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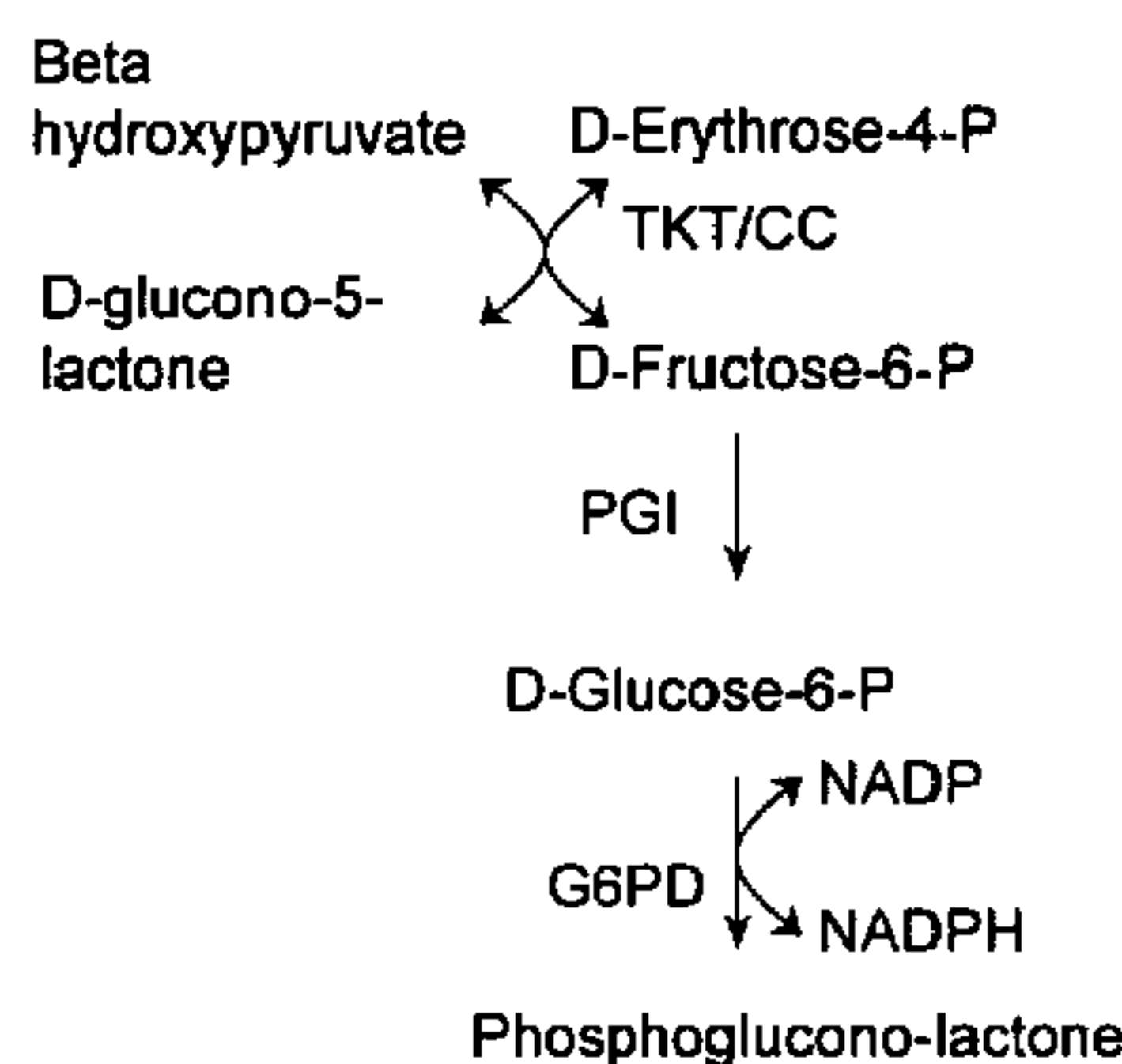
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