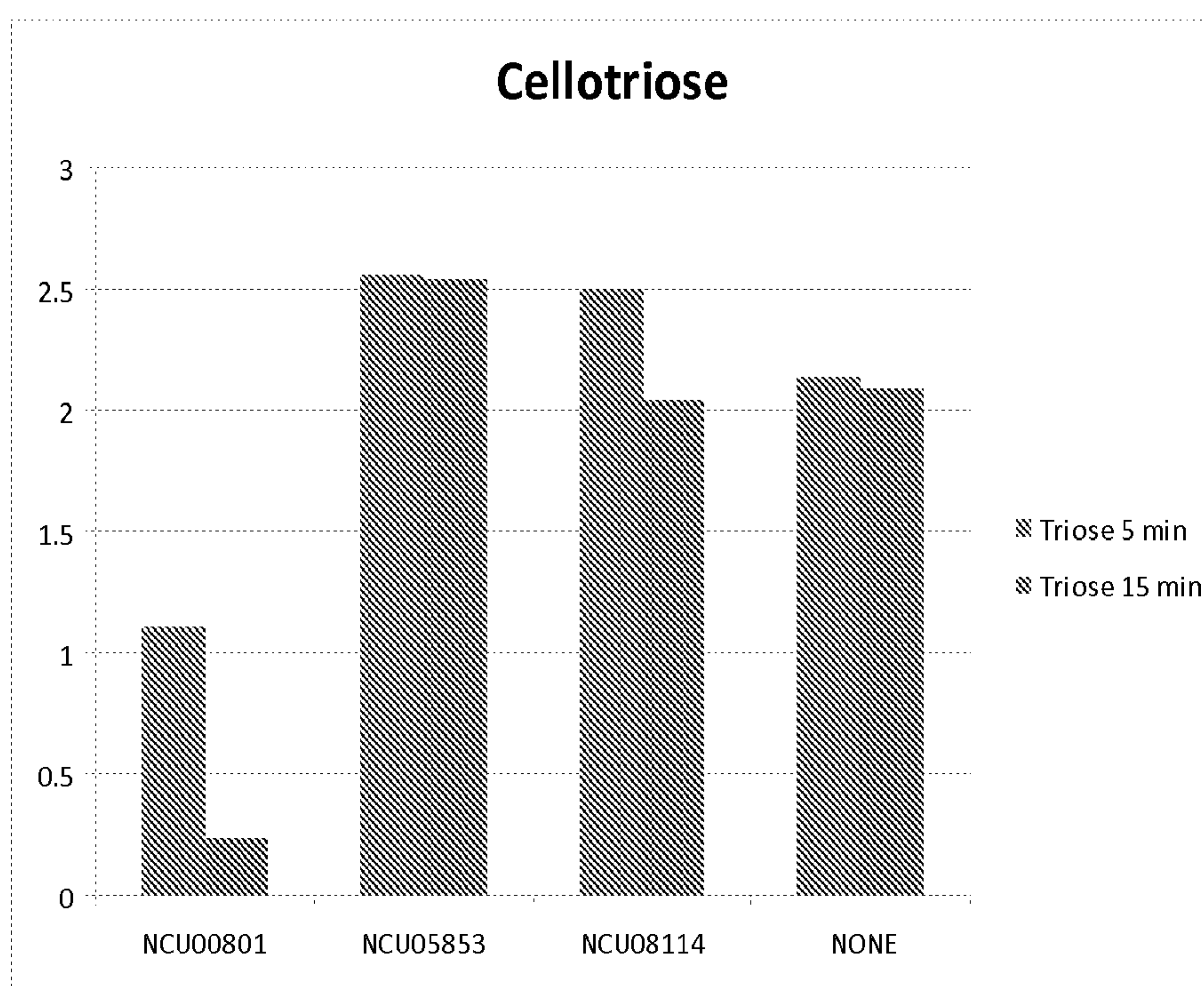
Fig. 12**A.****B.**

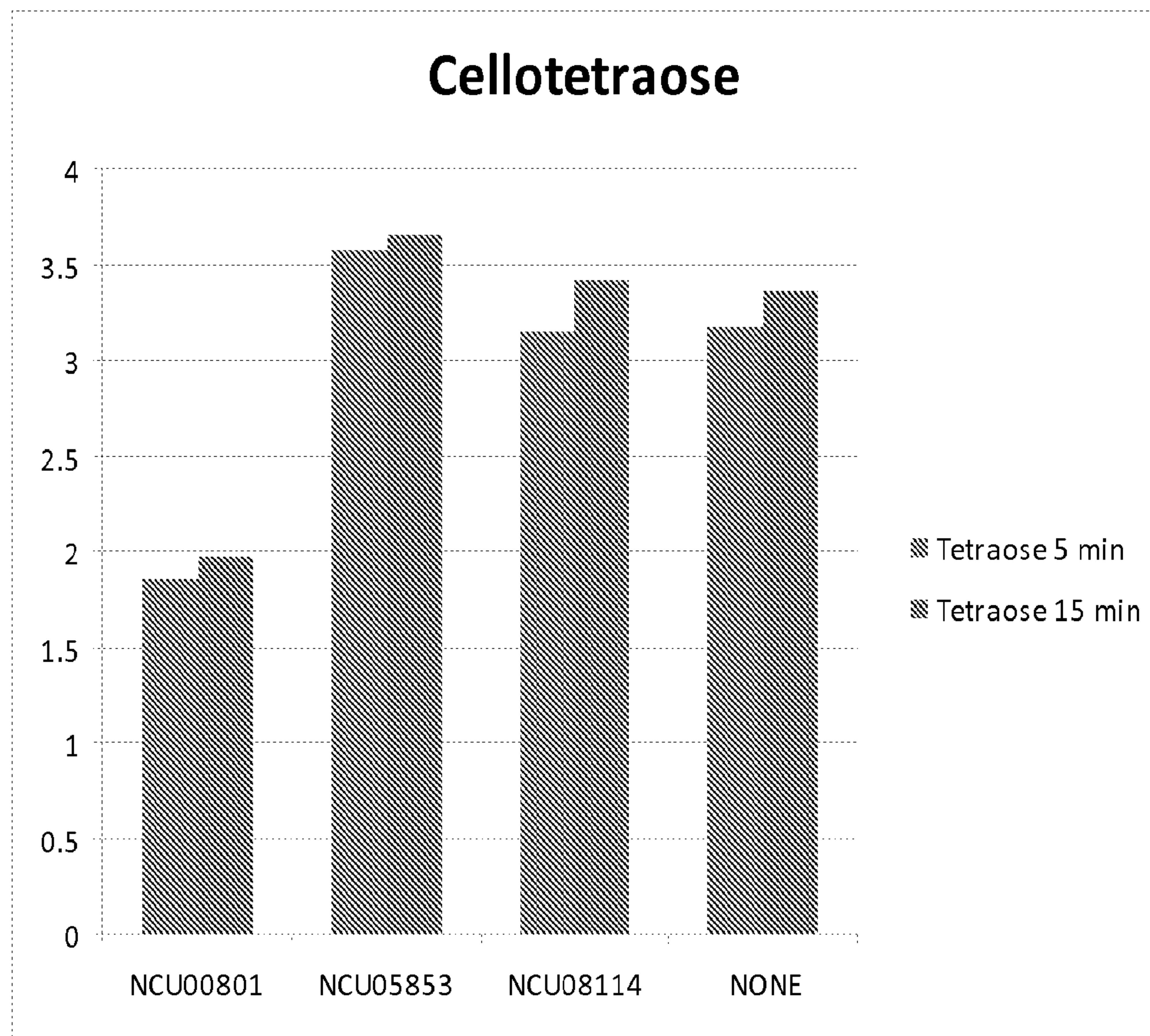
Fig. 12 (cont.)**C.**

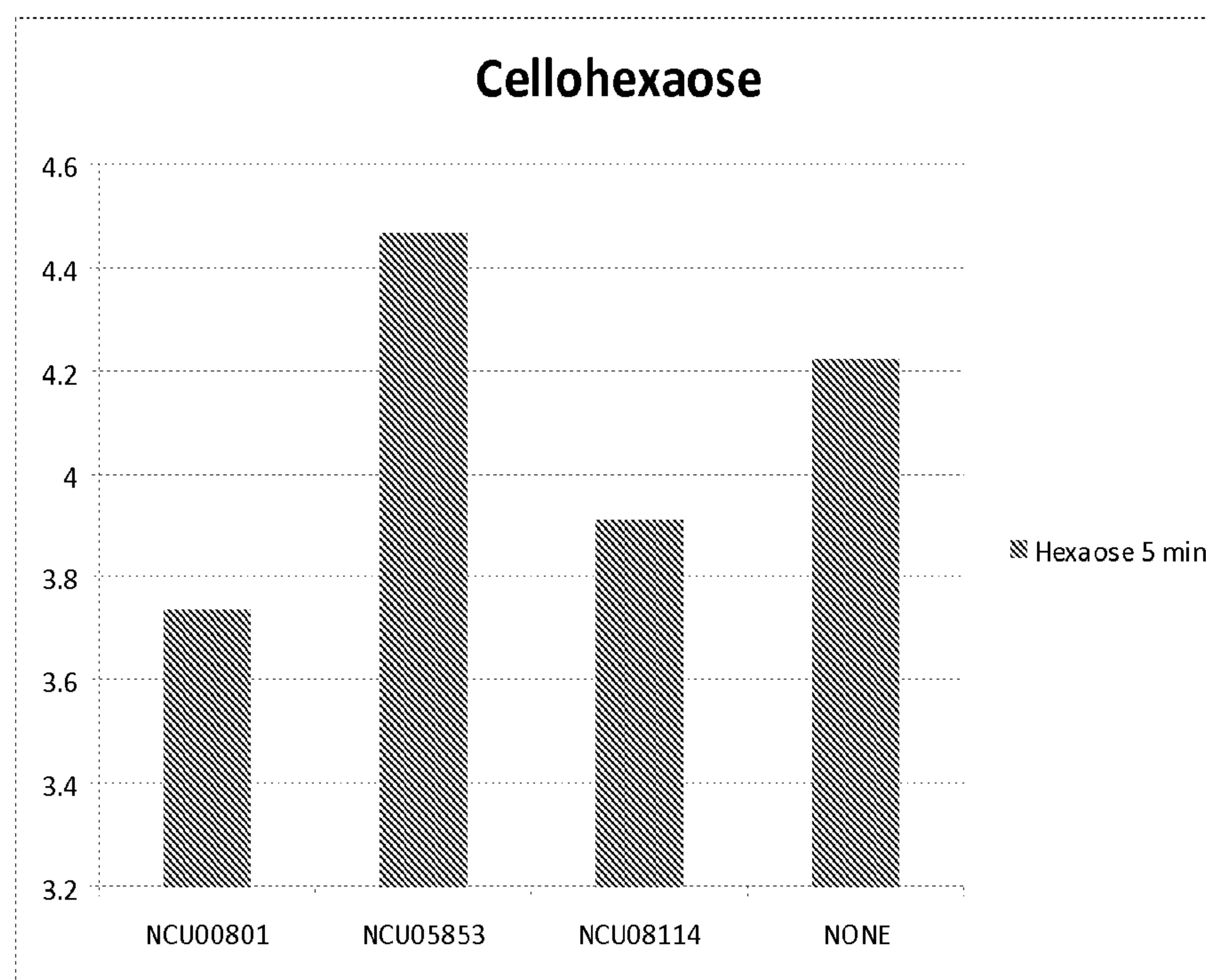
Fig. 12 (cont.)**D.**

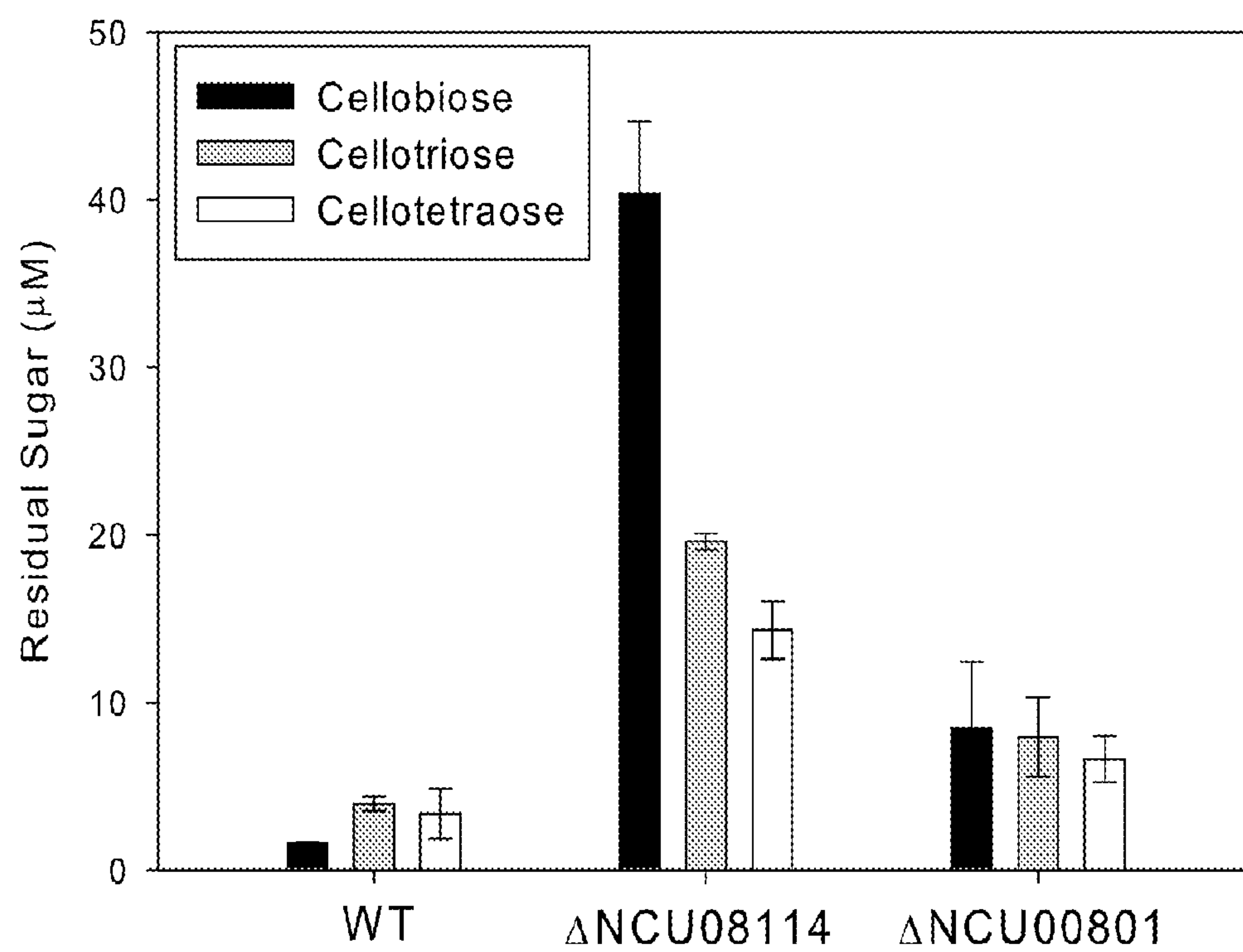
Fig. 13

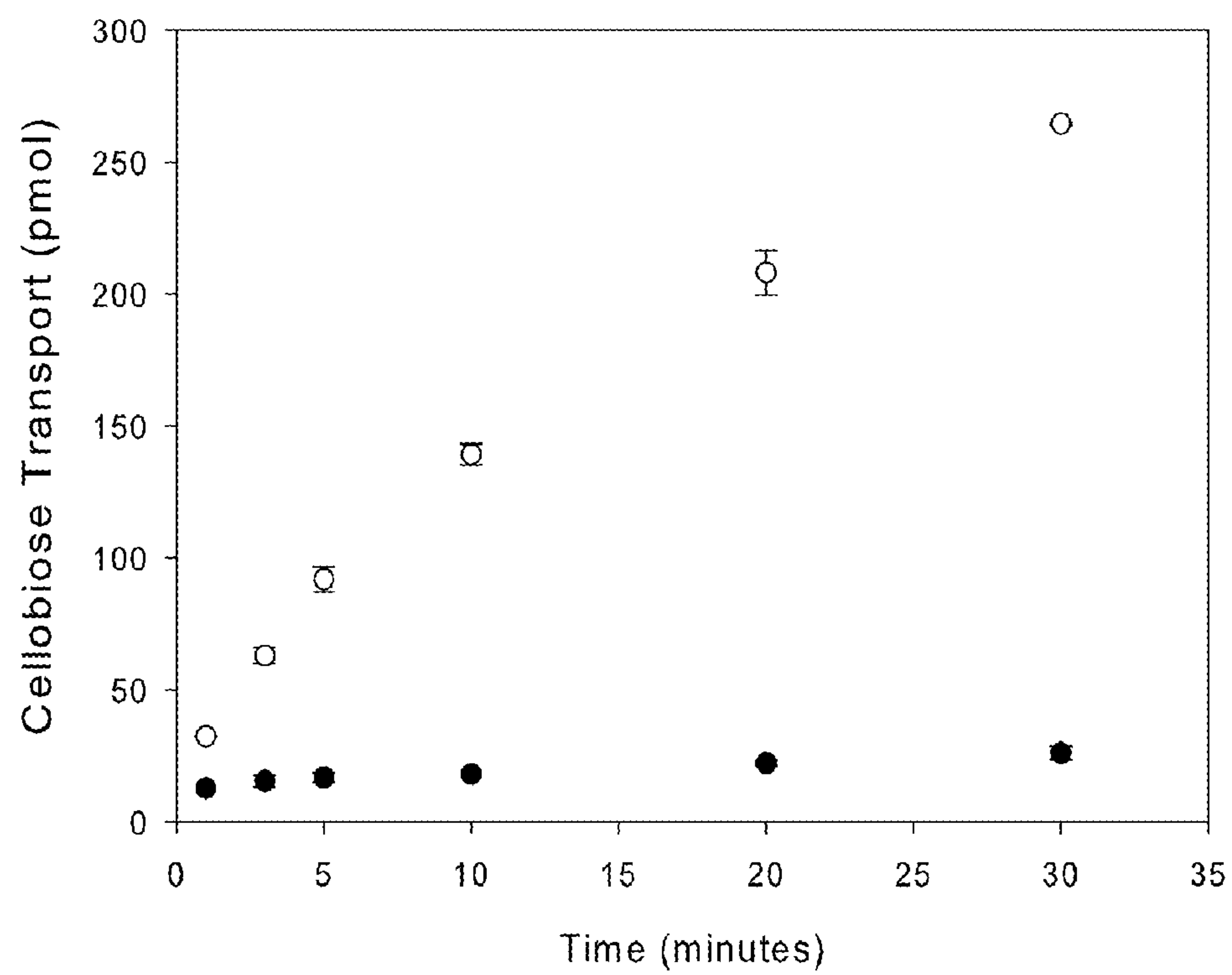
Fig. 14

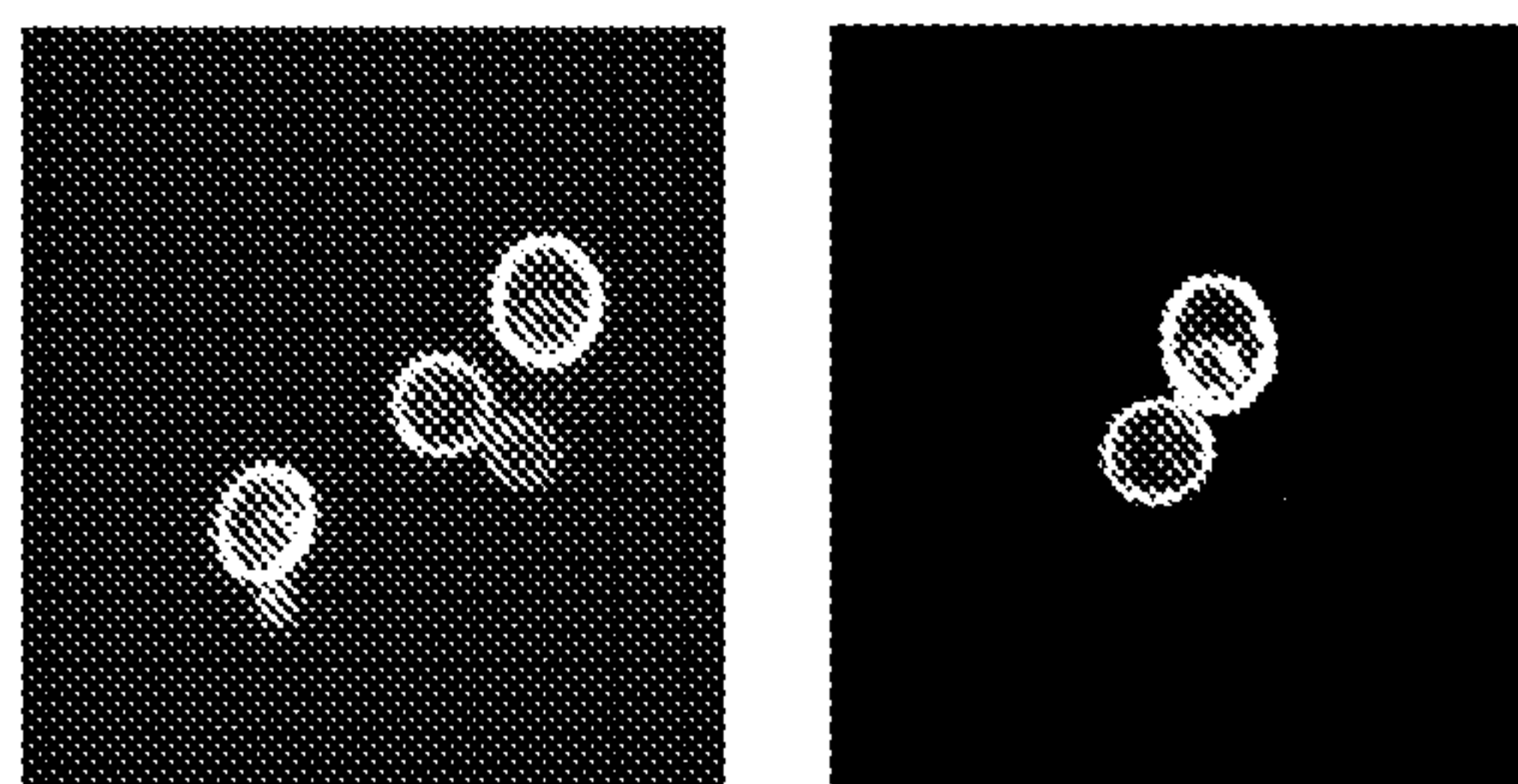
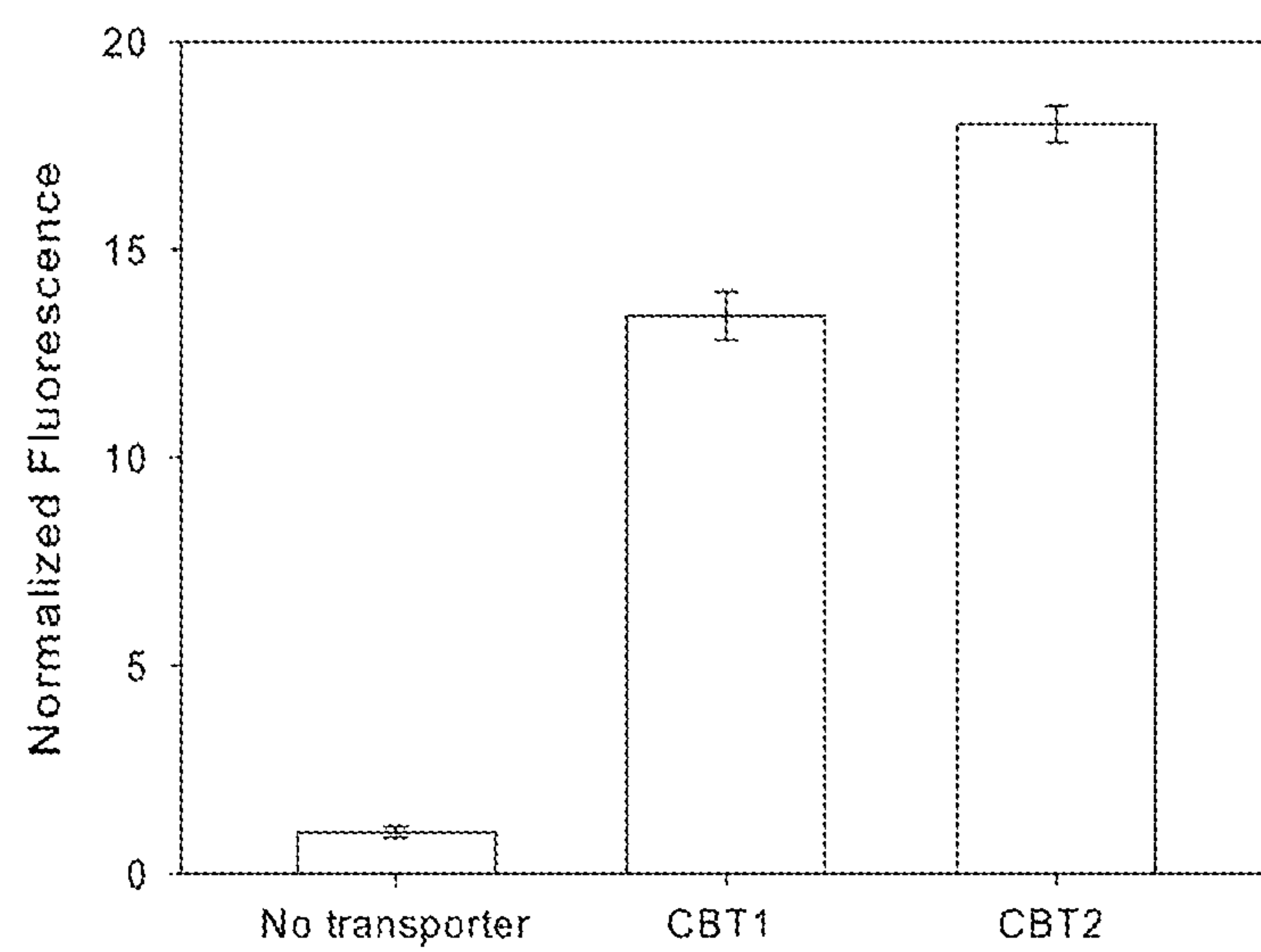
Fig. 15**A****B**

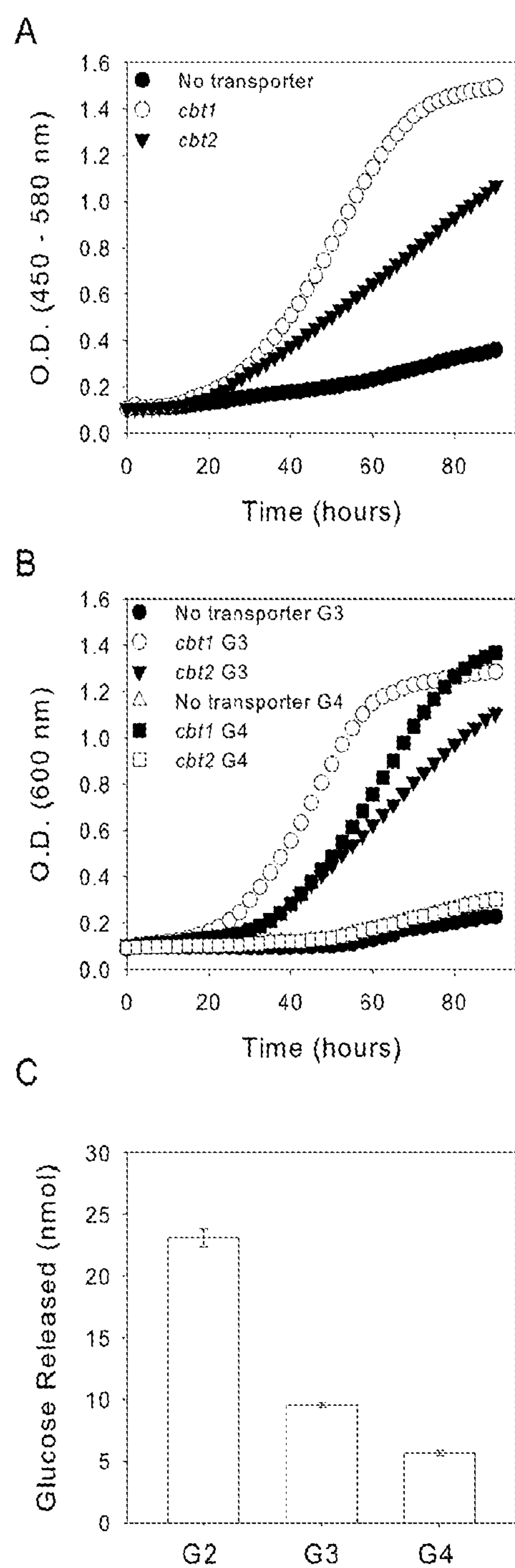
Fig. 16

Fig. 17

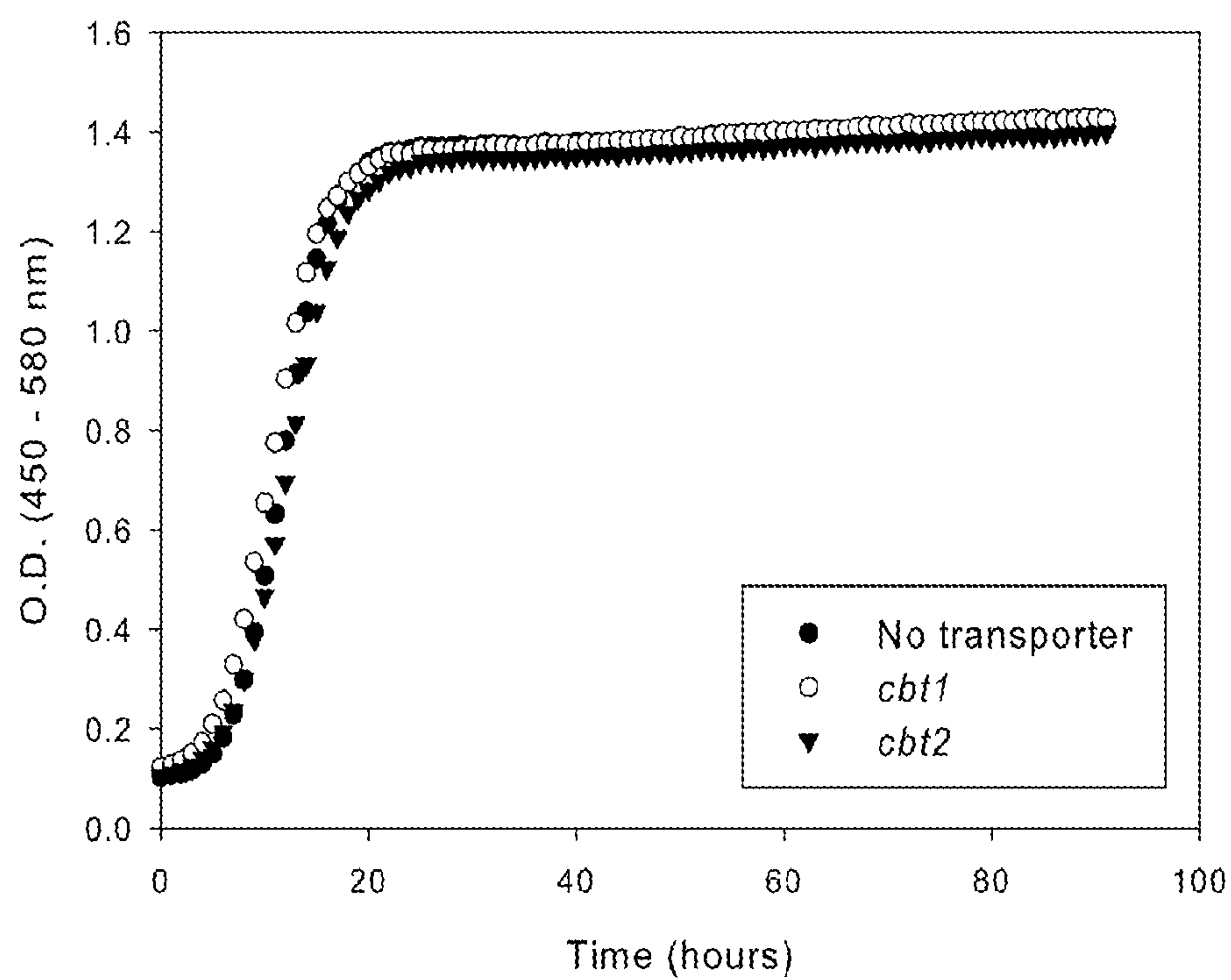


Fig. 18

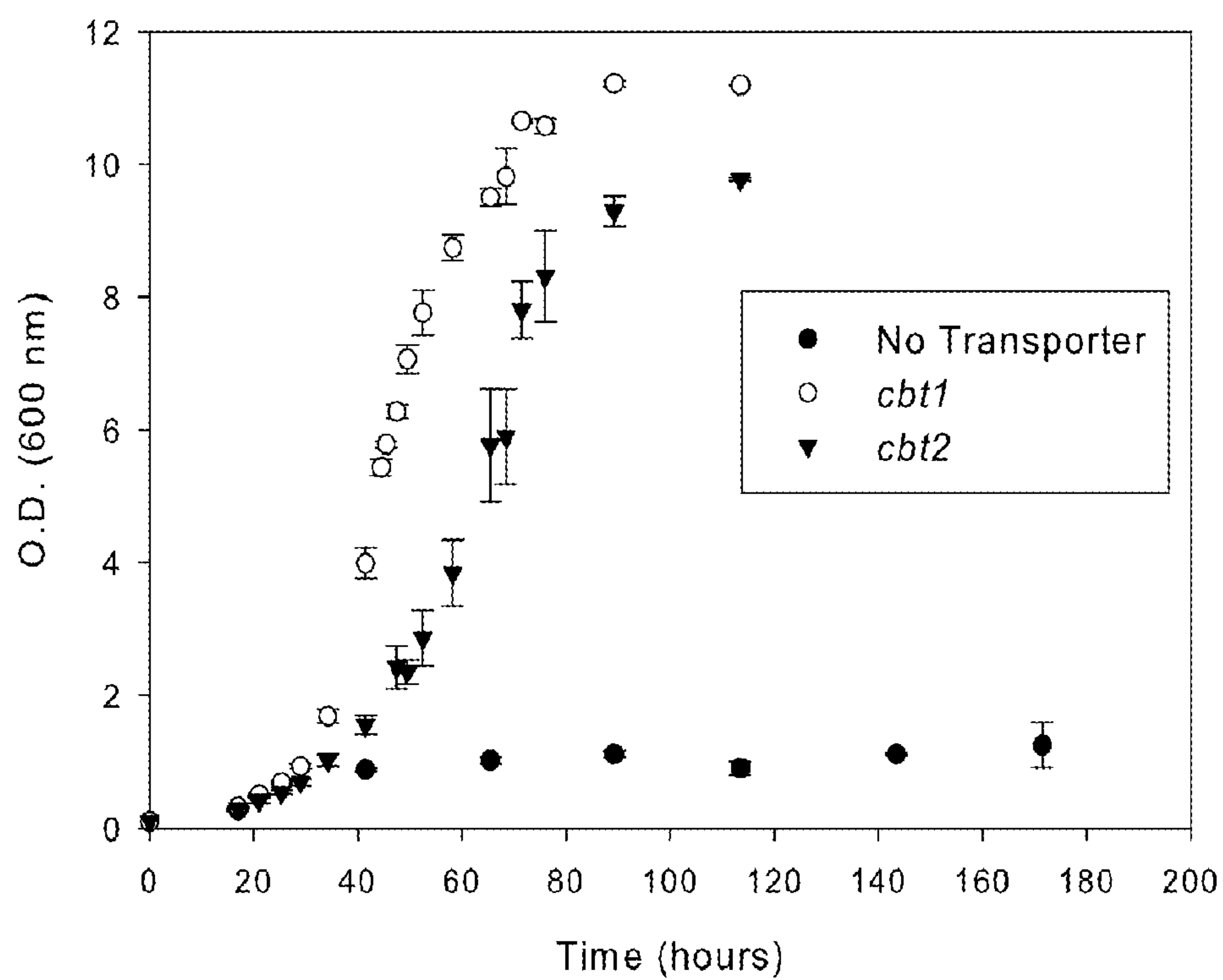


Fig. 19

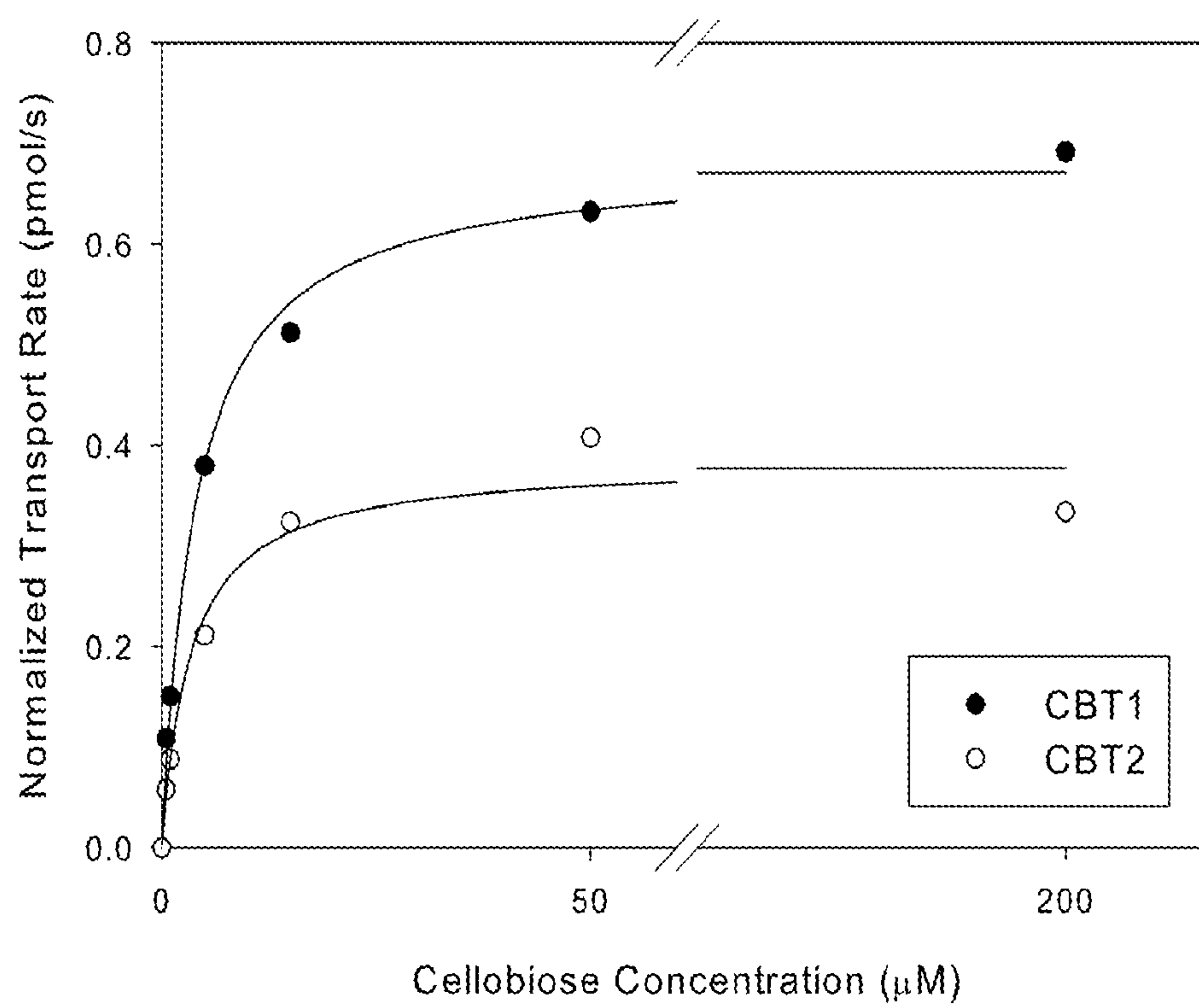


Fig. 20

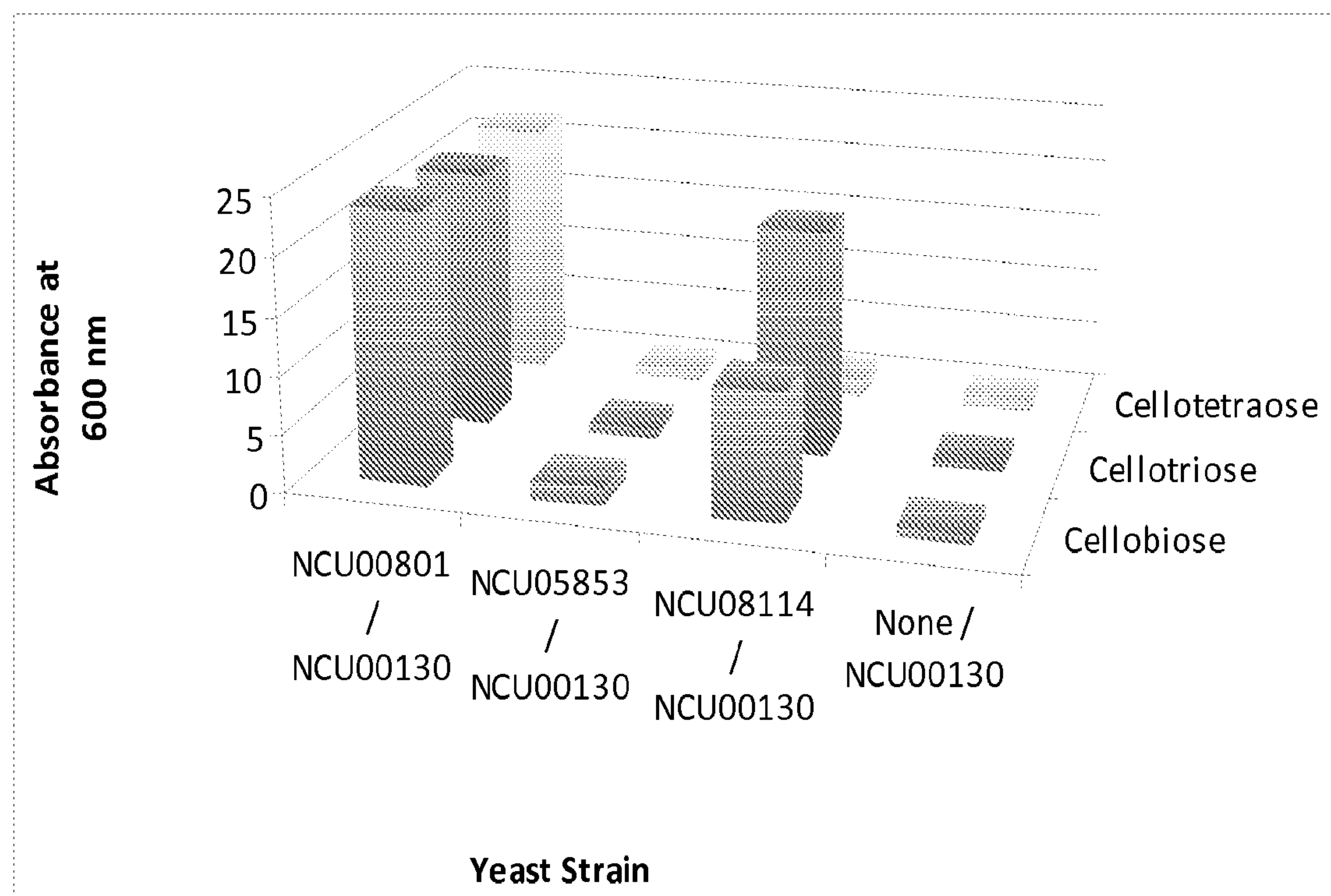


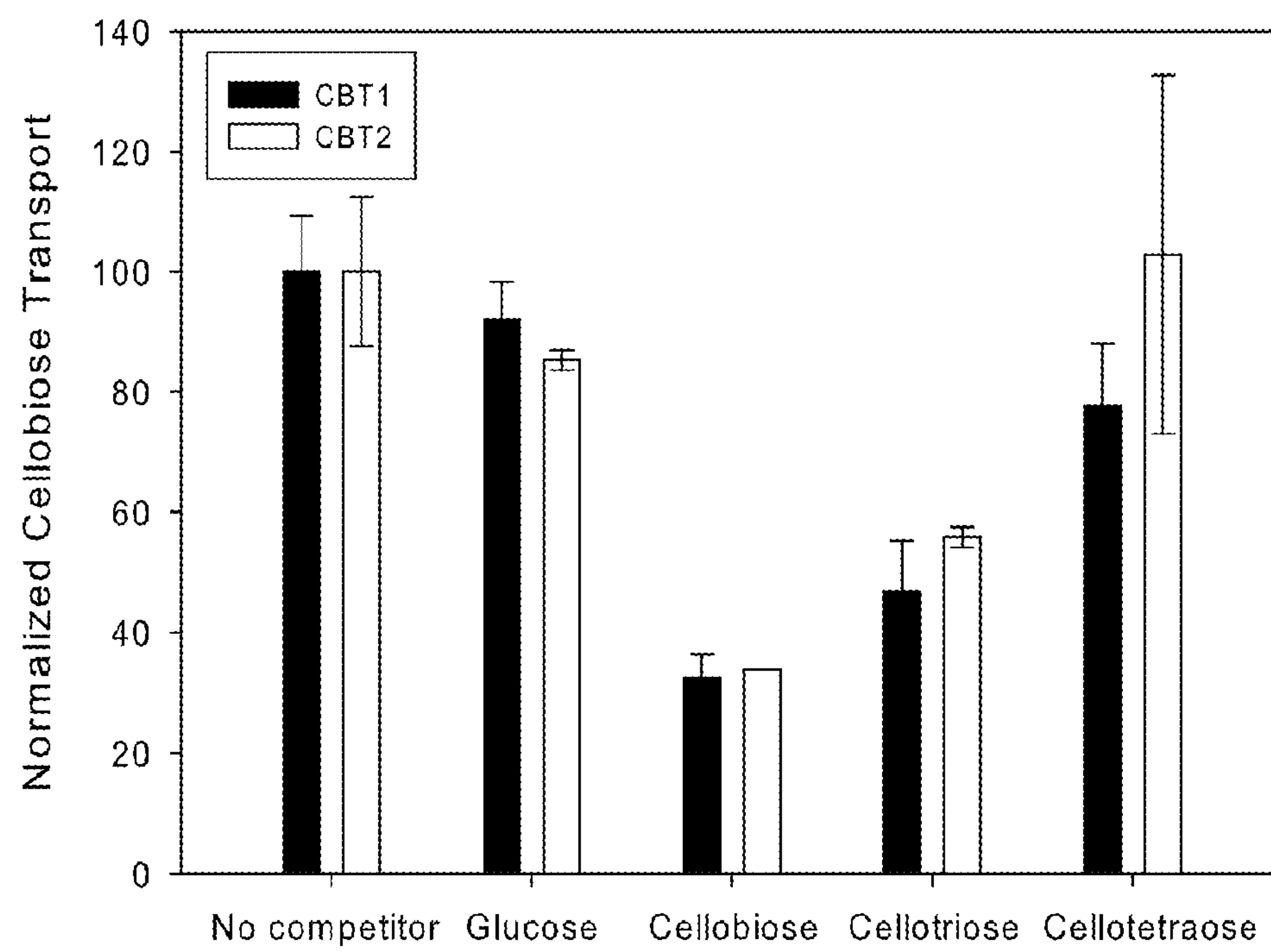
Fig. 21

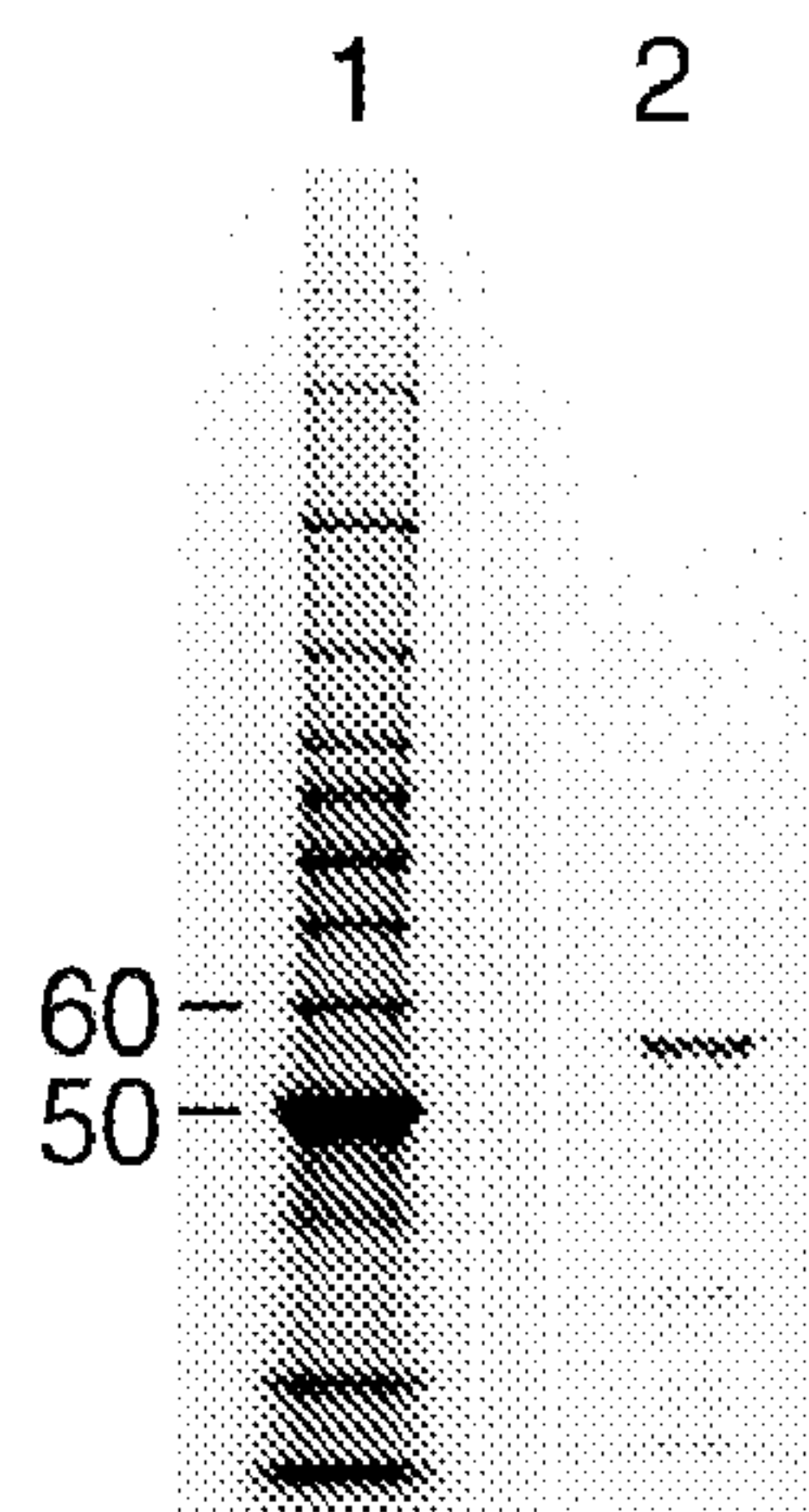
Fig. 22

Fig. 23

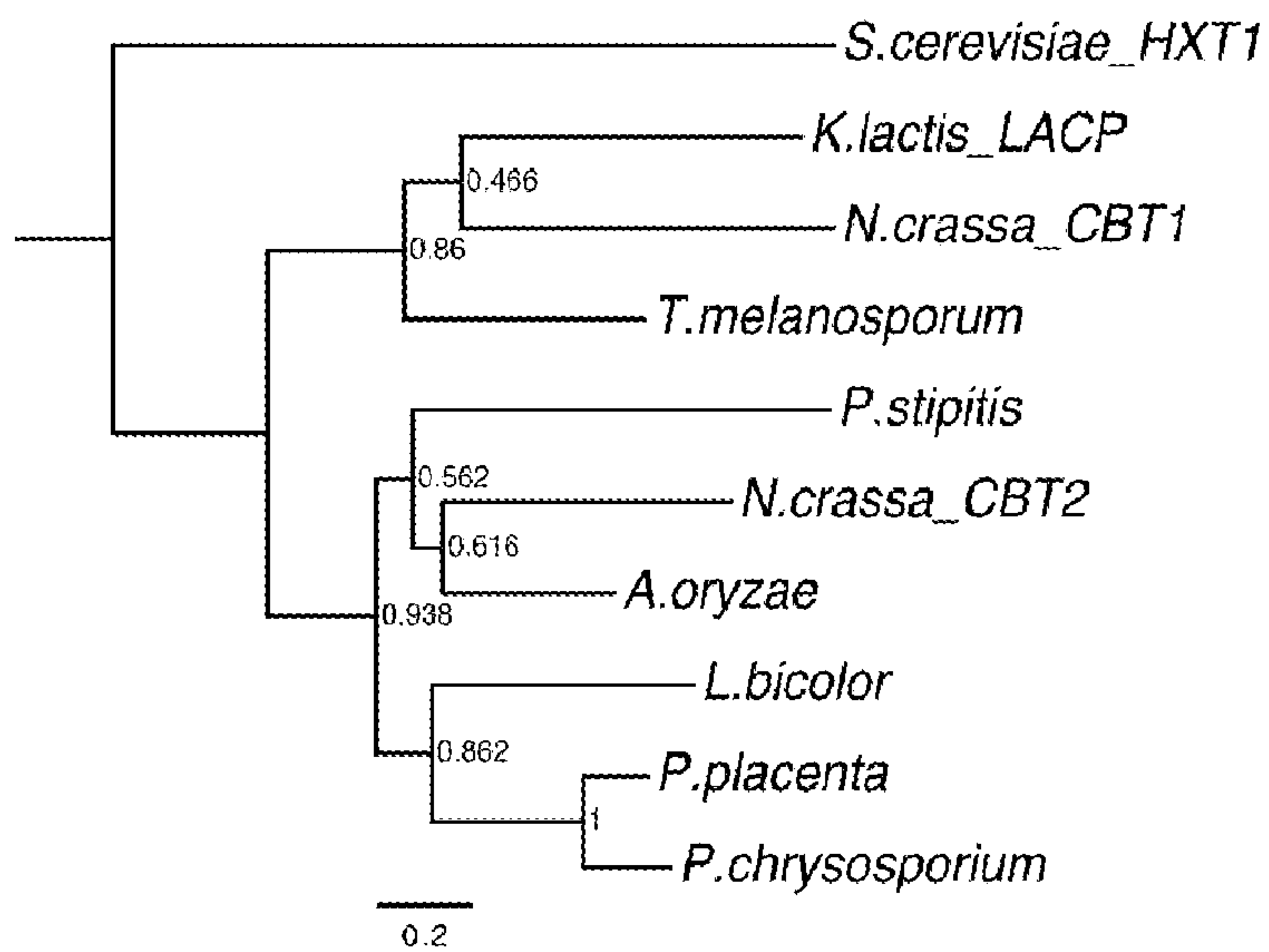
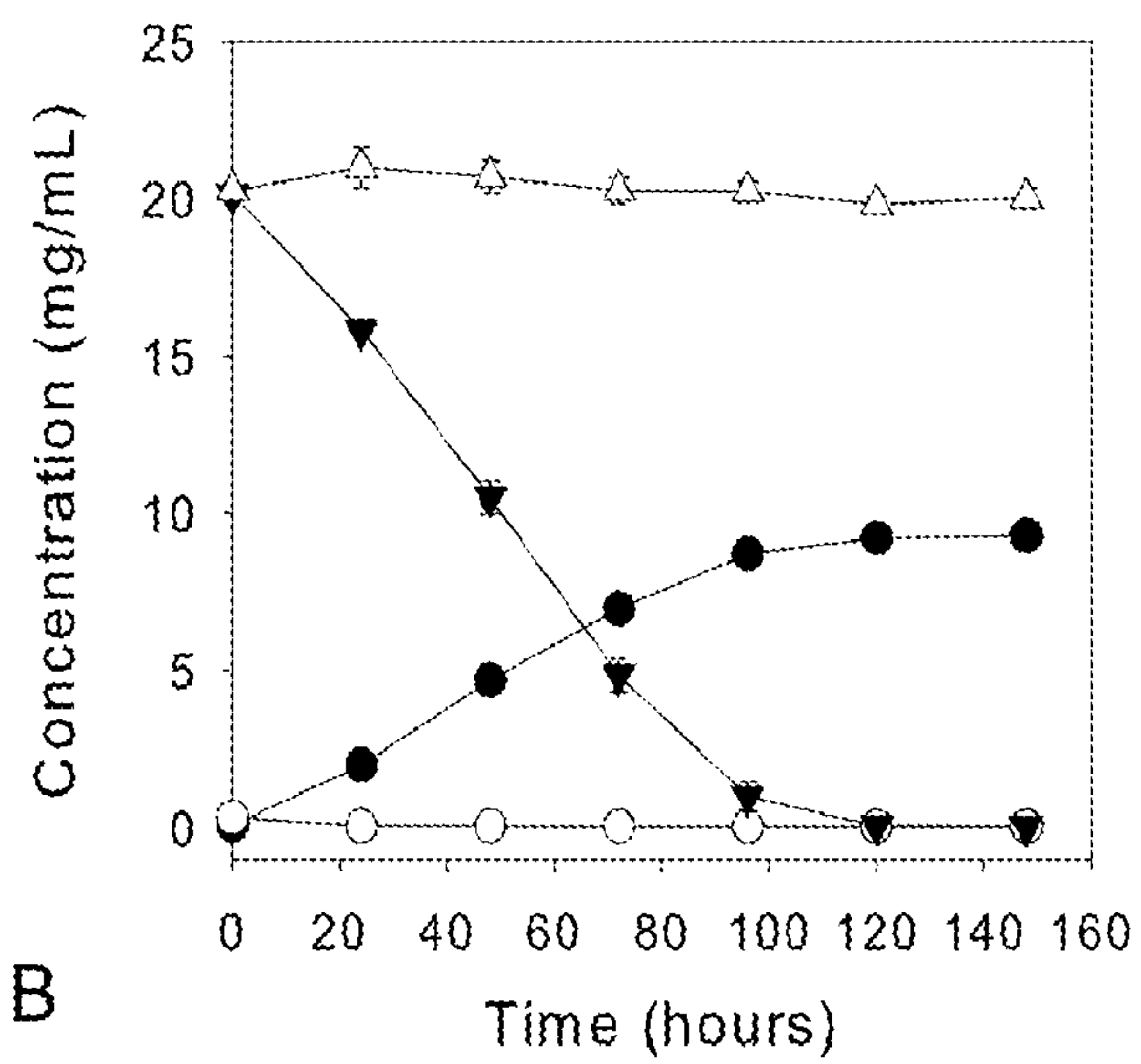
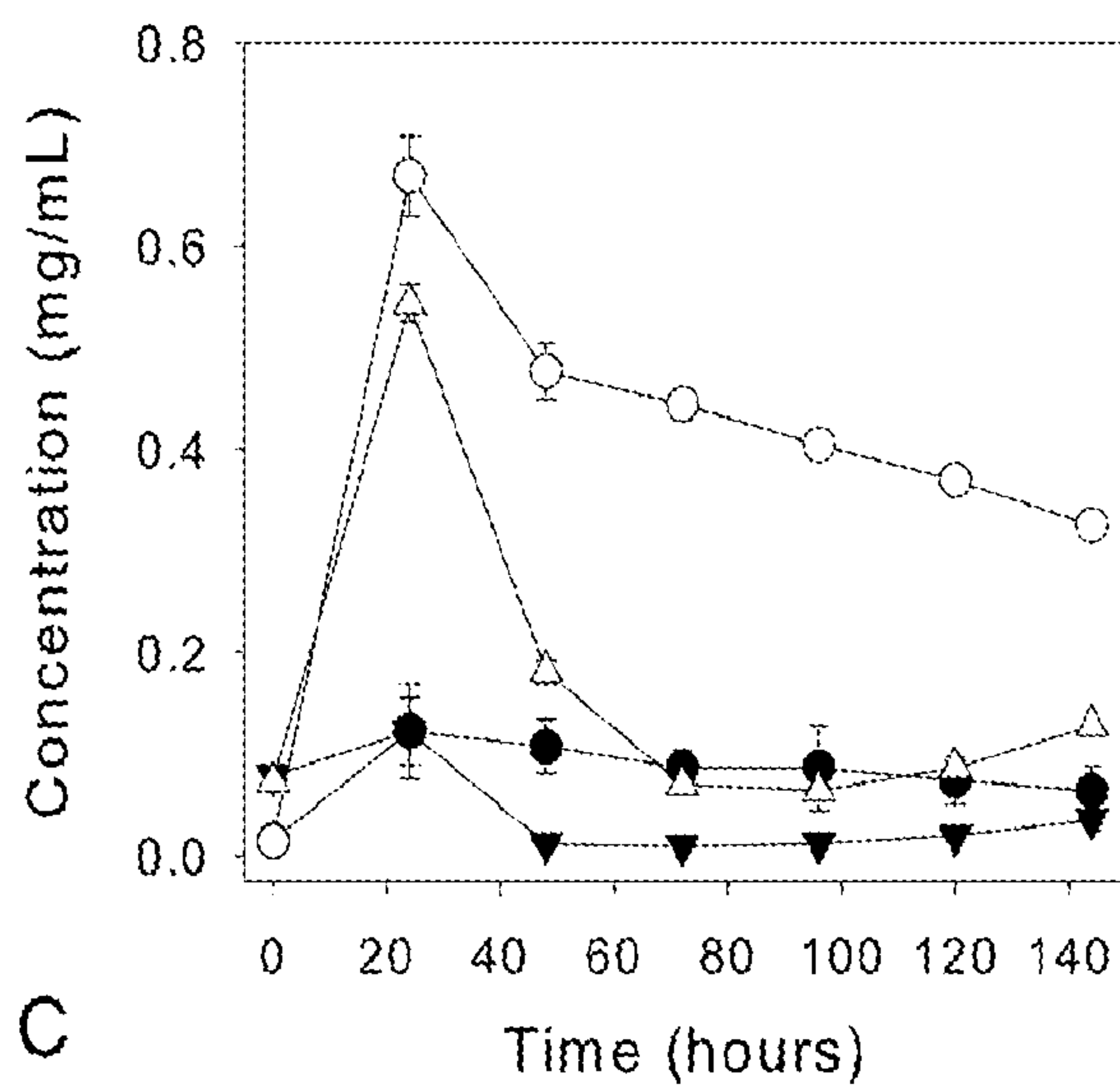