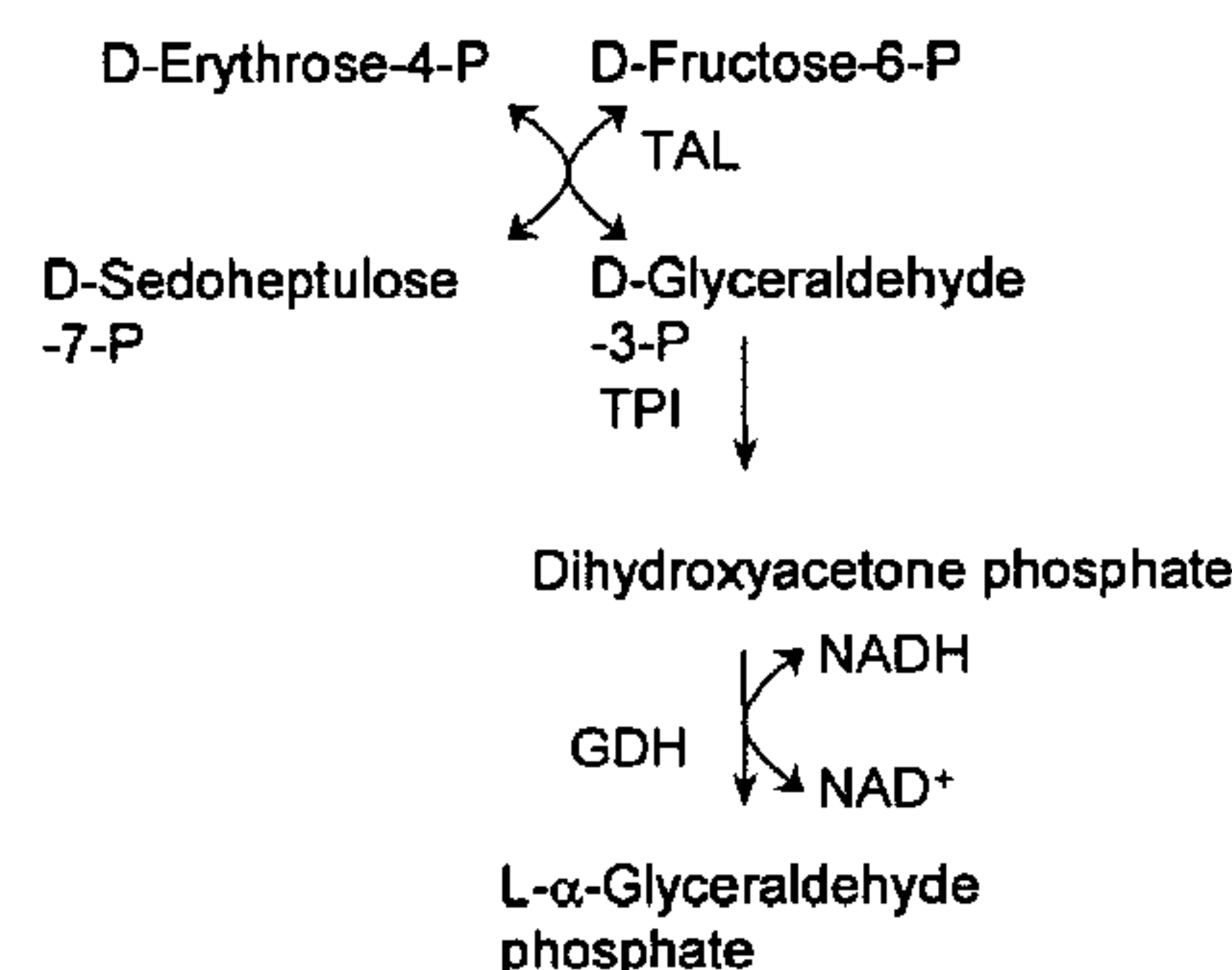
(54) Titre : PRODUCTION D'ETHANOL AMELIOREE DURANT LA FERMENTATION DE SUCRES MIXTES
CONTENANT DU XYLOSE