Fig. 24

A



B



C

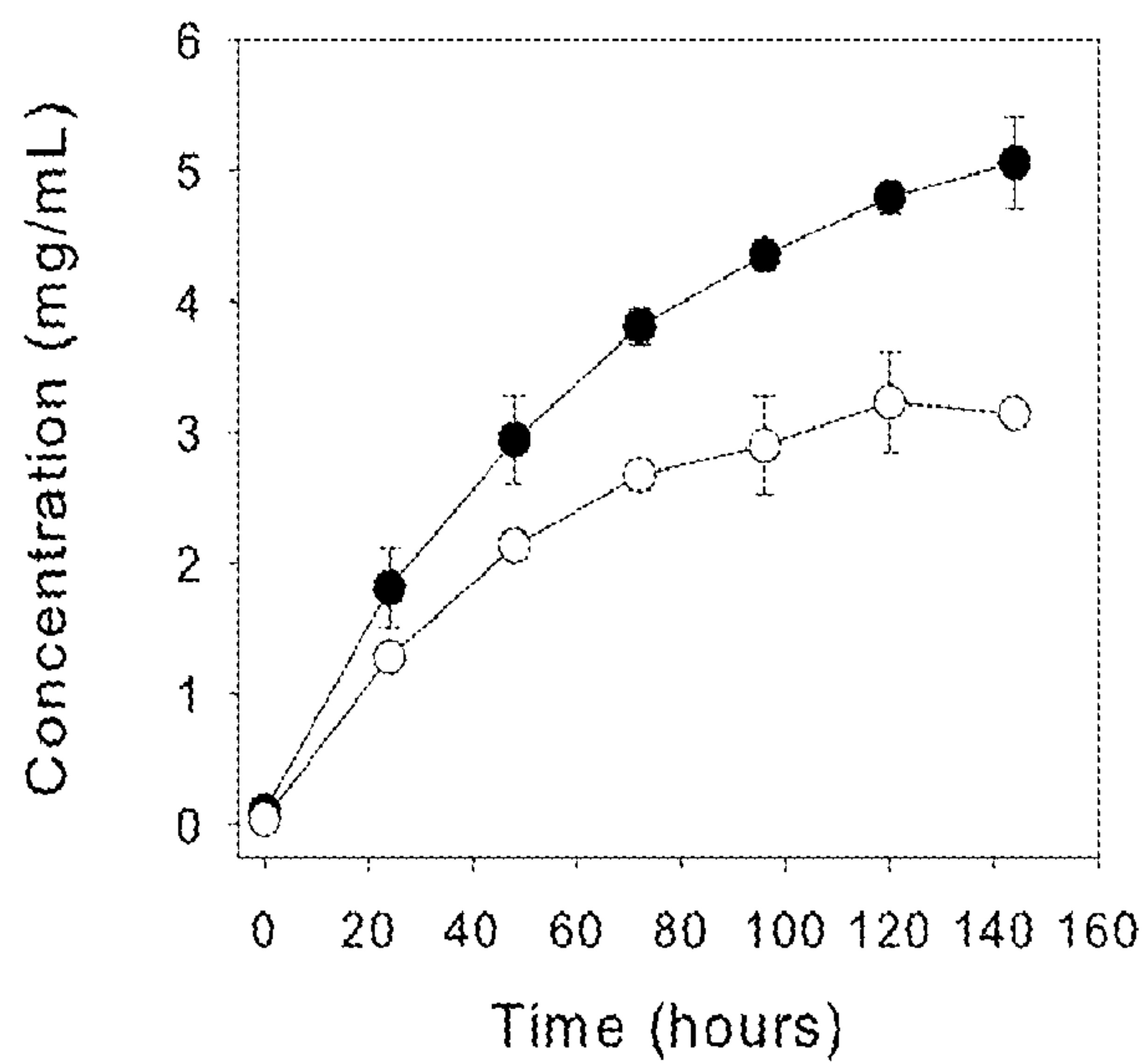


Fig. 25

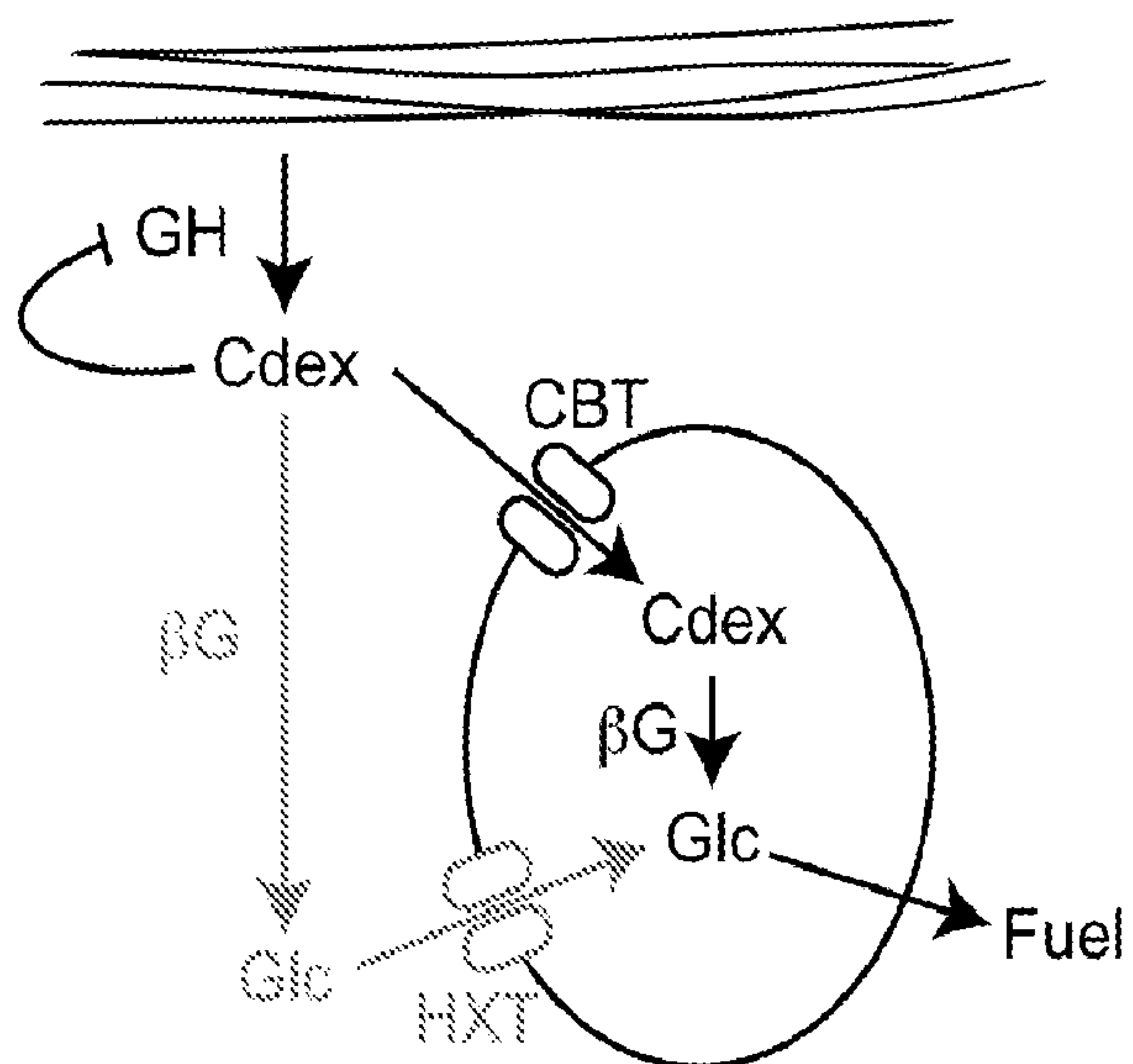


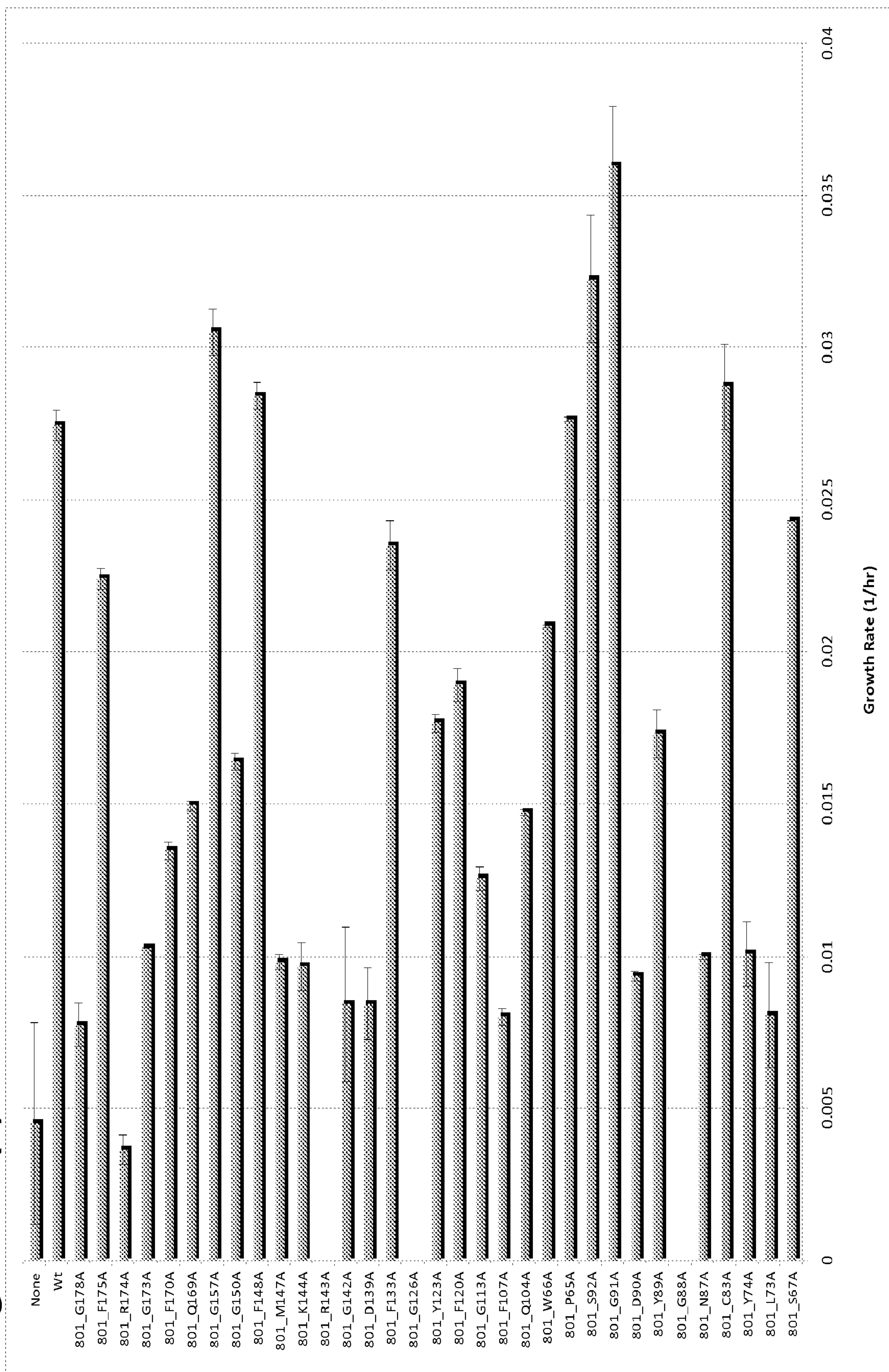
Figure 26 (a)

Figure 26 (a) (cont.)

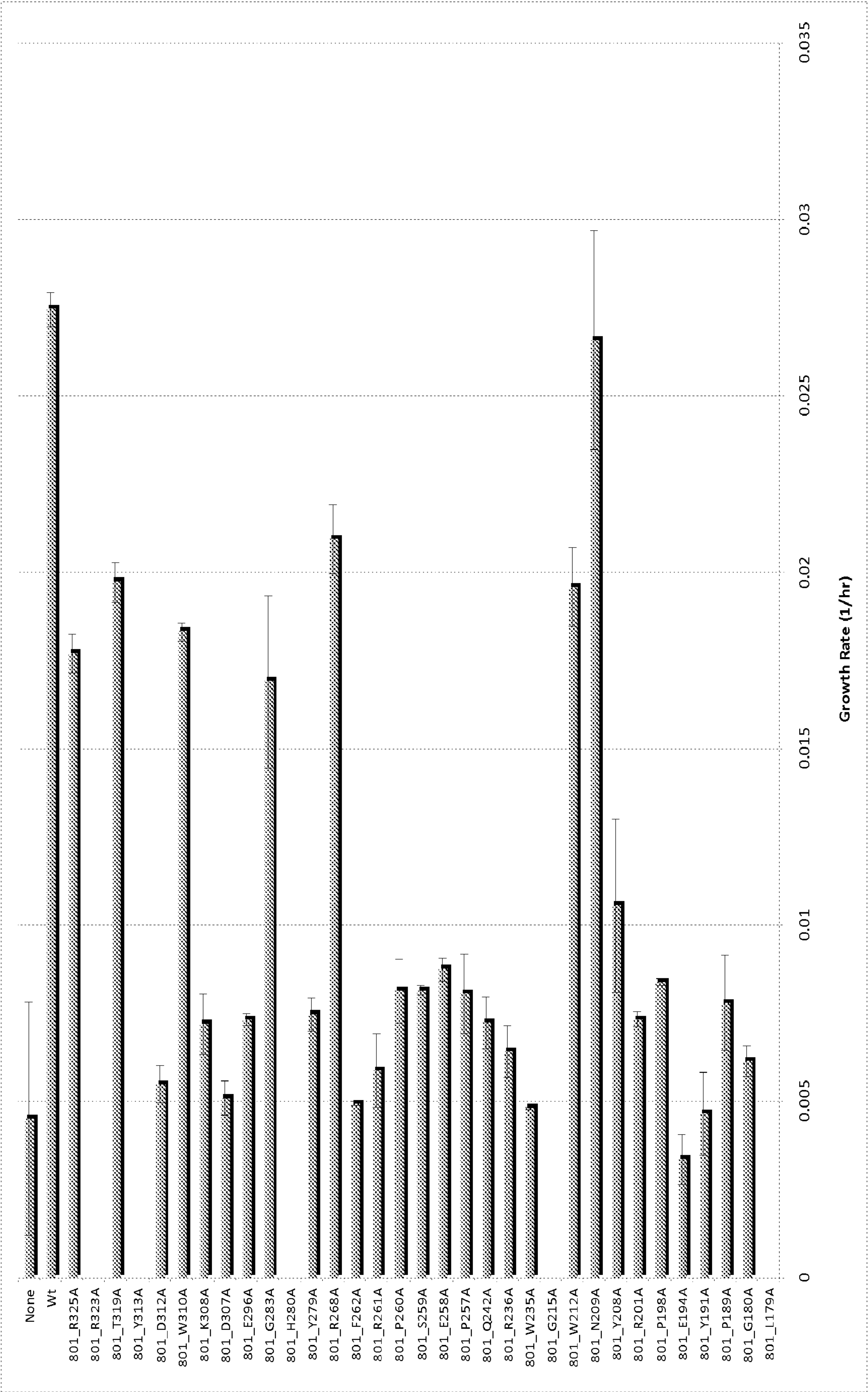


Figure 26 (a) (cont.)

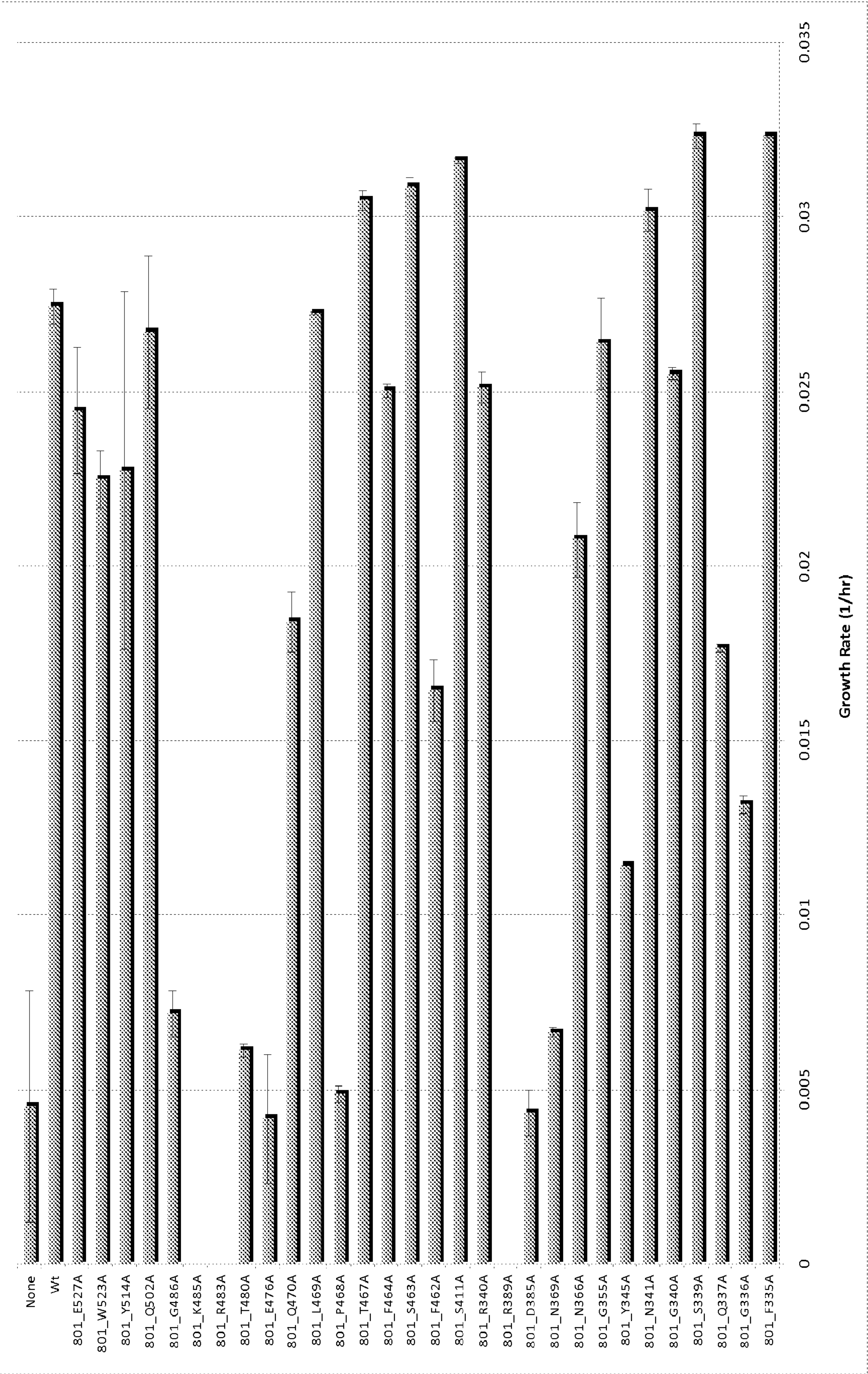


Fig. 26**(b)**

Polypeptide Sequence: NCU00801

MSSHGSHDGASTEKHLATHDIAPTHDAIKIVPKGHGQTATKPGAQEKEVR
 NAALFAAIKESNIKP[°]WSKESIHL[°]YFAIFVAFCCACAN[°]GY[°]**D**[°]GS[°]LMTGIIAM[°]
 DKF[°]QN[°]QFHTGDT[°]GPKVSVIF[°]SLTV[°]**G**AMVGAPFAAILS[°]DRF[°]GRKKGM[°]FI[°]G
 GIFIIIVGSIIVASSSKLA[°]QF[°]VV[°]GRFVL[°]GLGIAIM[°]**T**VAA[°]PAYSI[°]EIAPP[°]HW
RGRCT[°]GF[°]YNCGWFGGS[°]IPAACITYGCYFIKSN[°]WS[°]WRIPLIL[°]QAFTCLIVM[°]
 SSVFF[°]**L**[°]PESPR[°]FLFANGRD[°]AEAVAF[°]LVKY[°]HGN[°]GDPNSKLVL[°]LETE[°]EMRDG[°]
 IRTDGV[°]DKV[°]WWD[°]YRPLFM[°]THSGRWRMAQ[°]VLMI[°]SI[°]FG[°]QS[°]**G**[°]NGL[°]GYFNTVI[°]
 FKNIGV[°]TSTSQQ[°]LAYN[°]IL[°]NSVISAIGALTAVSMT[°]DRMPRR[°]AVLIIGTFMC[°]
 AAALATNS[°]GLS[°]ATLDKQTQ[°]RGTQINLNQGMNEQDAKDNAYLHVDSN[°]YAKG[°]
 ALAAY[°]FLENVIF[°]**S**[°]FTYT[°]PLQGV[°]IPTE[°]EA[°]LETTIRGK[°]GLALS[°]GFIVNAMGFI[°]
 NQFAGPI[°]ALHN[°]IGYKYIF[°]**V**[°]VGWD[°]LIETVAW[°]YFFGVESQGR[°]TLEQ[°]LEWV[°]Y
 DQPNPVKAS[°]LKVEKVVVQADGHVSEAI[°]VA

Italicized: All transporters

Double-underlined: β -linked transporters

Capped: NCU00801 clade

Bold: Essential in *S. cerevisiae* Hxt1/Hxt3

Underlined: functionally important according to alanine scanning experiment

Fig. 26 (cont.)

(c)

Polypeptide Sequence: NCU08114

MGIFNKKPVAQAVDLNQIQEEAPQFERVDWKKDPGLRKLYFYAFILCIAS
 ATTGYDGMFFNSVQNFETWIKYFGDPRGSELGLLGALYQIGSIGSIPFVP
 LLTDNFGRKTPIIIGCVIMIVGAVLQATAKNLDTFMGGRTMLGFGNSLAQ
 IASPMLLTELAHPQHRARLTTINCLWNVGALVSWLAFGTNYINNDWSW
 RIPALLQAFPSIIQLLGIWWVPESPRFLIAKDKHDEALHILAKYHANGDP
 NHPTVQFEFREIKETIRLEMESTKNSSYLDFFKSRGNRYRLAILLSLGFF
 SQWSGNAIISNYSSKLYETAGVIDSTAKLGLSAGQTGLALIVSVTMALLV
 DKLGRRLAFLASTGGMCGTFVIWTLTAGLYGEHRLKGADKAMIFFIWVFG
 IFYSLAWSGLLVGYAIEILPYRLRGKGLMVMNMSVQCALTLNTYANPVAF
 DYFGPDHSWKLYLIYTCWIAAEFVFVFFMYVETKGPTLEELAKVIDGDEA
 DVAHIDIHQVEKEVEIHEHEGKSVA

Italicized: All transporters

Double-underlined: β -linked transporters

Capped: NCU08114 clade

Bold: Essential in *S. cerevisiae* Hxt1/Hxt3

Underlined: functionally important according to alanine scanning experiment

Figure 27

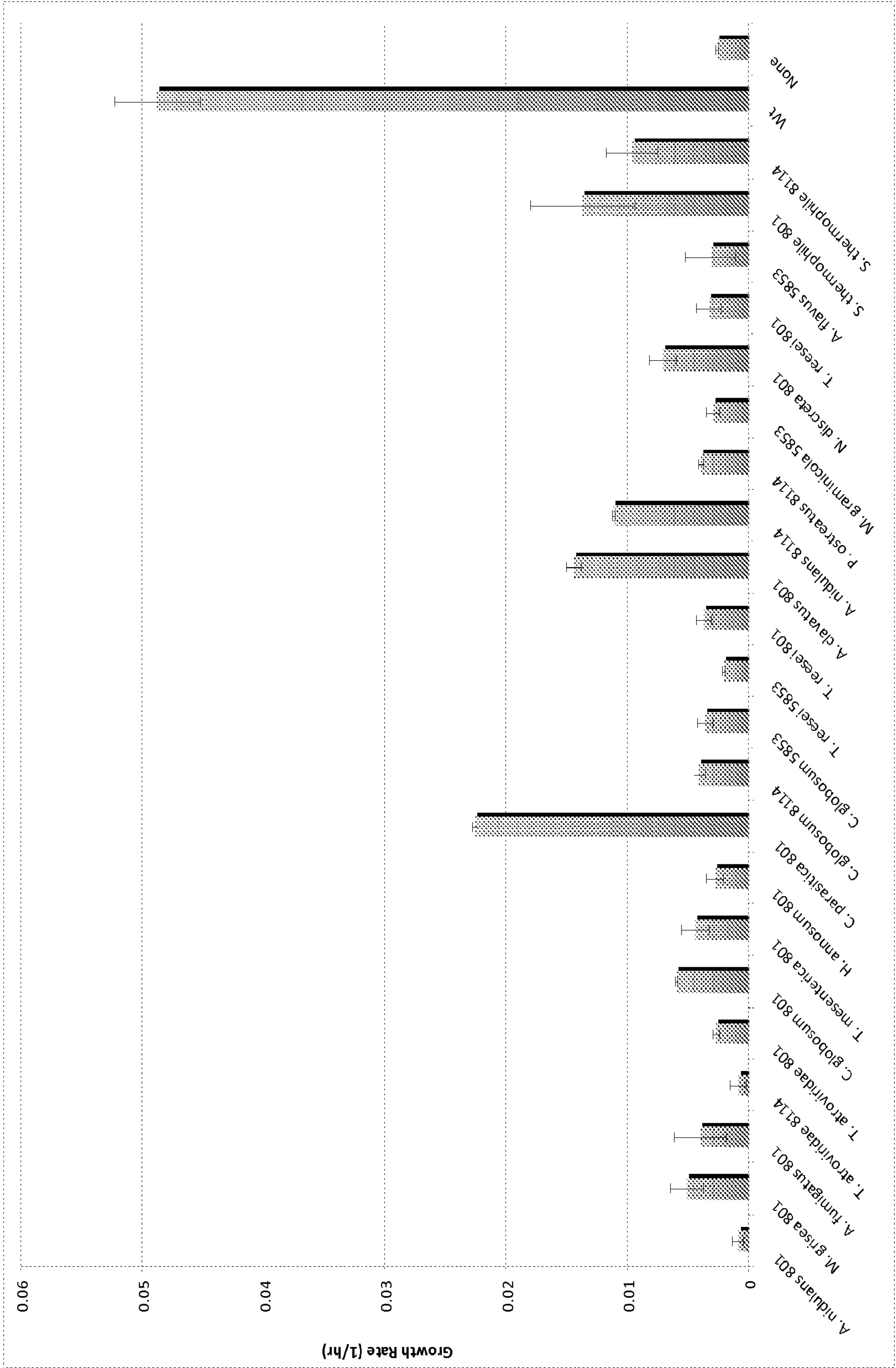


Figure 27 (cont.)

(b)

Cell growth in YPC medium

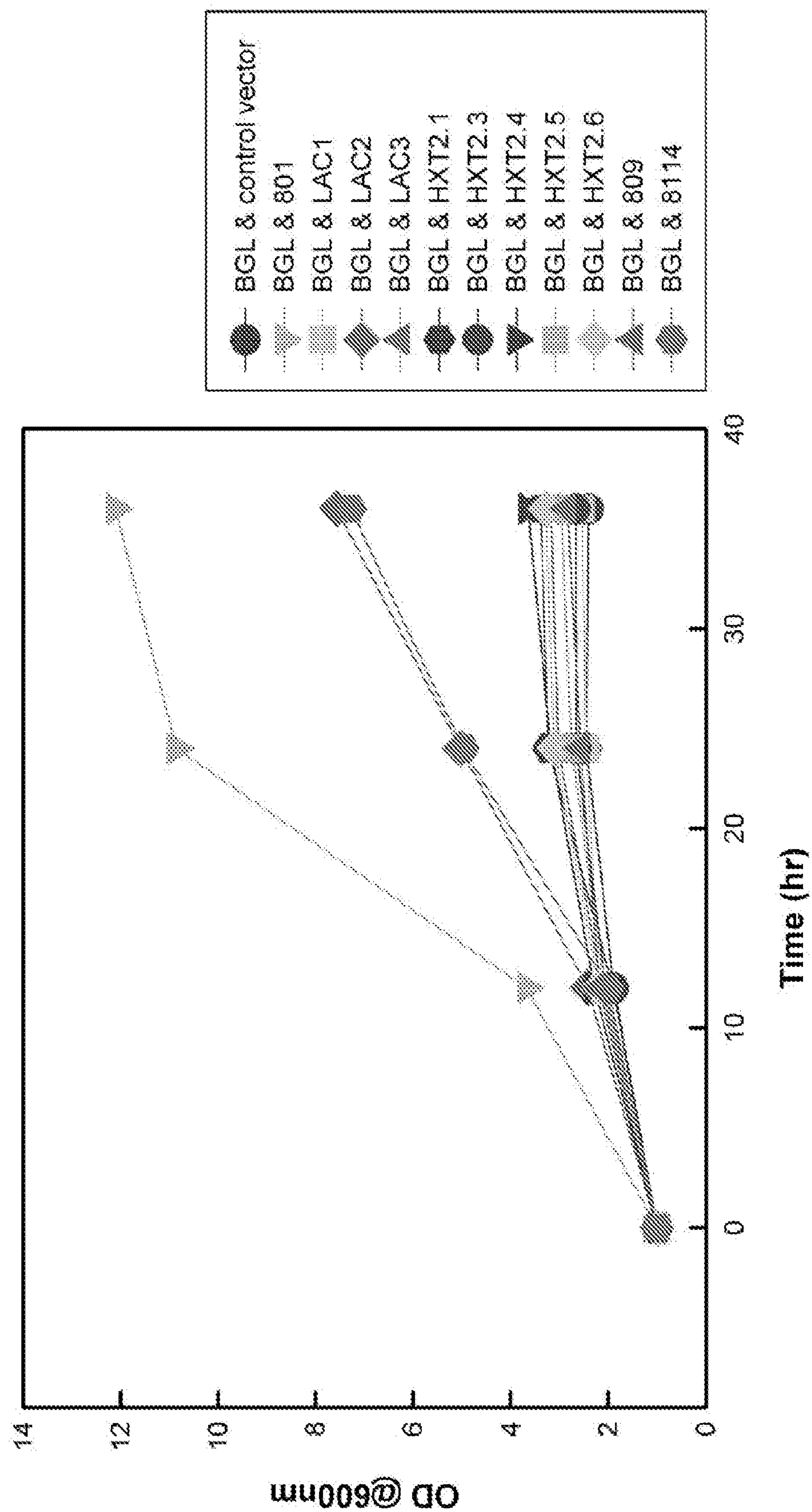


Figure 27 (cont.)

(c) Consumed xylose and produced ethanol

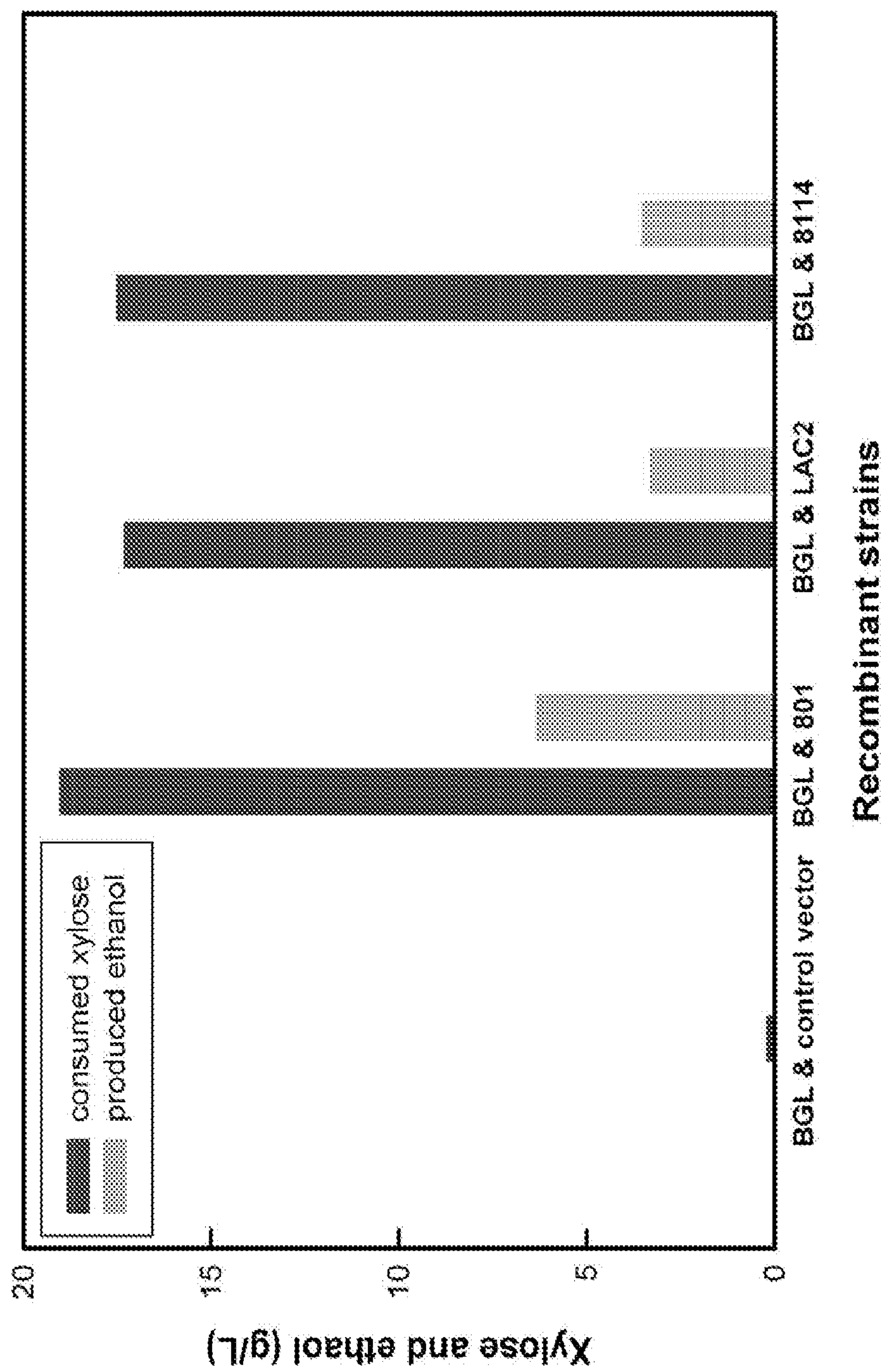


Fig. 28 (a)

Underlined: LYF-PESPR-KYH motifs

Capped: strictly conserved residues, critical to function (Ala-scan results)

```

NCU00801      -----MSSHGSHD----GASTEKH-LATHDIAPTHDA--IKIVPKGHG
NCU008114     -----MG
LAC2          -----MSTNSLND SYNPSSTKEKD-IVVQSEALADVA--I-----
C_parasiti    MILQDVQTTVFKRIDLIMSAEKTDP---VVDESNH-GISADV----Q--YPVVAEGTG
A_clavatus    -----MSSTDE-----KHGISVHHDSPGSDADFKPAVAAGQG
A_nidulans    -----MGKD-----TFTLA--G-----
S_thermo_l    -----MSSKGS-D---HIGDEKR-PVSPEIATAAVP--V-----S
S_thermo_i    -----MG

```

```

NCU00801      QTA-TK-PGAQEKEVRNAALFA-----AIKESNIKPWSKESIH-LYFAIFVAFCCACAN
NCU008114     IFN-KK-PVAQAVDLNQIQEEA-----PQFERVDWKKDPGLRK-LYFYAFILCIASATT
LAC2          ETAFET-DG-YKK-----IFQEHVPVRWTKLRLS-IYFTCLVIYLVSTTN
C_parasiti    KTL-DTSAGAKAREVHNAELFA-----AVQESNIERWSKSSIQ-LYFAVFIAFCCACAN
A_clavatus    ETA-KV-GGAKTRQVHNAELYA-----AITETPIEKWSKESLH-MYFAIFVAFCCACAN
A_nidulans    R-EWP-----KVNWWKMKGMRSVYLTTLGAAMVTSATN
S_thermo_l    HTP-KV-QG-KEKKVHNAELYA-----AILEAKIEPWSRTSIH-LYFSIFIAFCCACAN
S_thermo_i    IF-----AFNKQKPNAEATAVAQEEAPQFERVDWKRDPGLRK-LYFYAFVLCIASATT
                                     :*:          : ..

```

```

NCU00801      GYDGS LMTGIIAMD KFNQFH TGD TGPKVSVIFSLYTVGAMVGAPFAAILSDRFGRKKGM
NCU008114     GYDGMFFNSVQNFETWIKYFGDP-RGSELGLLGALYQIGSIGSIPFVPLLTDFNFGRRKTP I
LAC2          GYDGS LLSSLITMPEFISHLNK-SASGTGIVFAIFQVGQMVATLFV-WLGDFIGRRNAI
C_parasiti    GYDGS LIGSITAMKPFMDTFNSELGTGKVSIISSLSVGTIVTSPLAAMLSDRFGRRWSM
A_clavatus    GYDGS LMGAILAMDHFQNTFHTGMDGPKVSLVTSLYTVGSIATPFSAVLSDKLGRRKCM
A_nidulans    GYDGS LMNGLEALDDWKRSYNHP-EGATLGLLAASMSIGSILAIPVVPYVADLFGRRFGV
S_thermo_l    GYDGS LMTSIIAMPHFQQTFDVGKVGTA AVVFSLYVVGAMVGSPFAATLSDKFGRRKSM
S_thermo_i    GYDGMFFNSVQNFETWENYFNHP-TGSKLGV LGALYQIGSLASIPLVPIIADRVGRKIP I
****  ::  ::  :  :  ..  :::  :  :*  :  .  :  *  .**  :

```

```

NCU00801      FIGGIFIIVGSIIIVASSSKLAQFVVGRFVLGLGIAIMTVAAPAYSIEIAPPHWRGRCTGF
NCU008114     IIGCVIMIVGAVLQATAKNLDTFMGGRTMLGFGNSLAQIASPMLLTTELHPQHRARLTTI
LAC2          FIGSVIVCLGAIITSIANNTSTFIGGRFLLSFGSGISCALSTTYLLEITSPDERSALCAI
C_parasiti    FIGGWVIIIVGSVIACSSSTVAQITVGRFILGGGISIMTVAAPAYSIEIAPAHWRGRCTGF
A_clavatus    FVGAWVIIAGSIIIIATAKHLEQFYVGRVVLGFGIQIMVVSAPAYAVEIAPPHWRGRAVGF
A_nidulans    VLGCMIMIGGVAMVSIKYKIALFVVGR IILGFGLGIAQECSPLLVTETLVHPQHRAIYSTI
S_thermo_l    FAGGLTIIIGMILVSTAHLPLQFVVGRFVLGLGIAIMTVAAPAYAEVAPPHWRGRCTGF
S_thermo_i    AIGCVIMIVGAVLQAACRNLTGTFMGRFLLGFGNSLAQLCSPMLLTTELHPQHRGRLTTV
          *  :  *  :  .  :  **  :*  *  :  :  *  :  ..  *  .  .

```

```

NCU00801      YNCGWFGGSIPAACITYGCI--FIK-SNWSWRIPLILQAF-TCLIVM-SSVFFLPESPRF
NCU008114     YNCLWNVGALVVSWLAFGTN--YIN-NDWSWRIPALLQAF-PSIIQL-LGIWWVPESPRF
LAC2          YNSLYYIGSIIATWSSYATSISYAN-SVLSFRIPLWLQILCPALVVIGLLVGVAPESPRF

```


Fig. 28 (a) (cont.)

```

C_parasiti  YNCGWFGG SIPAA FVTFGCS--YMT-SNWA WRLPLL QCF-ACFIVI-ASVWFL PESPRF
A_clavatus  YNCGWFGG SIPAA VTYGTN--FID-NDFS WRIPFIL QCF-ACVIVI-FAVWFIP ES PRW
A_nidulans  YNSLWYVGSLIGACVALGTN--NIK GNDWS WRVPC LLQGV-PSVCQL-IFIWFVP ES PRW
S_thermo_l  YNCGWFGG AIPAA IITFGCN--YMD-NDYS WRVPLIF QAF-ACLIVM-VAVFFIP ES PRF
S_thermo_i  YNCLWNVGALVVAWVSFGTD--YLK-SDWS WRIPALI QAF-PSVIQL-LFIFWVP ES PRY
** . : *:: : : . . : : * : * . . . : : *****:

NCU00801    LFANGRDAEAVAFLVKYHGNGDPNSKLVLL ETEEMRDGIRTDGV-DKV-WWDYRPLFMTH
NCU008114   LIAKDKHDEALHILAKYHANGDPNHPTVQFEFREIKETIRLEME-STK-NSSYLDFFKSR
LAC2        YYLTGQPDKARAFFCKYHANGDEKHPIVEYEMAQLELSLLEVPKLRVRDYFDARILFKTK
C_parasiti  LMANGREEEAVAFLVKYHGNGDPSSRLVLL EIEEMRENIRQDGQ-DKV-WWDYRPMFLTH
A_clavatus  QMAHGQDEAALAFLTRYHGNGDPNARLVRL EIEEMREGIRIDGI-DKR-WWDYRPFFFTH
A_nidulans  LISKGKSEKAKKILAYVHAQGD EDELVNVEFDEIQQTIALEKQLEGS-GWS--ELWSTP
S_thermo_l  LMANGRDDEALAFLIK YHGNGDPNSRLVRLE YEEMKEGIRQDGI-DKV-WWDYRPFLLSH
S_thermo_i  LMAKDKHERALAILAKYHANGDANHPTVQFEYREIKETLRLEFE-ASK-SSSYLDFVRTR
          .: * :: *::** . * * ::. : . : :

NCU00801    SGRWRMAQVLMISIFGQFSGNGL-GYFNTVIFKNIGVTSTSQQLAYNILNSVISAIGALT
NCU008114   GNRYRLAILLSLGFFSQWSGNAIISNYSSKLYETAGVTDSTAKLGLSAGQTGLALIVSVT
LAC2        SRIYRSLVLCIAHSAFGQLSGNAVVGYYITNIFLELGITNPTRLLNGVNSILGFIFAMS
C_parasiti  NGRWRFGQIIMISVFGQFSGNGL-GYFNAAIFSLIGVKSTAQQLGFNVLSVLSAIGAGF
A_clavatus  SGRWRFLQVMMISVFGQWSGNGL-GYFNATIIYQRLGYTSSSMQLLMNLVNSIVSAIGALT
A_nidulans  GNRHRSIILISIGFFSQWSGN GIVSYFLPKVLELIGITDSHKVLTINSVLNAVNVVSATG
S_thermo_l  SGRWRIAQVLMISIFGQFSGNGL-GYFN TTI FENLGVTSPVQQLGYNILNQVLSAIGALT
S_thermo_i  GNRYRLAVLISLGIFSQWSGNAIISNYSSKLYDTAGVTGSTQKLGLSAGQTGLSLIISVT
          . * : . *. * ***.: . : . : * .. * . : : :

NCU00801    AVSMTDRMPRRAVLIIGTFMCAAALATNSGLSATL DKQTQRGTQINLNQGMNEQDAKDNA
NCU008114   MALLVDKLGRRLAFLASTGGMCGTFVIWTLTAGLYGEHR-L-----
LAC2        GSILVGRIGRRPILLYSTTG FVISFTIIAACIAAYTN NN---N-----
C_parasiti  GVSLSDKMNRRTVLVYGTFICACLLAVNGGTSTGLKKYETTDSSGNLNISA---D-----
A_clavatus  AVALTDRMPRRKVLVWGTLACAIAMAINAGVSEPMIKQAETPAGIN-----
A_nidulans  ICFFVDKLGRRKLFLT SVTCMLLCFISTTIALARFPHPG---GGAD-----
S_thermo_l  AVSLTDKMPRRPVLVFGTFMCAAALATNSGLSAVLDEQTQRG-----
S_thermo_i  MALLVDKFGRRPMFLTSTAGMFCTFI FWTLTSGLYEEHN-A-----
          : .:: ** ::. . : .

NCU00801    YLHVDSNYAKGALAAAYFLF-NVIFSFTYTP LQGVIPTEALET TIRGKGLALSGFIVNAMG
NCU008114   ----KGADKAMIFFI WVF-GIFYS LAWSGLLVGYAIEILPYRLRGKGLMVMNMSVQCAL
LAC2        ----QVAAKVGI AFIIYIFNNVFFSFGYTPLQPLYP AEILSSEMRAKGMALFQITQGTAS
C_parasiti  ----GKSLAQAAALAFYFLF-NIIYSFTYTP LQGIIPAEALDNLRAKGLAASGMIVGLEFG
A_clavatus  ----KTFGQTAVAFYFLF-NIIFSFTYTP LQGVIPAEALETTTRAKGLALSGLMVSGIG
A_nidulans  ----DHAGNAVIVFIFLY-YISYNIGFSGLLVSY SSEILPYRLRAKGLTLMFFCVALSL
S_thermo_l  --QIDLSYGRGALASYFLF-NIIFSFTYTP LQGAIPTEALET TTRAKGLALS AFIVNGMG
S_thermo_i  ----DGARYAMILFIWIH-GIFYSISWSGLLVGYAIEVLPYKLRAKGLMIMNLT VQAAL
          : .:: : ::: : * . * * .** :

```


Fig. 28 (a) (cont.)

NCU00801	FINQFAGP [.] IALHNI----GYKYIFV [.] FVGWDLIETVAWYFFGV [.] ESQGR [.] TLE [.] QLEWVYDQPN
NCU008114	TLNTYANP [.] VAFDYFGPDHSWKLYLIYTCWIAAEFV [.] FVFFMYVET [.] KGPTLE [.] EELAKVIDGDE
LAC2	FINTYAAP [.] VAMQNI----KYWYYV [.] FVFWDTFEV [.] IIIIYLYFVET [.] KNLTLE [.] EIEELIFESAT
C_parasiti	FINQFAGP [.] IALANI----NTNYVYV [.] FVAWDVIESILWWIFGV [.] ESQGR [.] TLE [.] QLEWVYNQPK
A_clavatus	FVSQYAS [.] PIGLRNI----STHYFWIFVGWDLFEALCWYLFGV [.] ESQGR [.] TLE [.] ELEWVYQQPN
A_nidulans	LFNQYVN [.] PIALLDI----GWKYYIV [.] YCVWLLFELFVWVKYYVET [.] KNTPLE [.] EIVKFFDGDQ
S_thermo_l	FINQFAGP [.] IALERI----GYKYIYV [.] FVAWDCIEALAWYLFGV [.] ESQGR [.] TLE [.] QLEWVYNQPN
S_thermo_i	TLNTYANP [.] VAFDAFE-GHSWKLYIIYTIWIFLELCFVWKMYIET [.] KGPTLE [.] EELAKIIDGDE
	 *:.. : .: * * : :*:.. .:~:~ . . :
NCU00801	PVKASLKV----EKVVVQA-----DGHVSEA-IV----A
NCU008114	ADVAHIDI----HQVEKEV-----EIHEHEGKSV----A
LAC2	PVKTSMII----SKPGHAANEKEL-RLANLKLGN-YVA----
C_parasiti	PVQASIKV----DKVVVQA-----DGKVTEK-IVADAAS
A_clavatus	PVKASLQV----DKVVVQA-----DGQVTEK-IT-D--A
A_nidulans	AVLGGAATEKIH [.] ELVT--VQARSAEETLGEKGPA-VSAEAR-
S_thermo_l	PVKASLKV----DKVILTD-----DGKVAEK-IV----A
S_thermo_i	AAVAHVDI----KQV--EK-----ETHINEE-KS----V
	. : .

Fig.28 (b)

NCU00801 MSSHGSHDGASTEKH-LATHDIAPTHDA--IKIVPKGHGQTATKPGAQEKEVRNAALFAA
NCU008114 -----MGIFNKKFVAQAVDLNQIQEEAP
LAC2 MST-----NSLNDSPNSSTKEKDIVVQSEALADVAIETAFETDGYK-----KI
A_clavatus MSS-----TDEKHGISVHHDSPGSDADFKPAVAAGQGETAKVGGAKTRQVHNAELYAA
.

NCU00801 IKESNKPWSKESIHLYFAIFVAFCCACANGYDGSLMTGIIAMDKEFQNFHTGDTGPKVS
NCU008114 QFERVDWKKDPGLRKLYFYAFILCIASATTGYDGMFFNSVQNFETWIKYFGD-PRGSELG
LAC2 FQEHVPVRWTKLRSLIYFTCLVIYLVSTTNGYDGSLSSLITMPEFISHLNI-KSASGTG
A_clavatus ITETPTEKWSKESLHMYFAIFVAFCCACANGYDGSLMGAILAMDHFQNTFHTGMDGPKVS
* : ** : : : :

NCU00801 VIFSPLYTVGAMVGAPFAAILSDRFGRKKGMFIGGIFIIVGSIIVASSSKLAQFVVGRFVL
NCU008114 LLGALYQIGSIGSIPFVPLLTDFGRKTPIIIGCVIMIVGAVLQATAKNLDTFMGGRTML
LAC2 IVFAIFQVGQMVATLF-VWLGDFIGRRNAIFIGSVIVCLGAIITSIANNTSTFEGGRFL
A_clavatus LVTSLYTVGSIAATPFSAVLSDKLGRKCMFVGAWVILAGSIIATAKHLEQFYVGRVVL
: : : : * : . * * * : : : : : : * * : *

NCU00801 GLGIAIMTVAAPAYSIEIAPPHWRGRCTGFYNCGWFGGSIPAACITYGC--YFICKSNWSW
NCU008114 GFGNSLAQIASPMLLTELAHPQHRARLTTIYNCLWNVGALVVSWLAFGT--NYINNDWSW
LAC2 SFGSGISCALSTTYLLEITSPDERSALCAIYNLSLYIGSIIATWSSYATSISYANSVLSF
A_clavatus GFGIQIMVVSAPAYAVEIAPPHWRGRAVGFYNCGWFGGSIPAAAVTYGT--NFIDNDFS
.: * : : . * : * . : * : : : : : : : * :

NCU00801 RIPLILQAFT-CLIVM-SSVFFLPESPRFLFANGRDAEAVAFLVKYHGNGDPNSKLVLL
NCU008114 RIPALLQAFP-SIIQL-LGIWVVPESPRFLIAKDKHDEALHILAKYHANGDPNHPTVQFE
LAC2 RIPLWLQILCPALVVIGLLVGVAPESPRFYLTGQPDKARAFFCKYHANGDEKHPIVEYE
A_clavatus RIPFILQCFA-CVIVI-FAVWFIPESPRWQMAHGQDEAALAFTRYHGNGDPNARLVRL
*** ** : : : : : * : : : * : * : *

NCU00801 TEEMRDGI-RTDGVDKV-WWDYRPLFMTHSGRWRMAQVLMISIFGQFSGNGL-GYFNTVI
NCU008114 FREIKETI-RLEMESTK-NSSYLDFKSRGNRYRLAILLSLGFFSQWSGNAIISNYSSKL
LAC2 MAQLELSLLEVPKLRVRDYFDARILFKTKSRIYRSLVCIAHSAFGQLSGNAVVGYYITNI
A_clavatus IEEMREGI-RIDGIDKR-WWDYRPFFFTHSGRWRFLQVMMISVFGQWSGNGL-GYFNATI
: : : : . : * : : : * : . * * * : : : :

NCU00801 FKNIGVTSTSQQLAYNILNSVISAIGALTAVSMTDRMPRAVLIIGTFMCAAALATNSGL
NCU008114 YETAGVTDSTAKLGLSAGQTGLALIVSVTMALLVDKLGRRLAFLASTGGMCGTFVIWT--
LAC2 FLELGITNPTTRLLLNQVNSILGFIFAMSGSILVGRIGRRPILLYSTTGTFVISFTIIAAC
A_clavatus YQRLGYTSSSMQLLMNLVNSIVSAIGALTAVALTDRMPRRKVLVWGTLACAIAMAINAGV
: * * : : * . : : : * : : : : * : : : :

NCU00801 SATLDKQTQRGTTQINLNQGMNEQDAKDQAYLHVDSNYAKGALAAYFLF-NVIFSFTYTP
NCU008114 -----LTAGLYGE-----HRLKGADKAMIFFIWFV-GIFYSLAWSG
LAC2 I-----AAYTN-----NNNQVAAKVGIAYIYIFNNVFFSFGYTP
A_clavatus SEPMIKQAETPAGIN-----KTFGQTAVAFYYLF-NIIFSFTYTP
: : : : * : : : * : *

NCU00801 QGVIPTEALETTIRGKGLALSGFIVNAMGFINQFAGPIALHNIG---YKYIFVFGWDL
NCU008114 LVGYAIEILPYRLRGKGLMVMNMSVQCALTNTYANPVAFDYFGPDHSWKLYLYTCWIA
LAC2 QPLYPAEILSSEMRAGMALFQITQGTASFINTYAAPVAMQNIK---YWYVFFVFWDT
A_clavatus QGVIPAEALETTTRAKGLALSGLMVSGIGFVSQYASPIGLRNIS---THYFWIFVFGWDL
* * * * * : : : : : : : : *

NCU00801 IETVAWYFFGVESQGRTELEWVYDQPNPVKASLKVEK-----VVVQADGHVSEAIV-
NCU008114 AEFVFFVFFMYVETKGPTEELAKVIDGDEADVAHIDIHQ-----VEKEVEIHEHEGKSV
LAC2 FEVIIYLYFVETKNLTLEEIEELIFESATPVKTSMIISKPGHAANEKRLRLANLKLKKNY
A_clavatus FEALCWYLFGVESQGRTELEWVYQPNPVKASLQVVK-----VVVQADGQVTEKITD
* : : : * : : : * : : : : :

NCU00801 -A
NCU008114 -A
LAC2 VA
A_clavatus -A
*

Fig. 28 (c)

BLASTP 2.2.24+

Reference: Stephen F. Altschul, Thomas L. Madden, Alejandro A. Schaffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Reference for compositional score matrix adjustment: Stephen F. Altschul, John C. Wootton, E. Michael Gertz, Richa Agarwala, Aleksandr Morgulis, Alejandro A. Schaffer, and Yi-Kuo Yu (2005) "Protein database searches using compositionally adjusted substitution matrices", FEBS J. 272:5101-5109.

RID: 4HJKWKG311R

Query= NCU00801
Length=579

	Score
E	
Sequences producing significant alignments:	(Bits)
Value	

1c1 47823	NCU08114	238
4e-67		

ALIGNMENTS

>1c1|47823 NCU08114
Length=525

Score = 238 bits (608), Expect = 4e-67, Method: Compositional matrix adjust.

Identities = 152/518 (29%), Positives = 257/518 (49%), Gaps = 35/518 (6%)

Query	66	WSKES--IHLYFAIFVAFCCACANGYDGLMTGIIAMDKFQNFHTGD-TGPKVSVIFSL	122
		W K+ LYF F+ + GYDG + + + F GD G ++ ++ +L	
Sbjct	30	WKKDPGLRKLYFYAFILCIASATTGYDGMFFNSVQNFETWIKYF--GDPRGSELGLLGAL	87
Query	123	YTVGAMVGAPFAAAILSDRFGRKKGMFIGGIFIIVGSIIVASSSKLAQFVVGRFVLGLGIA	182
		Y +G++ PF +L+D FGRK + IG + +IVG+++ A++ L F+ GR +LG G +	
Sbjct	88	YQIGSIGSIPFVPLLTDNFRKTPIIIGCVIMIVGAVLQATAKNLDTFMGGRTMLGFGNS	147
Query	183	IMTVAAPAYSIEIAPPHWRGRCTGFYNCGWFGGSIPAACITYGCFIKSNWSWRIPLILQ	242
		+ +A+P E+A P R R T YNC W G++ + + +G +I ++WSWRIP +LQ	
Sbjct	148	LAQIASPMLLTELAHPQHRARLTTIYNCLWNVGALVSWLAFGTNYINNDWSWRIPALLQ	207
Query	243	AFTCLIVMSSVFFLPESPRFLFANGRDAEAVAFLVKYHGNGDPNSKLVLLETEEMRDGIR	302
		AF +I + ++++PESPRFL A + EA+ L KYH NGDPN V E E+++ IR	
Sbjct	208	AFPSIIQLLGIWWVPESPRFLIAKDKHDEALHILAKYHANGDPNHPTVQFEFREIKETIR	267
Query	303	TDGVDKVWWDYRPLFMTHSGRWRMAQVLMISIFGQFSGNG-LGYFNTVIFKNIGVTSTSQ	361
		+ Y F + R+R+A +L + F Q+SGN + +++ +++ GVT ++	
Sbjct	268	LEMESTKNSSYLDFFKSRGNRYRLAILLSLGFFSQWSGNAIISNYSSKLYETAGVTDSTA	327
Query	362	QLAYNILNSVISAIGALTAVSMTDRMPRAVLIIGTFMCAAALATNSGLSATLDKQTQRG	421
		+L + + ++ I ++T + D++ RR A LA+ G+ T T	
Sbjct	328	KLGLSAGQTGLALIVSVTMALLVDKLGRR-----LAFLASTGGMCGTFVIWT---	374

Fig. 28 (c) (cont.)

Query	422	TQINLNQGMNEQDAKD NAYLHVDSNYAKGALAAYFLFNVIFSFTYTPLQGVIPTALET	481
		L G+ + H K + ++F + +S ++ L E L	
Sbjct	375	----LTAGLYGE-----HRLKGADKAMIFFIWVFGIFYSLAWSGLLVGYAIEILPYR	422
Query	482	IRGKGLALSGFIVNAMGFINQFAGPIALHNIG----YKYIFVFGWDLIETVAWYFFGVE	537
		+RGKGL + V +N +A P+A G +K ++ W E V +F VE	
Sbjct	423	LRGKGLMVMNMSVQCALT LNTYANPVAFDYFGPDHSWKLYLIYTCWIAAEFVFVFFMYVE	482
Query	538	SQGR TLEQLEWVYDQPNPVKASLKVEKVVVQADGHVSE	575
		++G TLE+L V D A + + +V + + H E	
Sbjct	483	TKGPTLEELAKVIDGDEADVAHIDIHQVEKEVEIHEHE	520

Figure 29

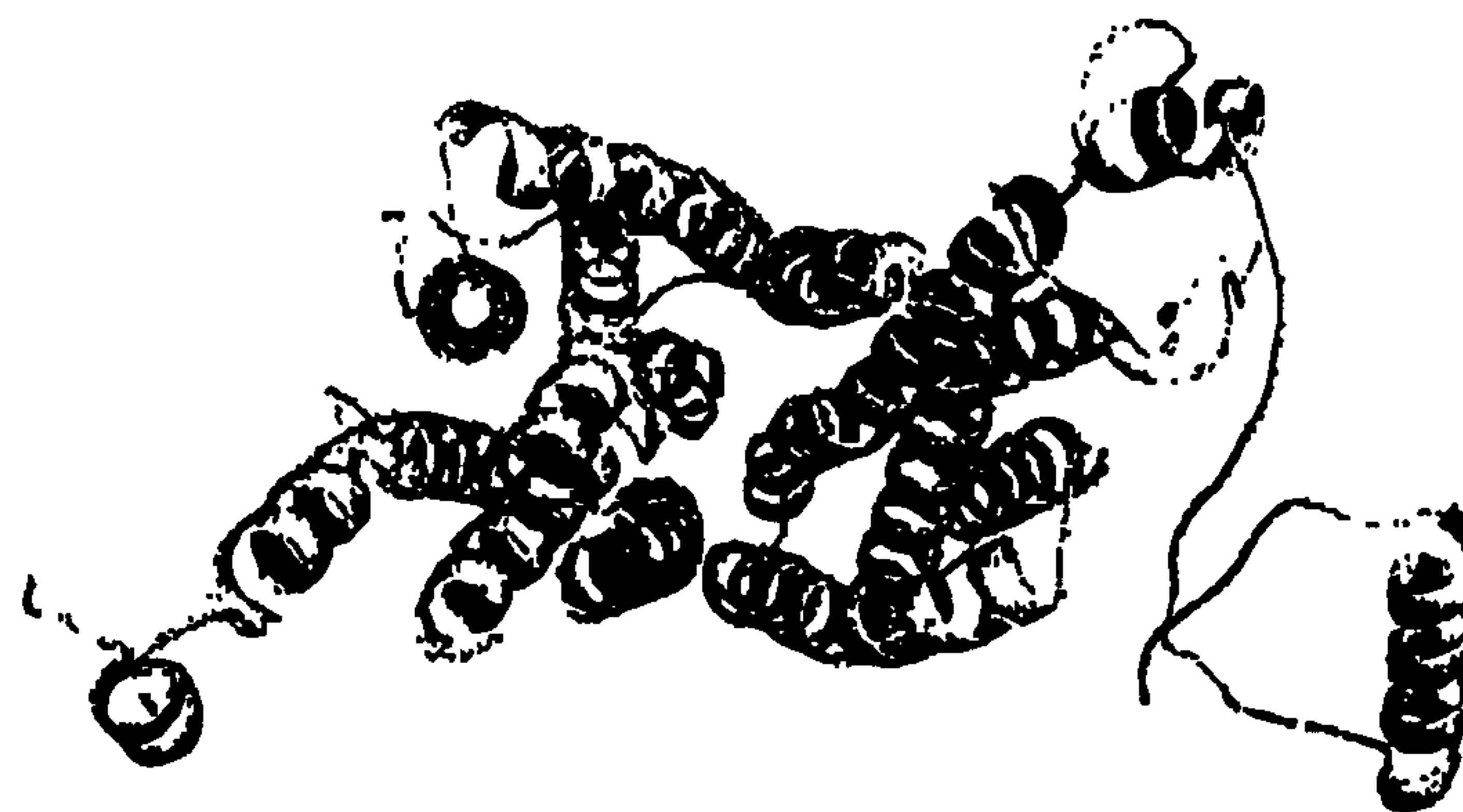
53 / 118

Part A

a.



b.



c.

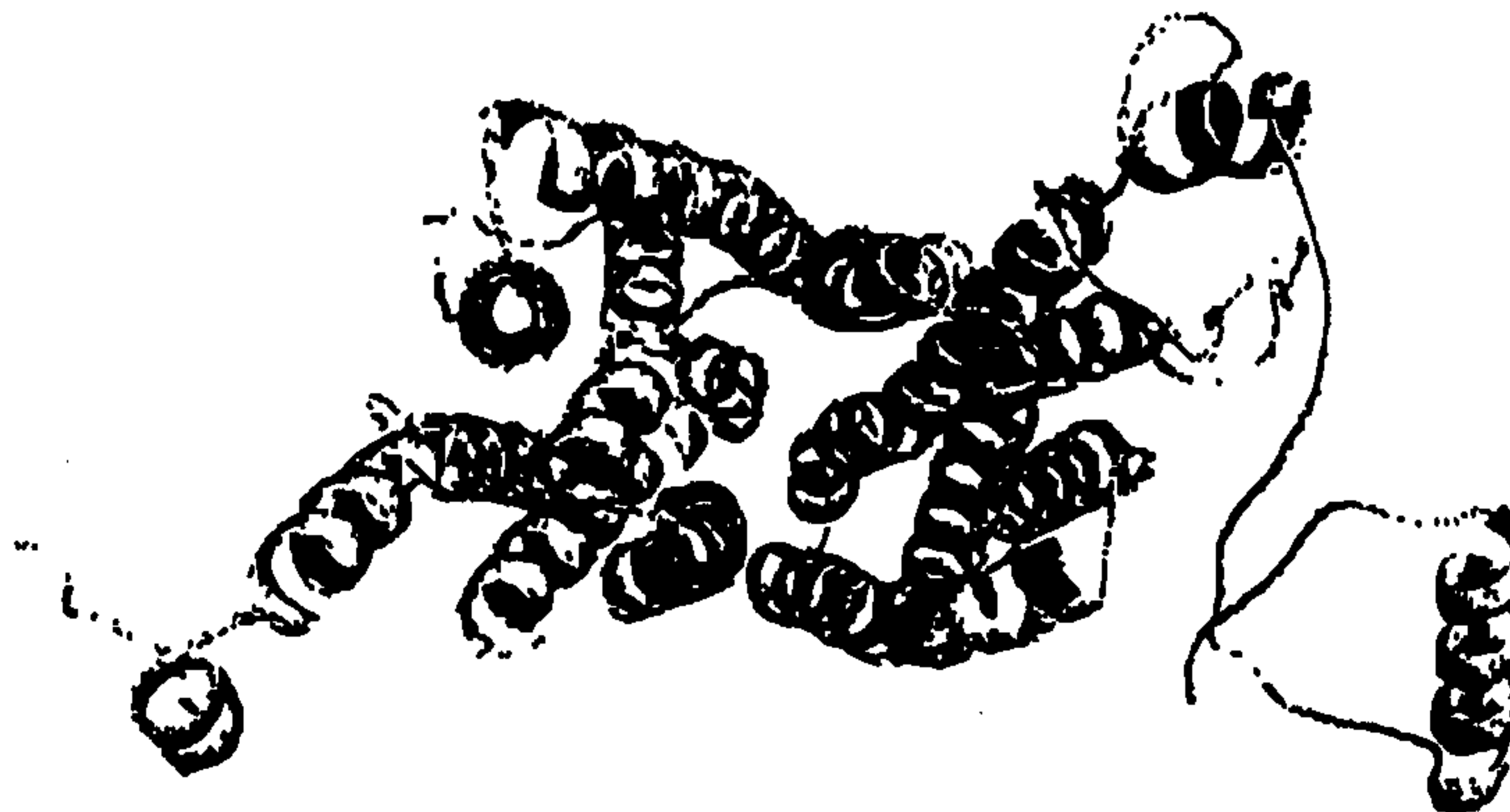
RECTIFIED SHEET

Figure 29

53 / 118 / 1



d.

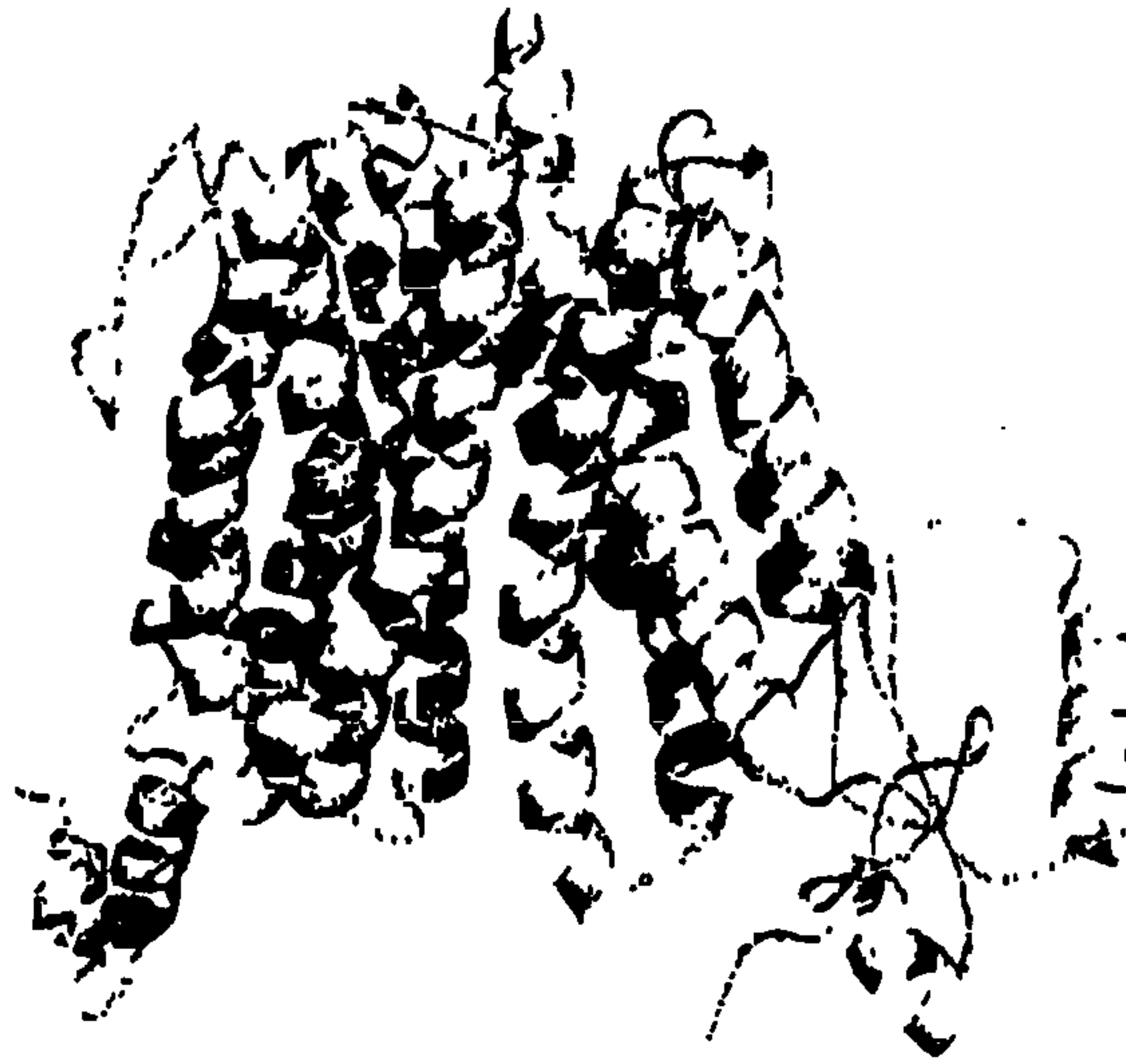


e.

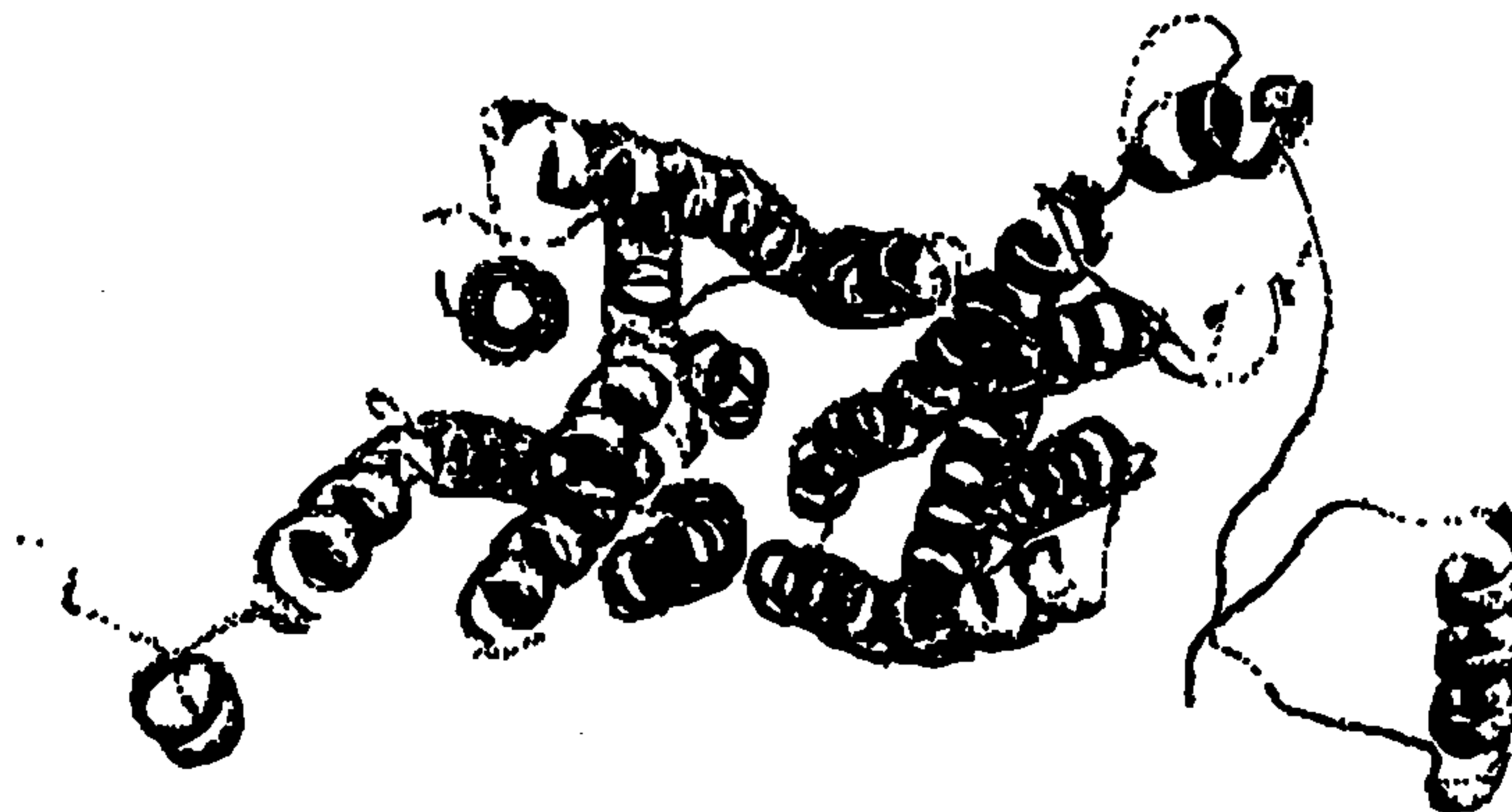
RECTIFIED SHEET

Figure 29

53 / 113 / 2



f.

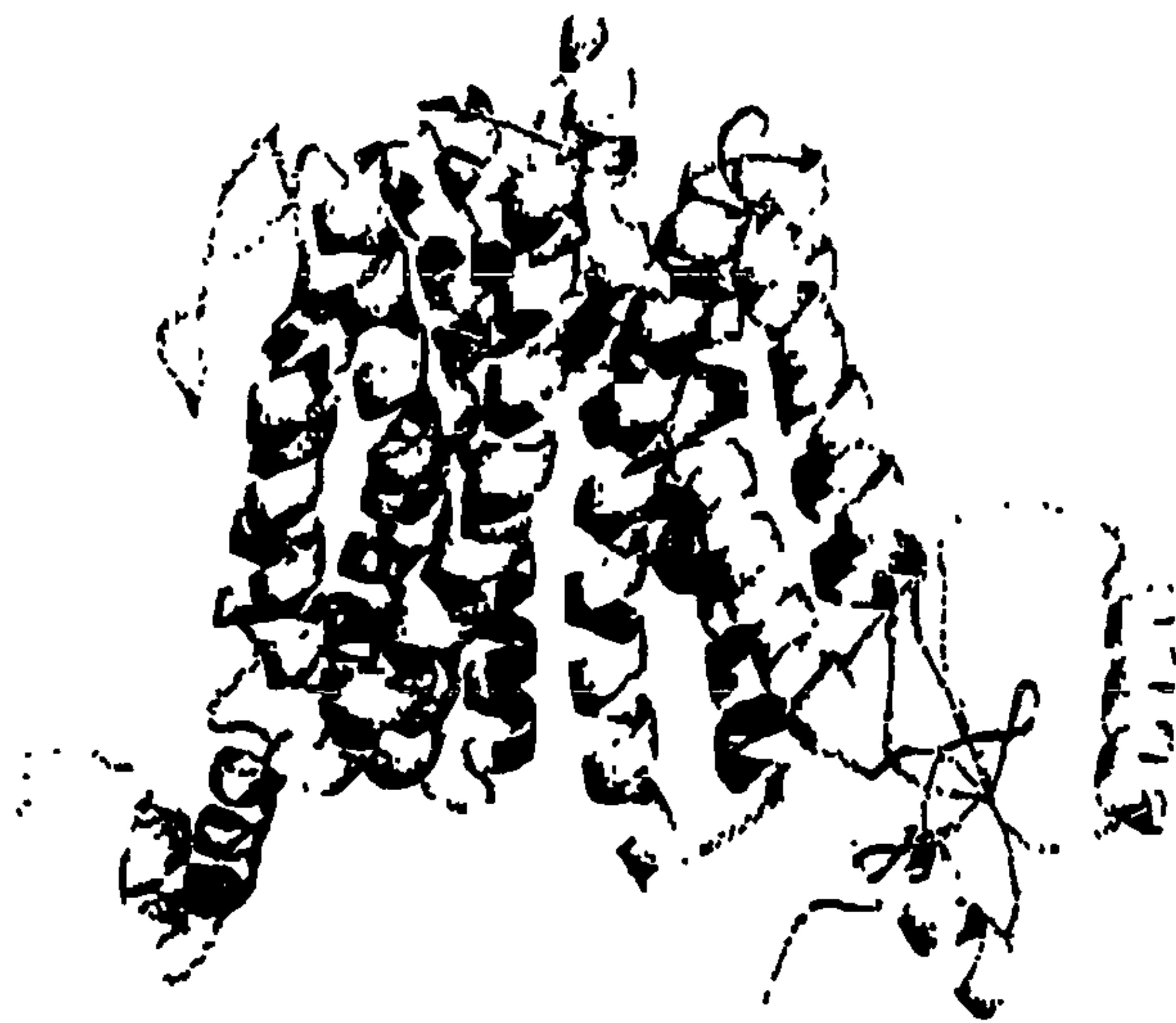


g.

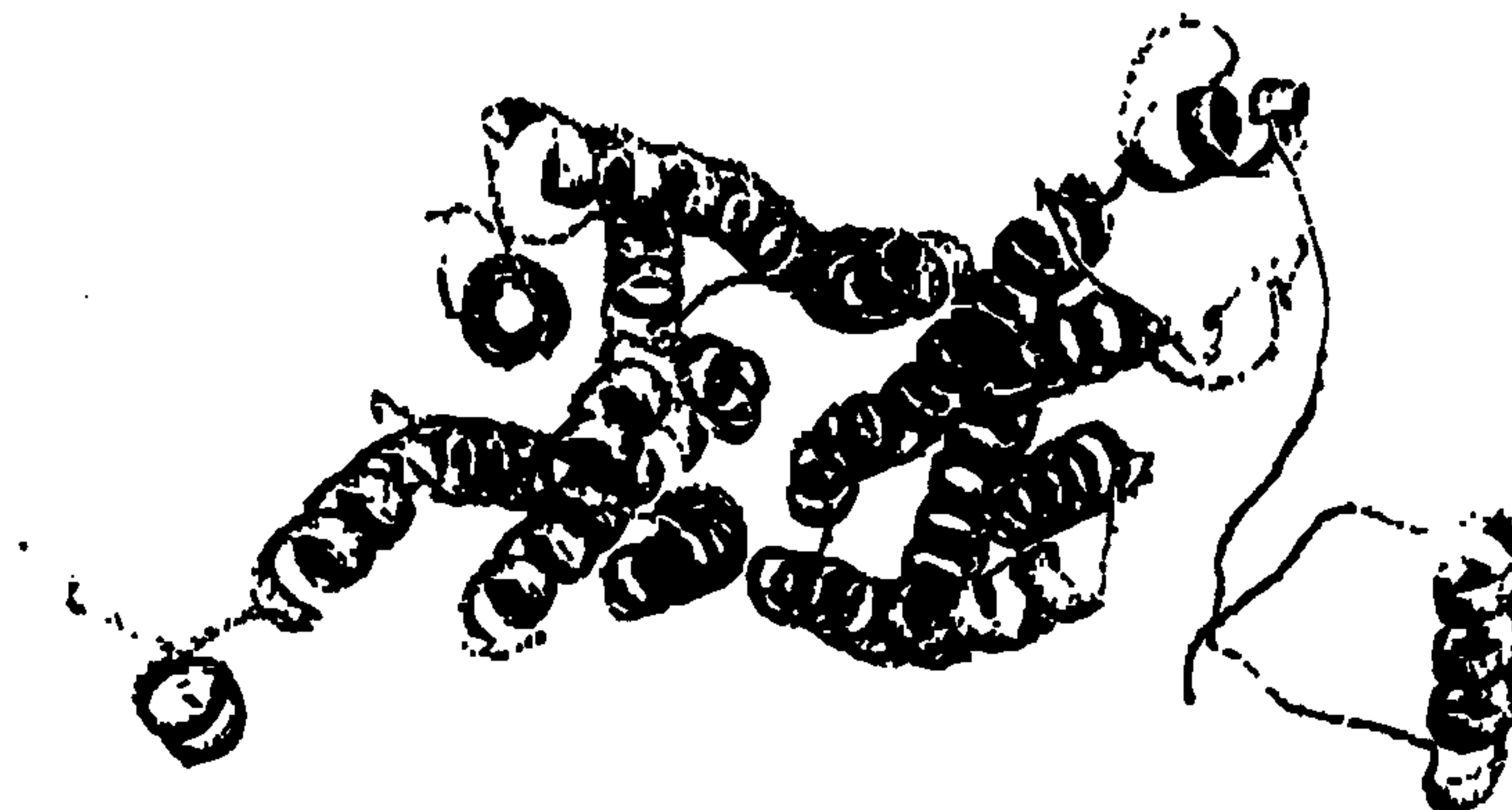
RECTIFIED SHEET

Figure 29

53 / 118 / 3



h.

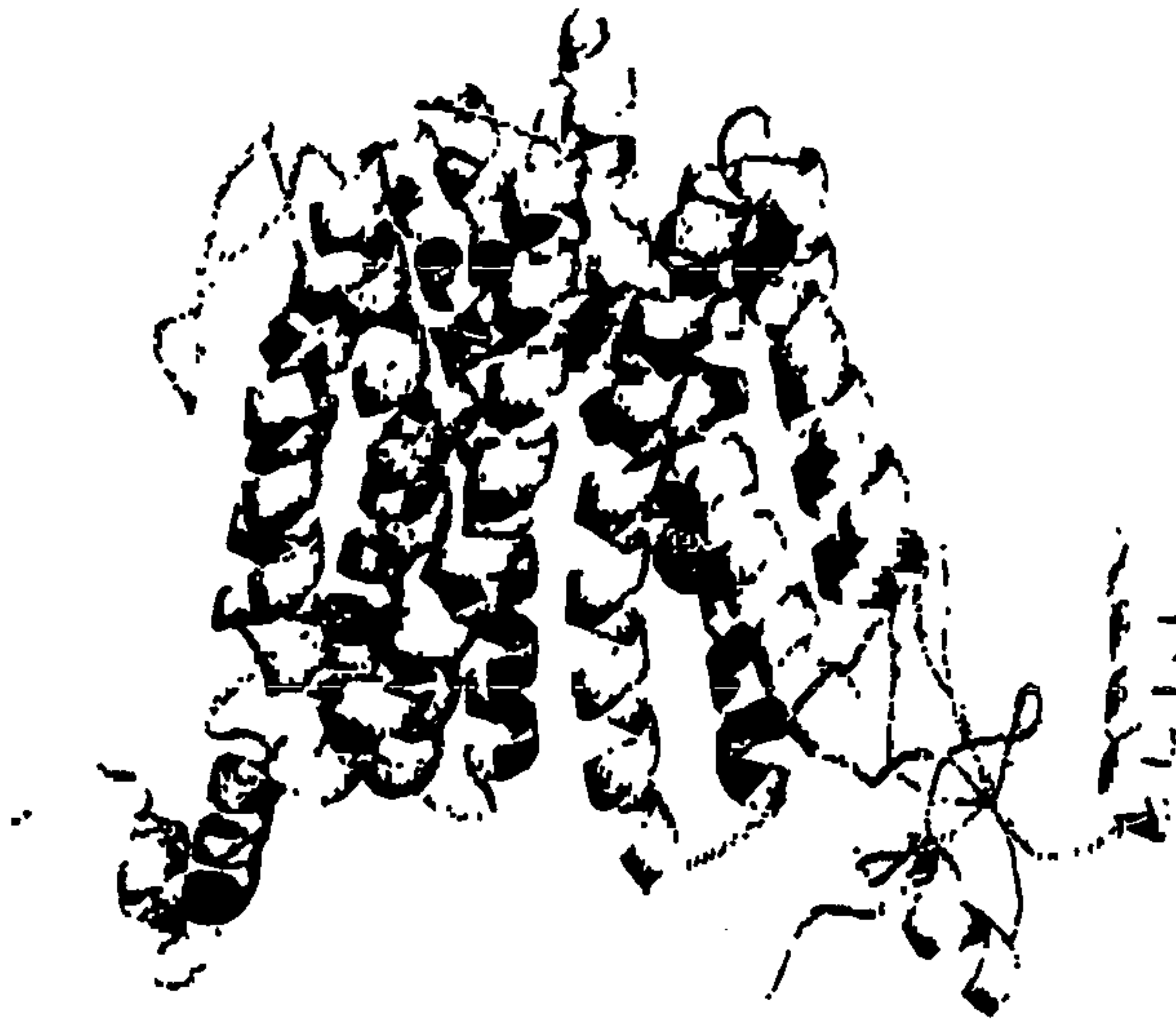


i.

RECTIFIED SHEET

Figure 29

53 / 118 / 4



j.



k

RECTIFIED SHEET

Figure 29

53 / 118 / 5



l.



m.

RECTIFIED SHEET

Figure 29

53 / 118 / 6



n.



o.

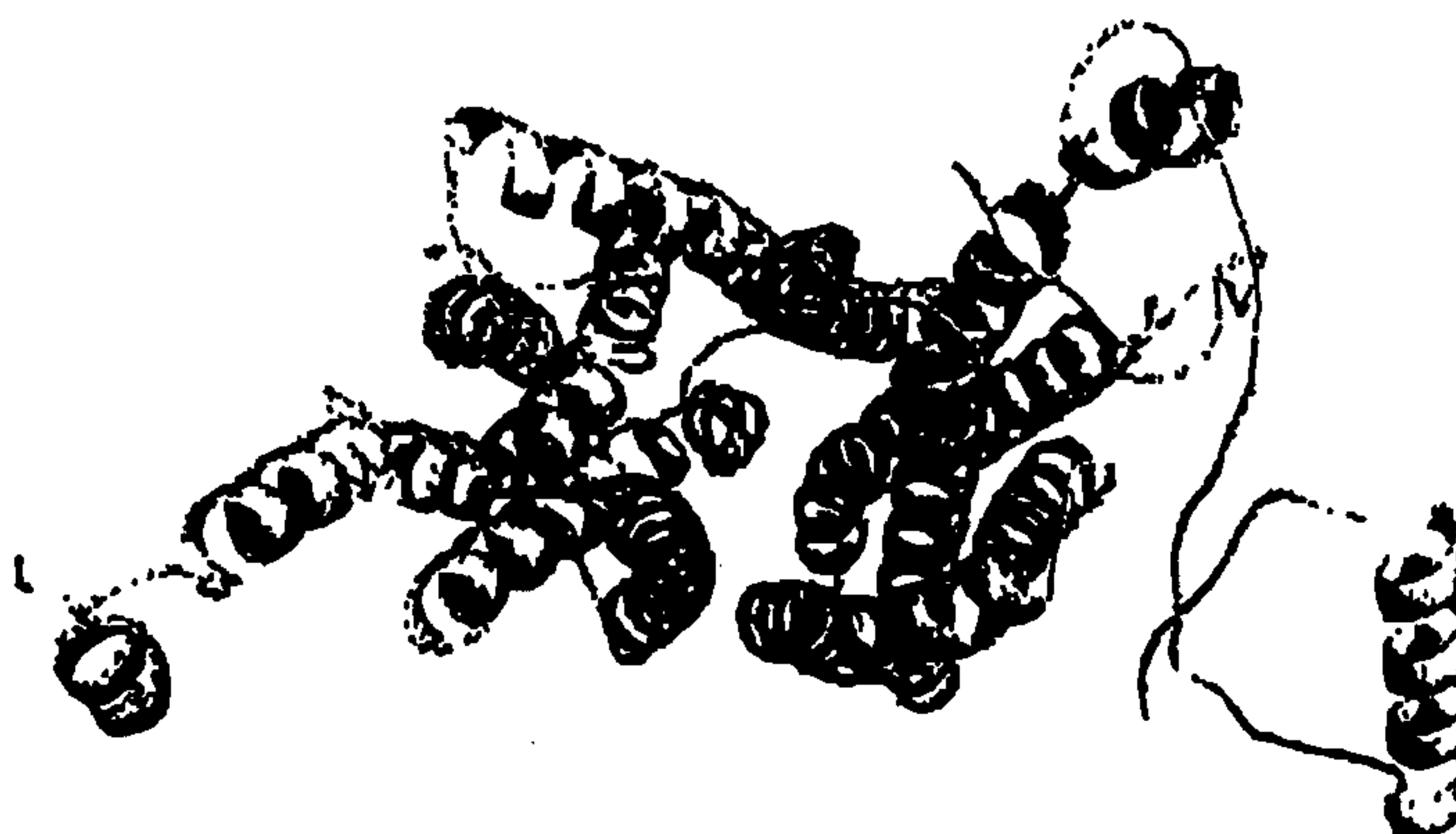
RECTIFIED SHEET

Figure 29

53/118/7



p.



RECTIFIED SHEET

Figure 29

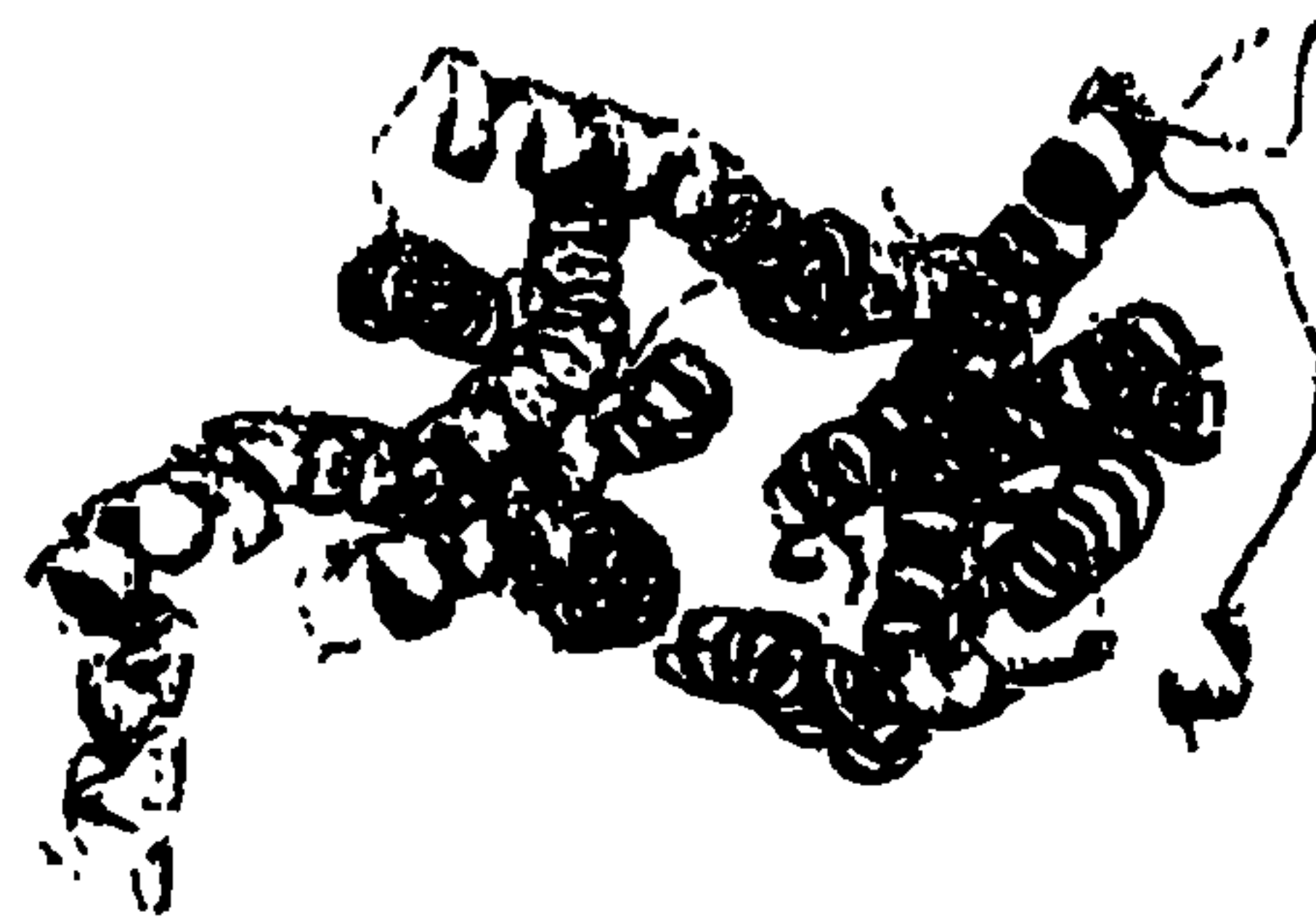
54 / 118

Part B

..



b.

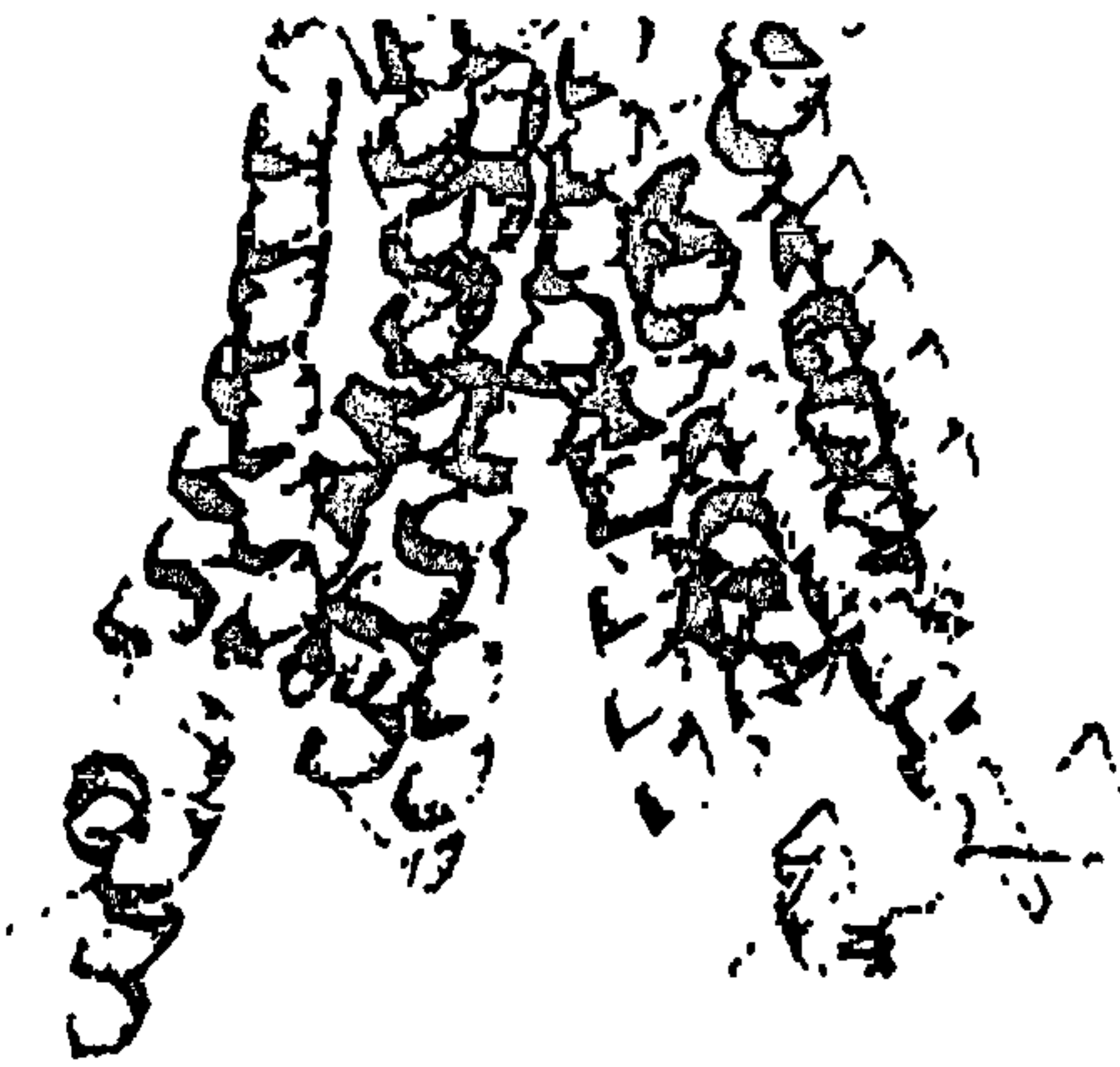


c.

RECTIFIED SHEET

Figure 29

54 / 118 / 1



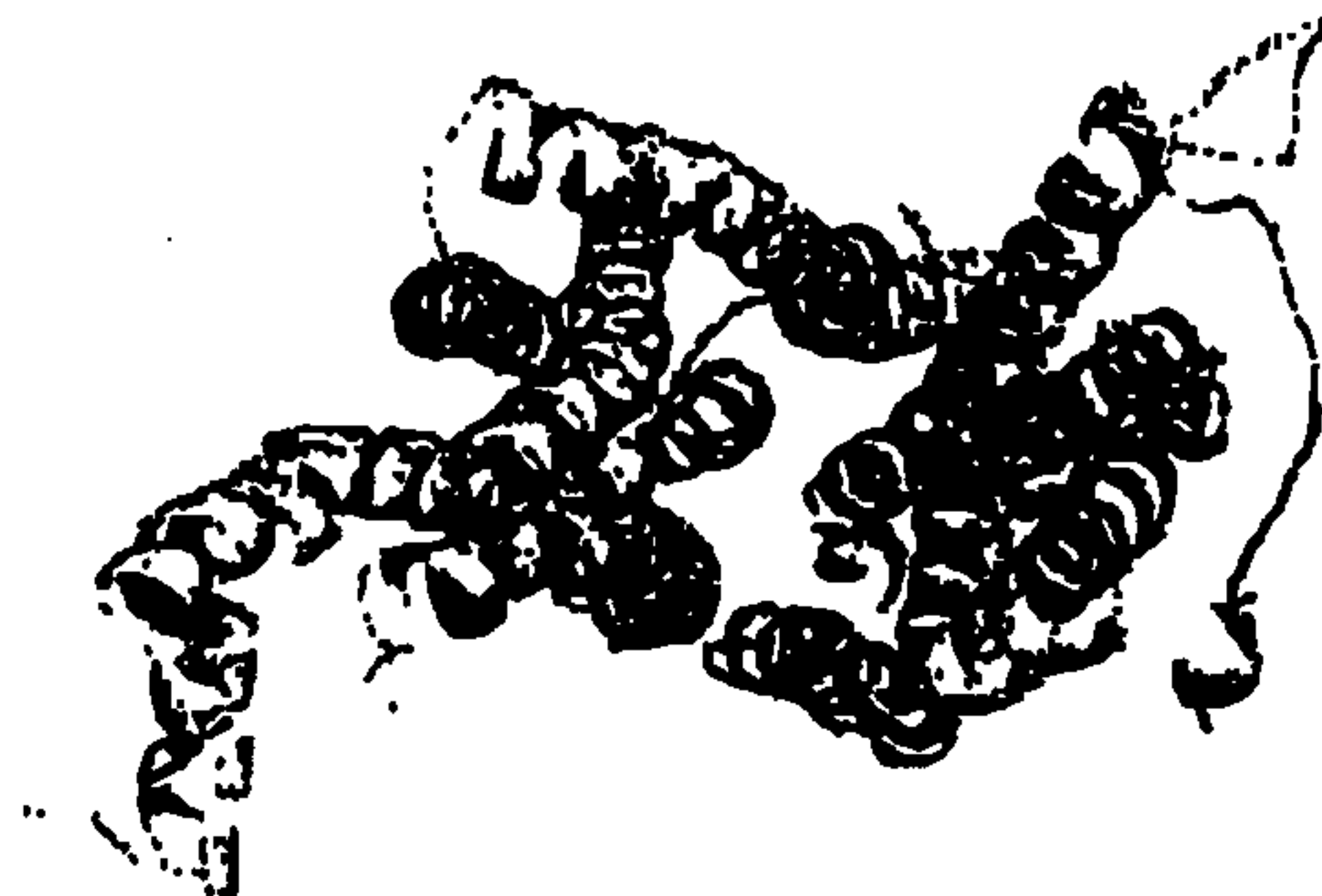
RECTIFIED SHEET

Figure 29

54 / 118 / 2



f.



g.

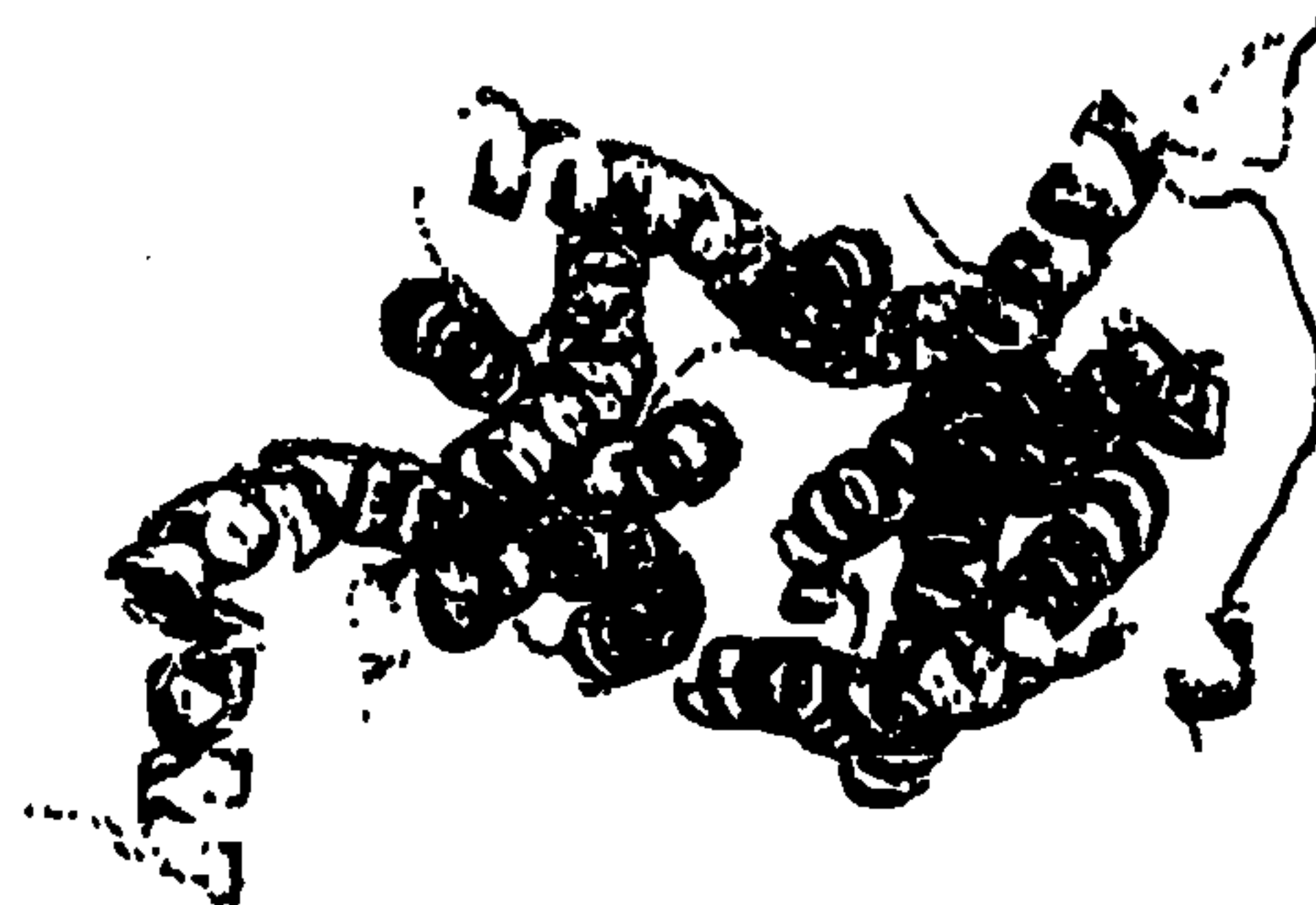
RECTIFIED SHEET

Figure 29

54 / 118 / 3



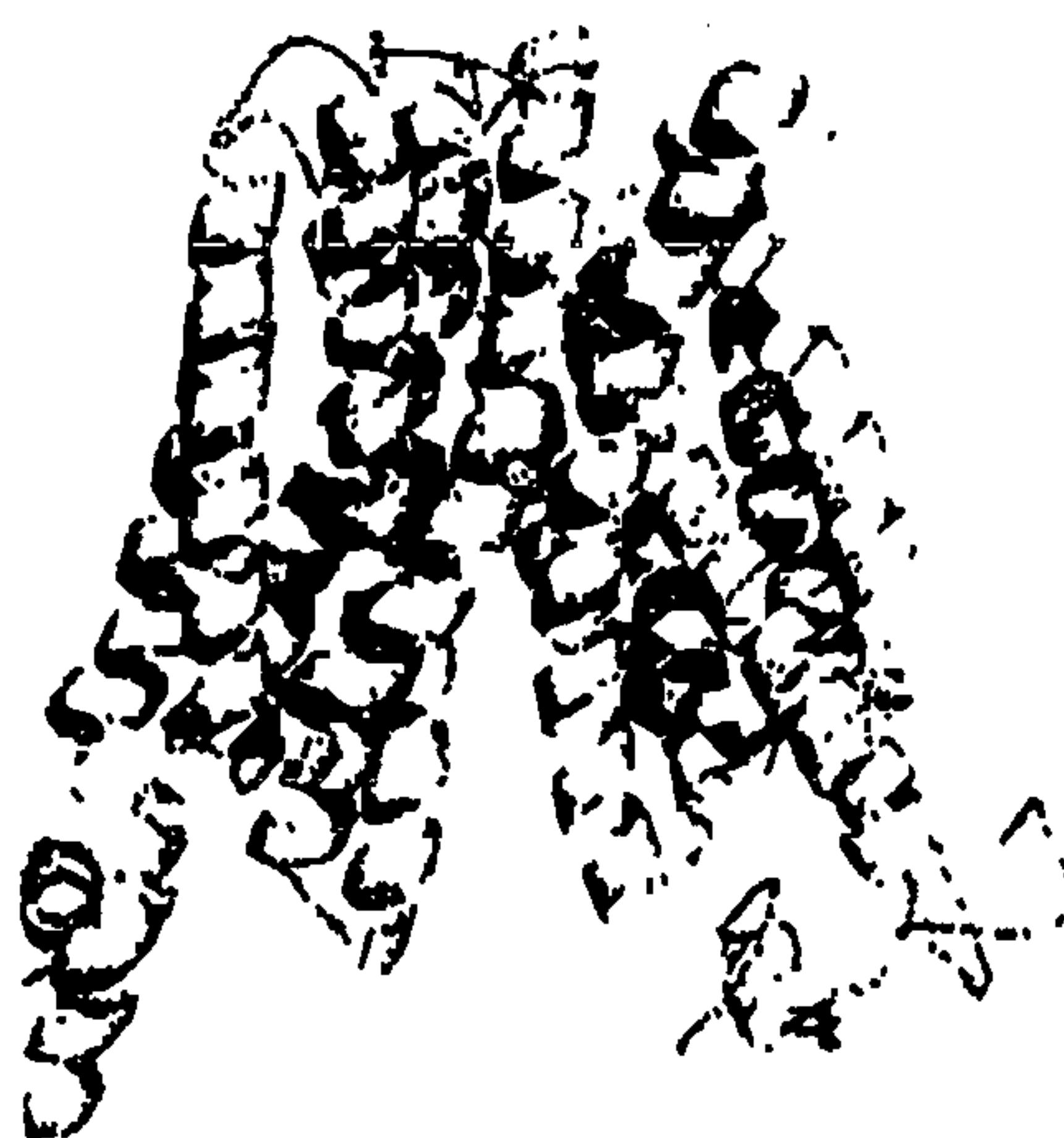
h.



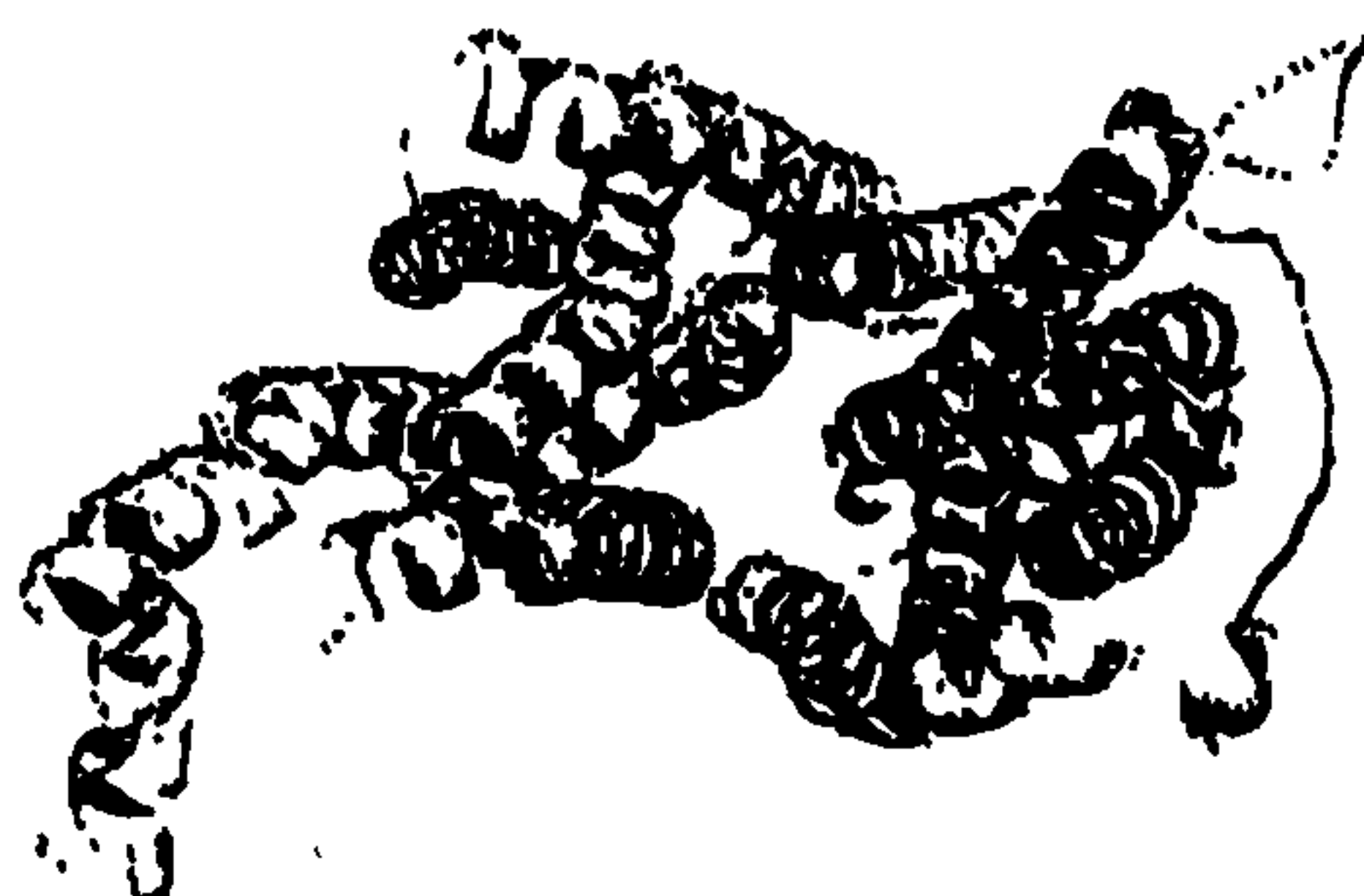
i.

Figure 29

54 / 118 / 4



j.



k.

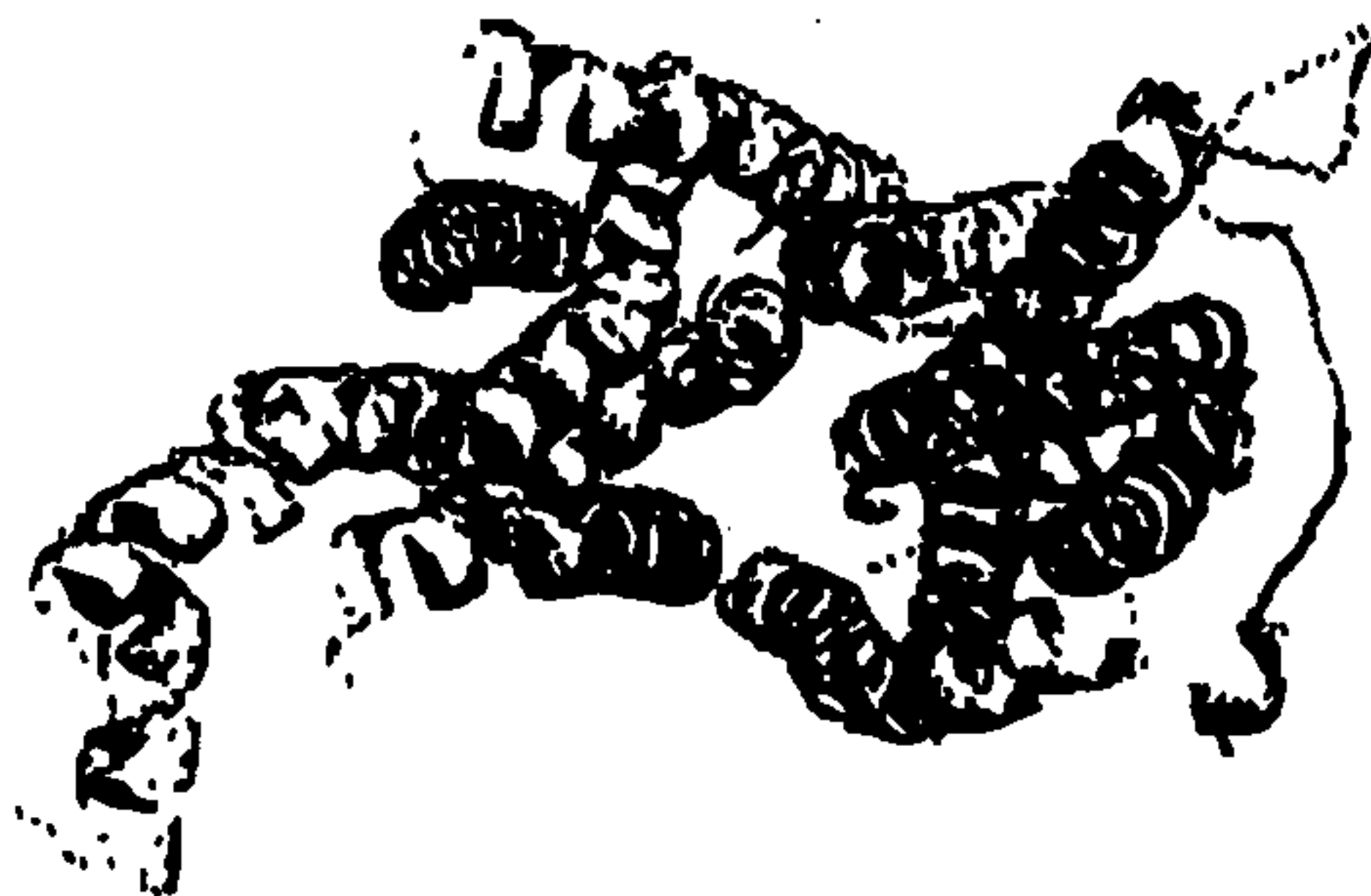
RECTIFIED SHEET

Figure 29

54 / 118 / 5



l.



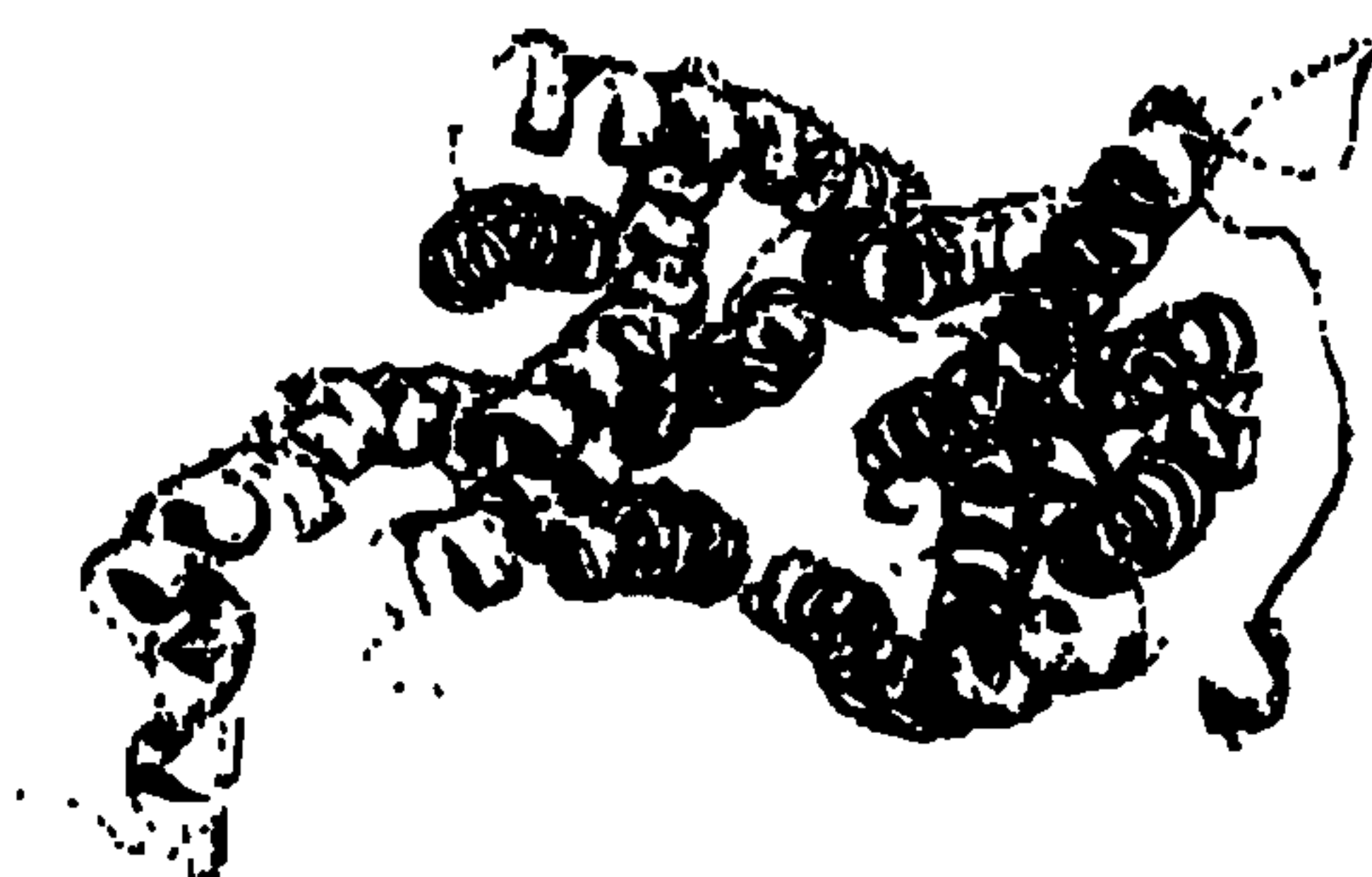
m.

Figure 29

54 / 118 / 6



n.



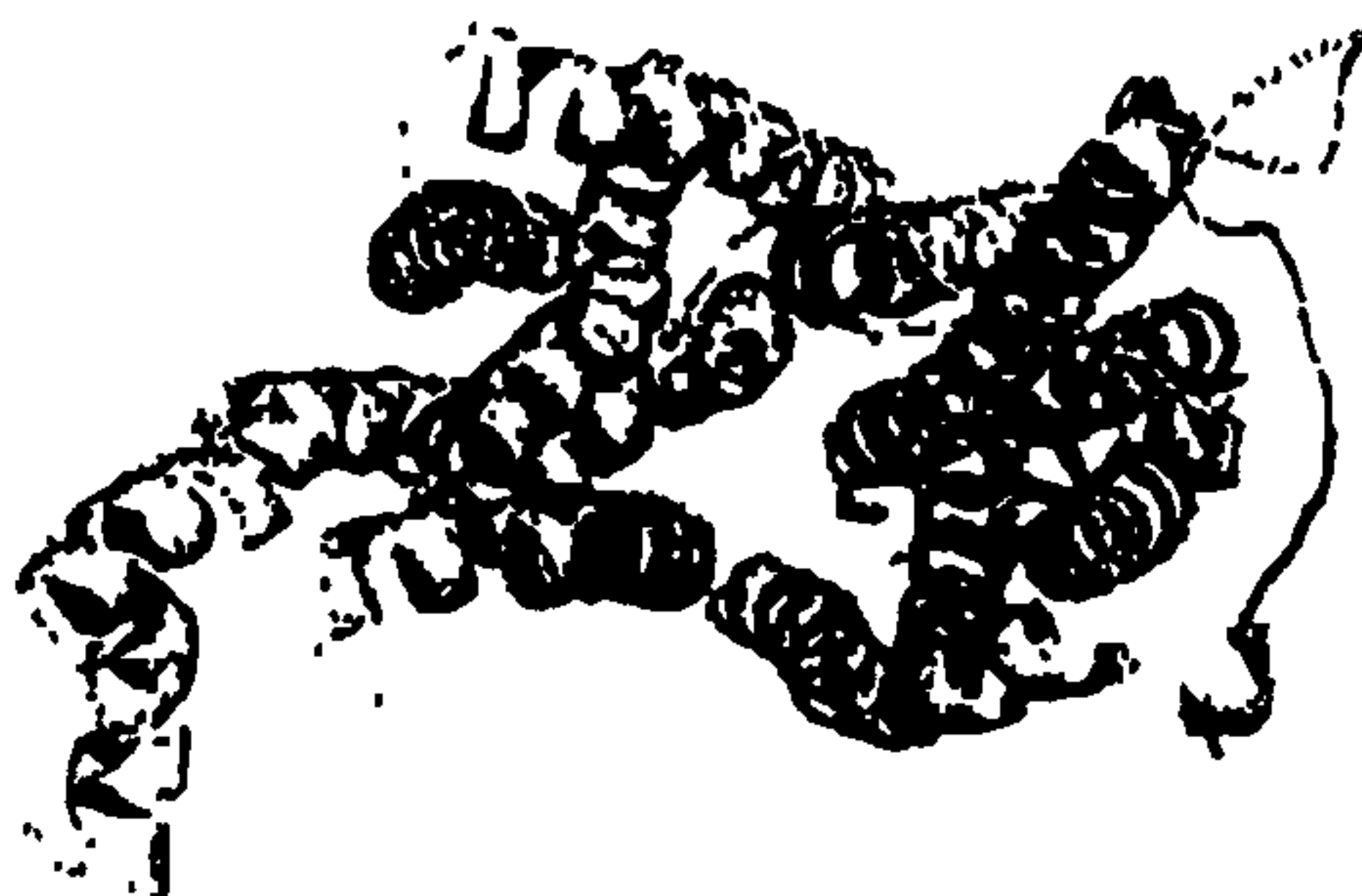
o.

Figure 29

54 / 118 / 7



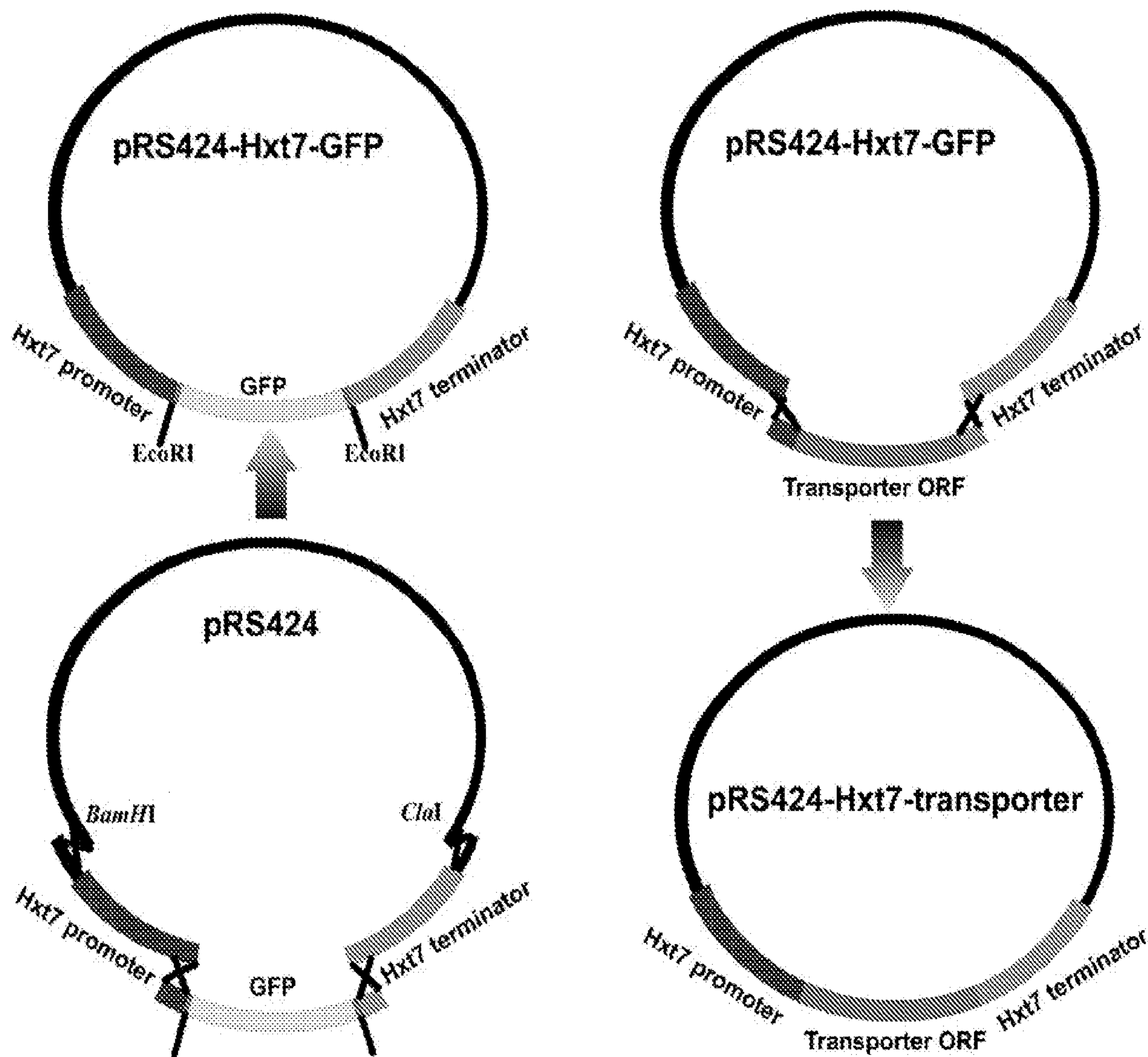
p.



RECTIFIED SHEET

Figure 30

A



B

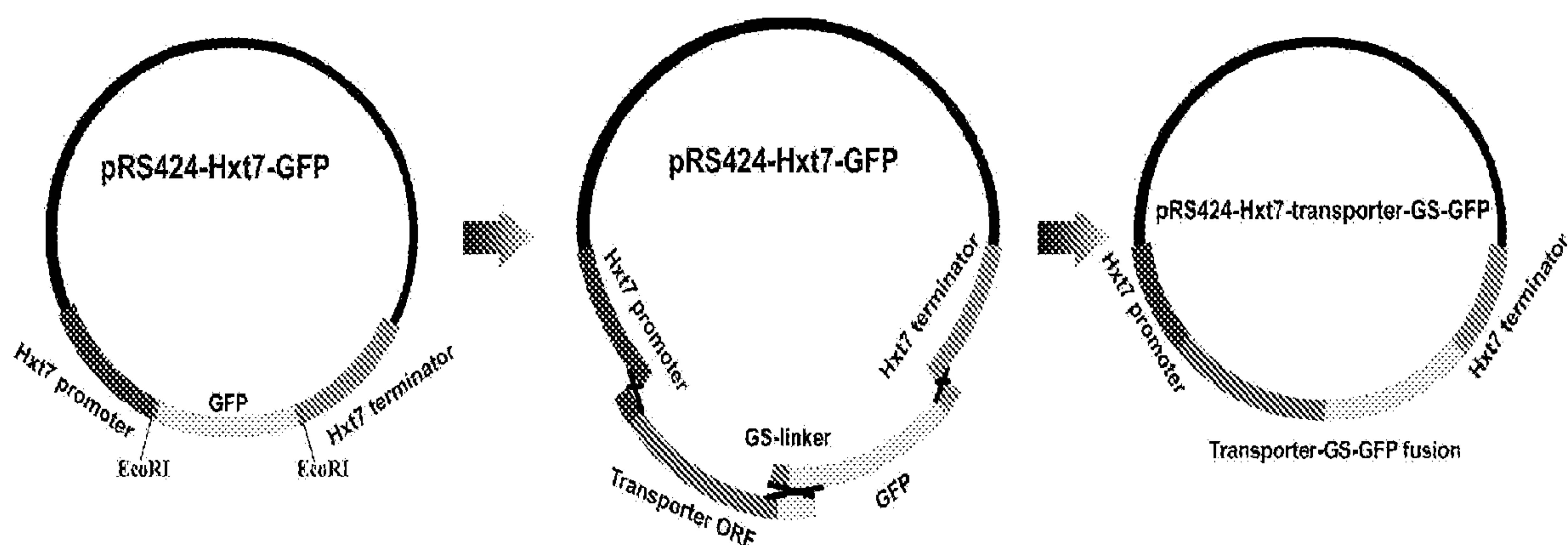


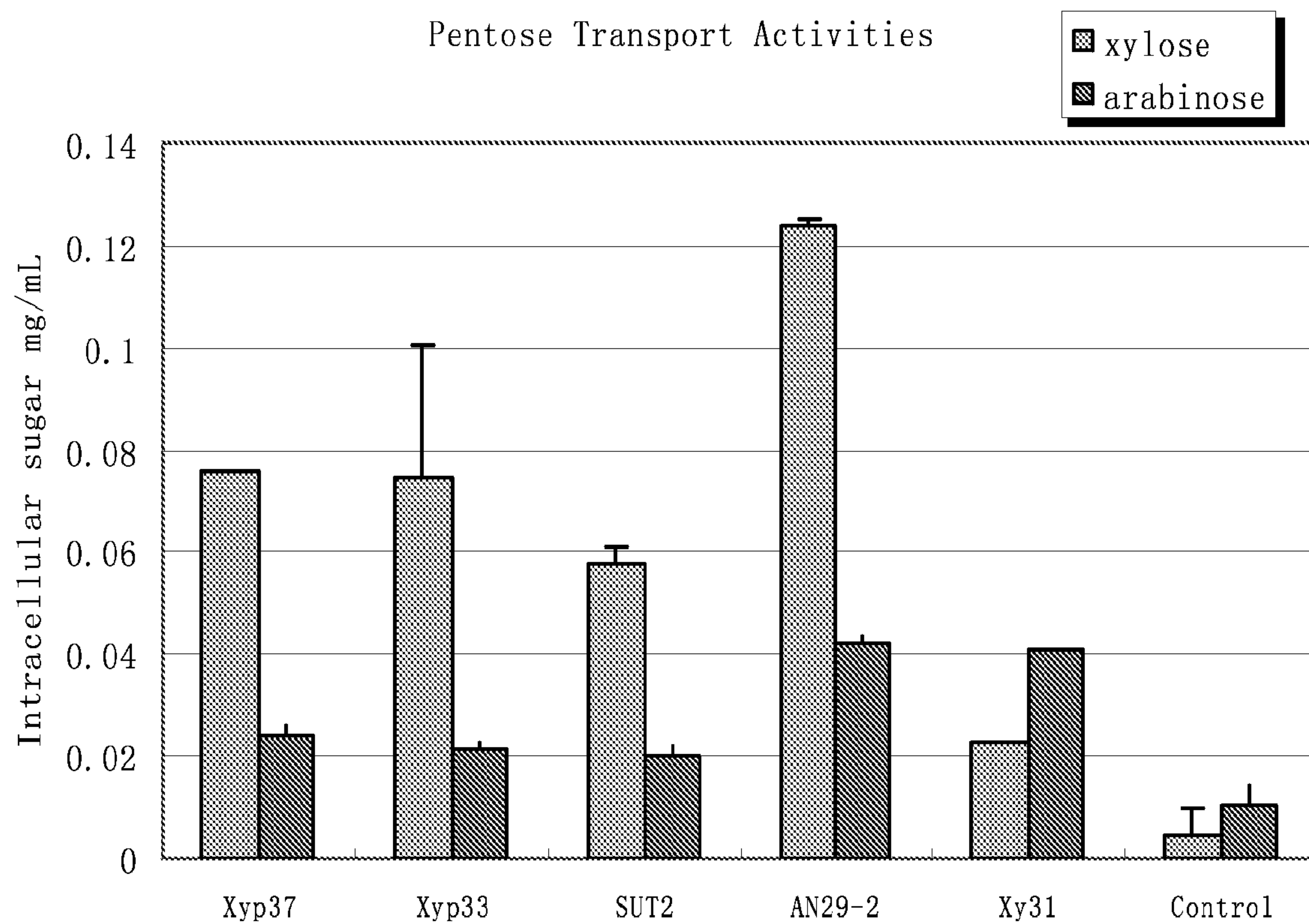
Figure 31

Figure 32

Pentose Transport Activities

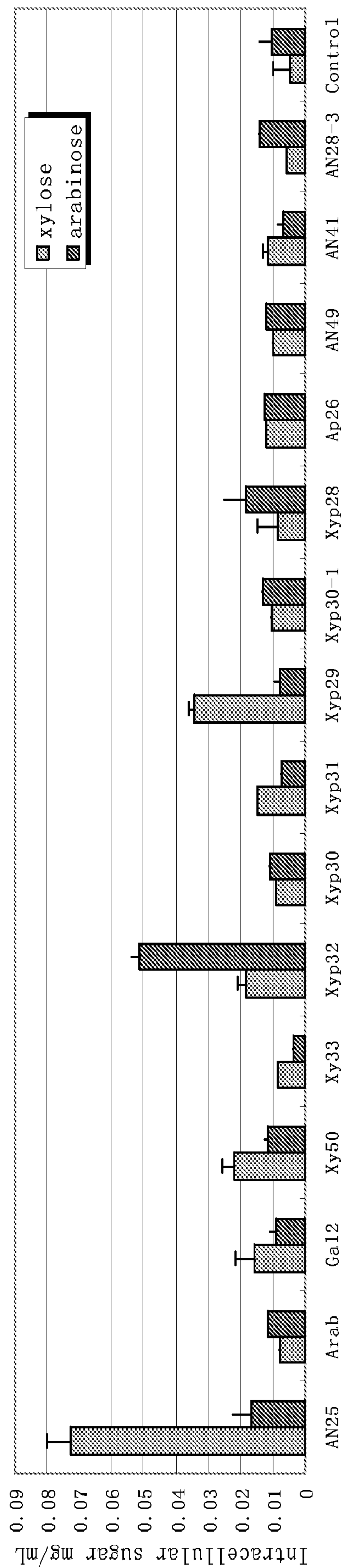


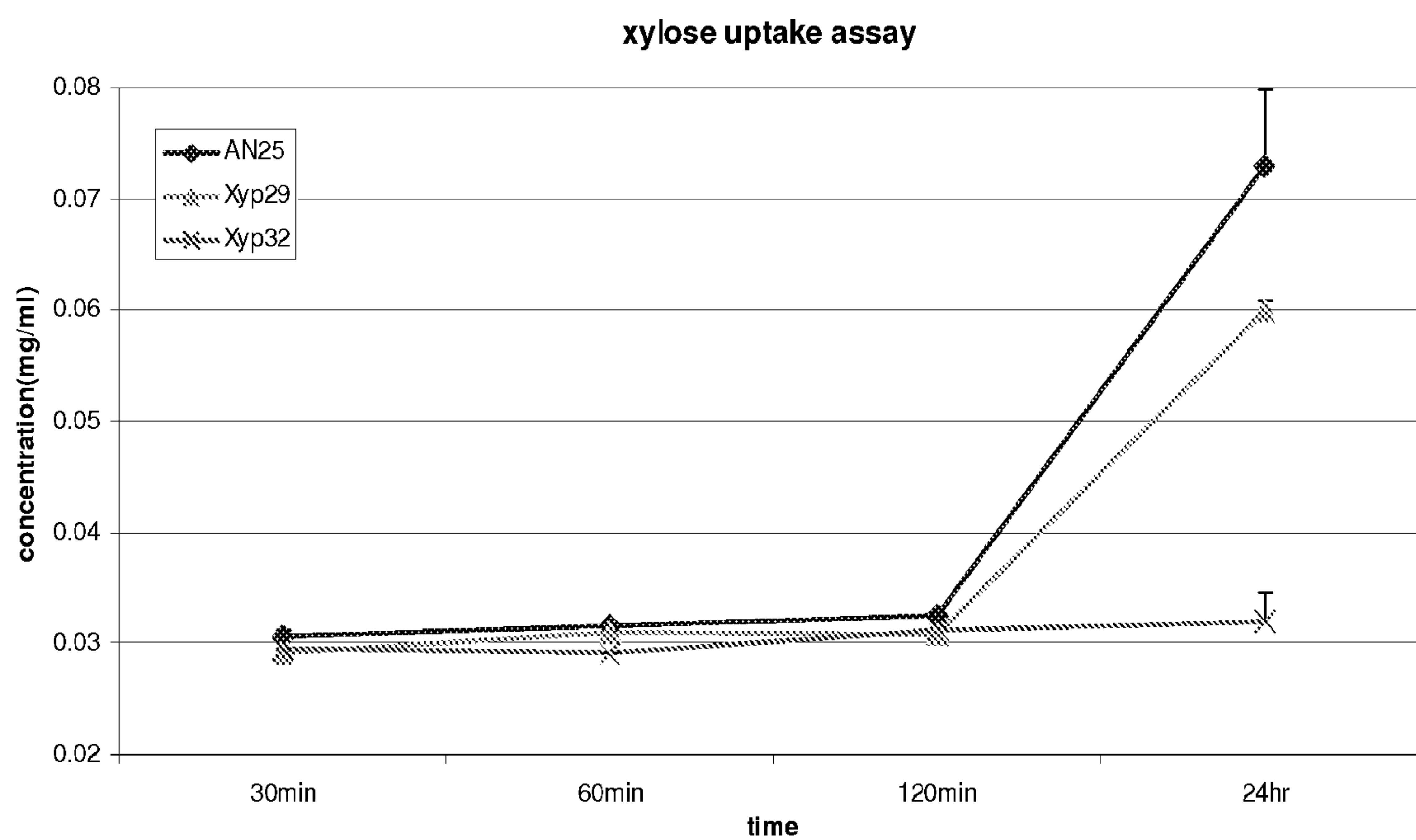
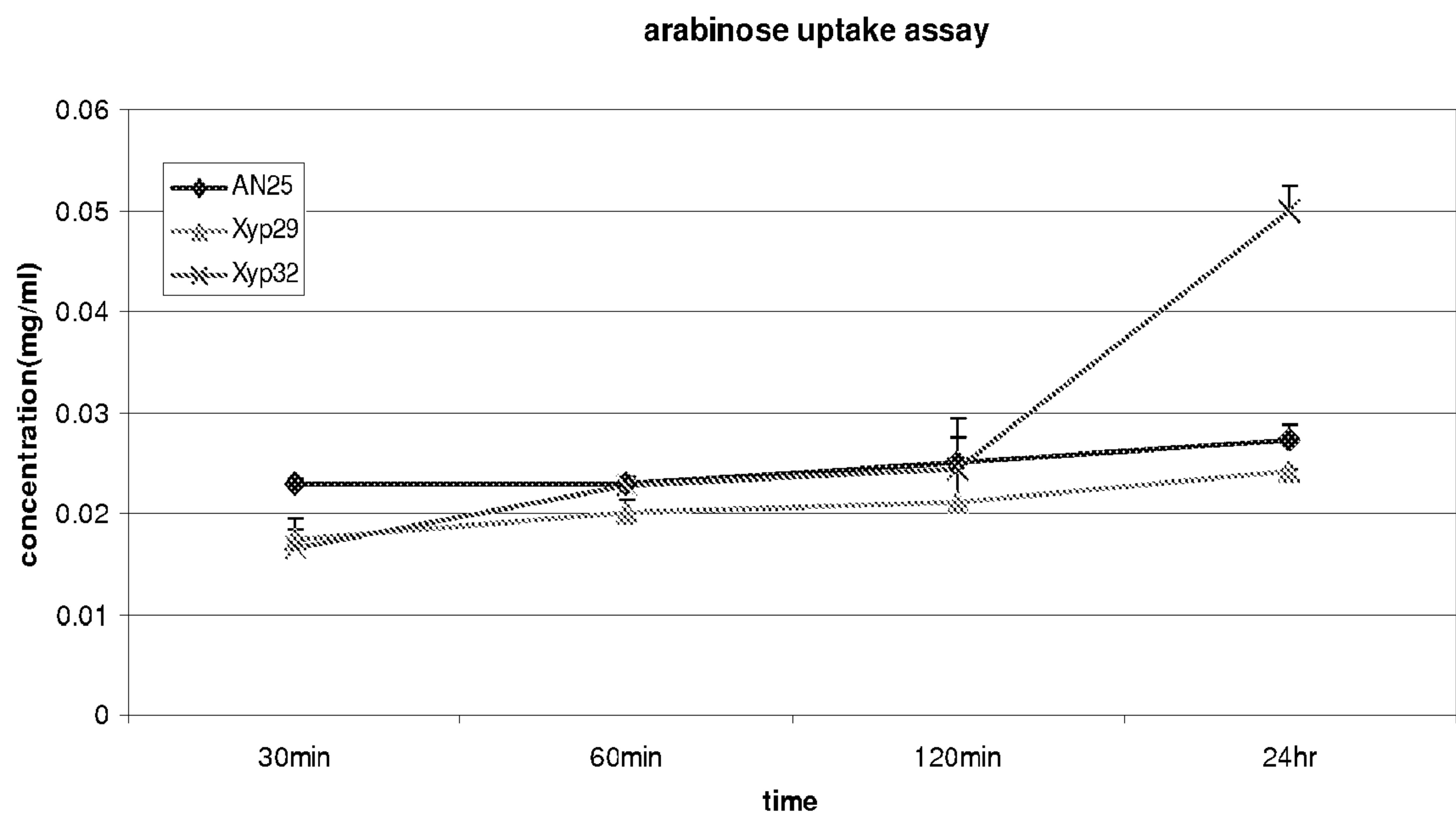
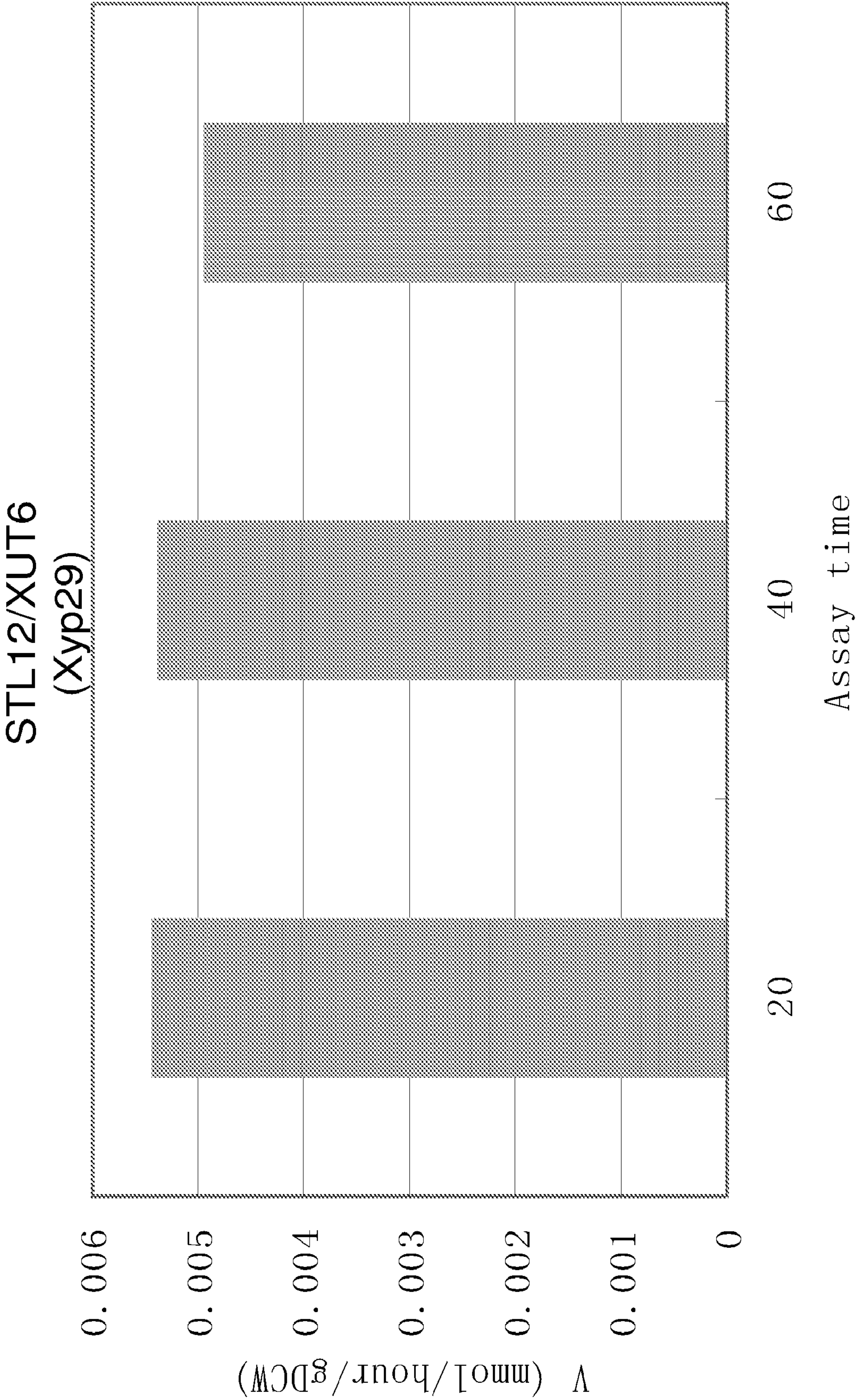
Figure 33**A****B**

Figure 34



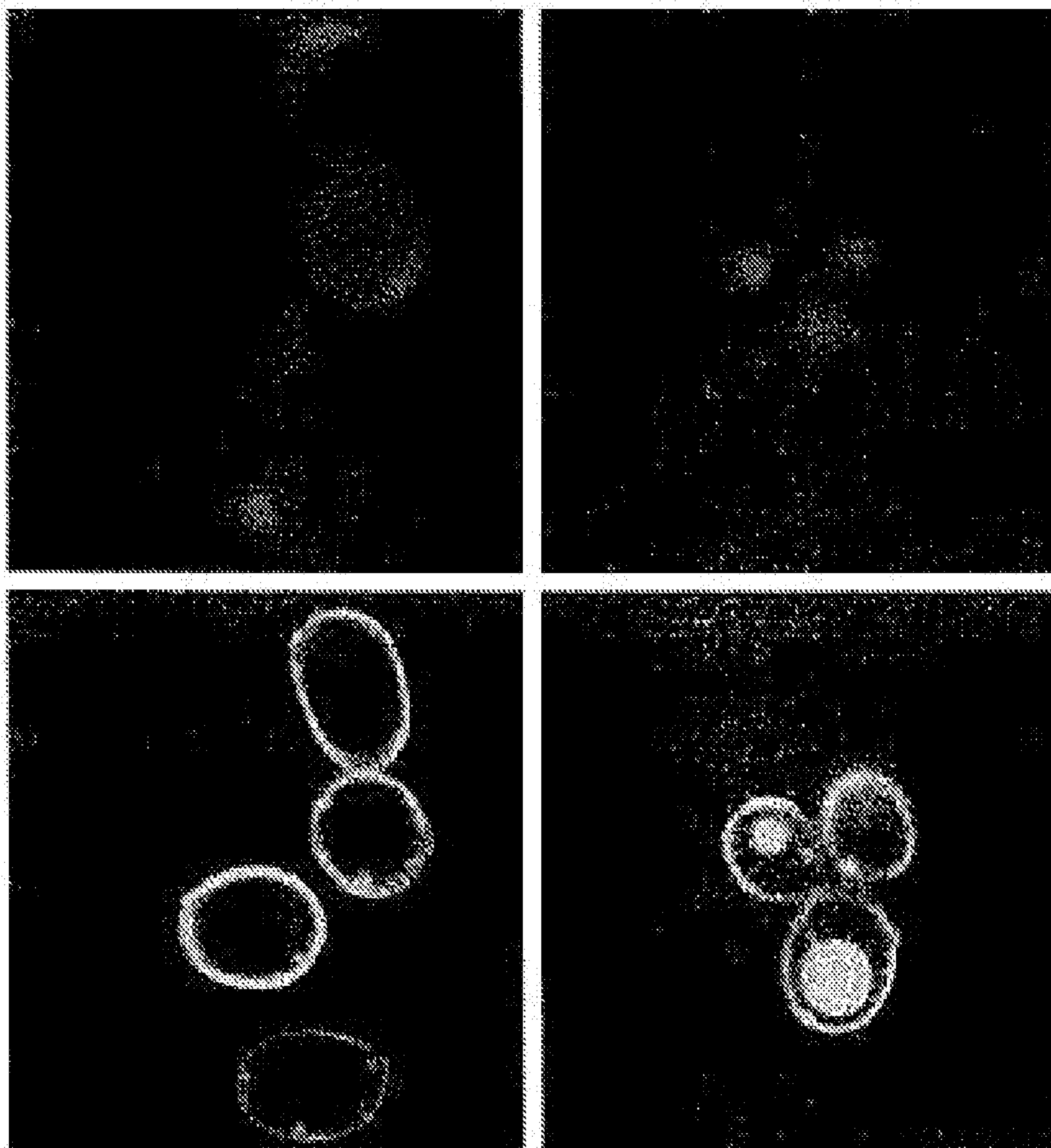
**Figure 35**

Figure 36

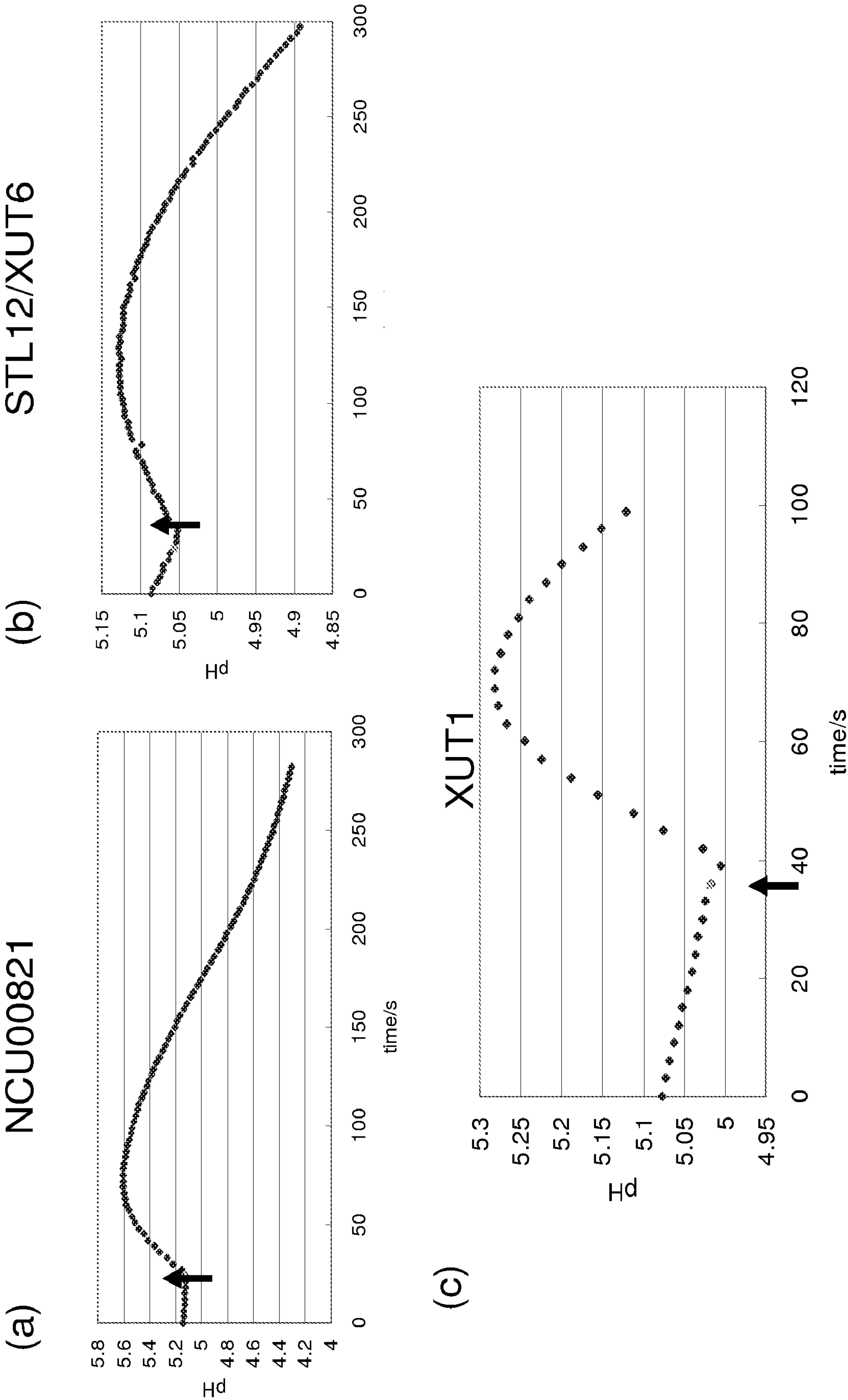


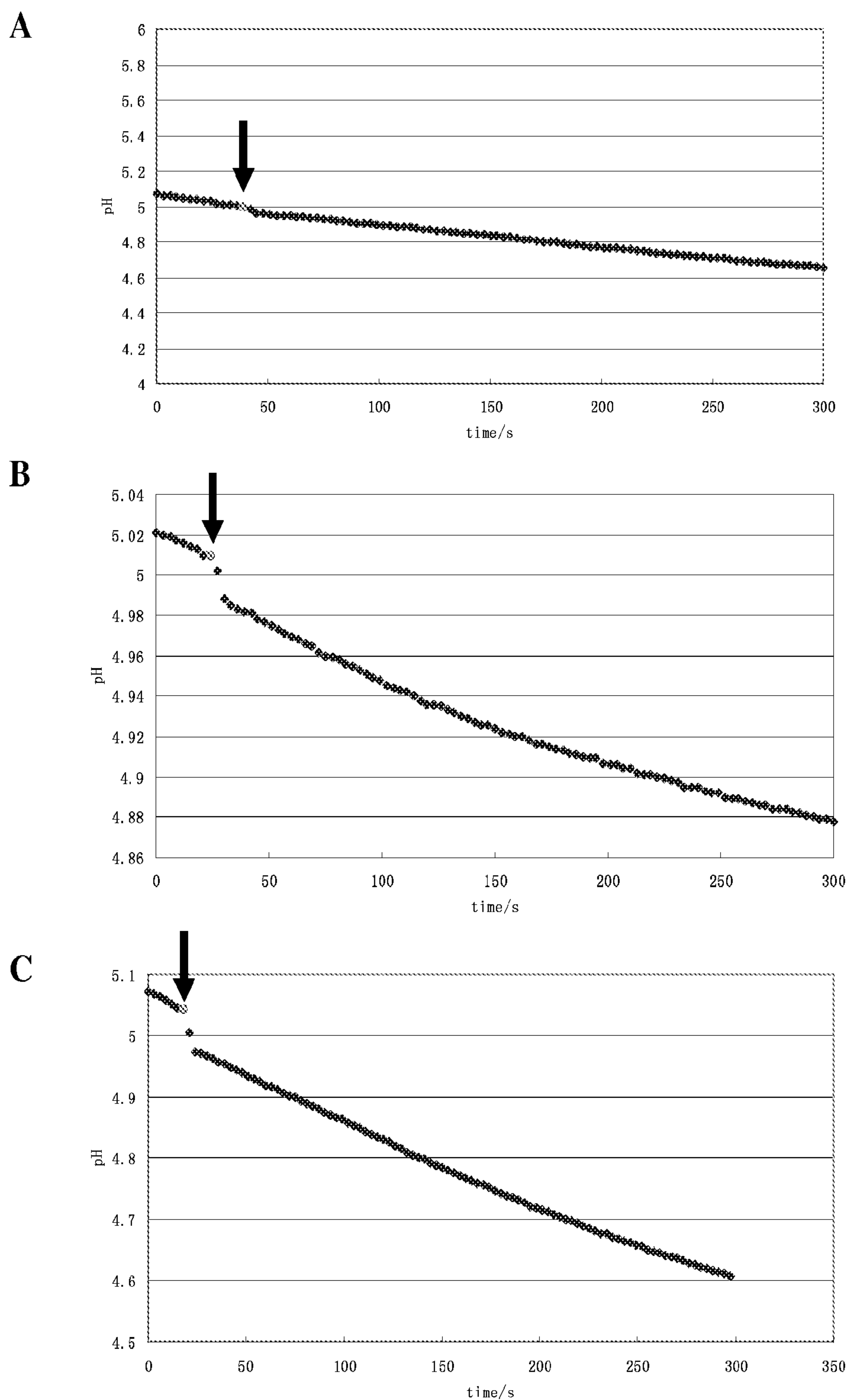
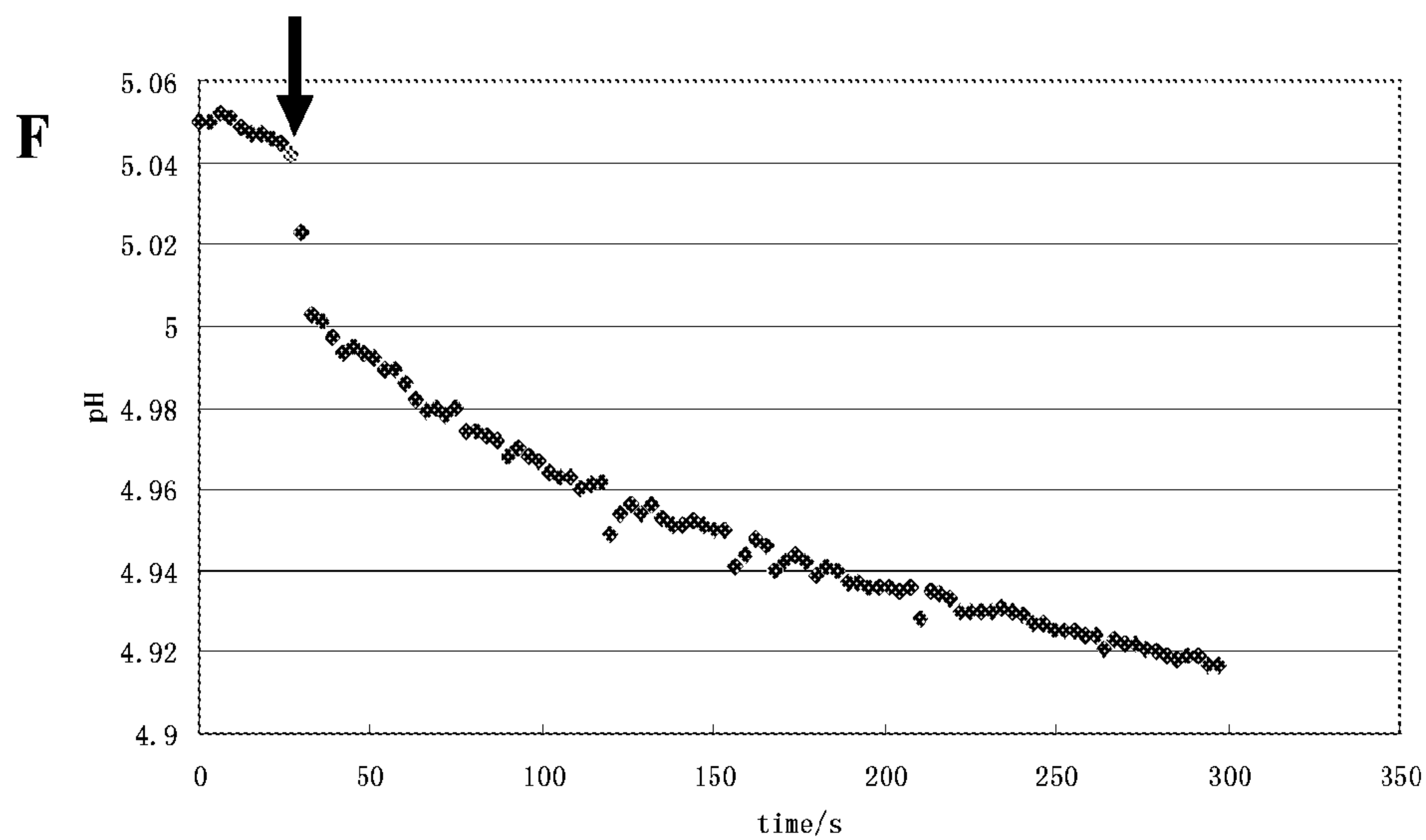
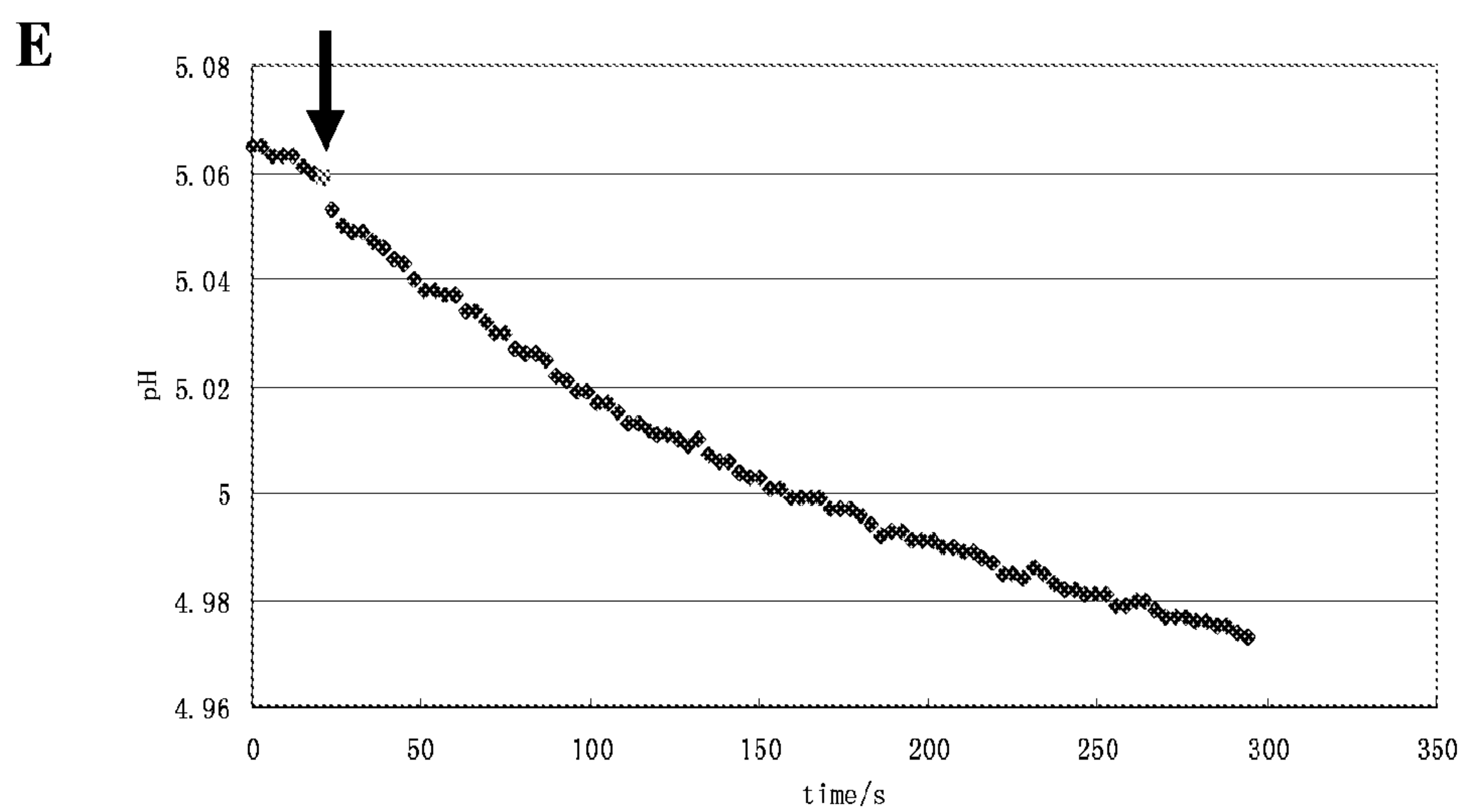
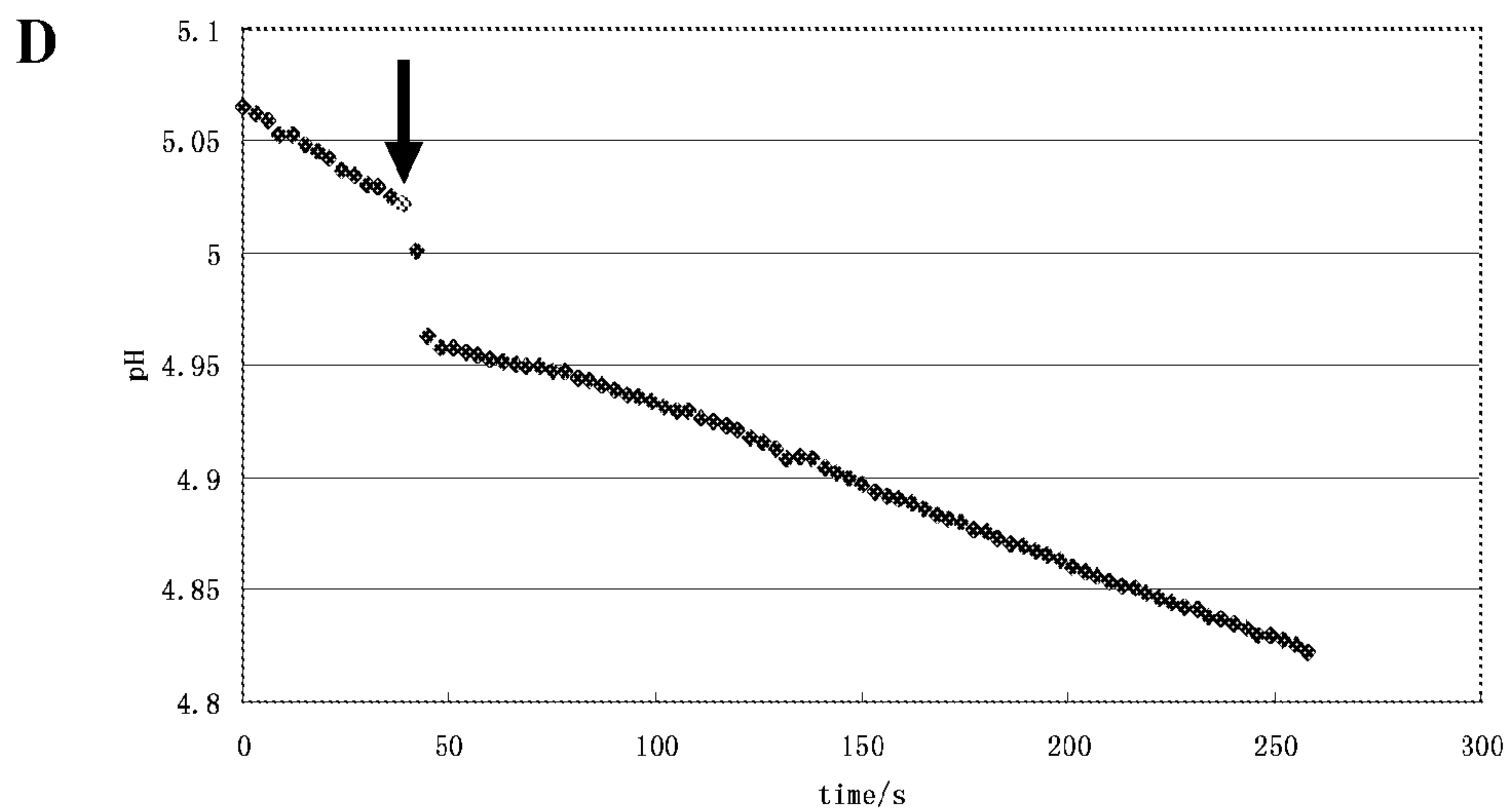
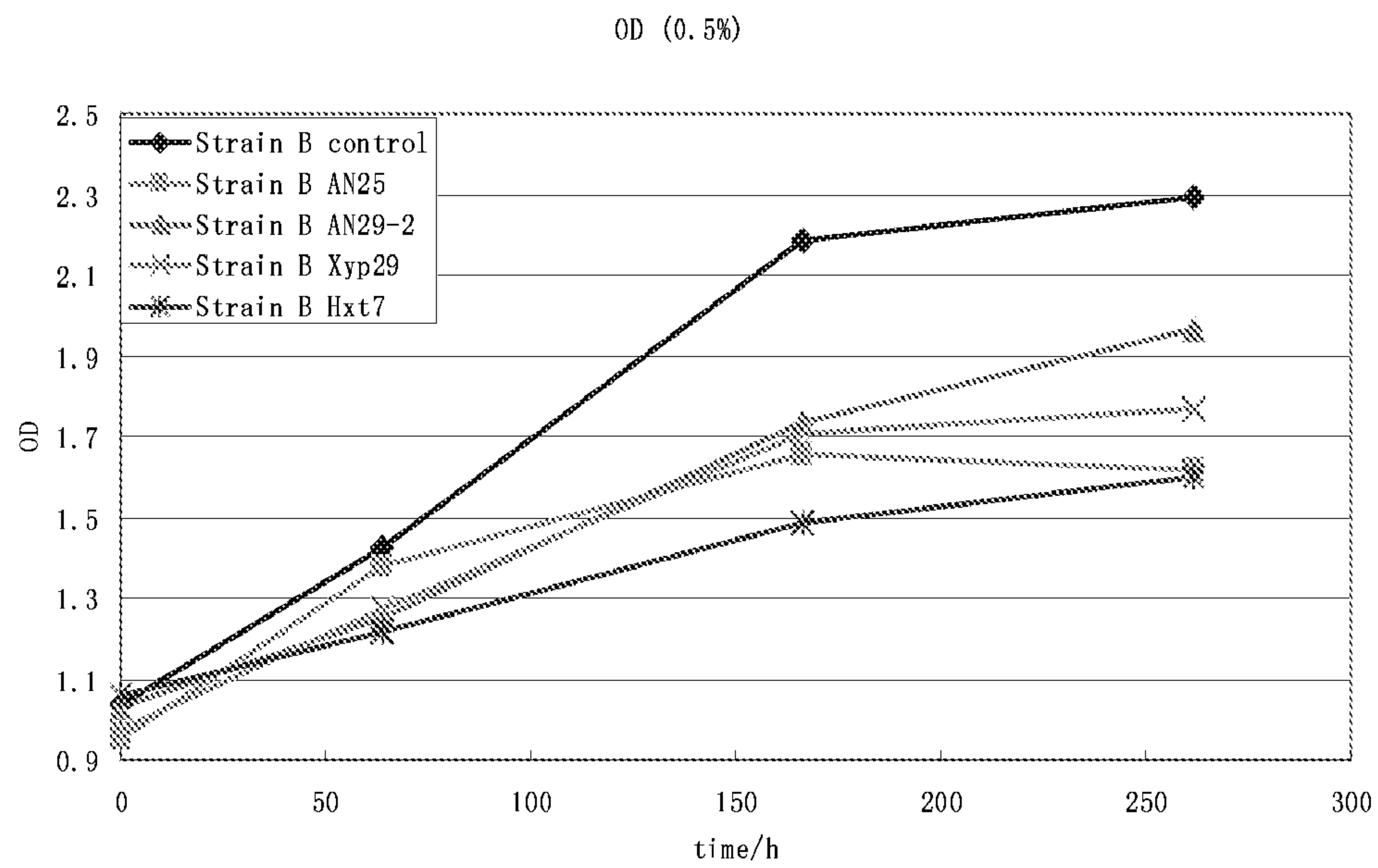
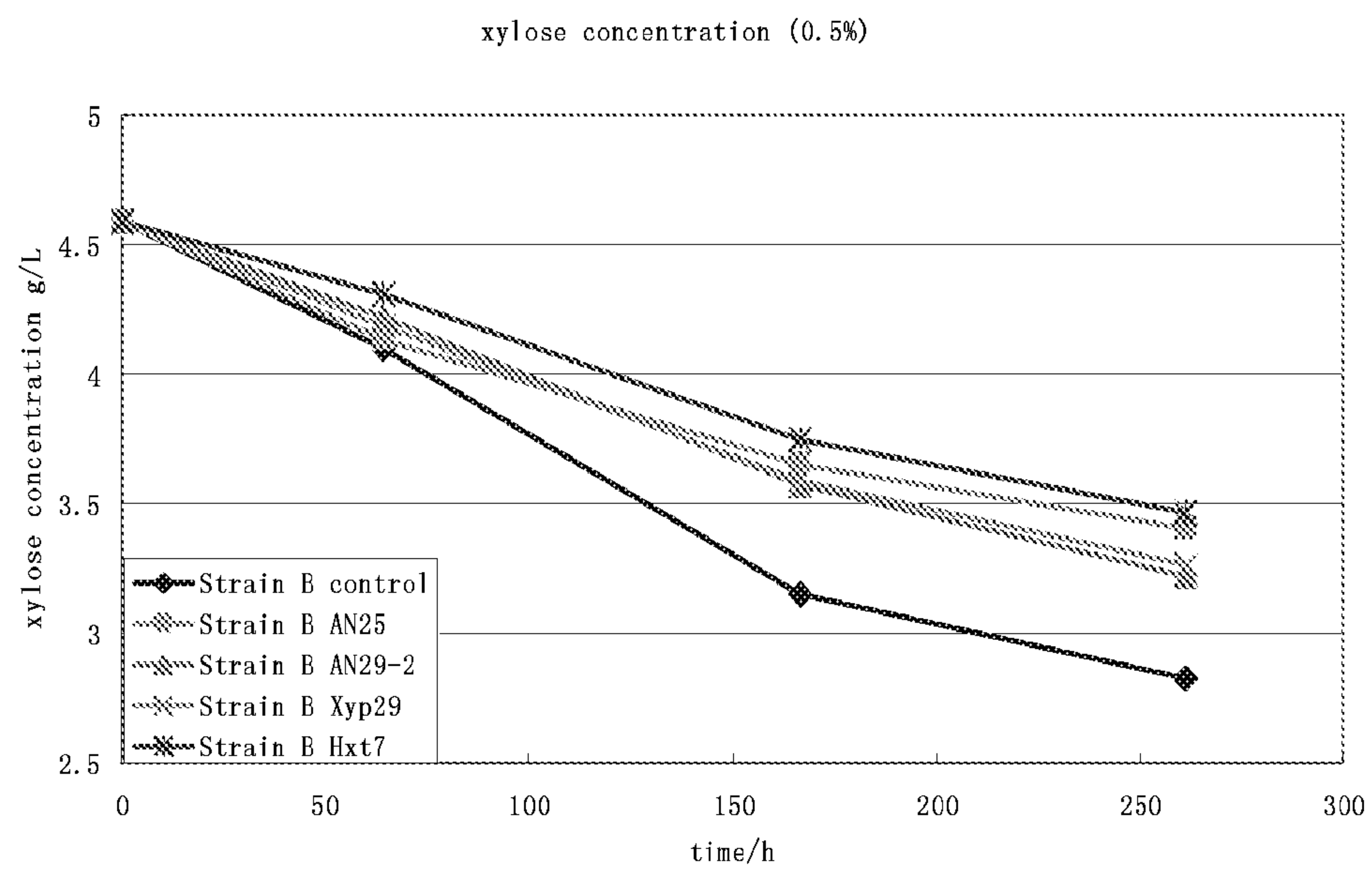
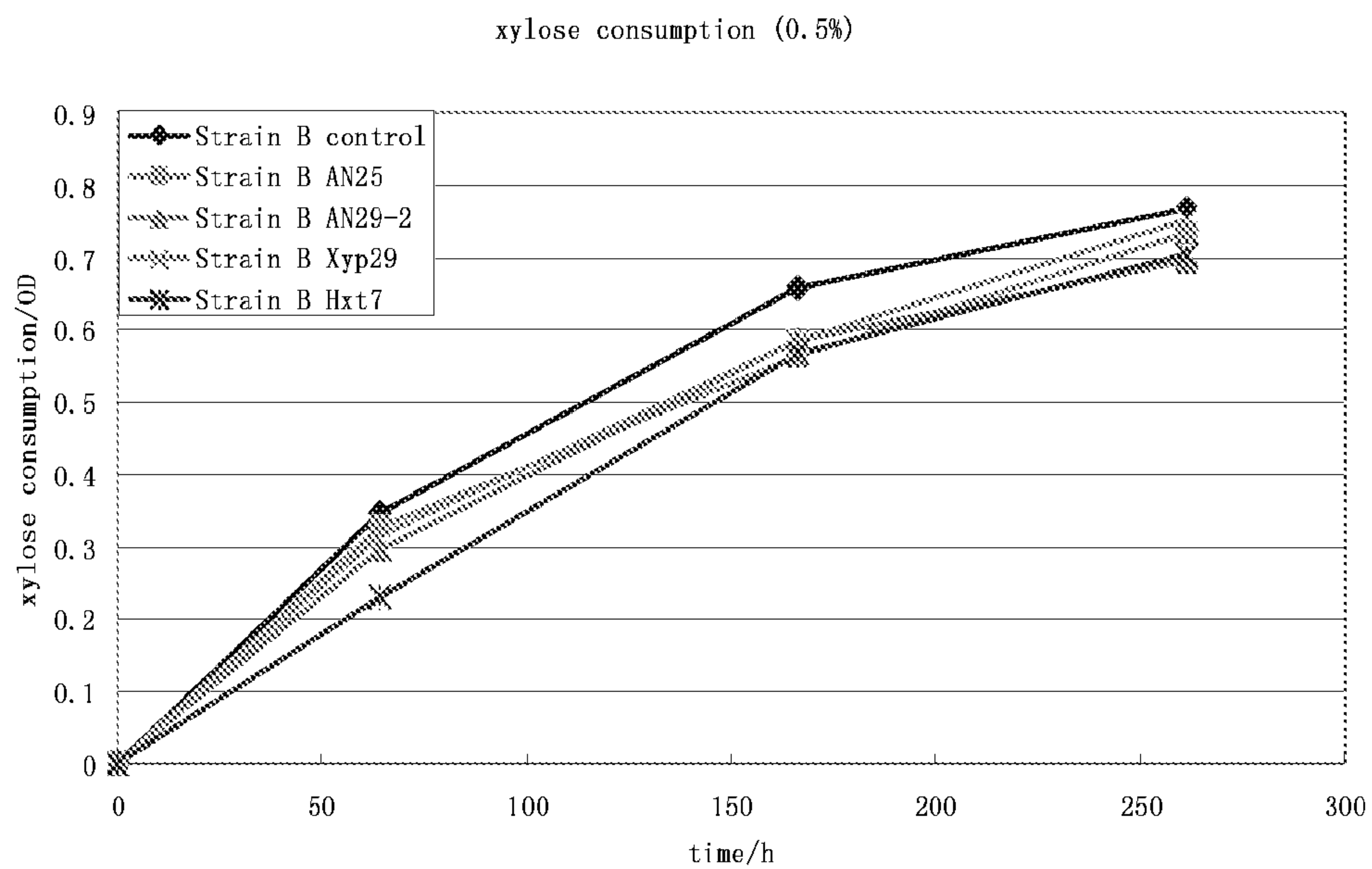
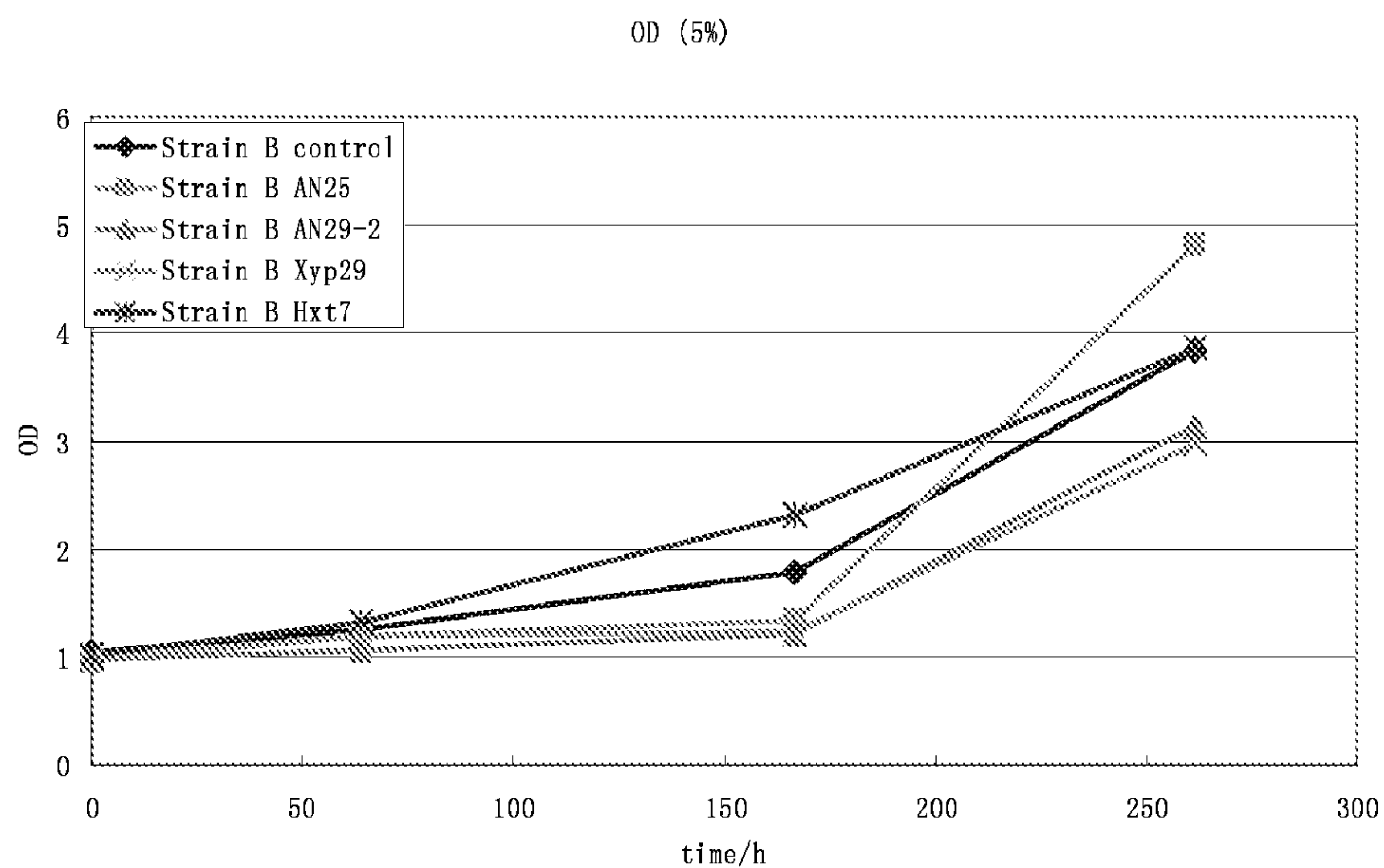
Figure 37

Figure 37 (cont.)



A**B****Figure 38**

C**D****Figure 38 (cont.)**

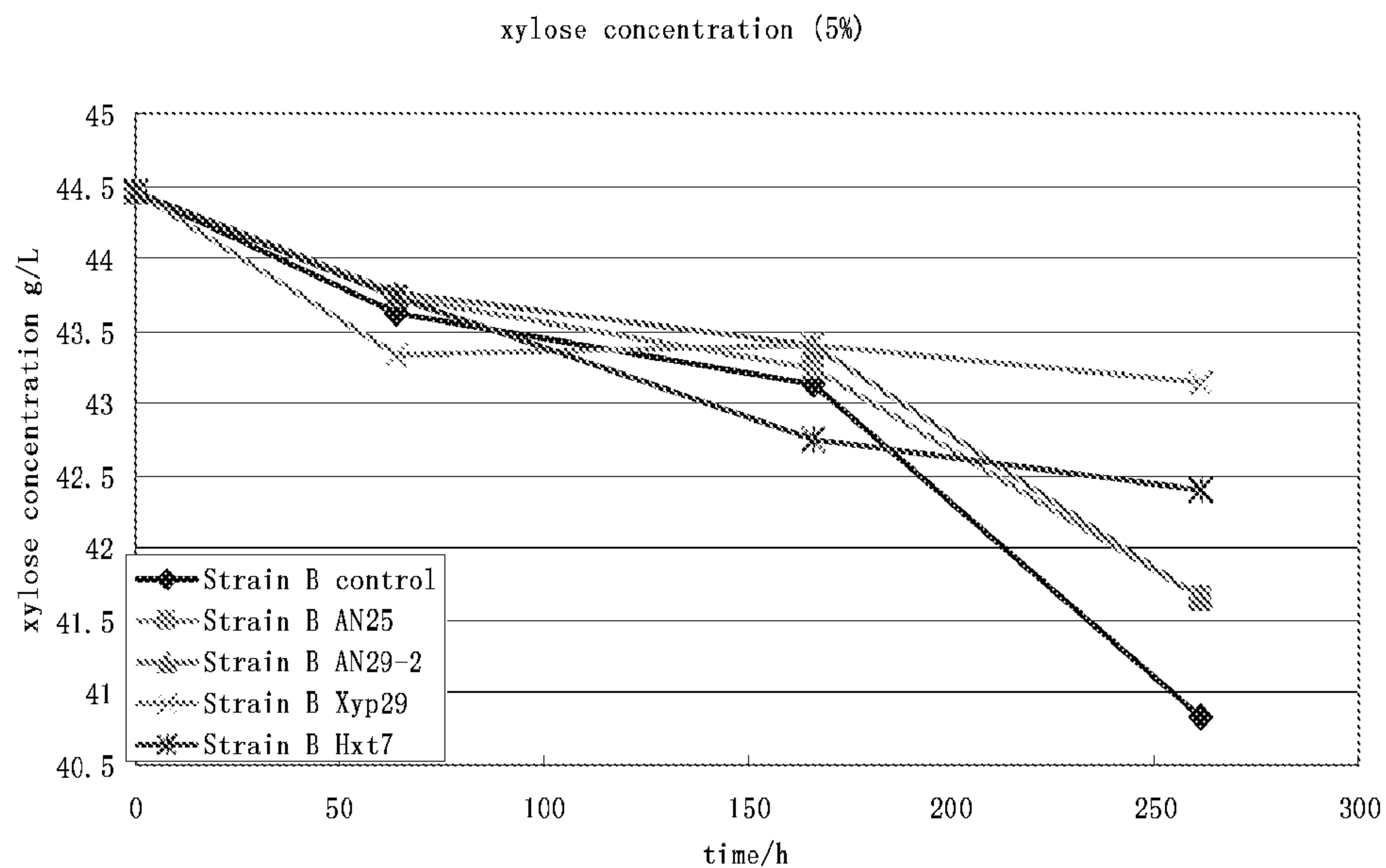
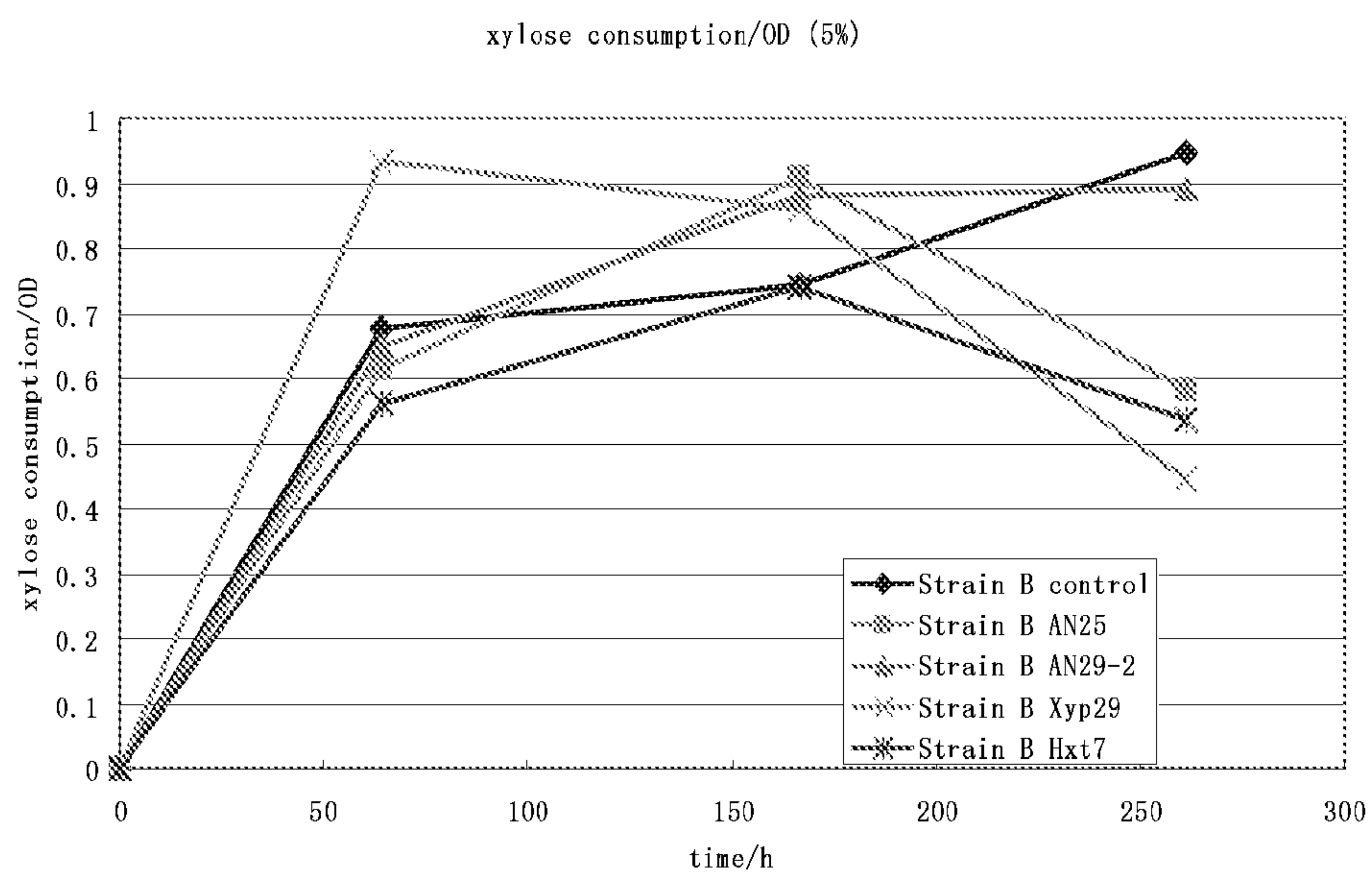
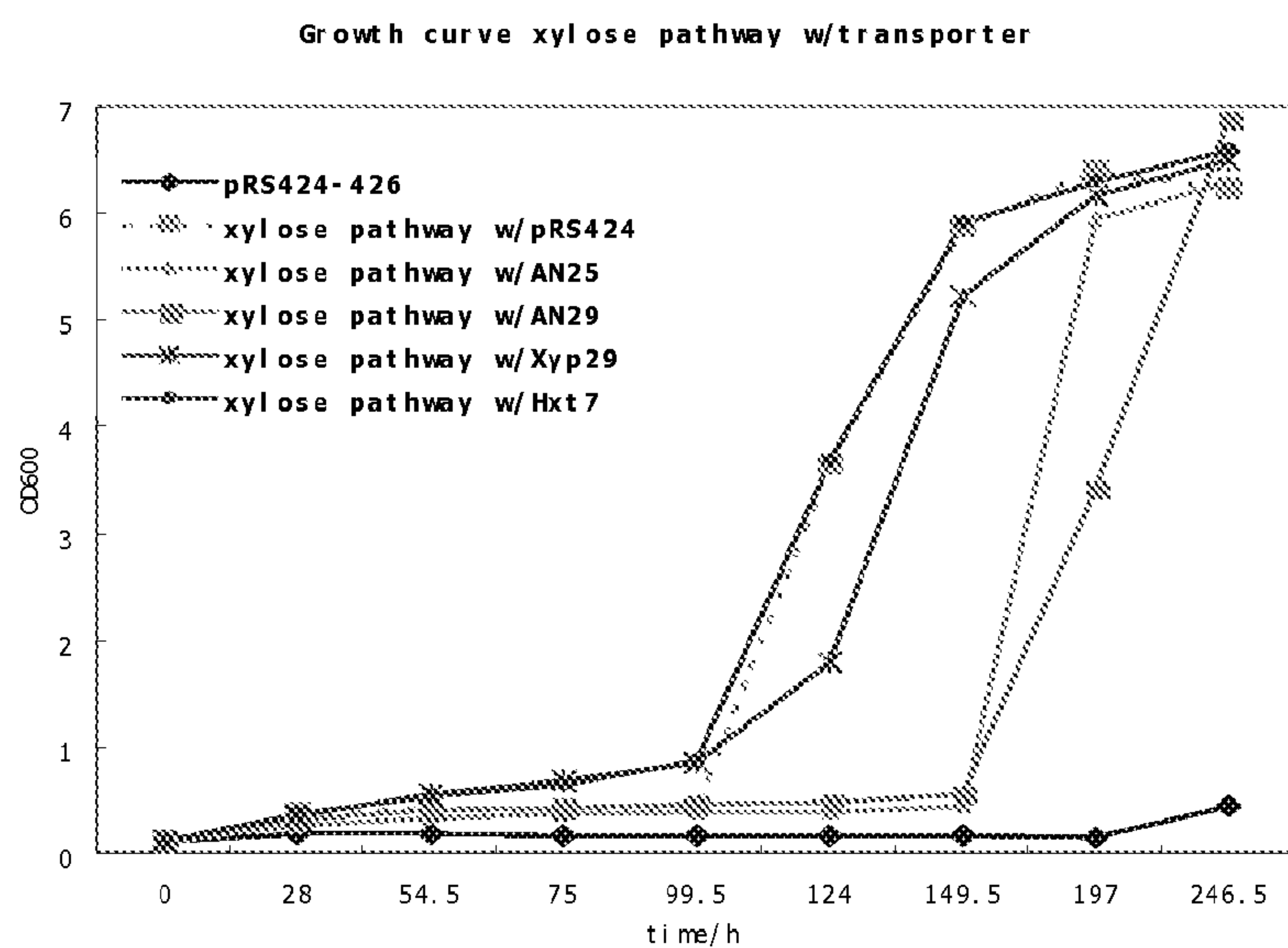
E**F****G**

Figure 38 (cont.)

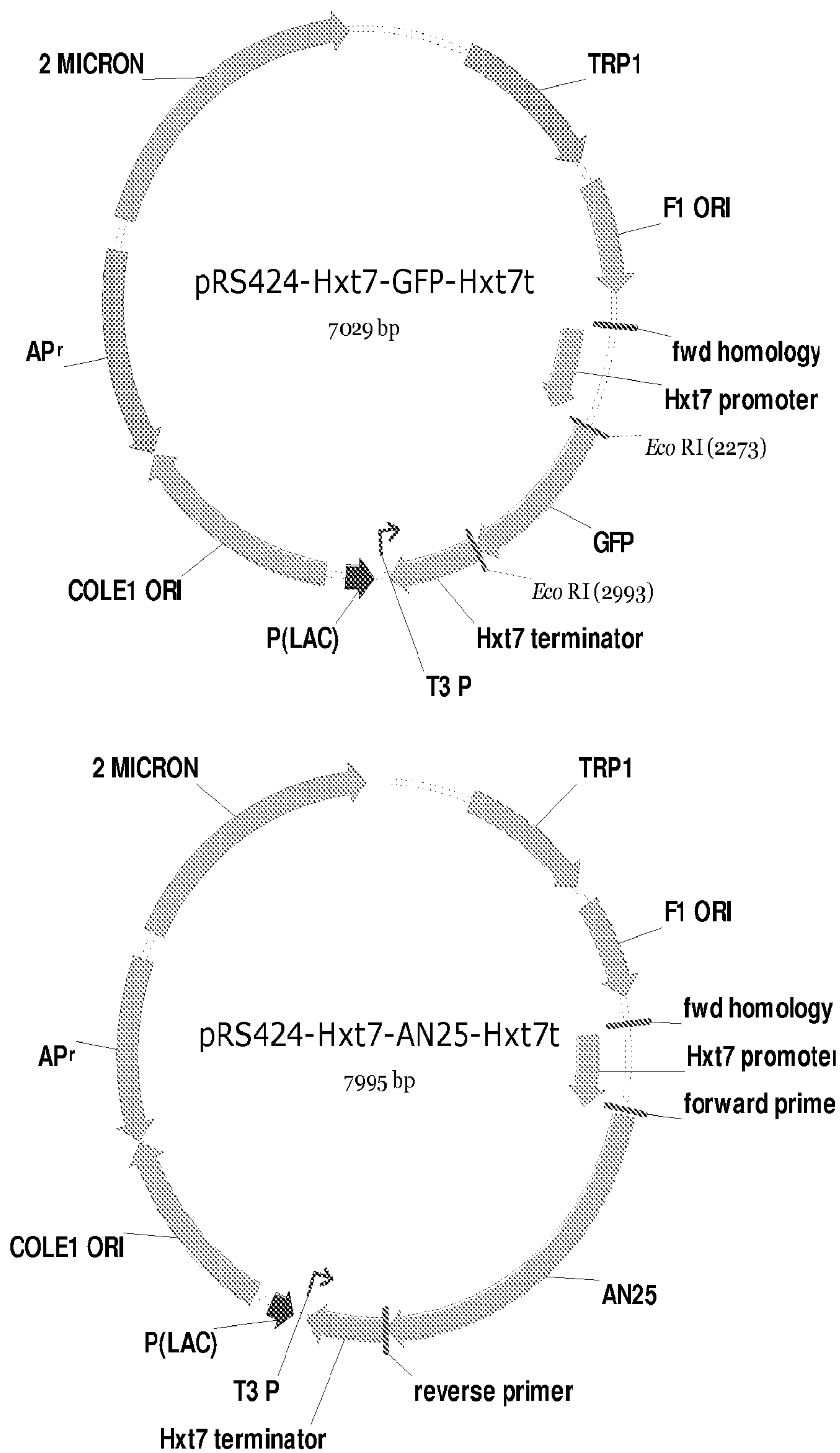
Figure 39

Figure 39 (cont.)

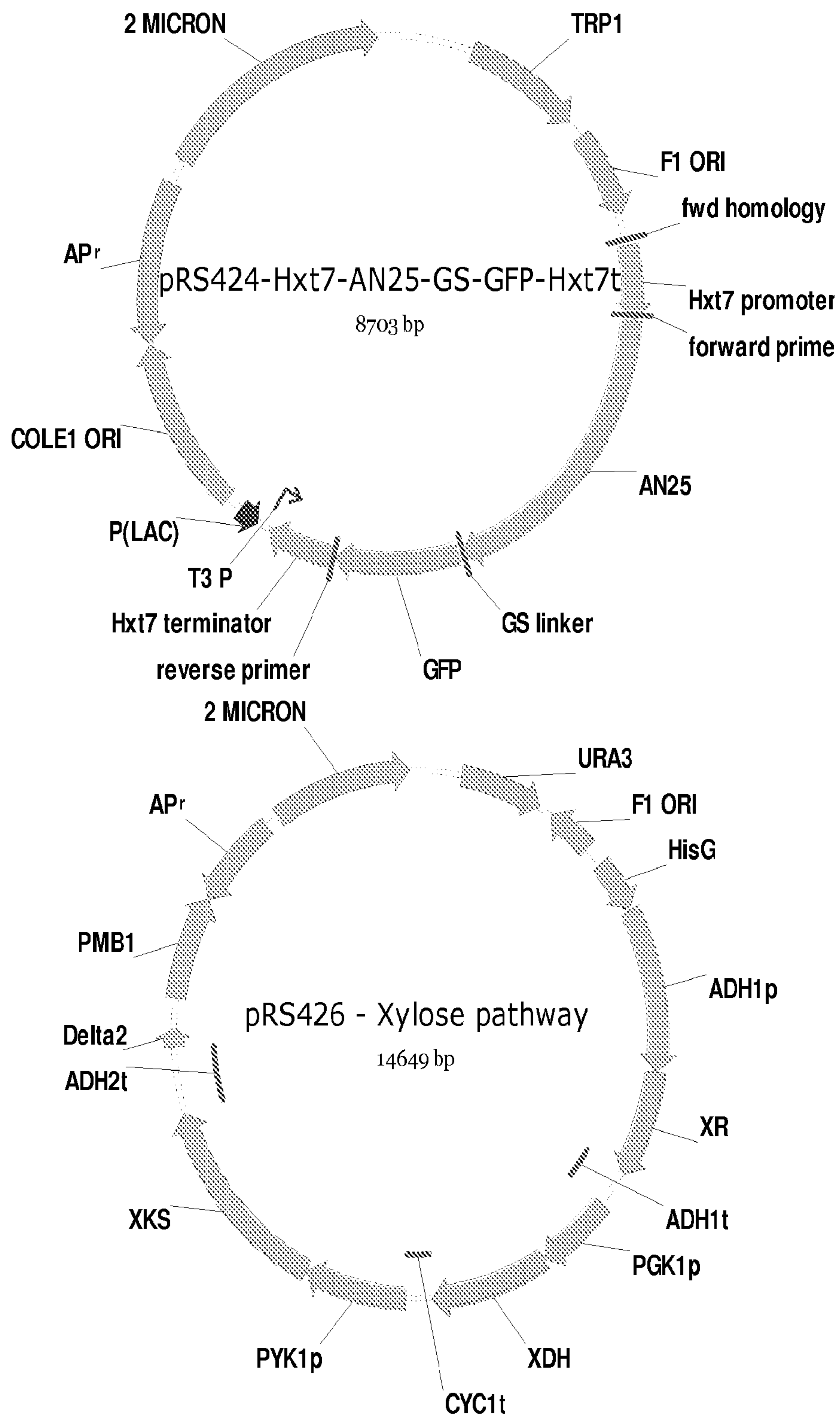


Figure 40

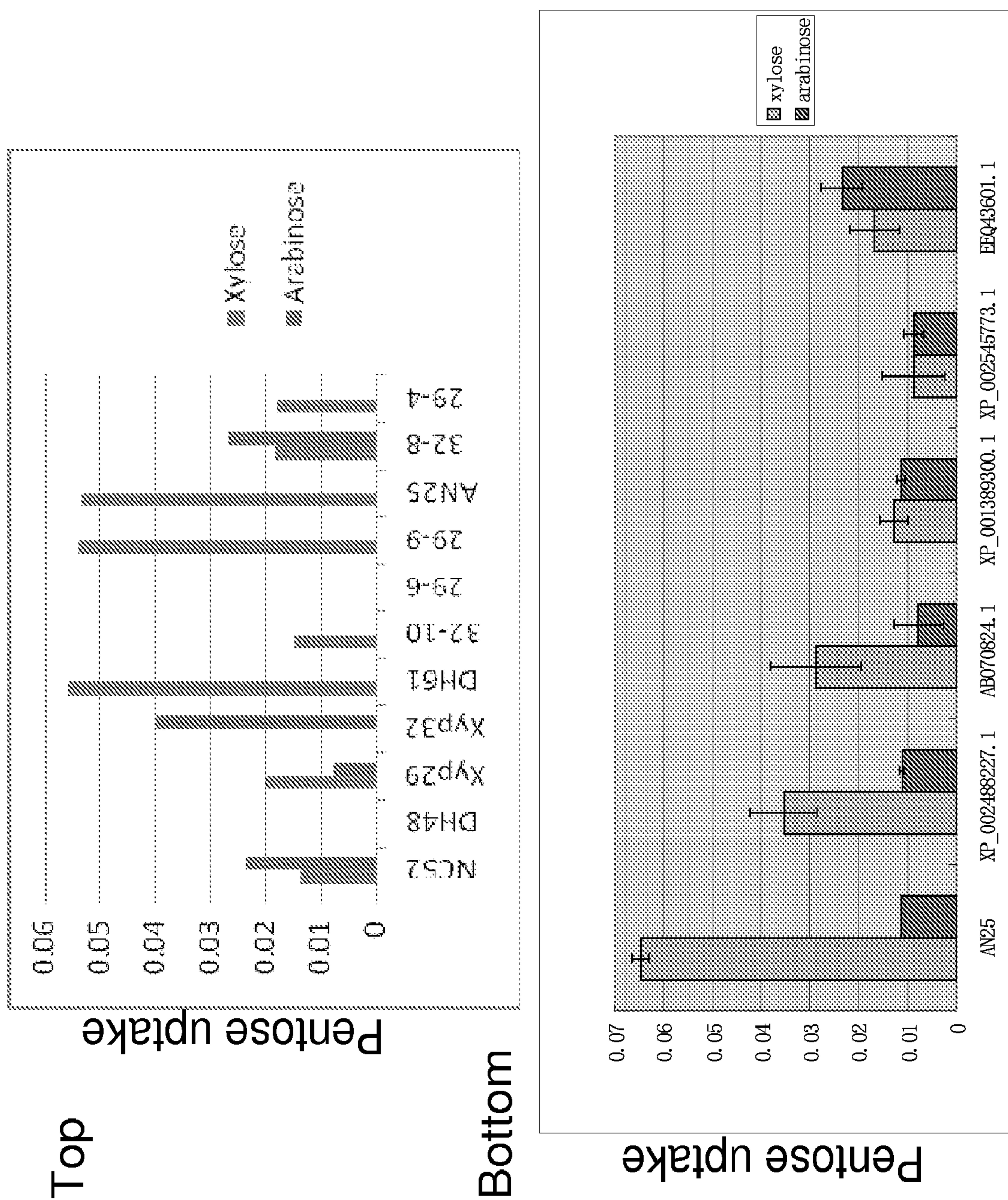


Figure 41**Consensus key**

- * -- single, fully conserved residue
- : -- conservation of strong groups
- . -- conservation of weak groups
- no consensus

CLUSTAL W (1.81) multiple sequence alignment

(a)

```

NCU00821      -----MAPPKFLGLSGRPLSLAVSTVATTGFLFLFGYDQGVM
XP_002488227.1 -----MAVPQFAGMSGGRKLSWSISTIATLGFLFLFGYDQGVM
XP_002382573.1 -----MAPTFAGLSGRPLSLAVST-----
XUT6          MEFSSVEKSAETASYTSQVSASGSAKTNSYLGLRGHKLNFAVSCFAGVGFLFLFGYDQGVM
                  : *: *: *, :;*

```

```

NCU00821      SGIITAPAFNNFFTPTKD--NSTMQGLITAIYEIGCLIGAMFVLWTGDLLGRRRNIMVGA
XP_002488227.1 SGIISAKPFNTVFKATED--DSTMQGTVTAIYEIGCLFGAMFILWFGDWLGRRKSVMLGA
XP_002382573.1 -----
XUT6          GSLTLPSFENTFPAMKASNNATLQGAVIALYEIGCMSSSLATIYLGDRLGRLKIMFIGC

```

```

NCU00821      FIMALGVIIQVTCQAGSNPFAQLFVGRVVMGIGNGMNTSTIPTTYQAECSTSNRGLLIC
XP_002488227.1 AIMILGVVIQVTAYTGHVPLAQFIVGRVVTGVGNGINTSTIPTTYQAECSTTNRGLLIC
XP_002382573.1 -----AECSKTSNRGLLIC
XUT6          VIVCIGAALQASAFT----IAHLTVARIITGLGTGFITSTVPVYQSECSPAKKRGQLIMM
                  :*** :. :** ** :

```

```

NCU00821      EGGVIAFGTLIAYWIDYGASYGPDD----LVWRFPIAFQLLFAIFICVPMFYLPESPRW
XP_002488227.1 EGGVIAFGTLIAYWIDFGASYGPDD----LTWRFPIAFQIVFGLGIIAGMFFLPESPRW
XP_002382573.1 EGGVIAIGTAIAYWIDFGAHYGPDD----LVWRFPIAFQIVFGVIIIVGMFFLPDSPRY
XUT6          EGSLIALGIAISYWIDFGFYFLRNDGLHSSASWRAPIALQCVFAVLLISTVFFFPEPRW
** :***: * :*****: : :*      ** :***: * : : : :* :***:

```

```

NCU00821      LLSHGRTQEADKVIAALRGYEIDGPETIQERNLIVDSLRA-----SG
XP_002488227.1 LFMREYQEGEAVIAALLNEETNSHHVQLQKTLVLDSTRA-----SG
XP_002382573.1 LISKDRIQEGEYVLAALGGYEVHDQETQTQKNLVIDSTRAYVCCLEVLPNKSVTNLDNSS
XUT6          LINKGRTEAREVFSALYDLPADSEKISIQIEETQAATDL-----ER
*: : * :*. * :*** . . . : : : :

```


Figure 41 (cont.)

NCU00821 GFGQKSTPFKALFTGCKTQHFRRLLLGSSSQPMQQVGGCNAVYYYFPILPQDSIGESHNM
 XP_002488227.1 QMG-KSTPLSAVFTGCKTQHFRRLMLGASSQPMQQIGGCNAVYYYFPILFENSIGQIHTM
 XP_002382573.1 GAG-ATTRYRDLLTGGRSQHLRRMLGSSSQIFQQLSGCNAVYYLPVLLKQSLHQSND
 XUT6 QAG-EGFVLKELFTQGPARNLQRVALSCWSQIMQQITGINIITYYAGTIFESYIGMSPFM
 * : * * : : : * : : . ** : ** : * * : ** : : . : :

NCU00821 SMLLGGINMIVYSIFATVSWFPIERVGRRRLFLIGTVGQMLSMV-IVFACLIPDD--PMK
 XP_002488227.1 SMLLGGVNMIVYSIFATMSWFLIERVGRRKLPLWGTIGQCGSMI-ITFACLIPGT--PGP
 XP_002382573.1 ALLIGGINMIVYAI FATPSWFFLEKIGRRKLPLGGSIIQTIAMV-ITFACLIPDD--TQV
 XUT6 SRILAAALNGTEYFLVSLIAPYTVBERLGRRLPLFWGAIAMALVMAGLTVTVKLAGEGNTHA
 : : : . : * * : .

NCU00821 ARGAAVGLFTYIAFGCATWLPLPWLPAEVPNPIRTRGKANAVSTCSNWMFNFLIVMVTPI
 XP_002488227.1 AKGAAVGLFTYIASFGCATWLPLPWLPAEISPIKTRAKANALSTCTNWLNFNFLIVMVTPI
 XP_002382573.1 SKGAVFGLFLYMAAFGAAWLPLPWLPAELSPIKTRAKANAVSTCSNWLNFNFTVVMITPV
 XUT6 GVGAAVLLFAFNSFFGVSWLGGSWLLPPELLSLSLRAPGAALSTASNWAFNFMVVMITPV
 . ** . ** : : ** : ** . ** * * : : : * . * : ** : ** *** : ** : ** :

NCU00821 MVDKIGWGTYLFFAVMNGCFLPIIYFFYPETANRSLEEIDIIFAKG-FVENMSYVTAAKE
 XP_002488227.1 MISNIKWGTYLFFAIVNACFLPVIYFWYPETARRSLEEIDIIFAKG-YTENISYVRAARE
 XP_002382573.1 MIAHIGWGTYLFFAALNALFIPIIWLFPETANRSLEEIDIIFAKG-YTENISYVKASKD
 XUT6 GFQSIGSYTYLIFAAINLLMAPVIYFLYPETKGRSLEEMDIIFNQCPVWEPWKVVQIARD
 . * *** : ** : : * :

NCU00821 LPHLTAEETESYANKYGLVDRDSNGEGGNRHDEEKTRDRPDQSDSDSPAHEIDVVDVDEHG
 XP_002488227.1 LPYLSDEDVERMAIQYGF GPAEVPSDSG-----EKASAHSEEF AETTGT PKQG
 XP_002382573.1 LPKLNDEETEQKANEYGF GNSTEDPEK-----ATAAEYSPSTSE-----
 XUT6 LPIMHSEVL DHEKNVI IKKSRIEHVEN-----IS-----
 ** : * : : : : : : : : : *

NCU00821 VESGFGDGINTKETR
 XP_002488227.1 EEH-----VSKMV-
 XP_002382573.1 -----
 XUT6 -----

(b)

EEQ43601.1	MSKGDLEELDIQKLIVEKRLEQSNQSGFATMKRNKRALGTCLFVSLGGILYGCYNQCMFGQ
XUT1	-MHGGGLGNDITETIAARRLQIAGKSGVAGLVANSRSFFIAVPASLGGLVYGCYNQCMFGQ
	:*, : ** :*, **: , *. * : *. *, : ,*, ****;*****
EEQ43601.1	VSSMHSFGETVVGIGKIQDNPTLQGLLTSILELGAWVGVLNMGYVADALGRRASVVIGCIL
XUT1	YSGMYSFSAIGVEKIQDNPTLQGLLTSILELGAWVGVLNMGYIADRLGRKKSIVVGVVF
	:*, *:*, :*: ** *****;** ***: **,* :
EEQ43601.1	FNIGVLIQAVARDADYGYILGGRFVIGLGVGVLSMVVPLYNSEISRAEIRGANTAIYQLS
XUT1	FFIGVIVQAVARGGNYDYILGGRFVVGIGVILSMVVPLYNAEISPPEIRGSLVALQQLA
	* ****;*****, ,*, *****;*,***;*****;*** ,****: ,*: **;
EEQ43601.1	ITFGIMISYWITYGTNFIGGTGDNQSQASWLVPNCIQAAPAILAVFIYSFPESPRWLIN
XUT1	ITFGIMISYWITYGTNYIGGTGSGQSKASWLVPICIQLVALLGVGIFFPESPRWLMN
	*****;*****, *,*****;*** ,*:**,* *: ;*****;*
EEQ43601.1	VGQEDKALEVLAWLRETEQENVGLQIEFLEMKAQKTFEQTLETEAYPHLQDGTKMSKFKI
XUT1	EDREDECLSVLSNLSLSKEDTLVQMEFLEMKAQKLFERELSAKYFPHLQDGSAKSNFLI
	,*:*,*,**:**, ,*: ,*:*****;*: *,*: :*****: **,*
EEQ43601.1	NLNQYKSMYTHLPTFKRVSVACLTMVPQQWTGAYNFILYYAPFIFASLGLSGNTTSLLAS
XUT1	GFNQYKSMITHYPTFKRVAVACLIMTFQQWTG-VNFILYYAPFIFSSLGLSGNTISLLAS
	,*****;** *****;*** *,***** *****;***** *****
EEQ43601.1	GVVGIVMFLCTIPAVMWVDKVGCRKPLLISGALVMGLCHFVVAGTLGGYSDNIGSHKAAGW
XUT1	GVVGIVMFLATIPAVLWVDRLCRKPVLLISGATIMGTCHFVVAAILGQFGGNFVNHSAGW
	*****, *****,***;*****,*****;*,*****.*** ,. ,*: ,*, ,**
EEQ43601.1	VAVVFIWIFAGAFGYSWGPCAWVIVAEEVPLGMRAKGVSLGSSFNWLMNFSVAISTPKFV
XUT1	VAVVFVWIFAIGFGYSWGPCAWVLVAEEVPLGLRAKGVSIGASSNWLNNFAVAMSTPDFV
	*****,*****, *****;*****;*****;*,* **** **;***,***, **
EEQ43601.1	ANAKYGAYIFLGLMCVIGSMYVYFMVPETKNTLDELDEVFGDFTGTSKKESELREXILK
XUT1	AKAKFGAYIFLGLMCIFGAAYVQFFCPETKGETLEEDELFGDTSGTSKMEKEIHEQKIK
	*:**;*****;*, ** *: ****, :*,*:*,*** ***** *,*:*: *

Figure 41 (cont.)

```
EEQ43601.1    QVGLVDLLVGSDKELDSFRSKPEVEYKEKEAHSE
XUT1          EVGLLQLLG-EENASESENSKADVYHVEK-----
              [***]:** . : : [* .**.* : **
```


Fig. 41 (cont.)**(c)**

NCU00821 -----MAPPKFLGLSGRPLSLAVSTVATTGFLFLFGYDQGVM
 XP_002488227.1 -----MAVPQFAGMSGRKLSWSISTIATLGFLFLFGYDQGVM
 XP_002382573.1 -----MAPTFAGLSGRPLSLAVST-----
 XUT6 MEFSSVEKSAETASYTSQVSASGSAKTNSYLGURGHKLNFAVSCFAGVGFLFLFGYDQGVM
 EEQ43601.1 --MSKGDLEELDIQKLIVEKRLEQSNQSGFATMKRNKRALGTCLFVSLGGILYGYNQGMF
 XUT1 ---MHGGGDGNDITEIIAARRLQIAGKSGVAGLVANSRSFFIAVFASLGGLVYGYNQGMF

: . . .

NCU00821 SGIITAPAFNNFFTPTKD--NSTMQGLITAIYEIGCLIGAMFVLWTGDLLGRRRNIMVGA
 XP_002488227.1 SGIISAKPFNTVFKATED--DSTMQGTVTAIYEIGCLFGAMFILWFGDWLGRRKSVMLGA
 XP_002382573.1 -----
 XUT6 GSLLTLPSFENTFPAMKASNNATLQGAVALYEIGCMSSSLATIYLGDRLGRLKIMFIGC
 EEQ43601.1 GQVSSMHSFGETVGIGIKIQDNPTLQGLLTSILELGAWVGVLNMGYVADALGRRASVVIGC
 XUT1 GQISGMYSFSAIGVEKIQDNPTLQGLLTSILELGAWVGVLNMGYIADRLGRKKSVMVVG

NCU00821 FIMALGVIIQVTCQAGSNPFAQLFVGRVVMGIGNMNTSTIPTTYQAECSTSNRGLLIC
 XP_002488227.1 AIMILGVVIQVTAYTGHVPLAQFIVGRVVTGVNGINTSTIPTTYQAECSTTNRGLLIC
 XP_002382573.1 -----AECSKTSNRGLLIC
 XUT6 VIVCIGAALQASAFT----IAHLTVARIITGLGTGFITSTVPVYQSECSPAKKRGQLIMM
 EEQ43601.1 ILFNIGVIIQAVARDAD--YGYILGGRFVIGLVGVLSMVVPLYNSEISRAETRGANTAI
 XUT1 FFFFIGVIVQAVARGGN--YDYILGGRFVVGIGVILSMVVPLYNAEISPPETRGSLVAL

: * * , ** :

NCU00821 EGGVIAFGTLIAYWIDYGASYGPDD-----LVWRFPPIAFQLLFAIFICVPMFYLPESPR
 XP_002488227.1 EGGVIAFGTLIAYWIDFGASYGPDD-----LTWRFPPIAFQIVFQLGIIAGMFFLPESPR
 XP_002382573.1 EGGVIAIGTALAYWIDFGAHYGPDD-----LVWRFPPIAFQIVFGVIIIVGMFFLPESPR
 XUT6 EGSLIALGIALSYWIDFGFYFLRNDGL-HSSASWRAPIALQCVFAVLLISTVFFFPESPR
 EEQ43601.1 YQLSITFGIMISYWITYGTNFIGGTGDNQSQASWLVPMCIQAAPAILAVFIYSFPESPR
 XUT1 QQLAITFGIMISYWITYGTNYIGGTGSGQSKASWLVPICIQLVPALLGVGIFFPESPR

:; :*** :* : , * :;:* .: : :; :****

NCU00821 WLISHGRTQEADKVIAALRGYEIDGPETIQERNLIVDSLRA-----S
 XP_002488227.1 WLFMRERYQEGEAVIAALLNEETNSHHVQLQKTLVLDSIRA-----S
 XP_002382573.1 YLISKDRIQEGEYVLAALGGYEVHDQETQTQKNLVIDSIRAYVCCLEVLPNKSVTNLDNS
 XUT6 WLUNKGRTTEAREVFSALYDLPADSEKISIQIEETQAAIDL-----E
 EEQ43601.1 WLVNQGQENKALEVLAWLRETEQENVGLQIEFLEMKAQKIFEQTLETEAYP-----H
 XUT1 WLMNEDREDECLSVLSNLRSLSKEDTLVQMEFLEMKAQKLFERELSAKYFP-----H

*: : : : * : : *

Figure 41 (cont.)

NCU00821	VEIDVVDEHGVESGFGDGINTKETR
XP_002488227.1	AETTGTPKQGEEH-----VSKMV-
XP_002382573.1	SE-----
XUT6	-----
EEQ43601.1	VEYKEKEAHSE-----
XUT1	VYHVEK-----

Figure 42

Strain backgrounds vs. xylose fermentation

- Construction of engineered strains using an identical construct and different laboratory strains

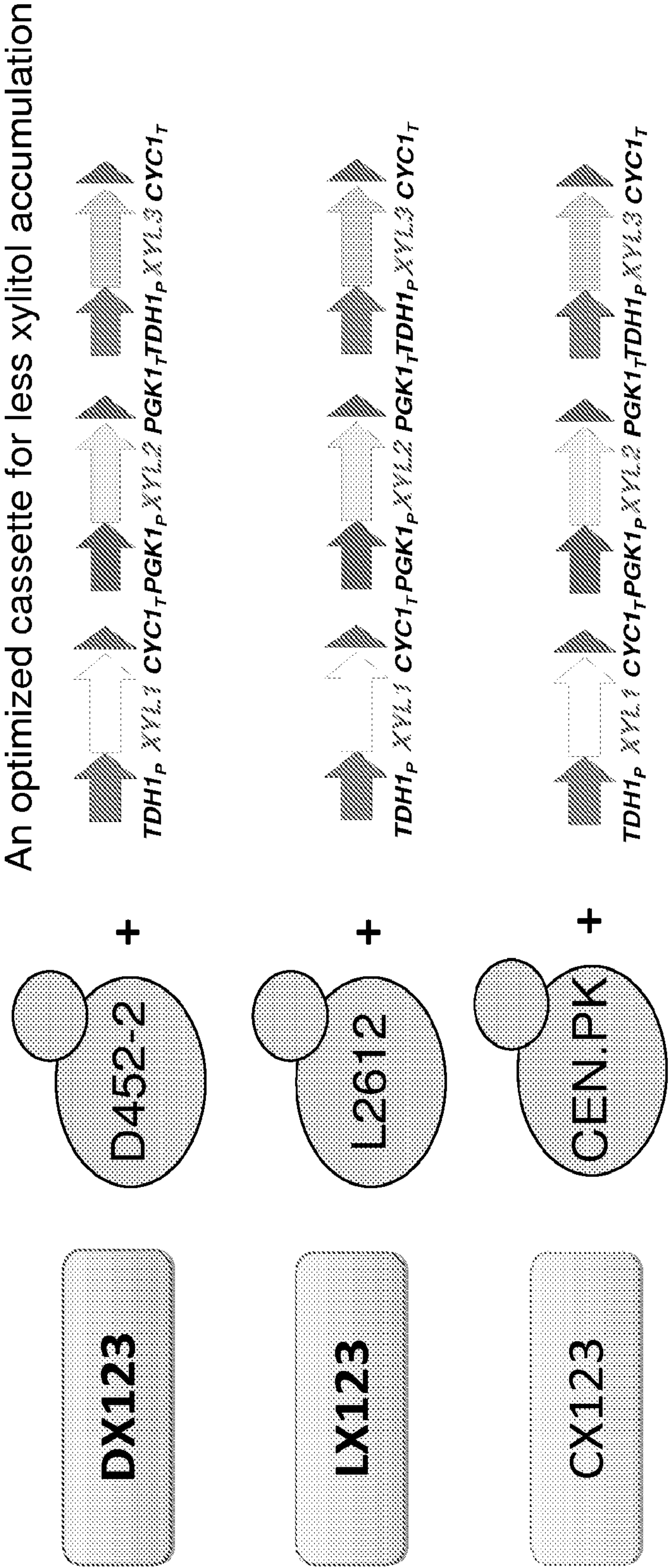
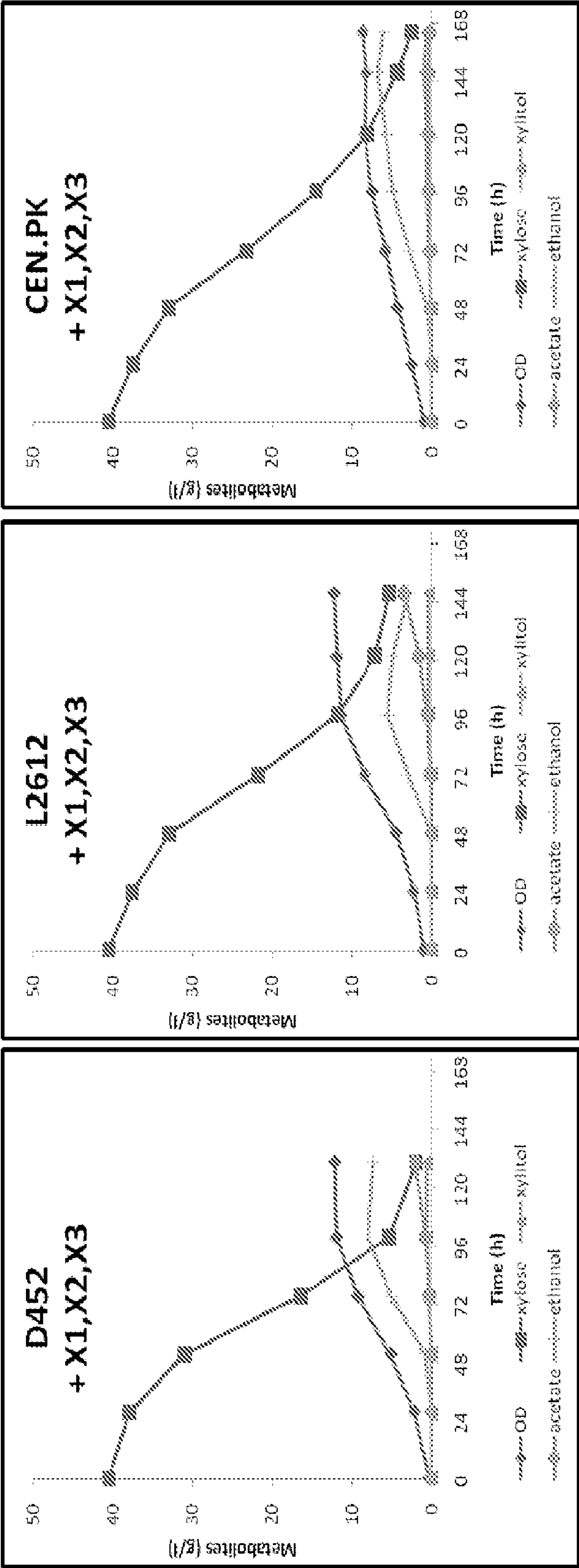


Figure 43
Identical expression cassette under different strain backgrounds



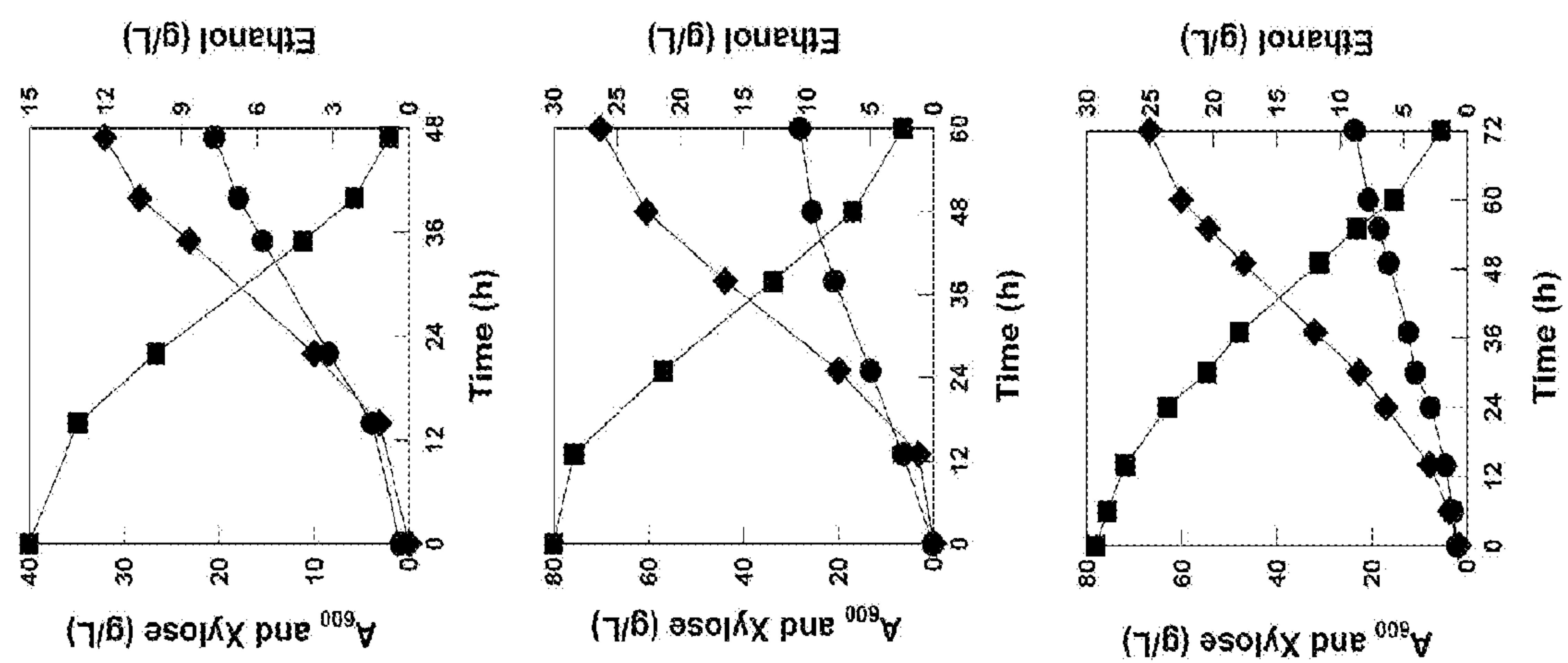


Figure 45 (a)

(b)

(c)

Figure 46

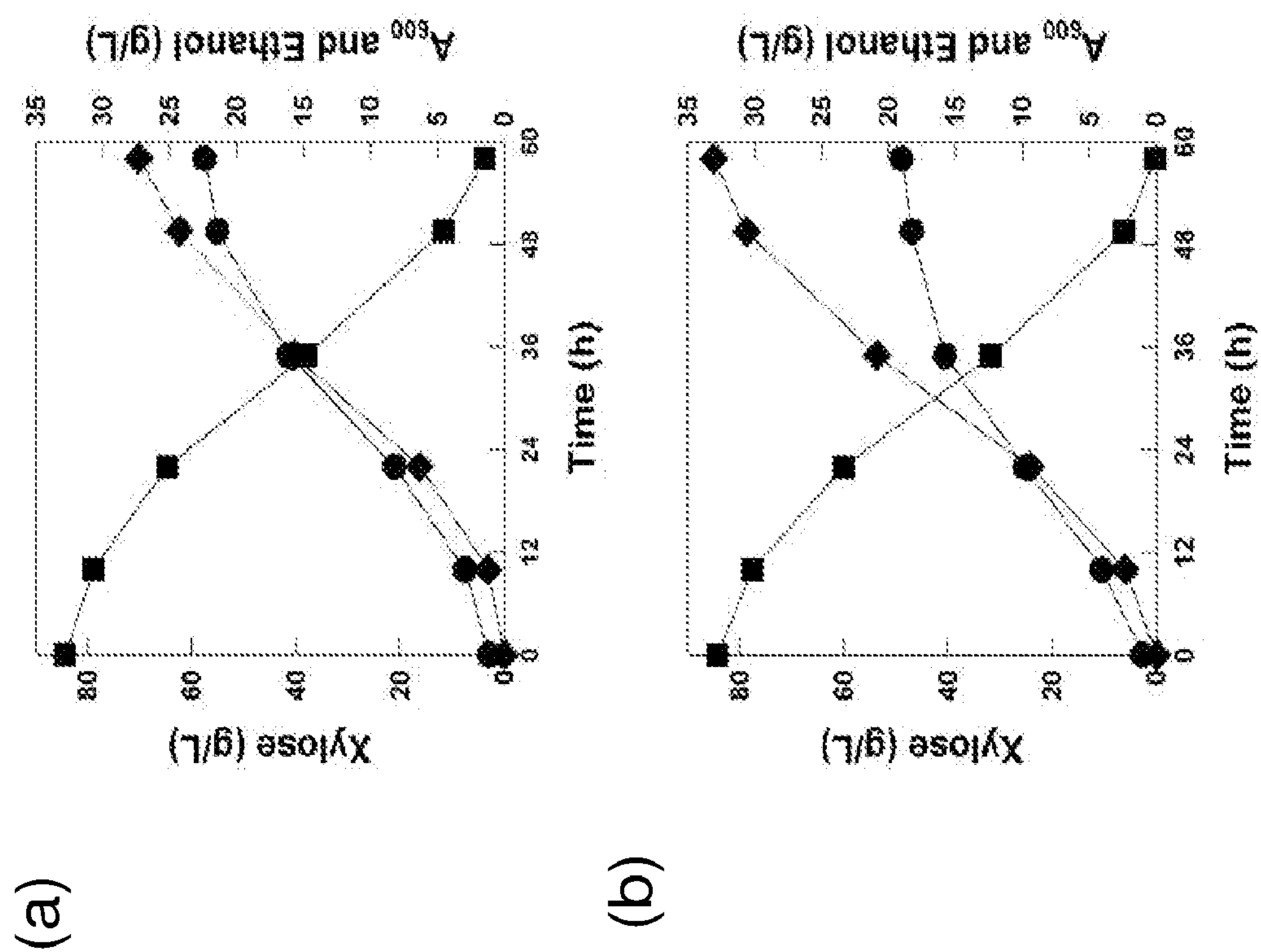
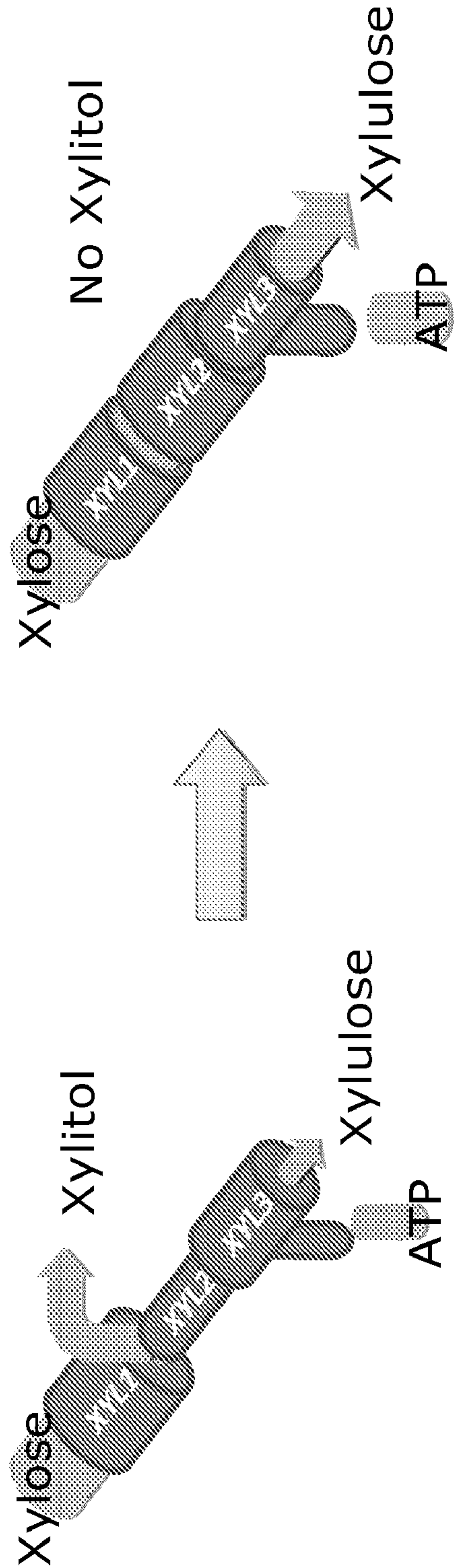


Figure 47
Hypothesis and experimental design



WT	Parental xylose-fermenting strain	Integration vector	Overexpression levels of XYL2
L2612	YSX3 (TDHp-XYL1, TDHp-XYL2, and native-XYL3)		+
		p404 TDHp-XYL2	++
		p404 PGKp-XYL2	+++

Figure 48
Additional *XYL2* integration improves xylose
fermenting ability

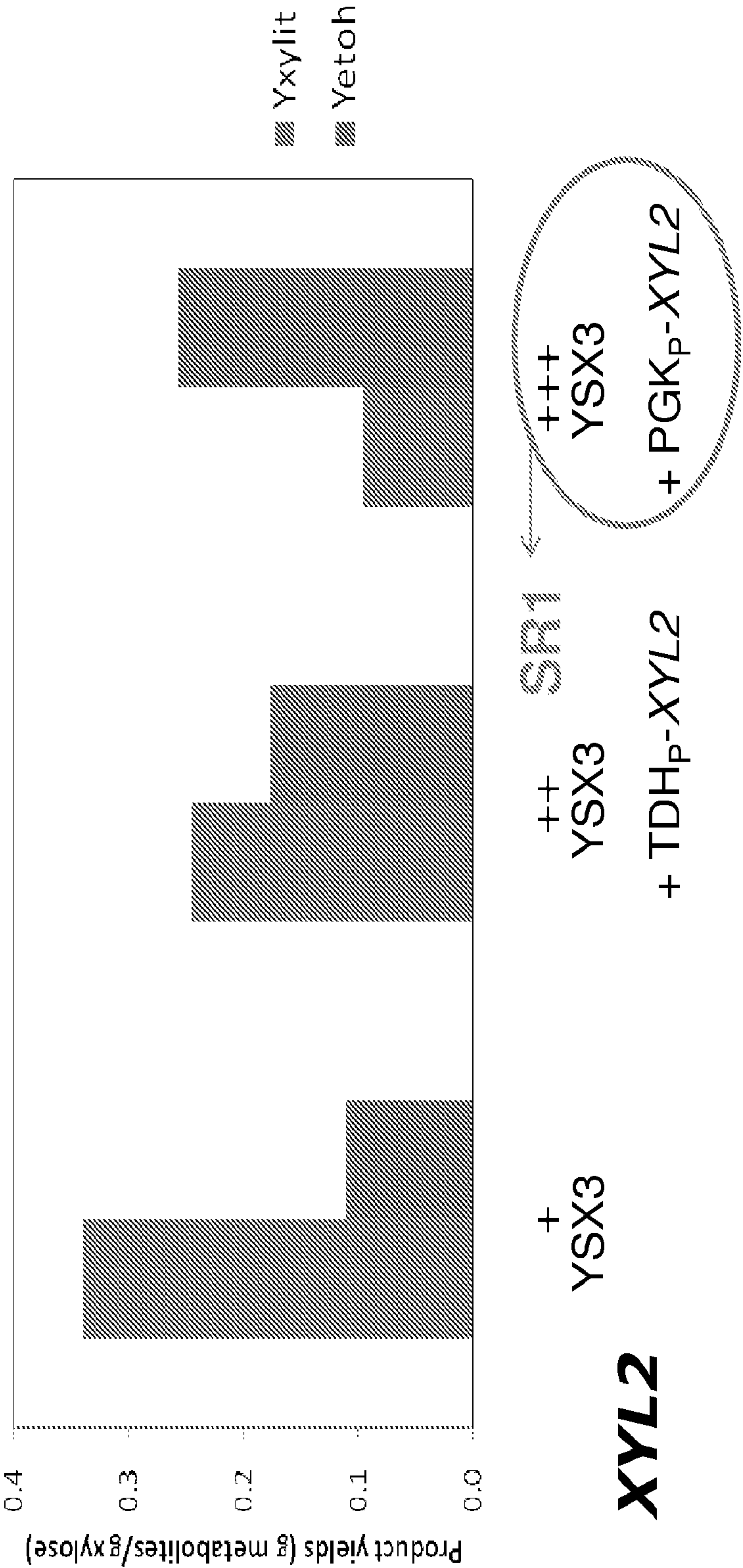
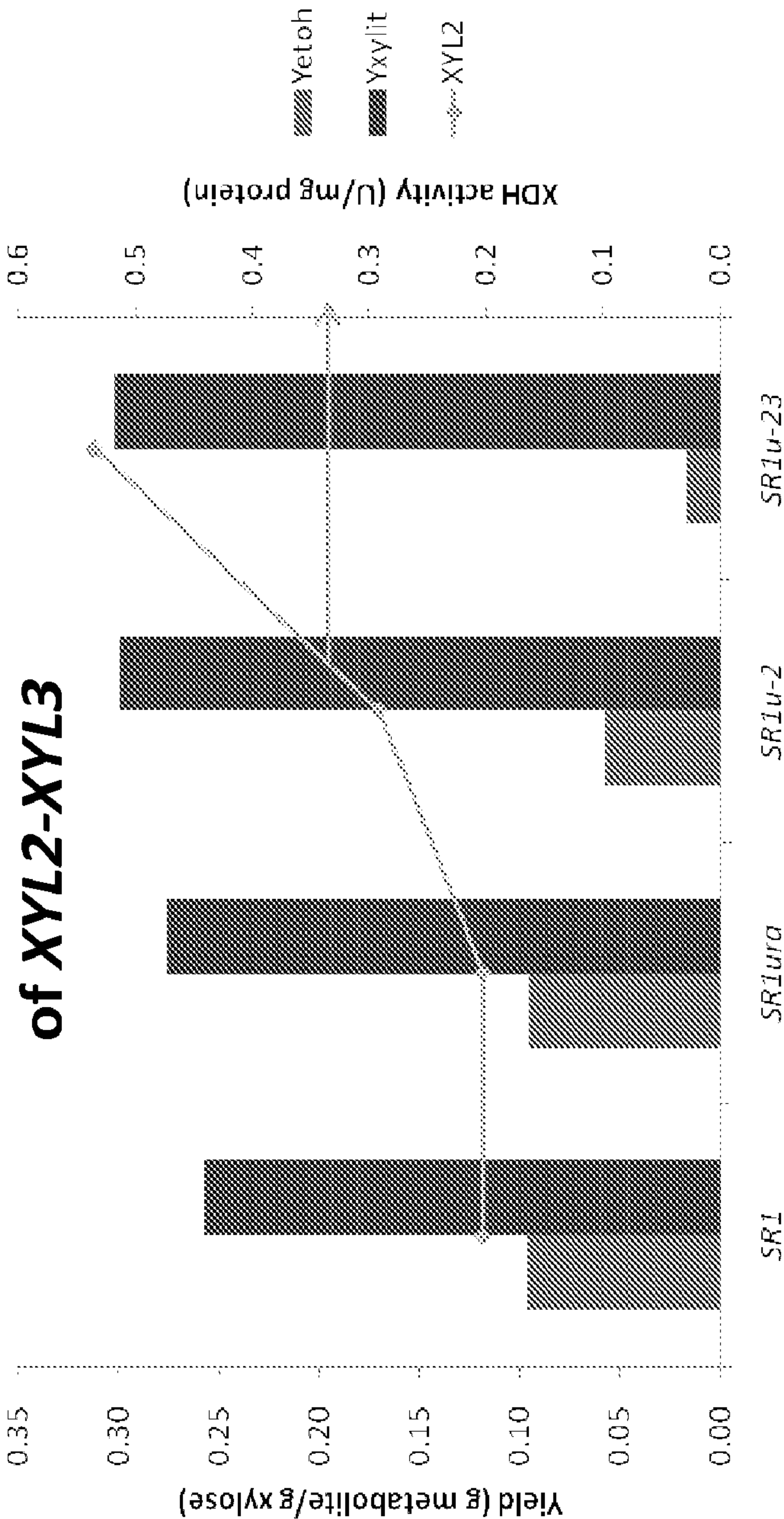


Figure 49
Negligible amounts of xylitol were accumulated after additional overexpression of XYL2-XYL3



FOA selection + XYL2 ↑ + XYL2 ↑ and XYL3 ↑

XDH activity	++	++	++++	++++ / XK ↑

Figure 50
Construction of engineered strains exhibiting
different XR activities

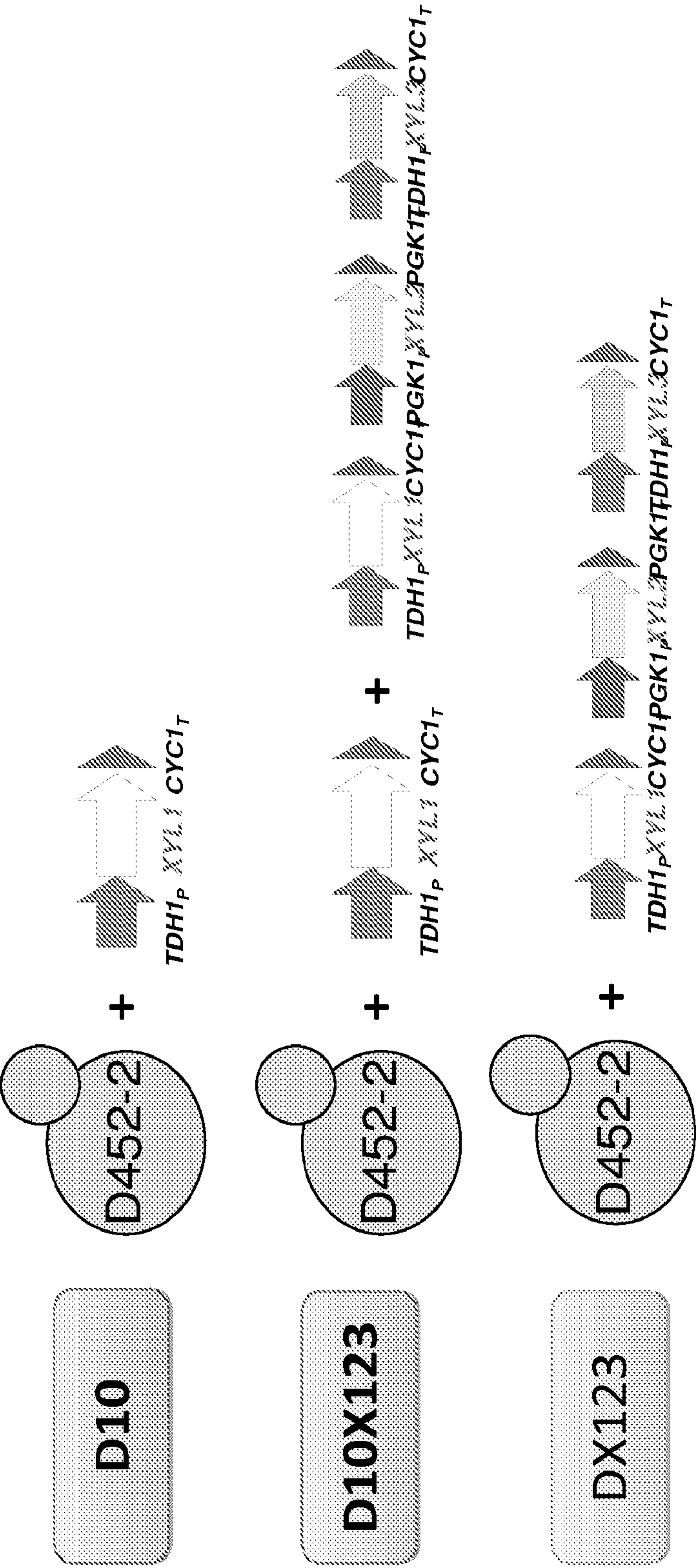


Figure 51
Strong *XYL1* with *XYL2/XYL3* caused xylitol
accumulation

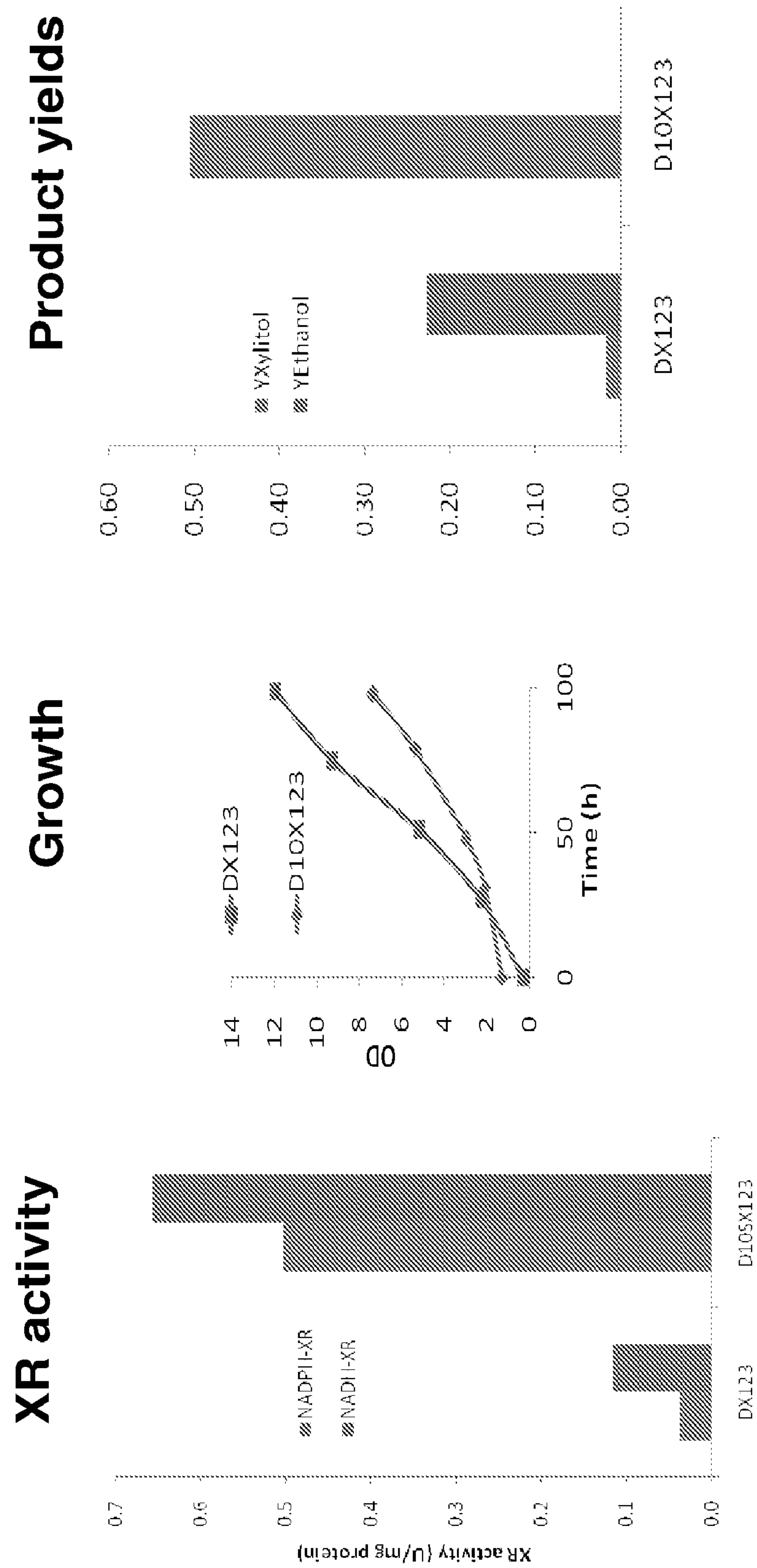


Figure 53
Xylose fermentation with high OD
(OD=10) inoculations

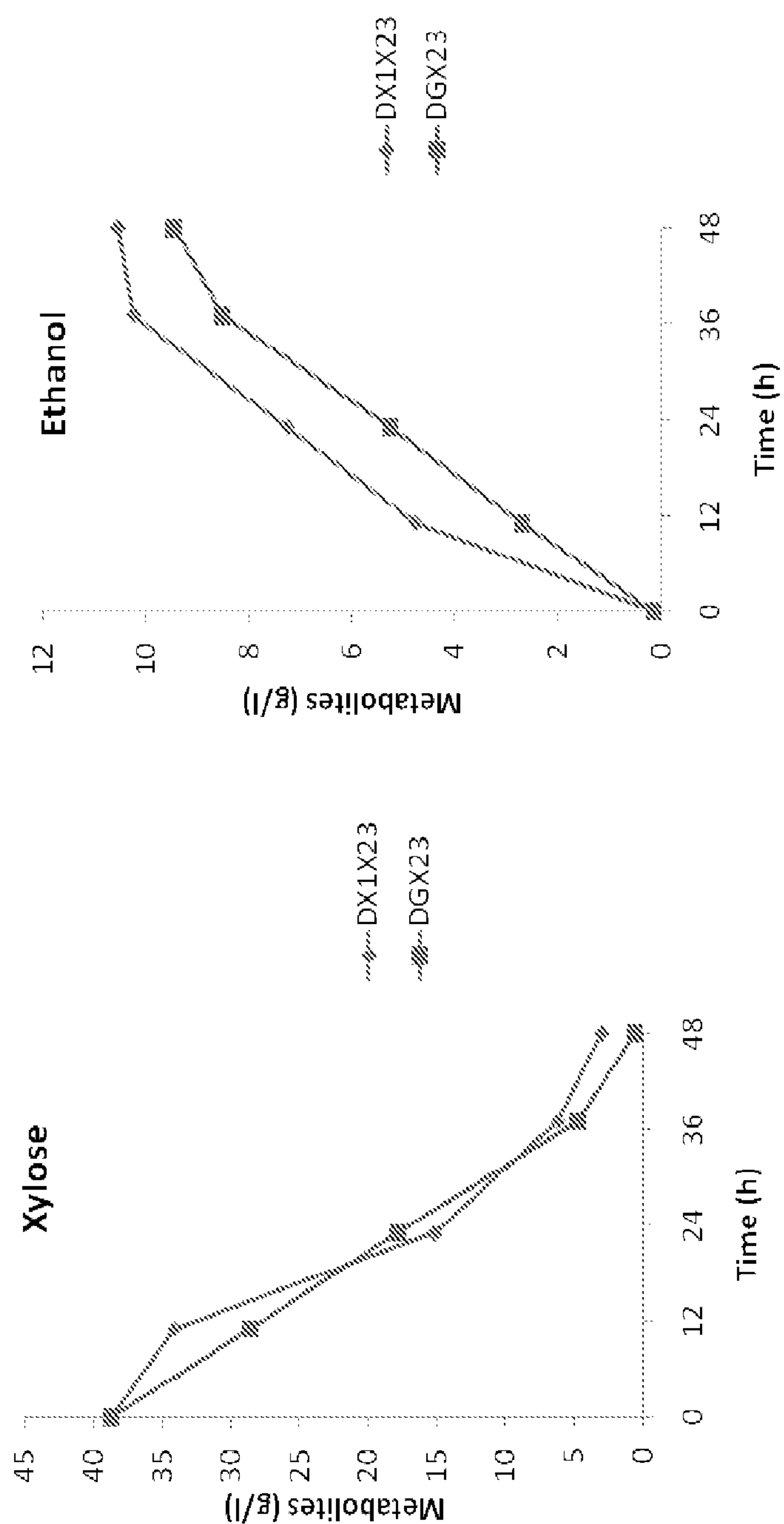


Figure 54
80 g/L of xylose fermentation with
high OD (OD=15) inoculations

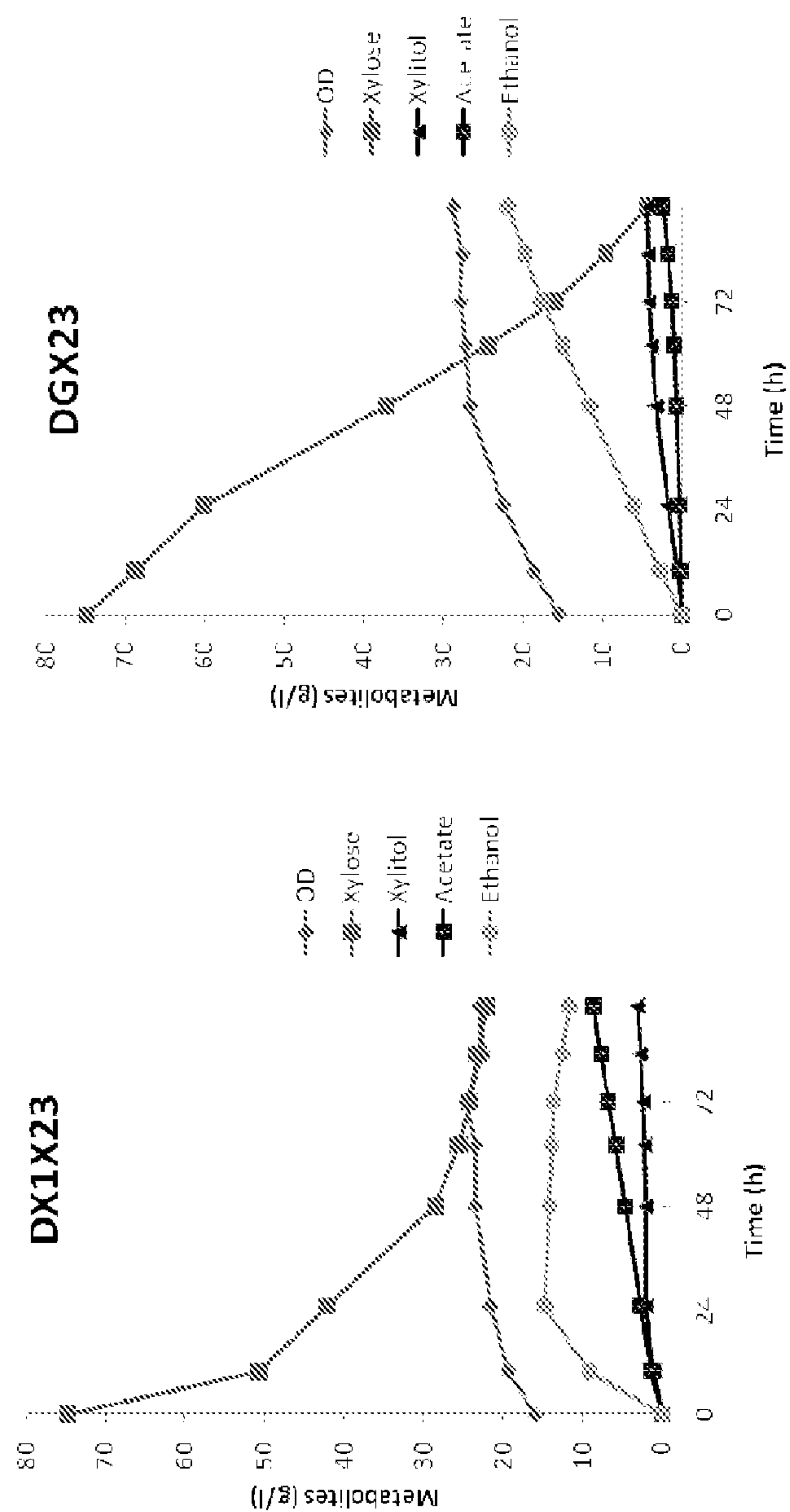


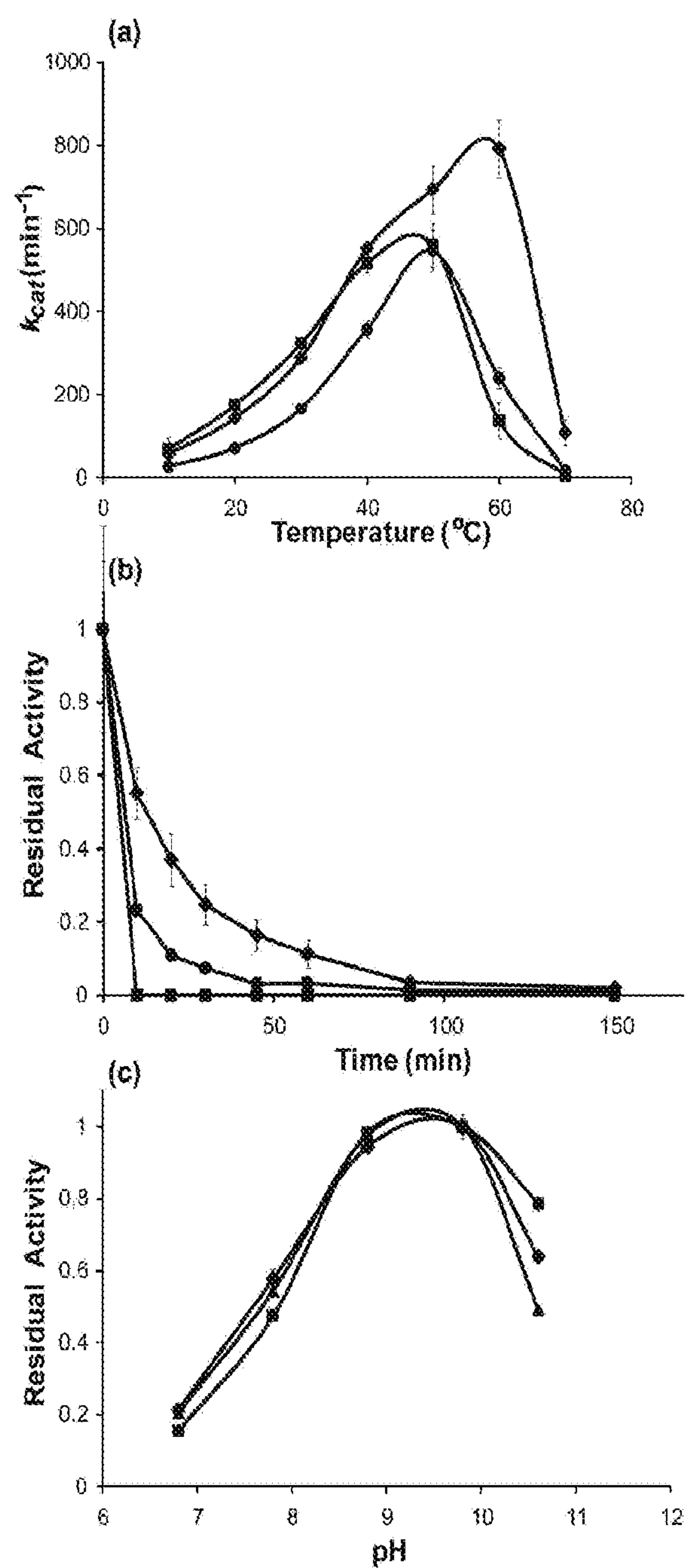
Figure 55

Figure 56

Score = 299 bits (765), Expect = 3e-79, Method: Compositional matrix adjust.

Identities = 169/365 (46%), Positives = 222/365 (60%), Gaps = 22/365 (6%)

```

psXDH ANPSLVLNKIDDISFETYDAPEISEPTDVLVQVKKTGICGSDIHFYAHGRIGNFVLTKPM 62
      +N S VLNK D- F+ P+I+ P DVLV V TGICGSD+H-+ HG IG+FV+ PM
ncXDH SNLSFVLNKPLDVCFQDKPVPKINSPHDVLVAVNYTGICGSDVHYWLHGAIGHFVVKDPM 66

psXDH VLGHESAGTVVQVGKGVTSCLKVGDNVAIEPGIPSRFSDEYKSGHYNLCPHMAFAATPNSK 122
      VLGHESAGT+V VG V +L VGD VA+EPG P R SGHYNLCP M FAATP
ncXDH VLGHESAGTIVAVGDAVKTLVSGDRVALEPGYPCRRCVHCLSGHYNLCPMRFAATPPYD 126

psXDH EGEPNPPGTLCKYFKSPEDFLVKLPDHVSLELGALVEPLSVGVHASKLGSVAFGDYVAVF 182
      GTL -+ +P DF KLP+ VSL+ GAL+EPL+V VH +K + G V V
ncXDH -----GTLTGFWTAPADFCYKLPETVSLQEGALIEPLAVAVHITKQAKIQPGQTVVVM 179

psXDH GAGPVGLLAAAVAKTFGAKGVIVVDIFDNKLKMAKDIGAATHTFNSKTGGSEE-----LI 237
      GAGPVGLL AAVAK +GA V+ VDI +KL+ AK AATH+ S+ EE +
ncXDH GAGPVGLLCAAVAKAYGASKVVSVDIVPSKLEFAKSF-AATHTYLSQRVSPEENARNIIA 238

psXDH KAFGGNVPNVVLECTGAEPCKLGVDAIAPGGRFVQVGNAAGPVSEFPITVFAMKELTLFG 297
      A G +V+- +GAEP I+ + + GG +VQ G +-FPI +KE+T G
ncXDH AADLGEGADAVIDASGAEPSIQALHVVRQGGHYVQGGMGKDNITFPIMALCIKEVTASG 298

psXDH SFRYGFNDYKTAVGIFDTNYQNGRENAPIDFEQLITHRYKFKDAIEAYDLVRAGKGAVKC 357
      SFRYG DY+ A- + E +D ++L+ FK+A EA+ V+ G+ +K
ncXDH SFRYGSGDYRLAQLV-----EQGKVDVKLVNGVVPFKNAEEAFKKVKEGE-VIKI 349

psXDH LIDGP 362
      LI GP
ncXDH LIAGP 354

```


FIG. 57

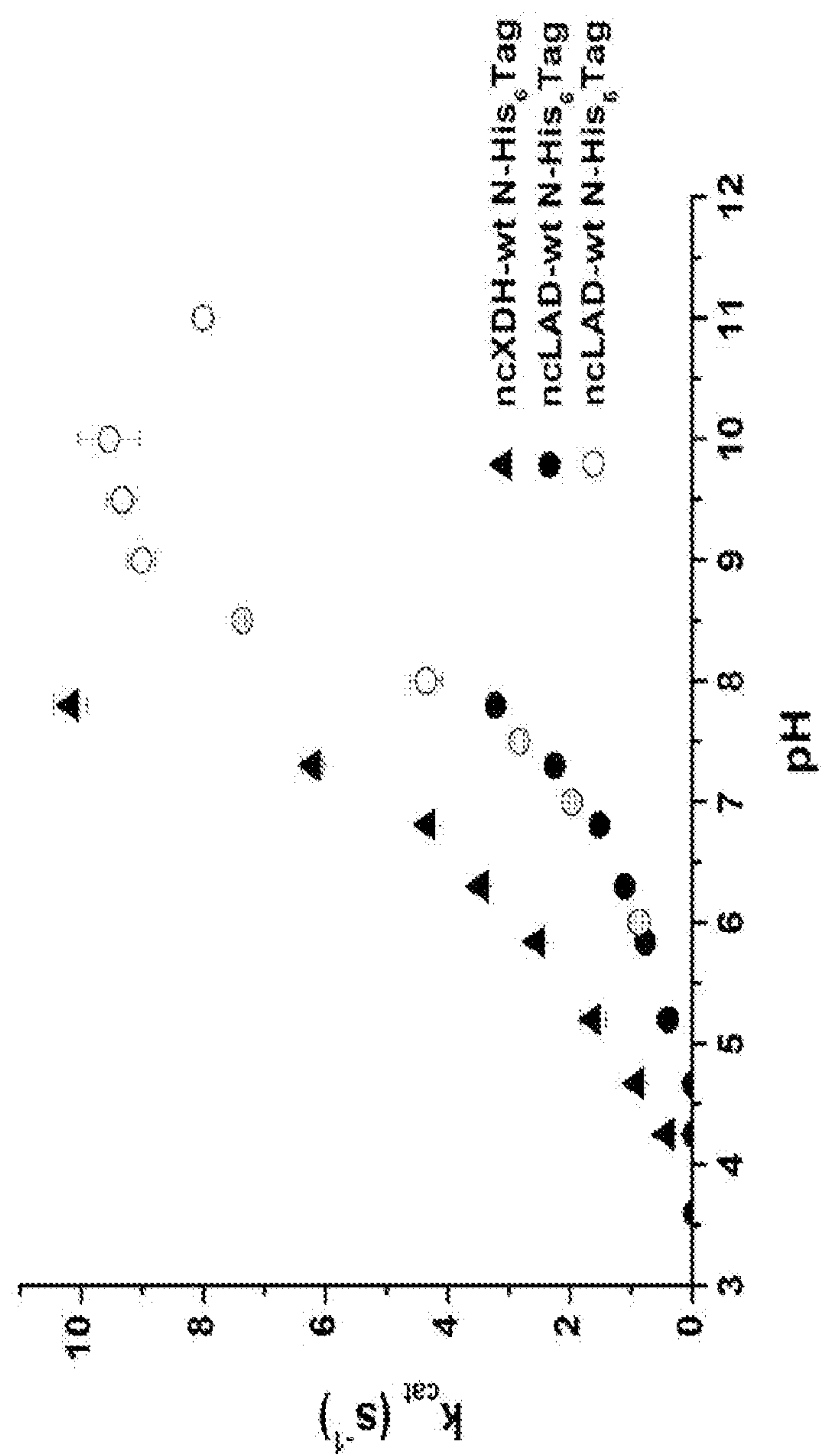
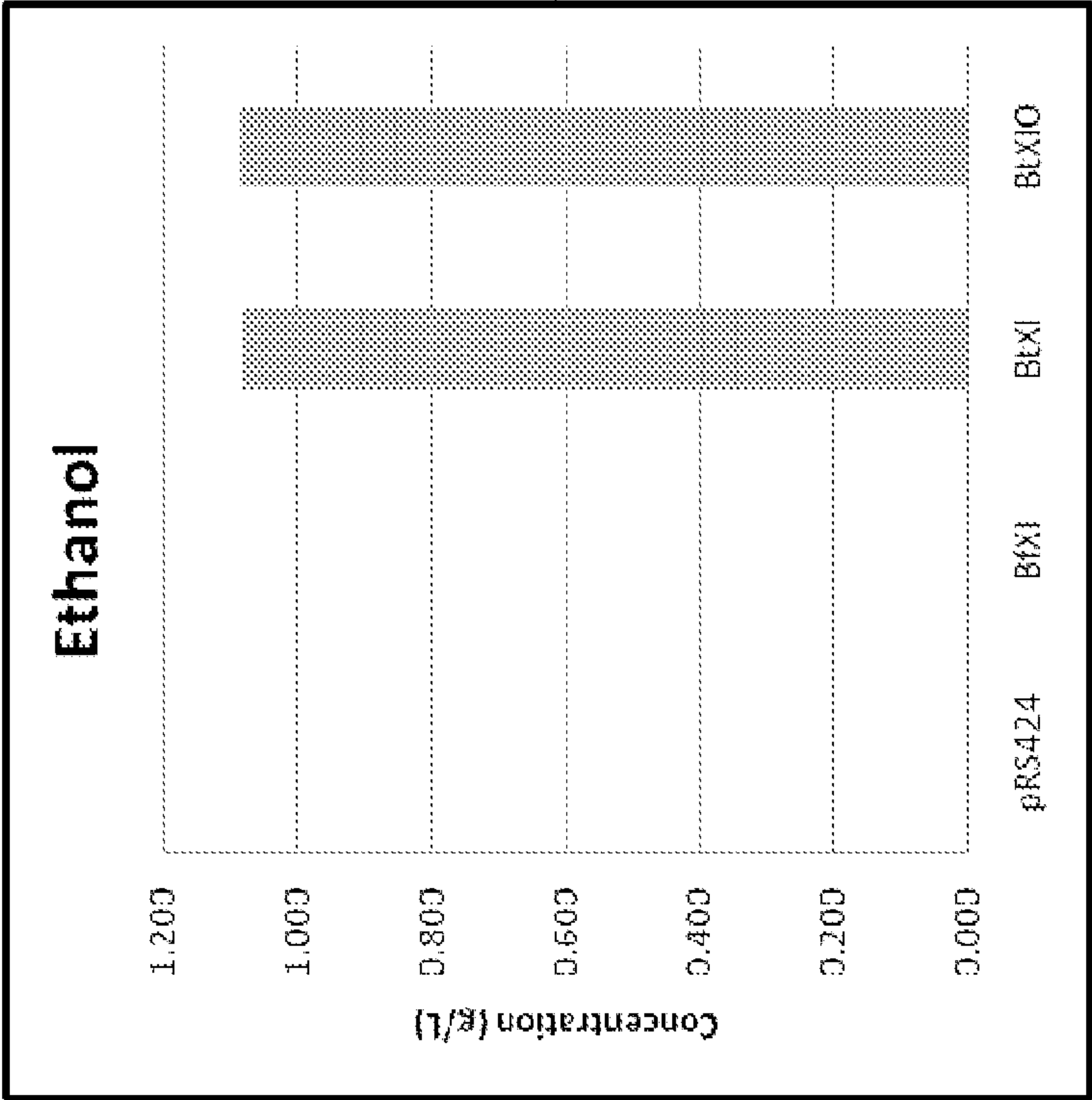
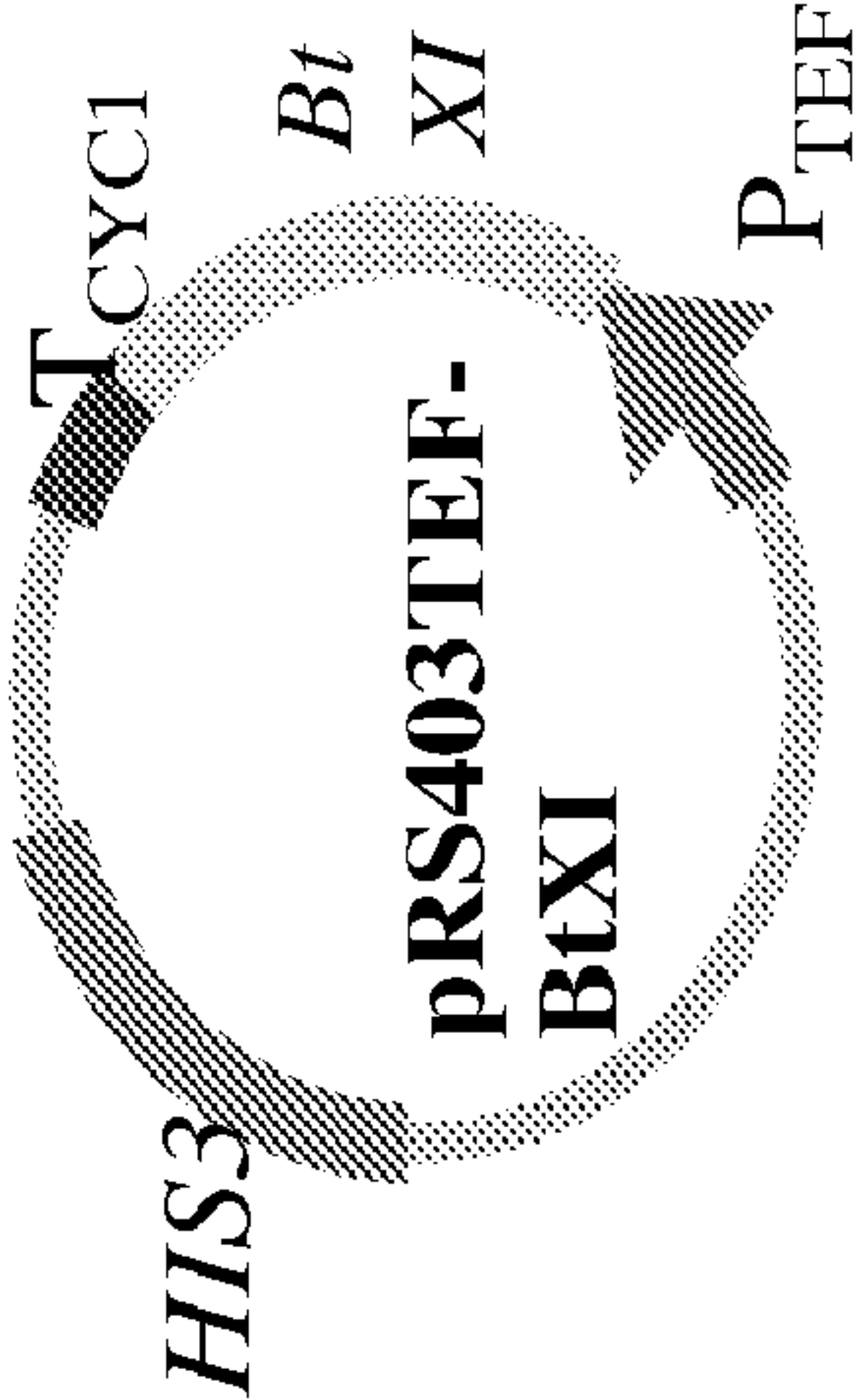


Figure 58



- ✓ As a result of high cell density fermentation in minimum medium under xylose and anaerobic condition
- ✓ Only BtXI and BtXIO have activity in *S. cerevisiae*
- ✓ No activity with BfXI

Figure 59



	Plasmid (L2612)		Integration (D452-2)	
	OD 48	OD 96	OD 51	OD 96
Ethanol (g/L)	4.92	5.15	7.67	7.88
Yield (EtOH)	0.15	0.34	0.40	0.40
Productivity (g/L-hr)	0.04	0.04	0.05	0.06
Xylitol (g/L)	3.62	3.61	2.80	2.74
Yield (Xt)	0.26	0.24	0.15	0.14

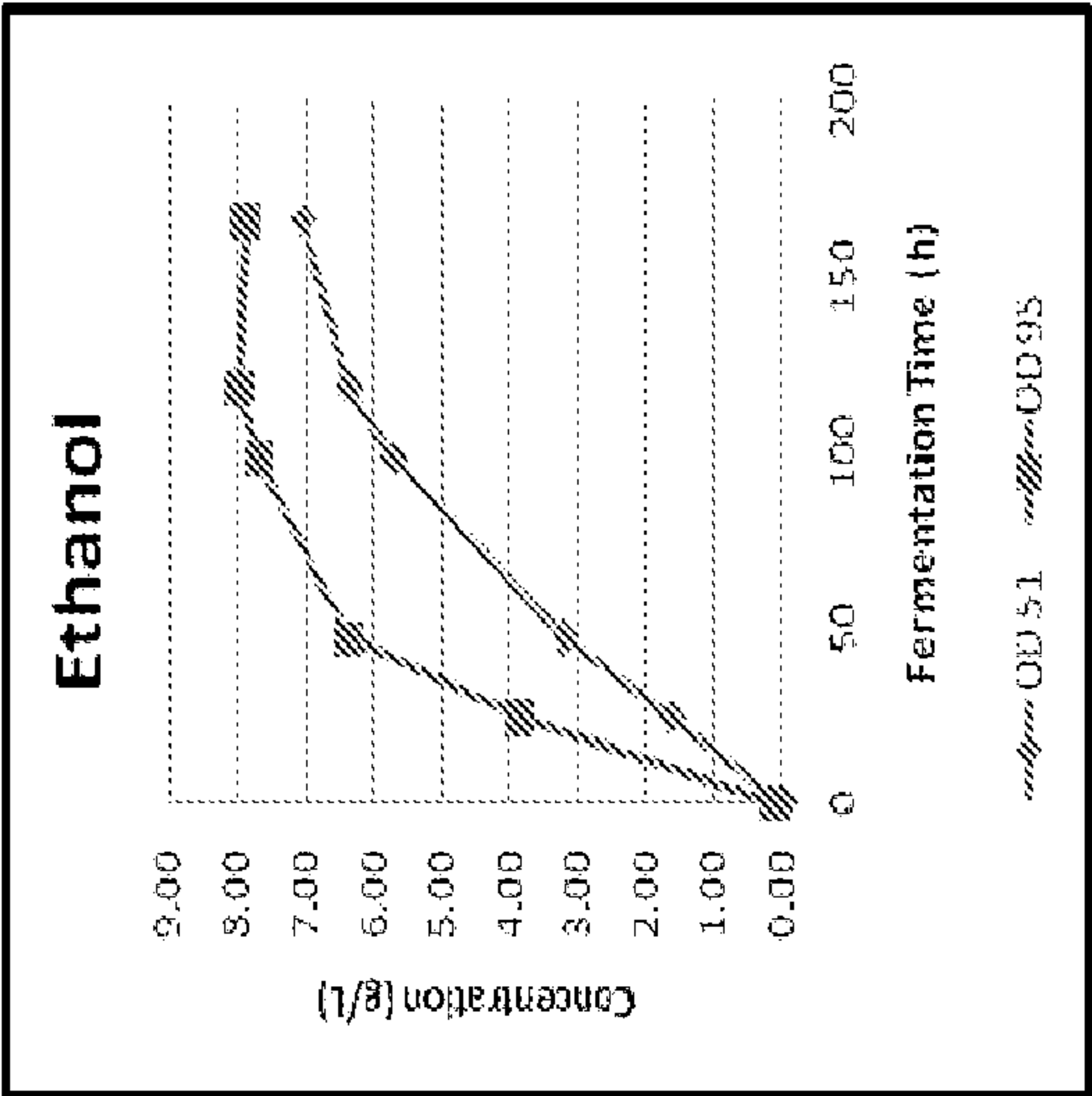
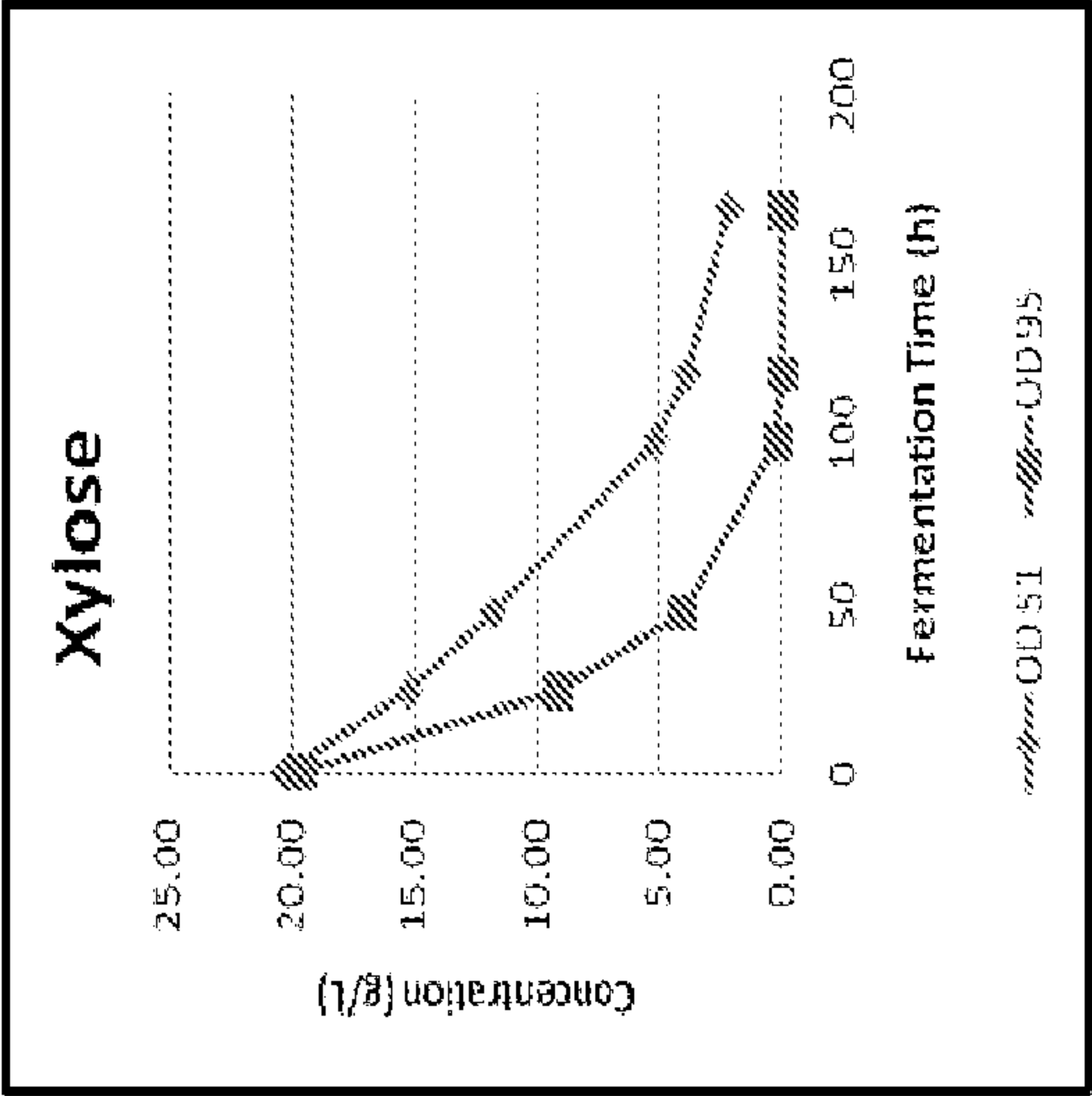
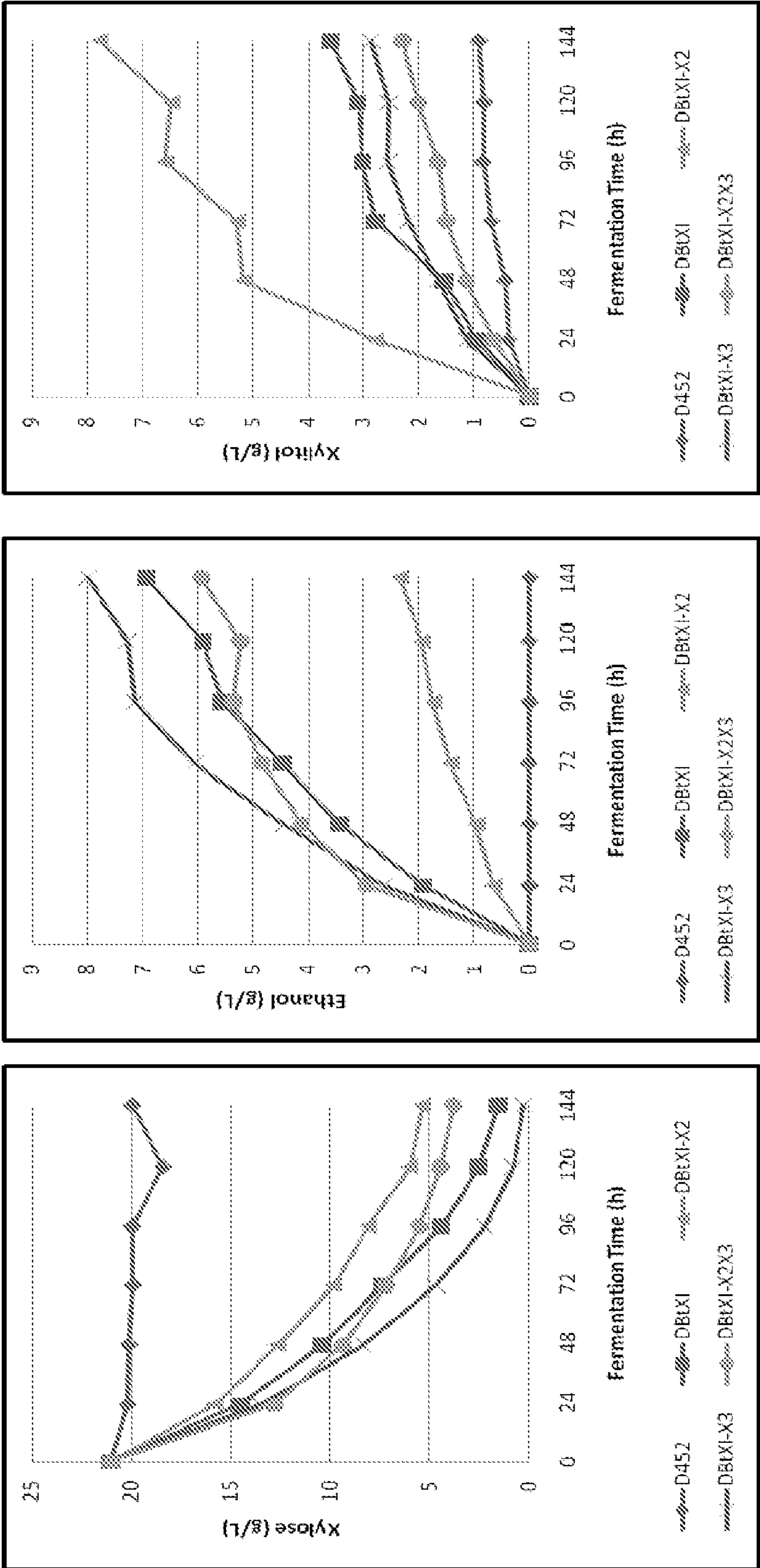


Figure 60



	DBtXI	DBtXI+X2	DBtXI+X3	
Y_{EtOH}	0.34	0.15	0.41	0.37
Y_{XOH}	0.18	0.50	0.14	0.12

Figure 61
Cell growth on xylulose

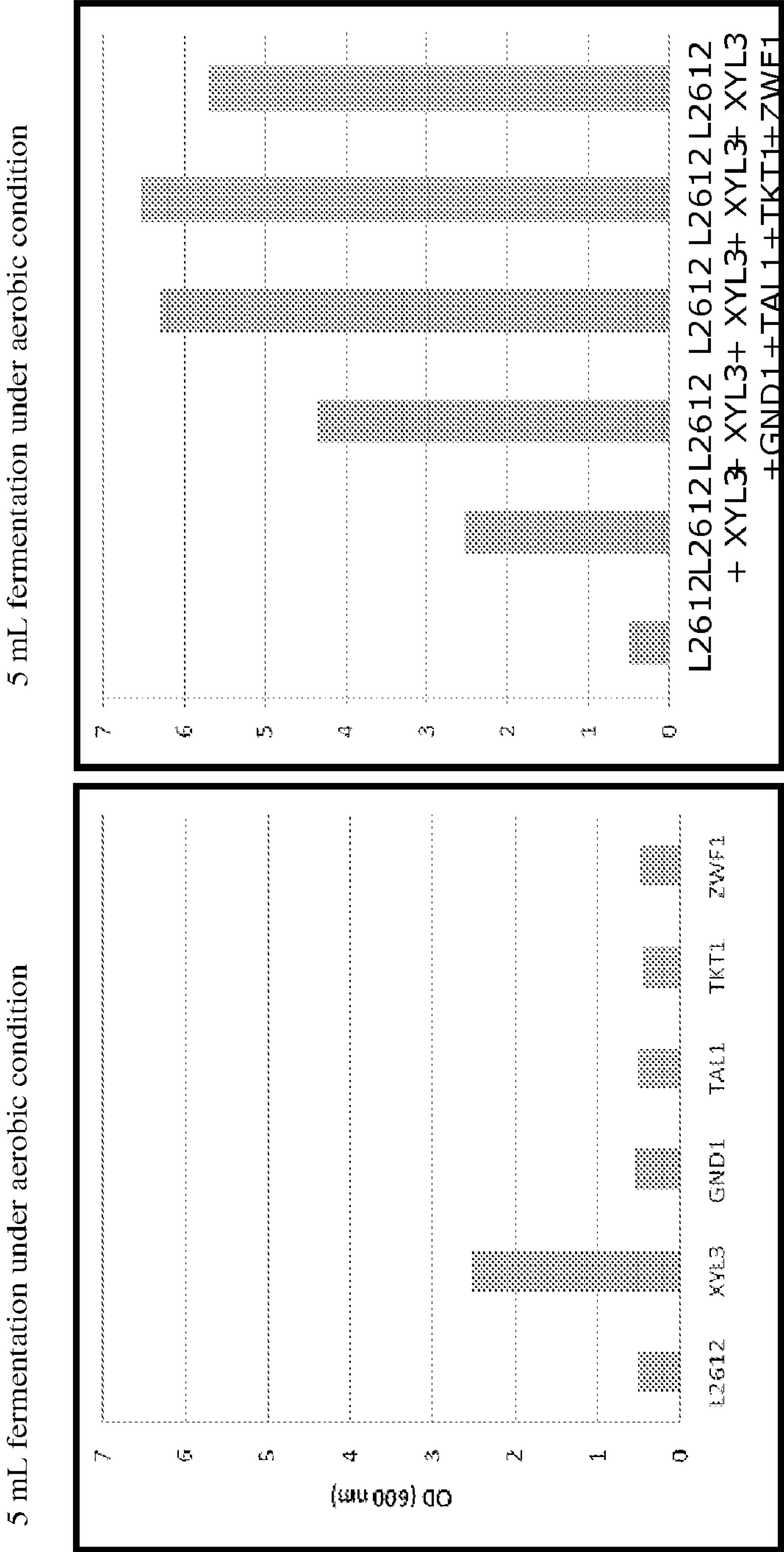


Figure 62

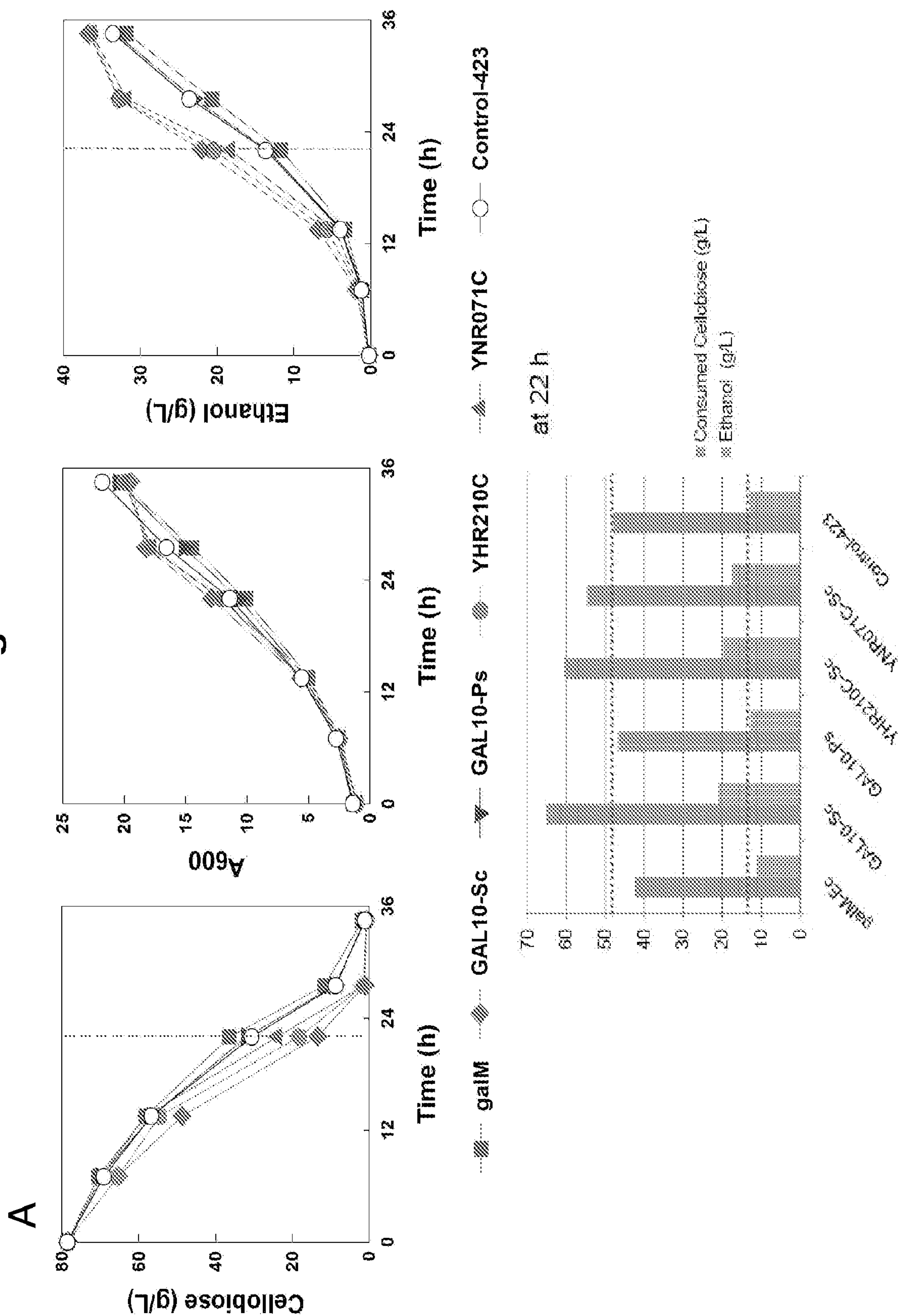


Figure 62 (cont.)**B**

	OD (A ₆₀₀)	Ethanol (g/L)	Y _{EtOH} (g/g)	P _{EtOH} (g/L-hr)
galM-Ec	20.4	30.3	0.40	0.88
GAL10-Sc	19.5	34.0	0.45	1.01
GAL10-Ps	20.5	31.7	0.41	0.92
YHR210C-Sc	20.0	34.7	0.45	1.01
YNR071C-Sc	20.0	34.0	0.45	1.00
Control-423	21.8	32.0	0.42	0.93

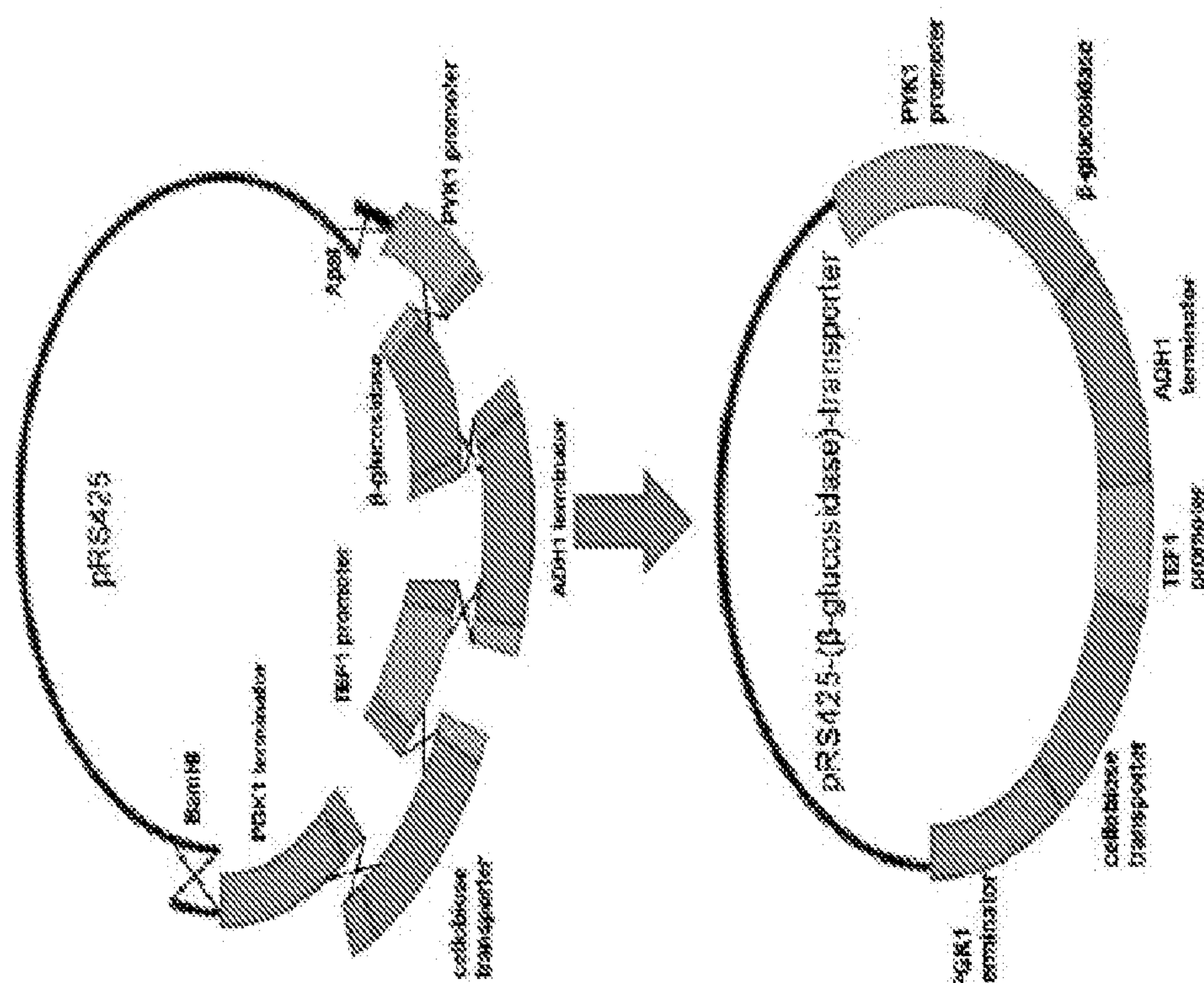
Figure 64

Figure 65

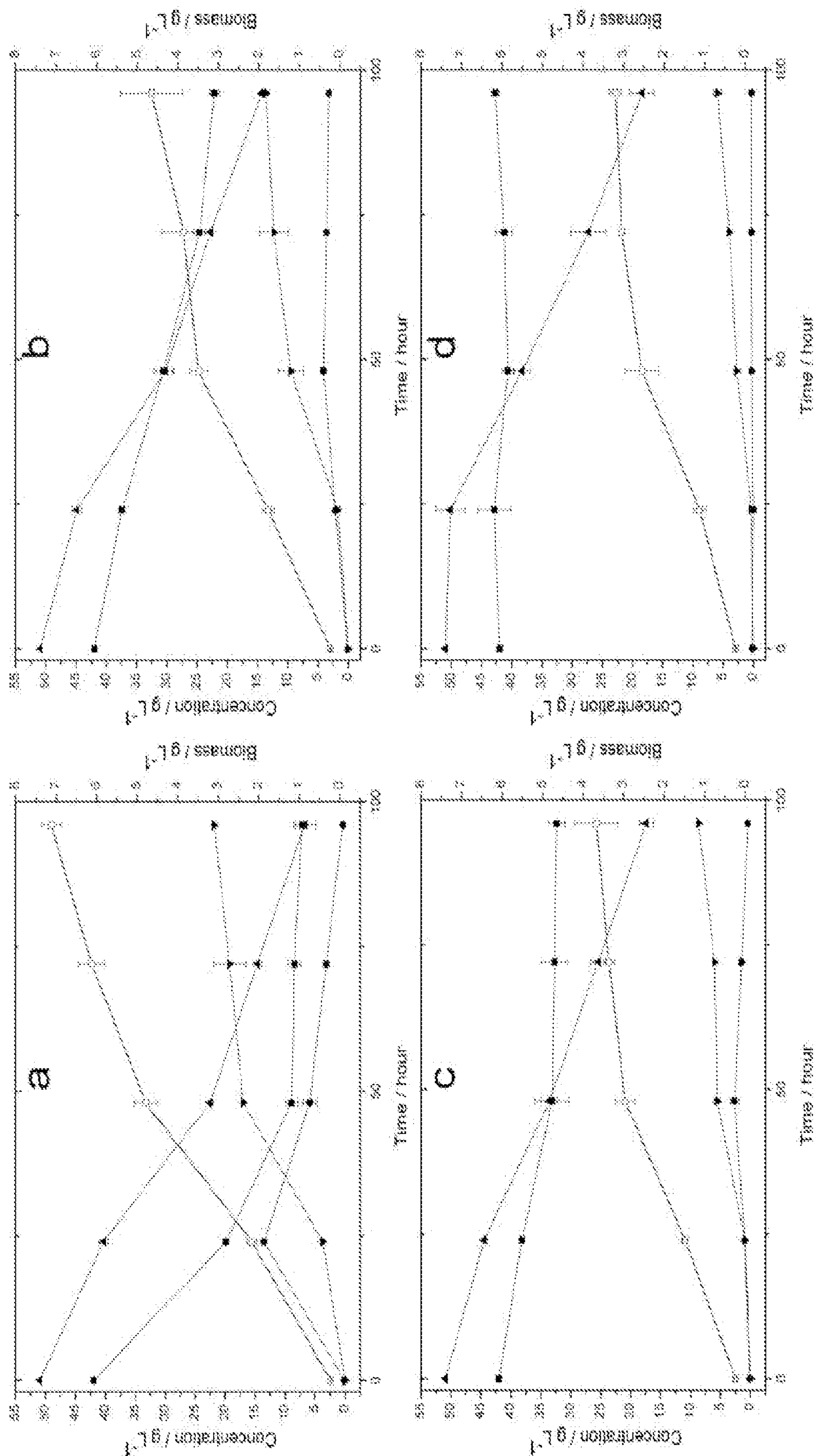


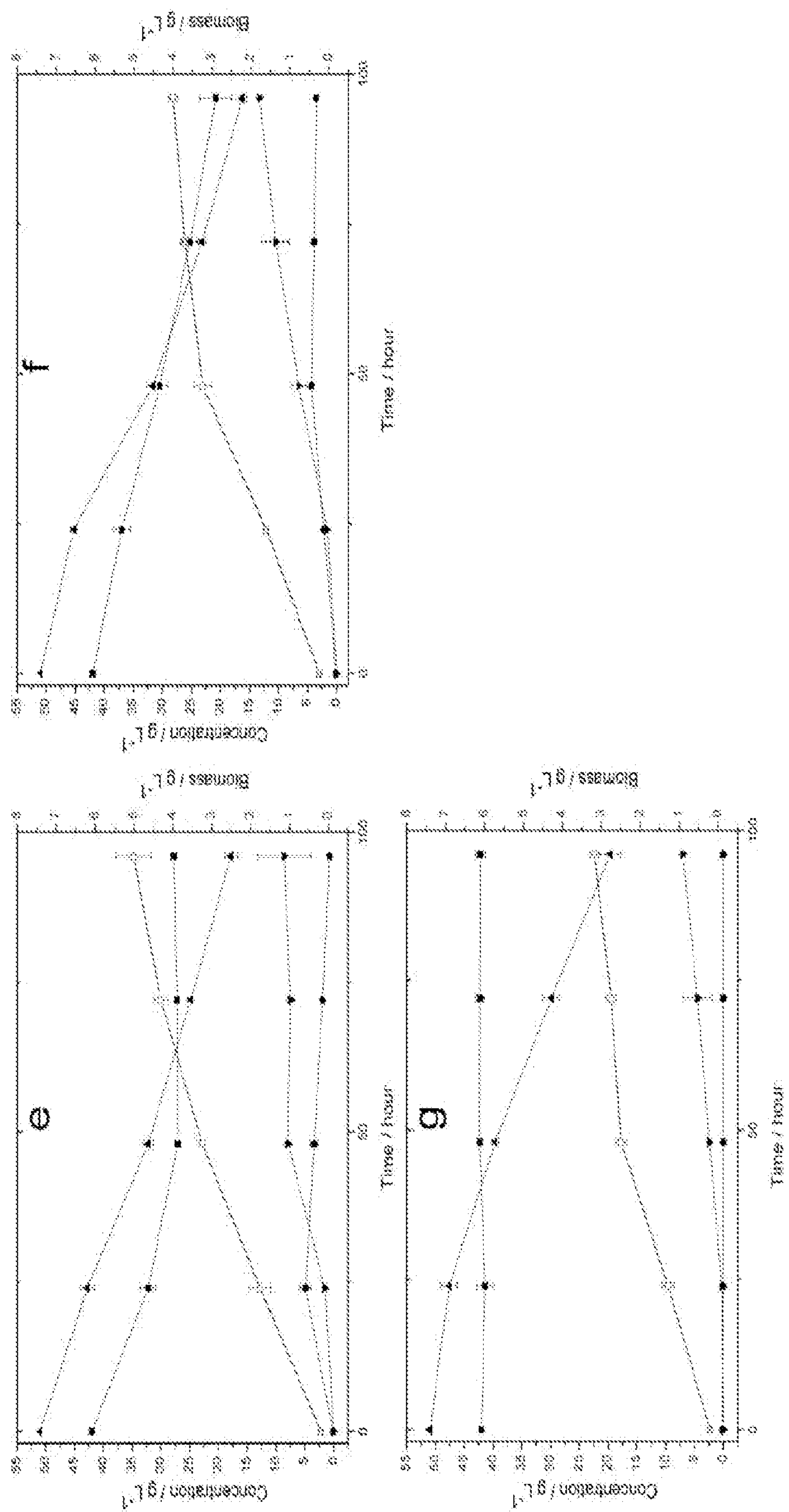
Figure 65,
cont.

Figure 66

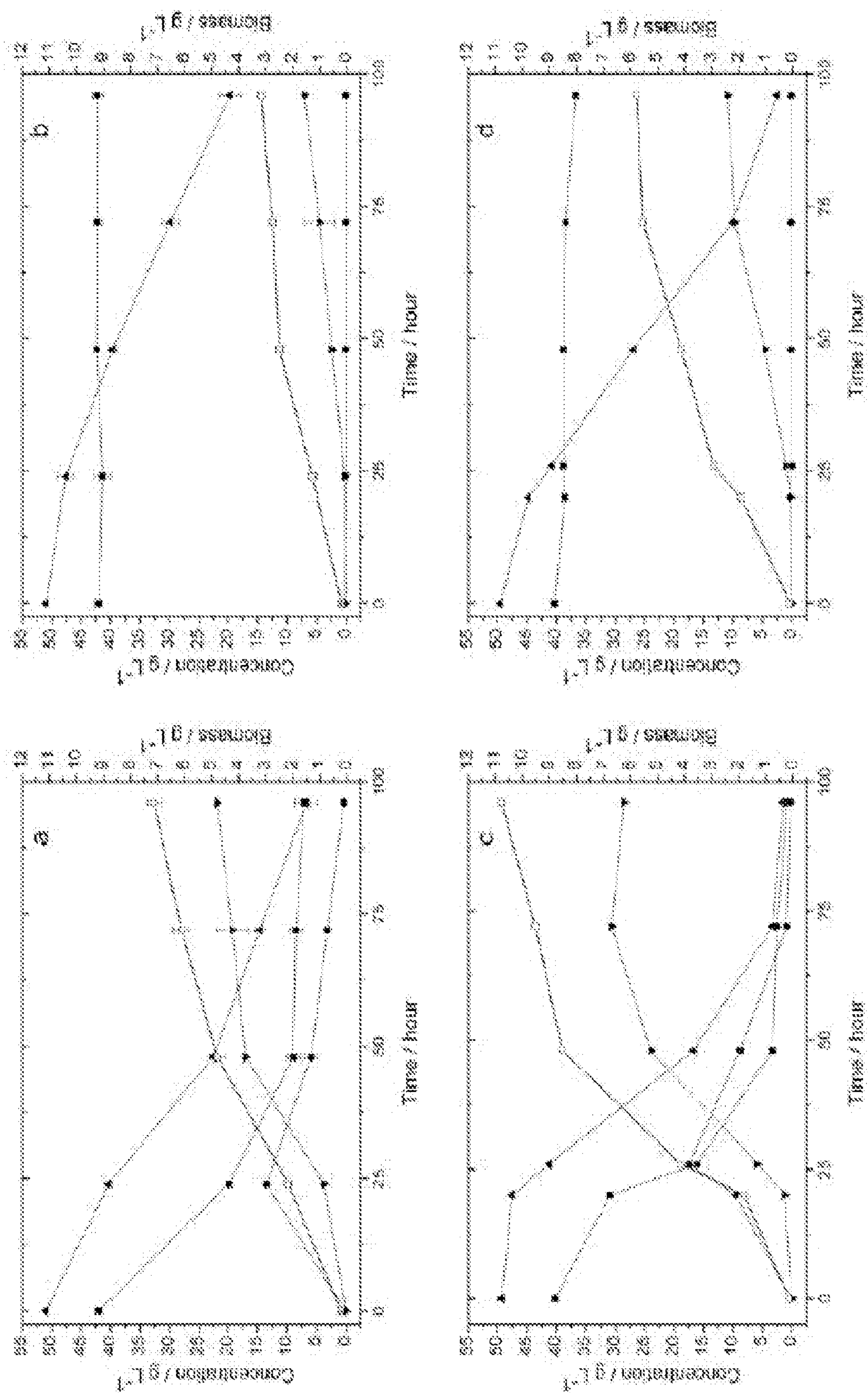
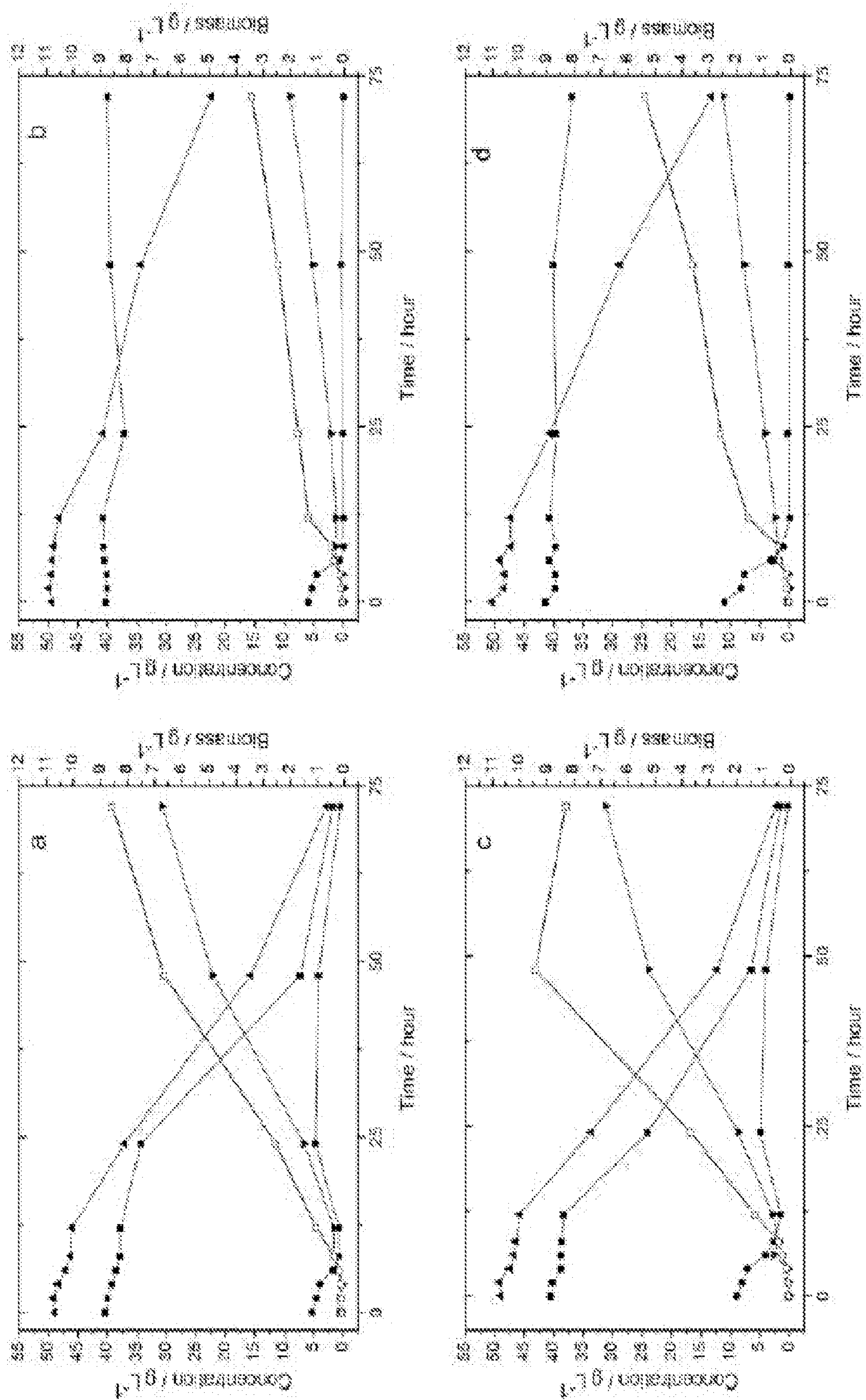
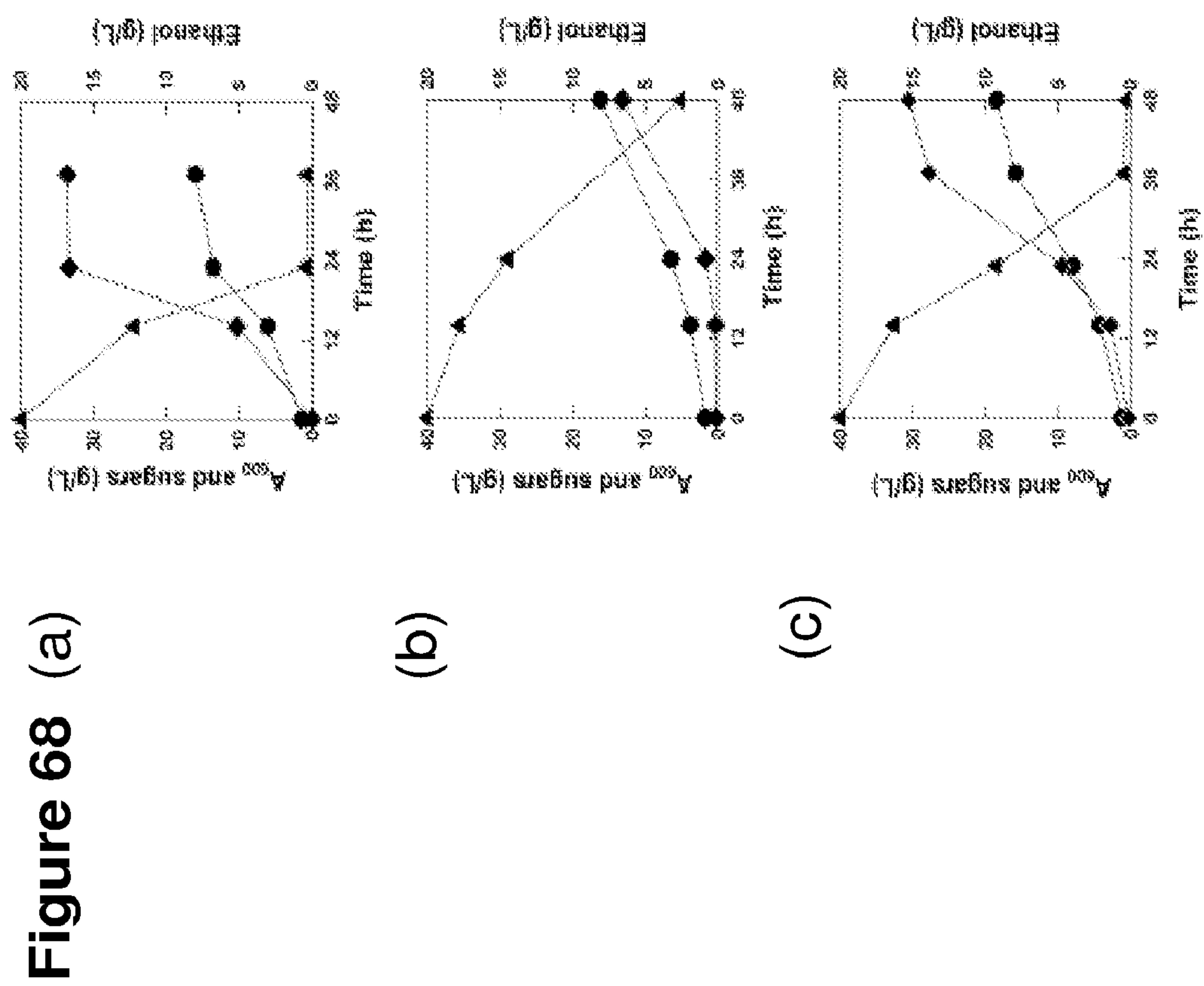


Figure 67





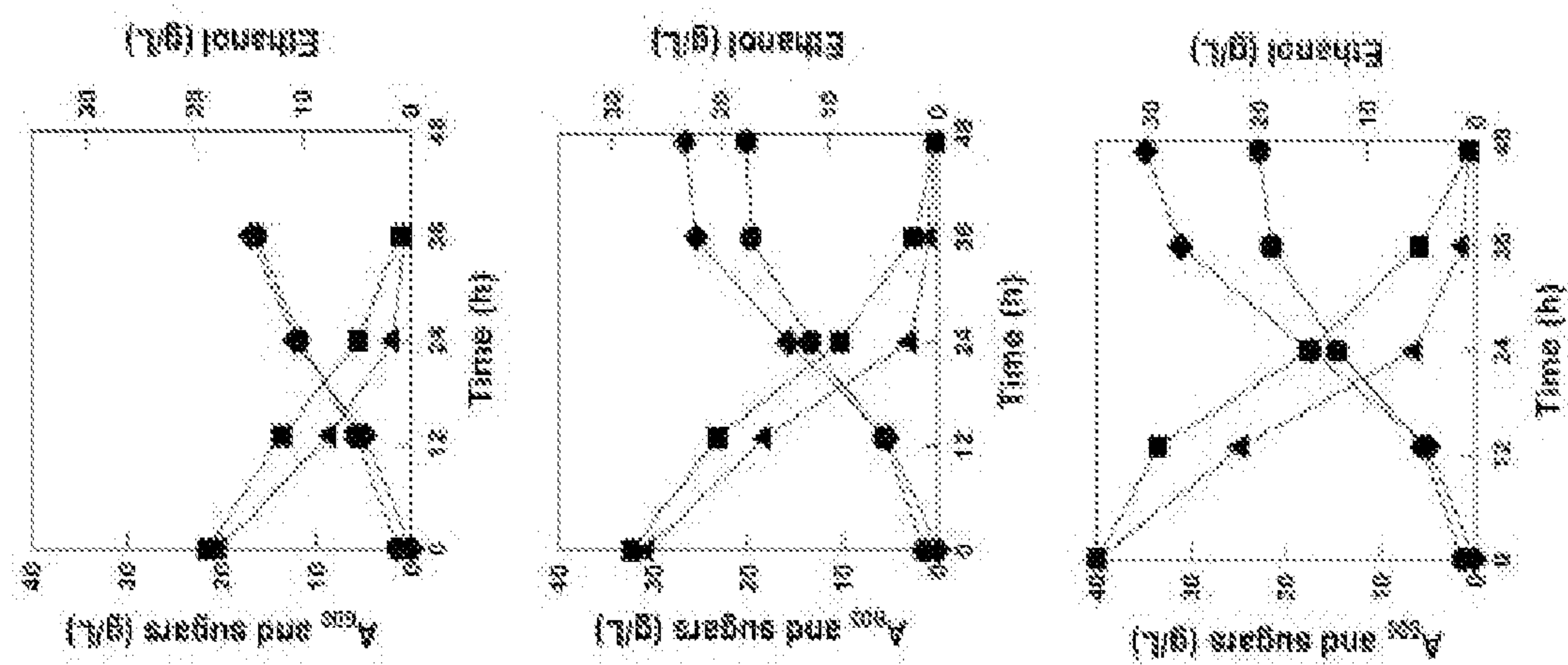
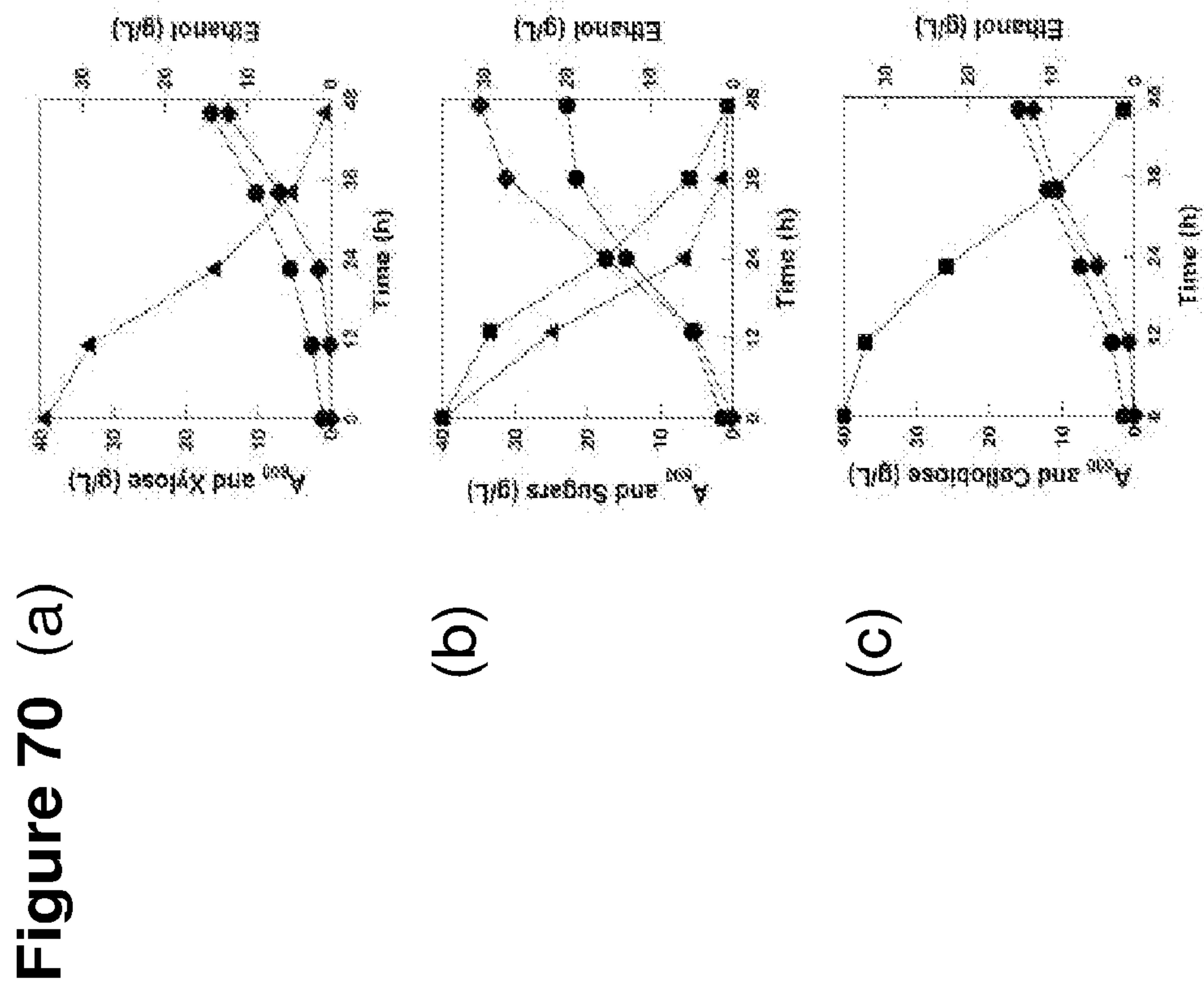


Figure 69 (a)

(b)

(c)



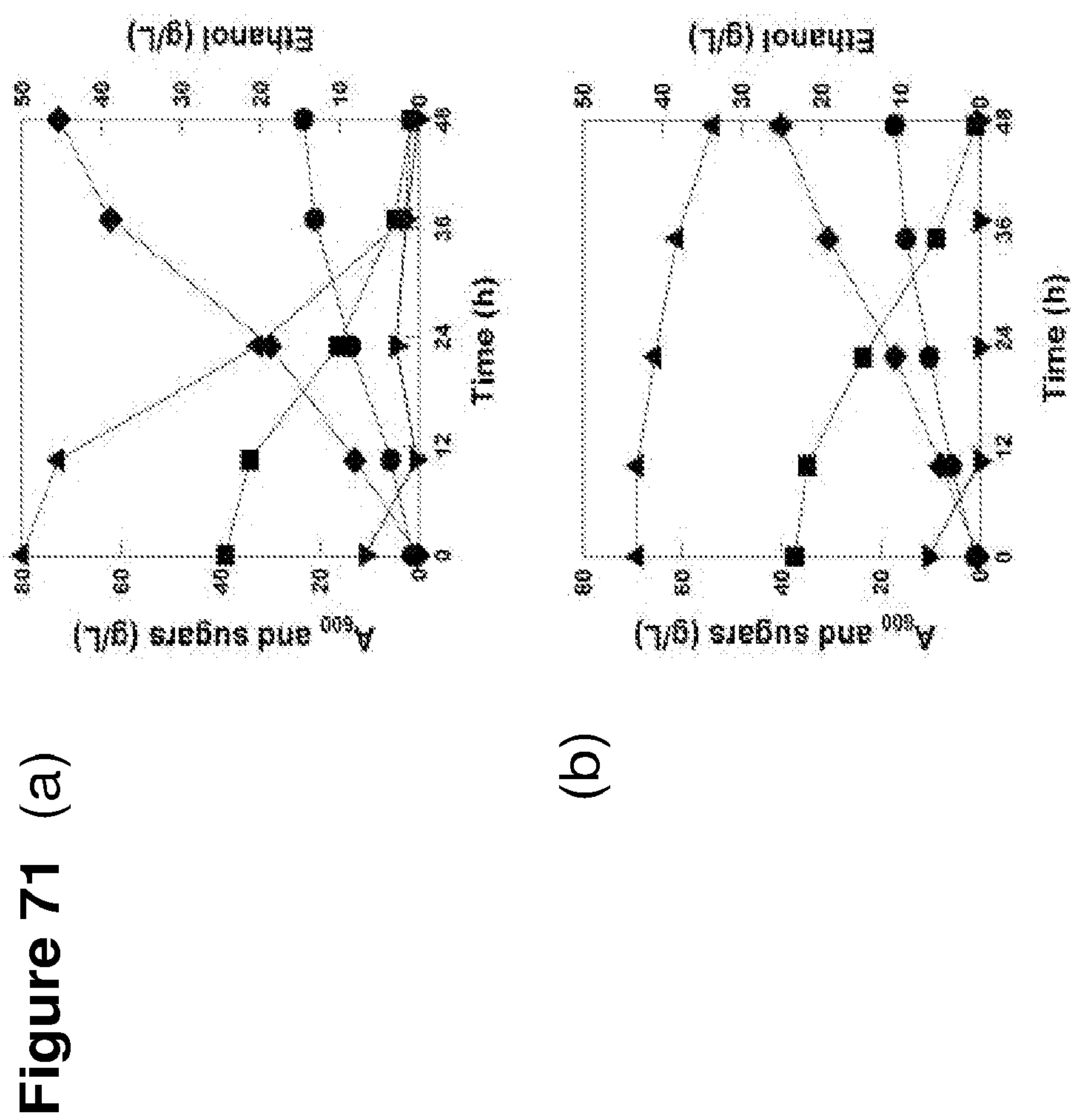


FIGURE 72

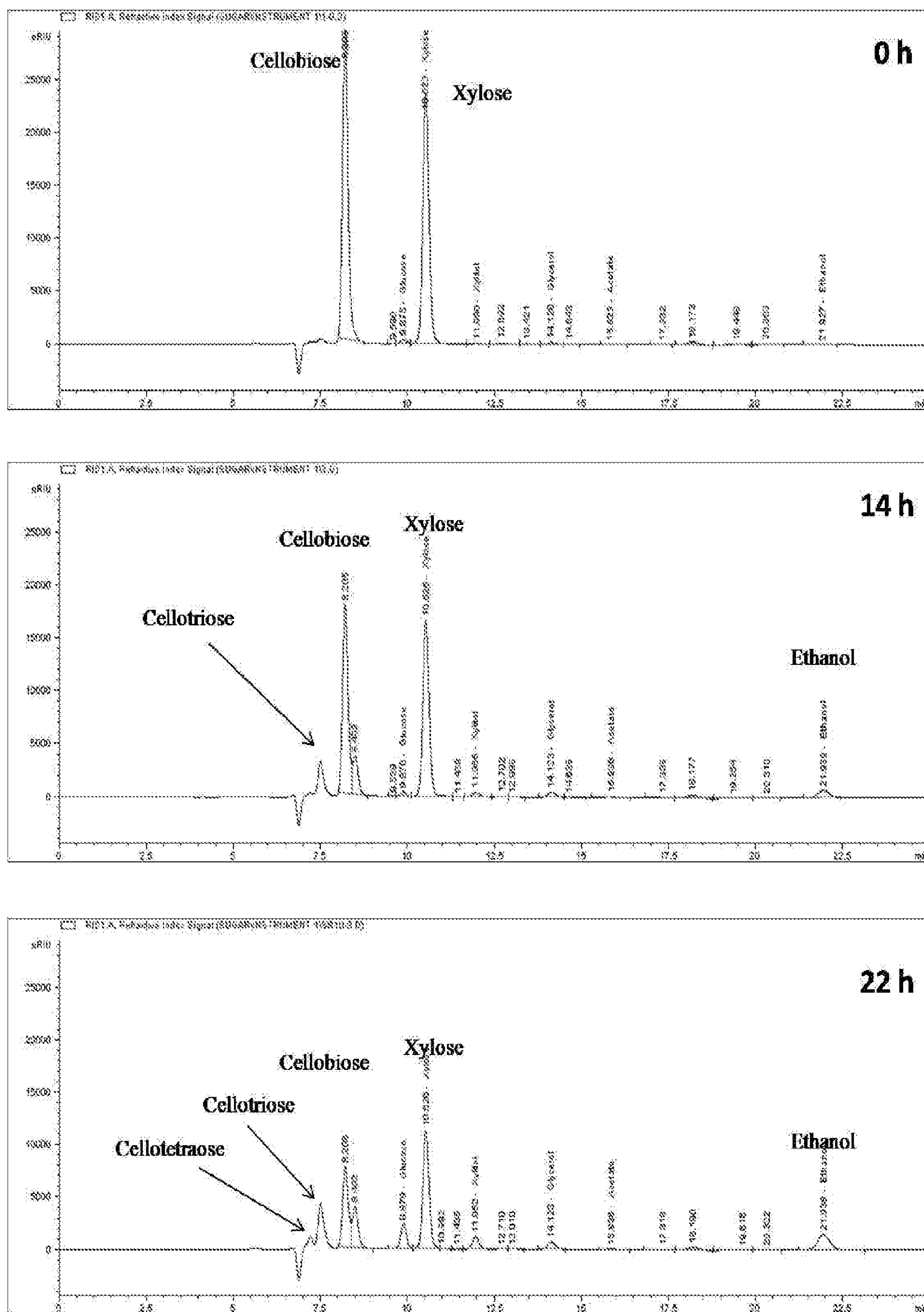


FIGURE 72, cont.

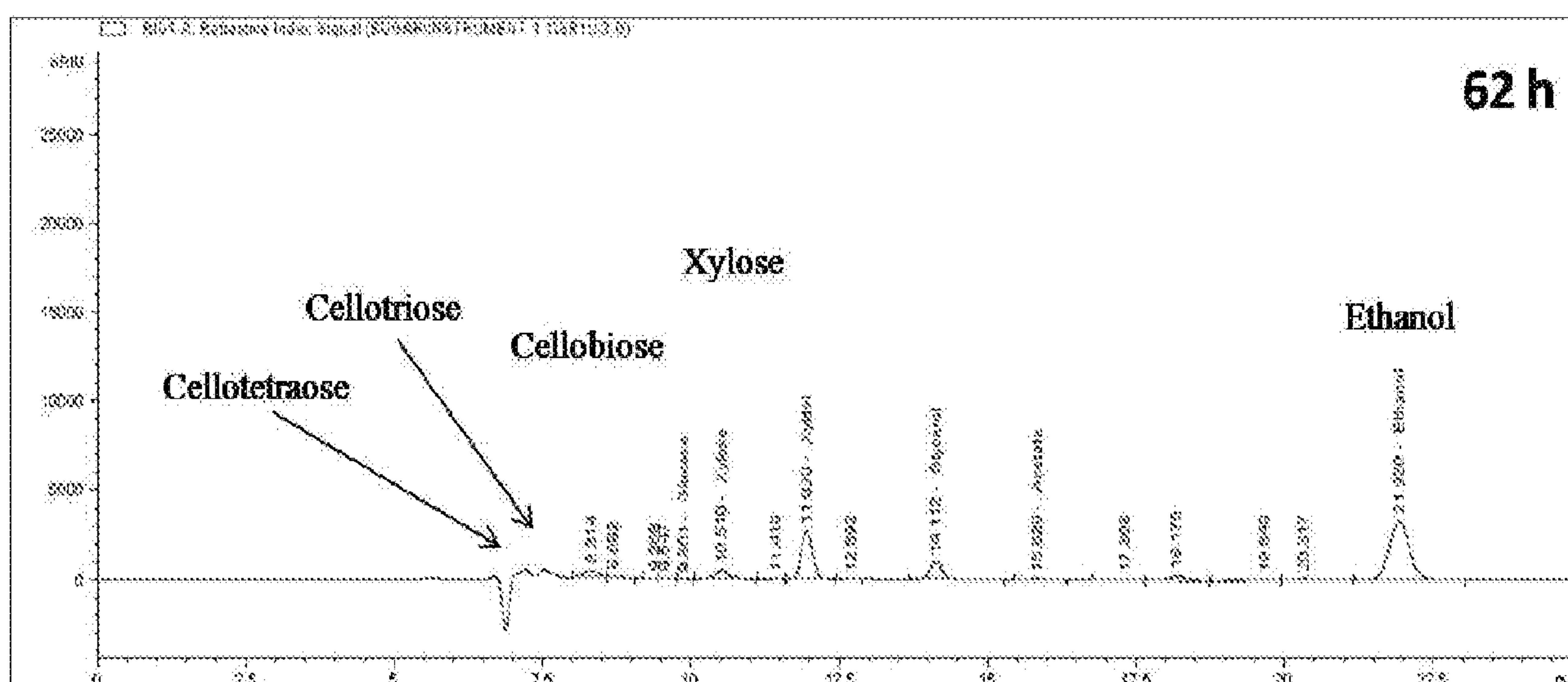
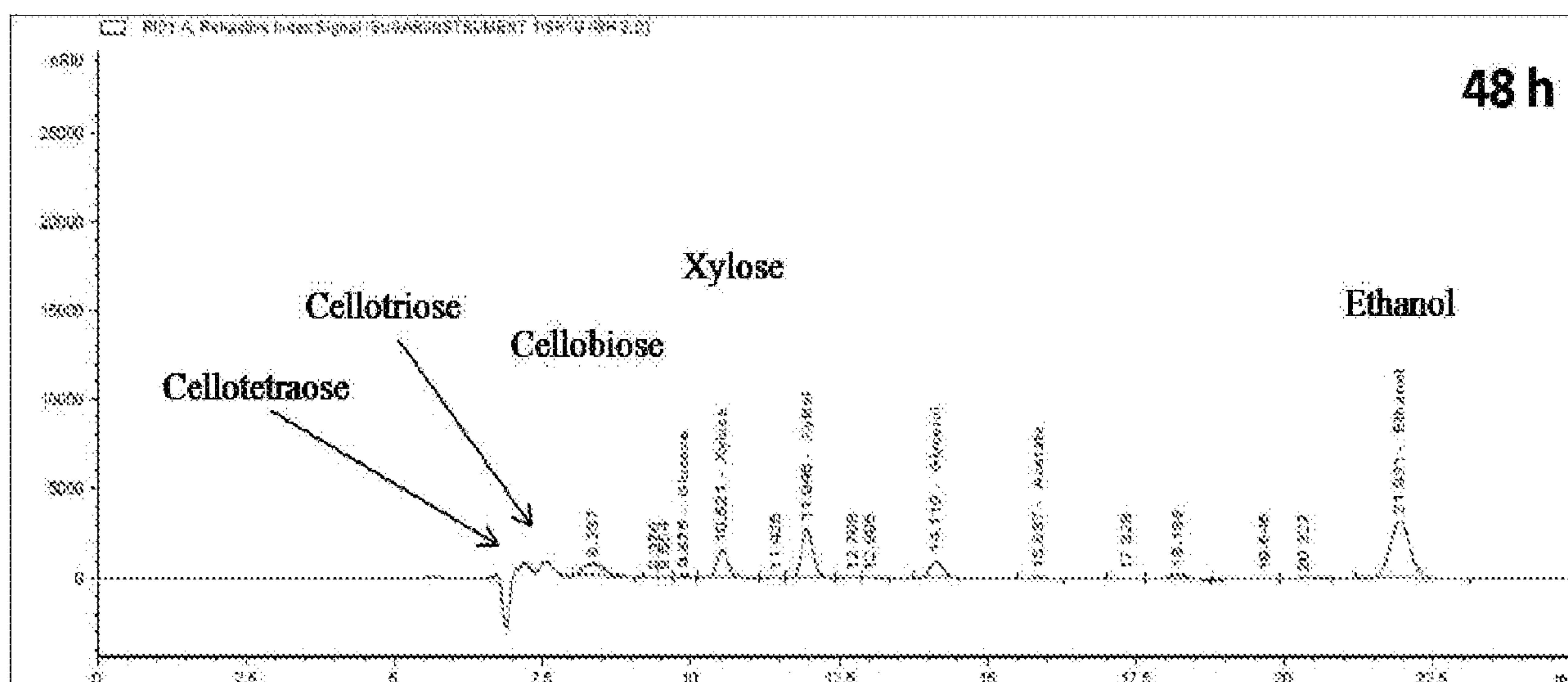
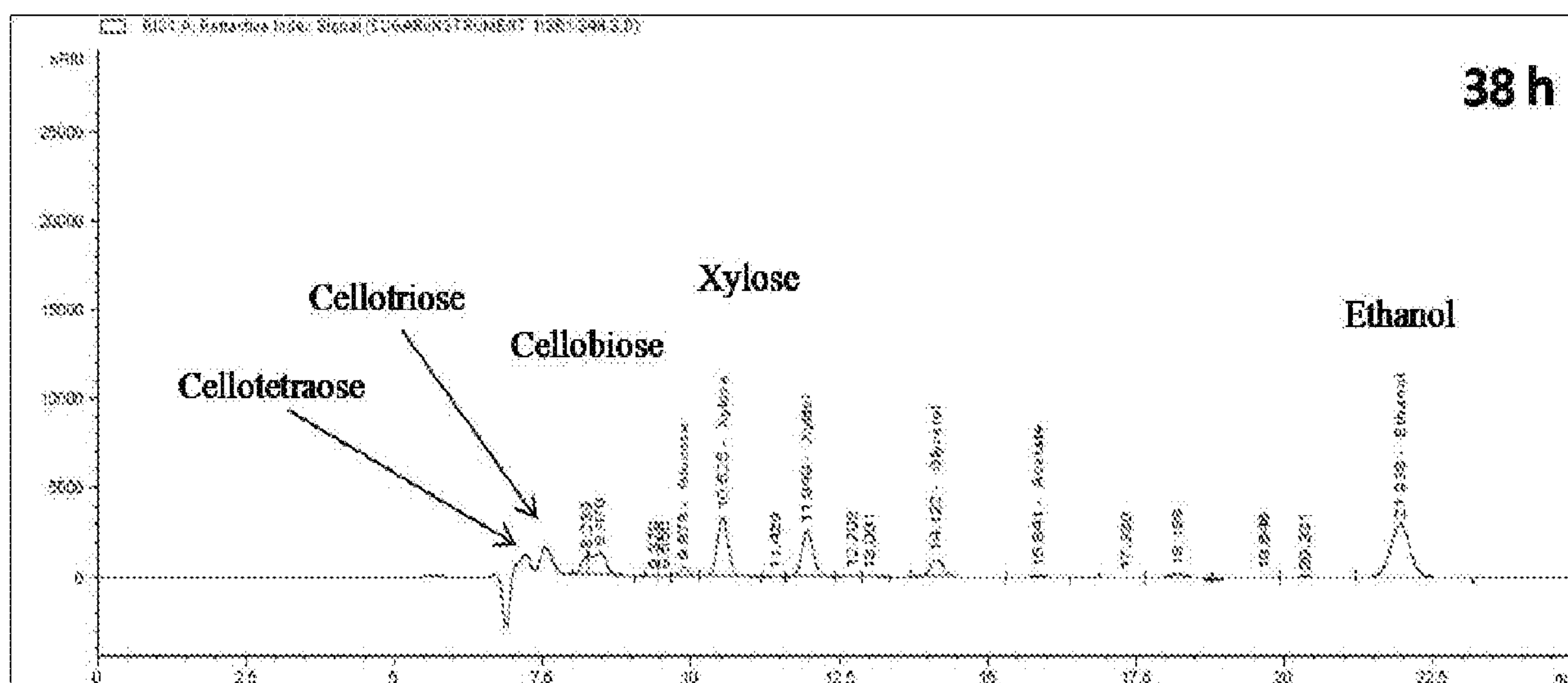


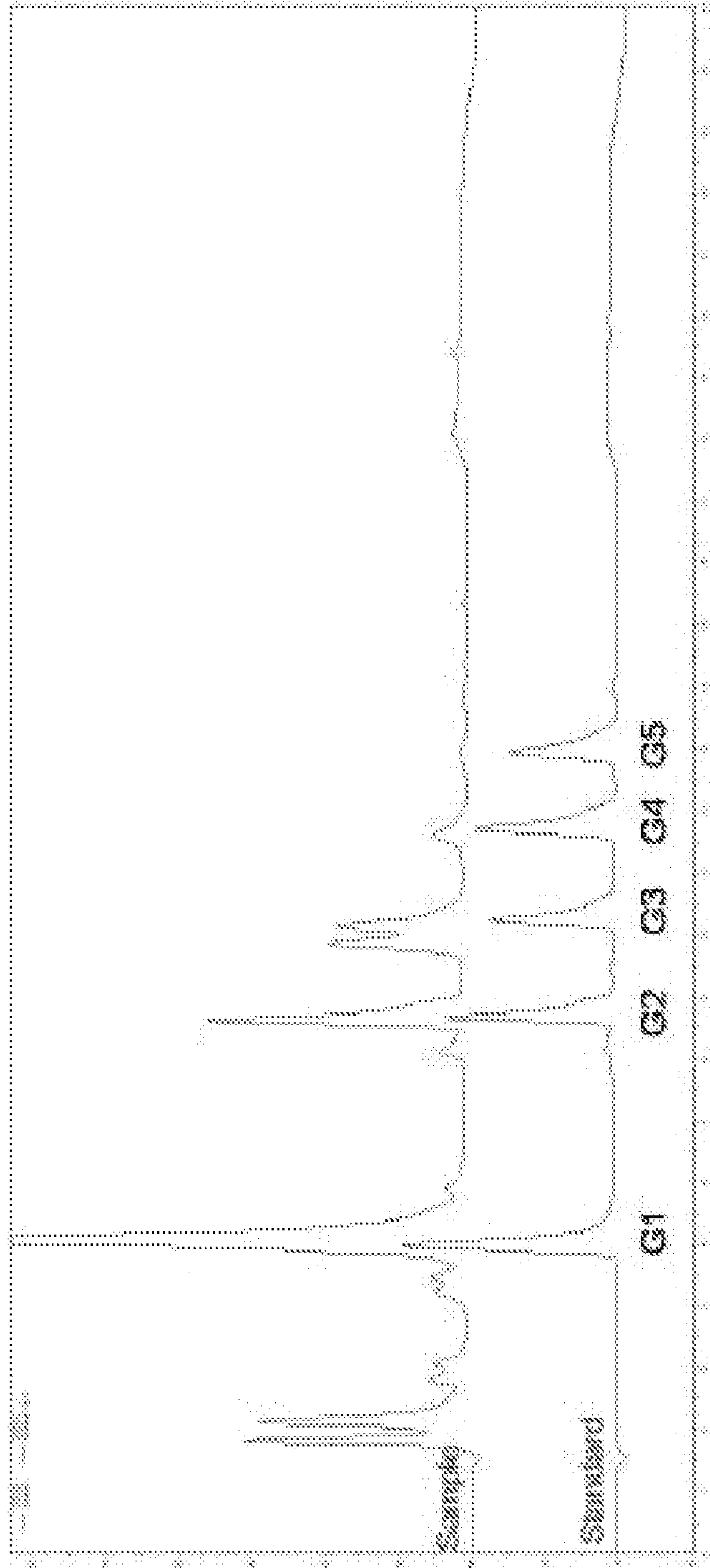
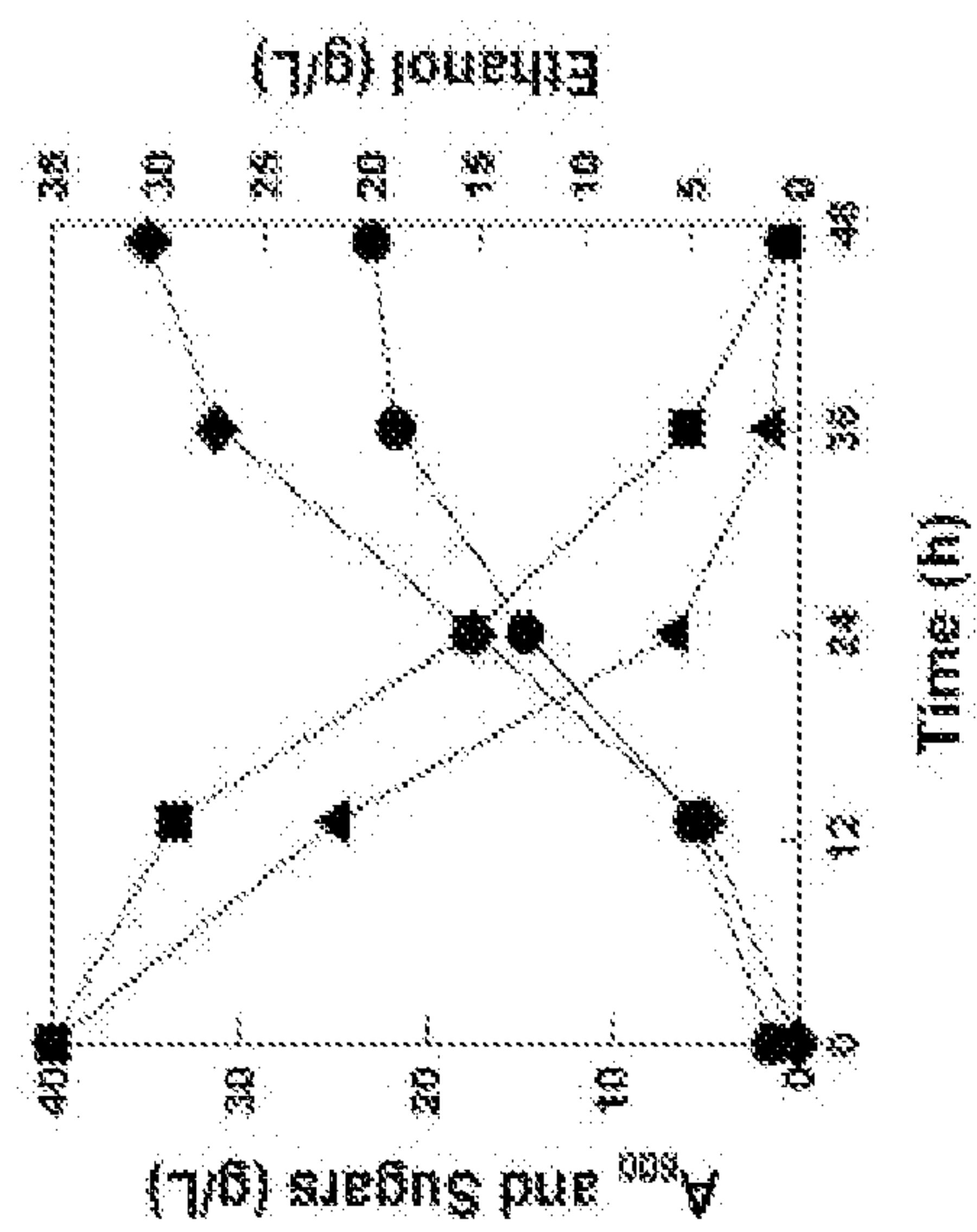
Figure 73

Figure 74 (a)



(b)

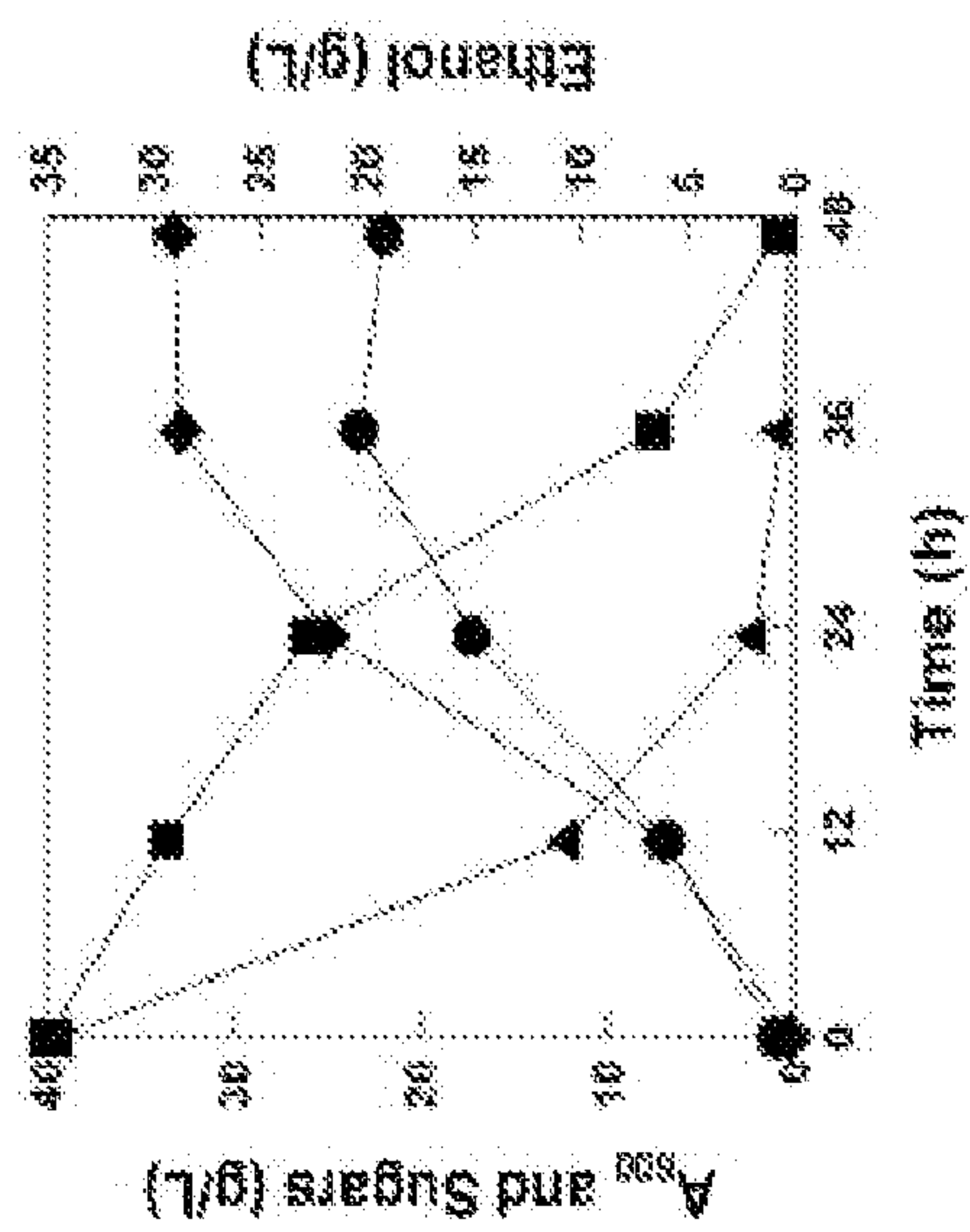
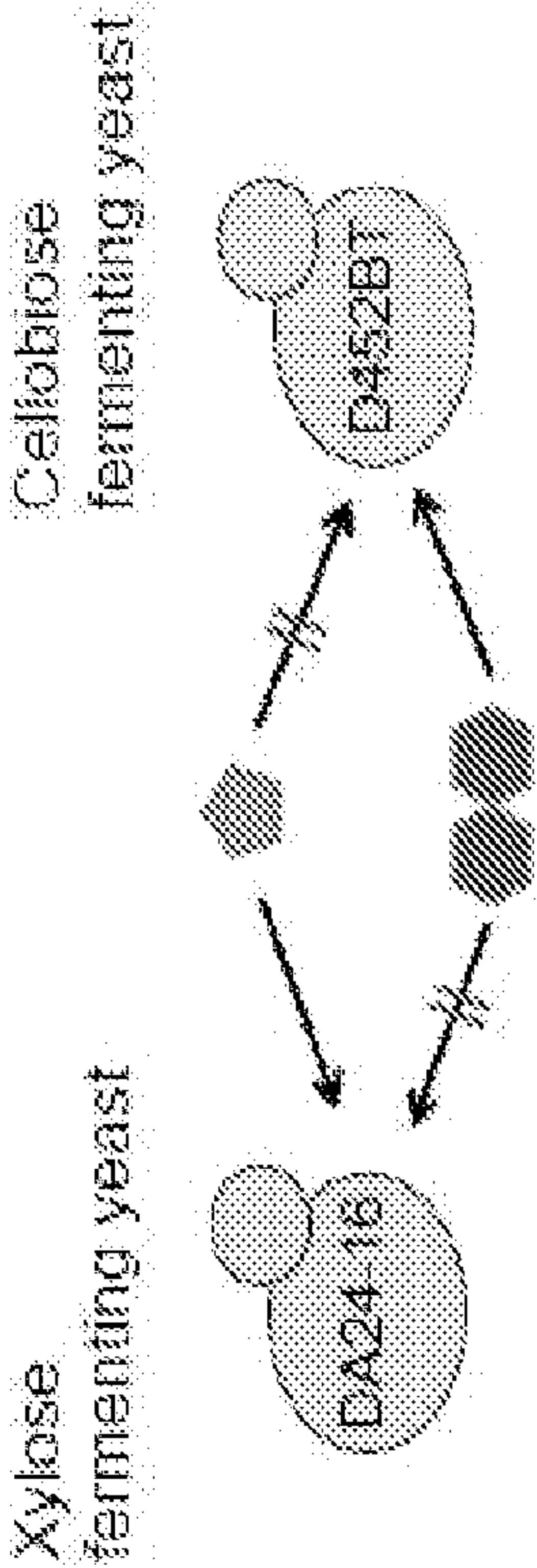


Figure 75

(a)



(b)

D452-BT and DA2416

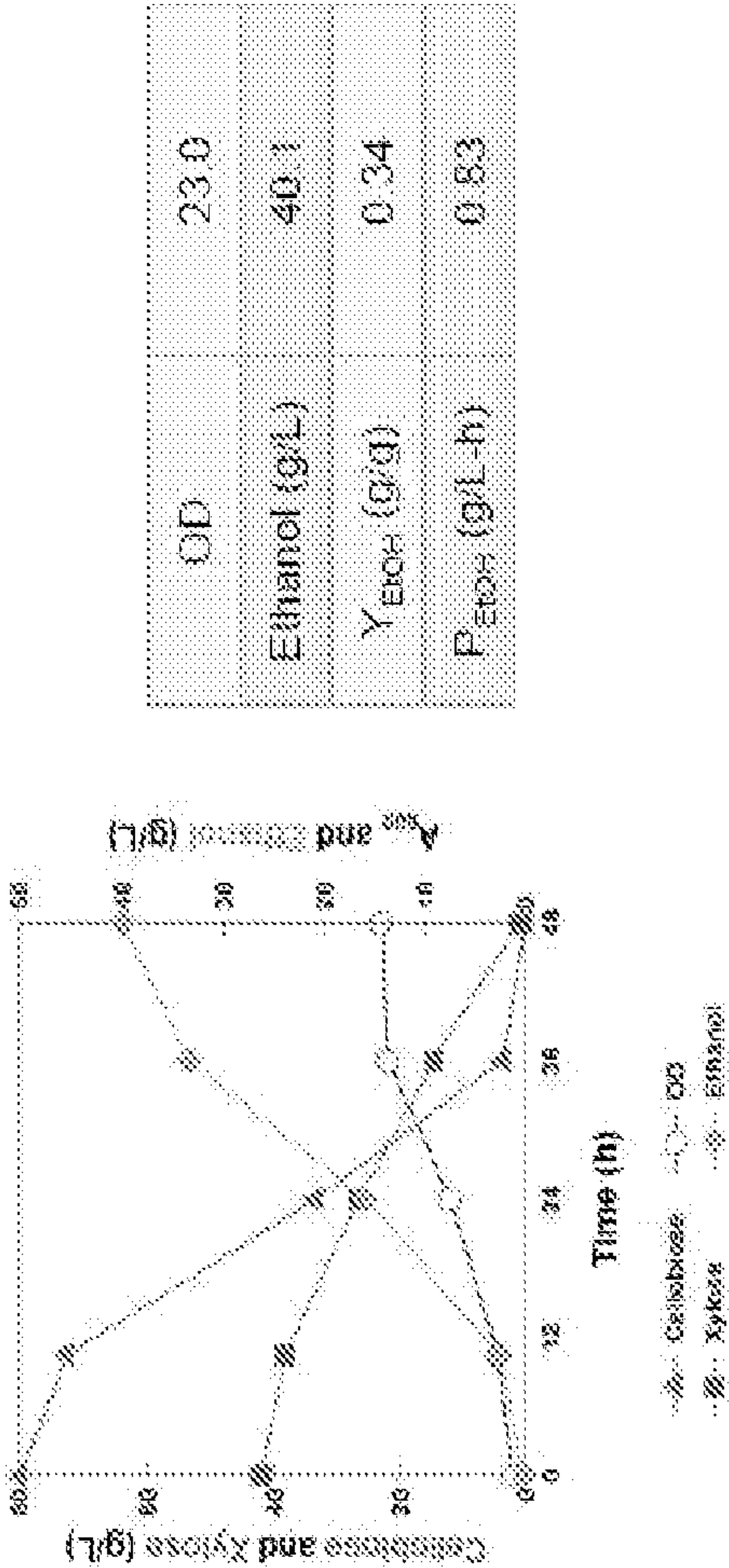


Fig. 76

NCU00010.2	NCU01906.2	NCU04373.2	NCU06656.2	NCU09051.2b
NCU00028.2	NCU01940.2	NCU04388.2	NCU06681.2	NCU09115.2
NCU00060.2	NCU01970.2	NCU04394.2	NCU06707.2	NCU09133.2
NCU00073.2	NCU01989.2	NCU04400.2	NCU06752.2	NCU09138.2
NCU00102.2	NCU02018.2	NCU04401.2	NCU06803.2	NCU09169.2
NCU00111.2	NCU02027.2	NCU04415.2	NCU06977.2	NCU09175.2
NCU00122.2	NCU02097.2	NCU04417.2	NCU06999.2	NCU09209.2
NCU00124.2	NCU02124.2	NCU04460.2	NCU07027.2	NCU09210.2
NCU00130.2	NCU02136.2	NCU04475.2	NCU07063.2	NCU09266.2
NCU00173.2	NCU02179.2	NCU04491.2b	NCU07064.2b	NCU09267.2
NCU00292.2	NCU02188.2	NCU04510.2	NCU07133.2	NCU09285.2
NCU00299.2	NCU02238.2	NCU04521.2	NCU07143.2	NCU09316.2
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NCU00608.2	NCU02397.2	NCU04781.2	NCU07287.2b	NCU09682.2
NCU00611.2	NCU02453.1	NCU04801.2	NCU07310.2	NCU09705.2
NCU00642.2	NCU02455.2	NCU04815.2	NCU07311.2	NCU09763.2b
NCU00643.2	NCU02512.2	NCU04905.2	NCU07313.2b	NCU09783.2
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NCU01181.2	NCU03415.2	NCU05850.2	NCU08333.2	NCU03156.2
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NCU01866.2	NCU04298.2	NCU06597.2	NCU09027.2	
NCU01900.2	NCU04349.2a	NCU06603.2	NCU09034.2	
NCU01904.2	NCU04369.2	NCU06650.2	NCU09041.2	
NCU01905.2	NCU04371.2	NCU06652.2	NCU09043.2	

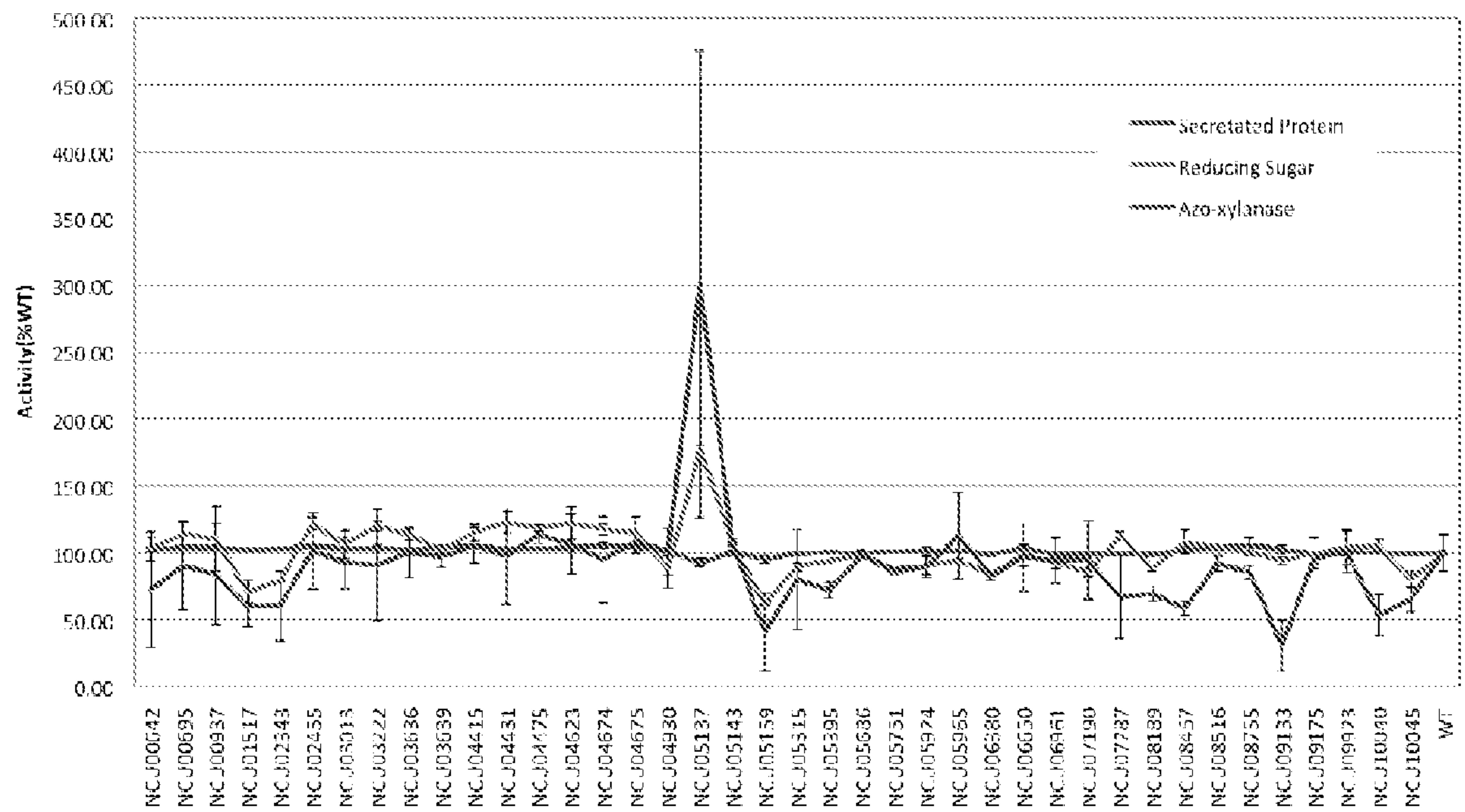
Figure 77

Figure 78**A**

	Bradford	CMC- activity
Wt-1	0.57	0.50
Wt-2	0.58	0.53
Ko-1	0.89	0.76
Ko-2	0.89	0.65
GFP	0.56	0.54
GFP	0.60	0.58

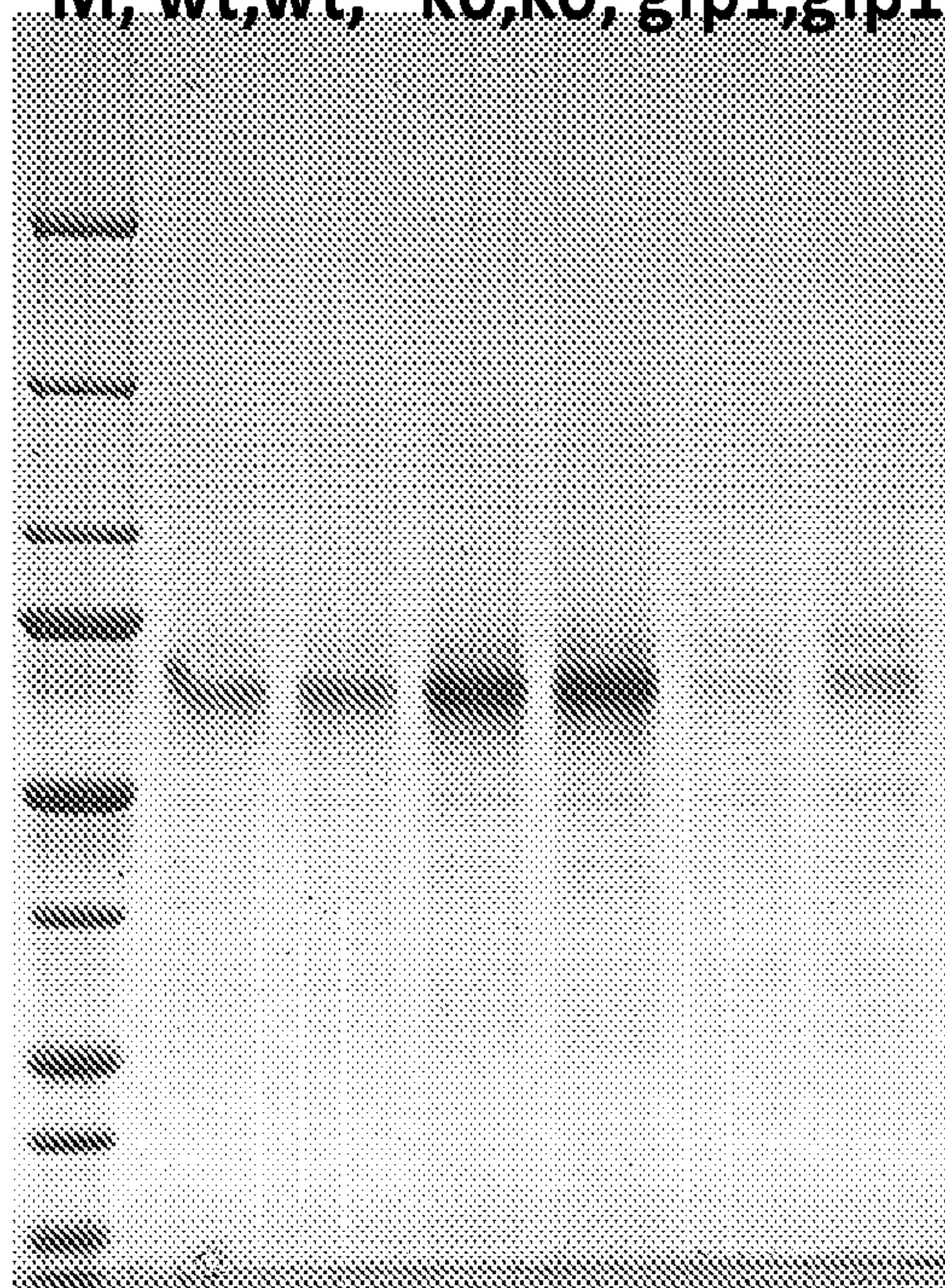
B**M, wt,wt, ko,ko, gfp1,gfp1**

Figure 79

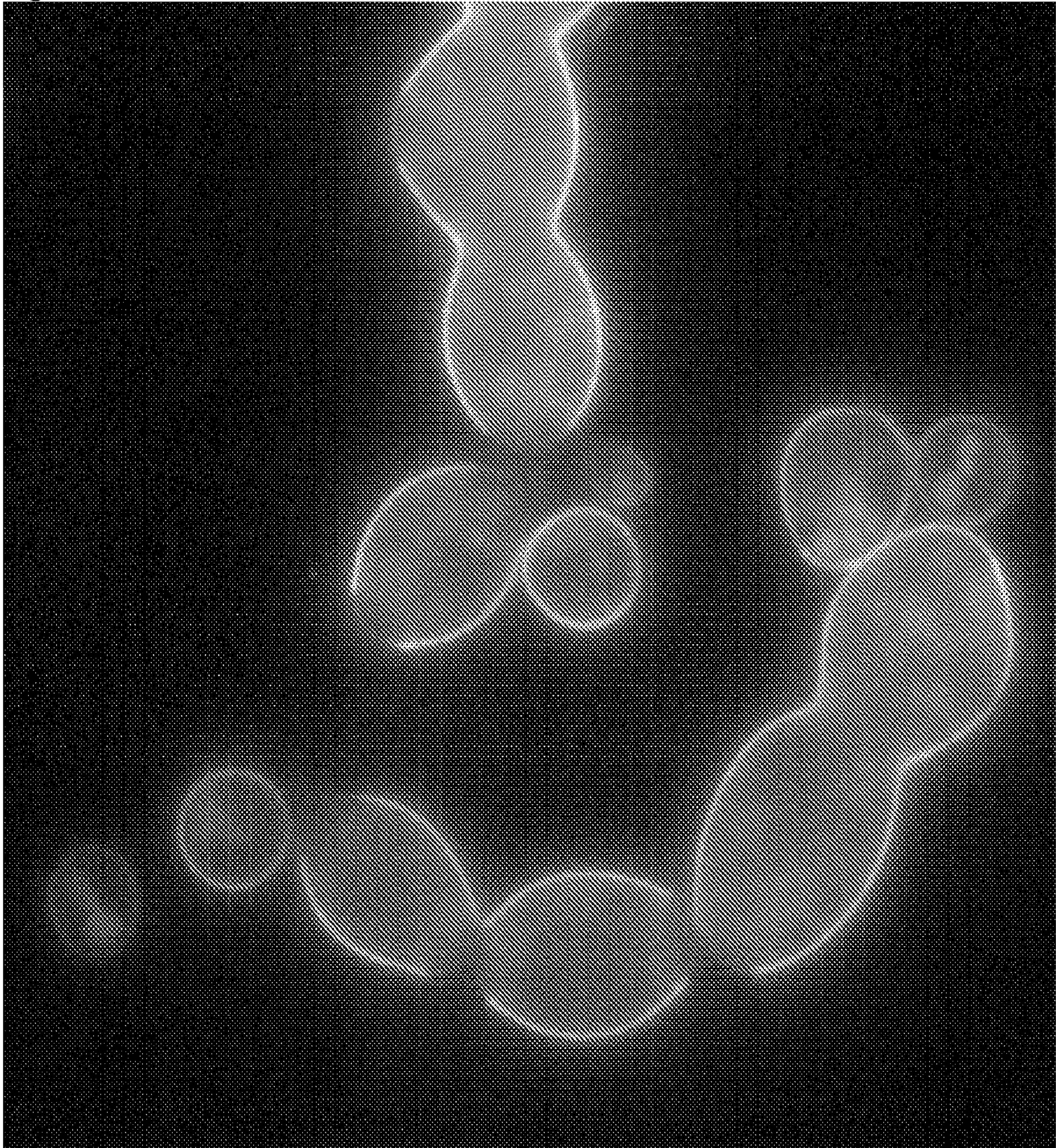


Figure 80

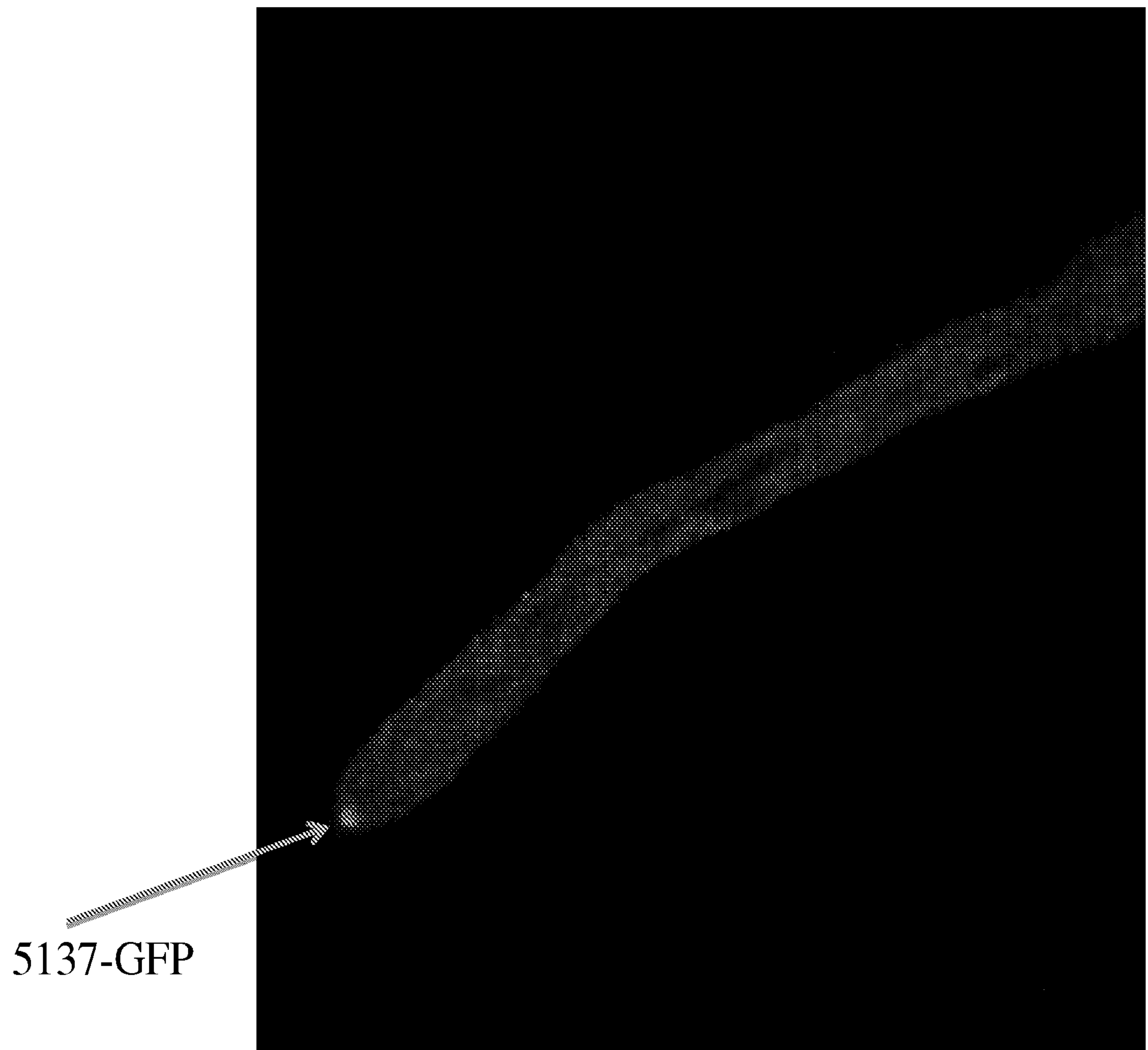


Fig. 25