(54) Title: IMPROVED ETHANOL PRODUCTION IN FERMENTATION OF MIXED SUGARS CONTAINING XYLOSE

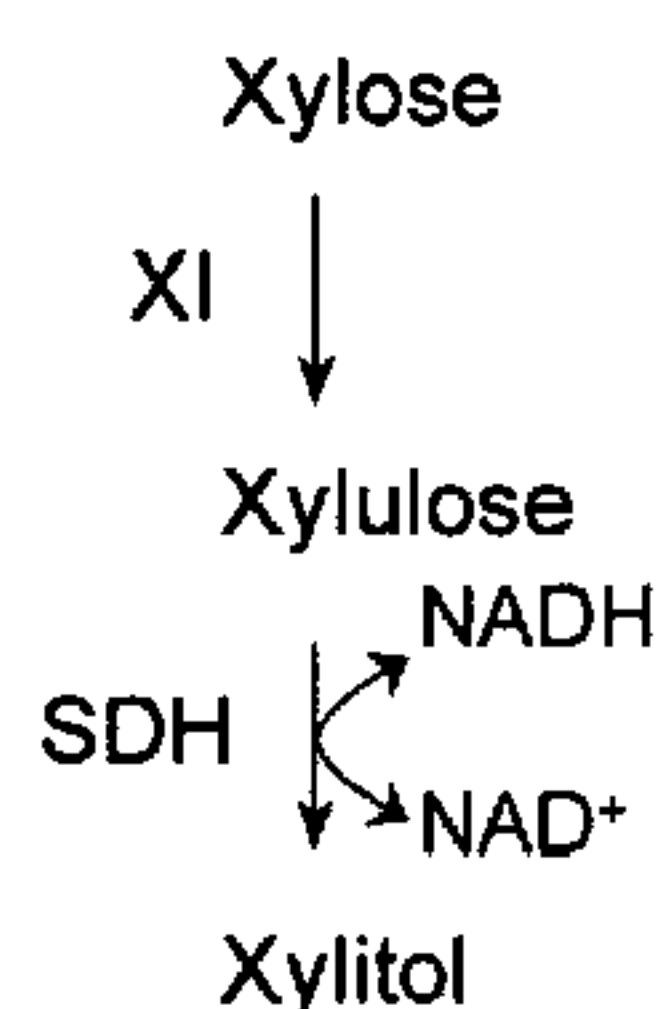
A Transketolase Assay



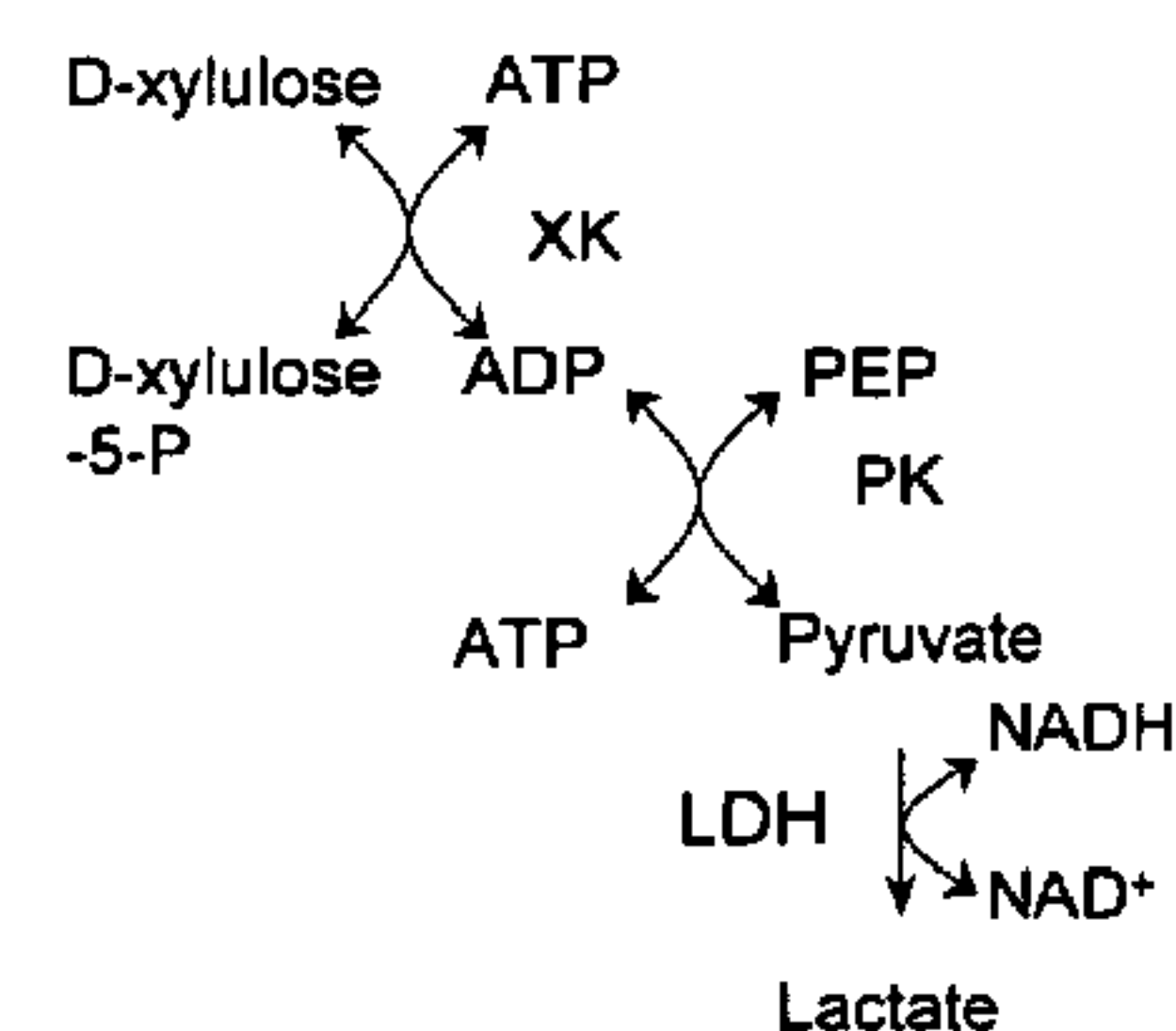
B Transaldolase Assay



C Xylose Isomerase Assay

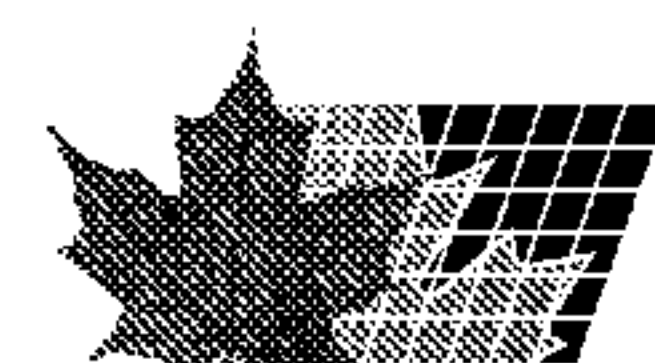


D Xyulokinase Assay



(57) Abrégé/Abstract:

Xylose-utilizing *Z. mobilis* strains were found to have improved ethanol production when grown in medium containing mixed sugars including xylose if sorbitol or mannitol was included in the medium. The effect was seen in concentrations of mixed sugars where no growth lag period occurs, as well as in higher sugars concentrations.



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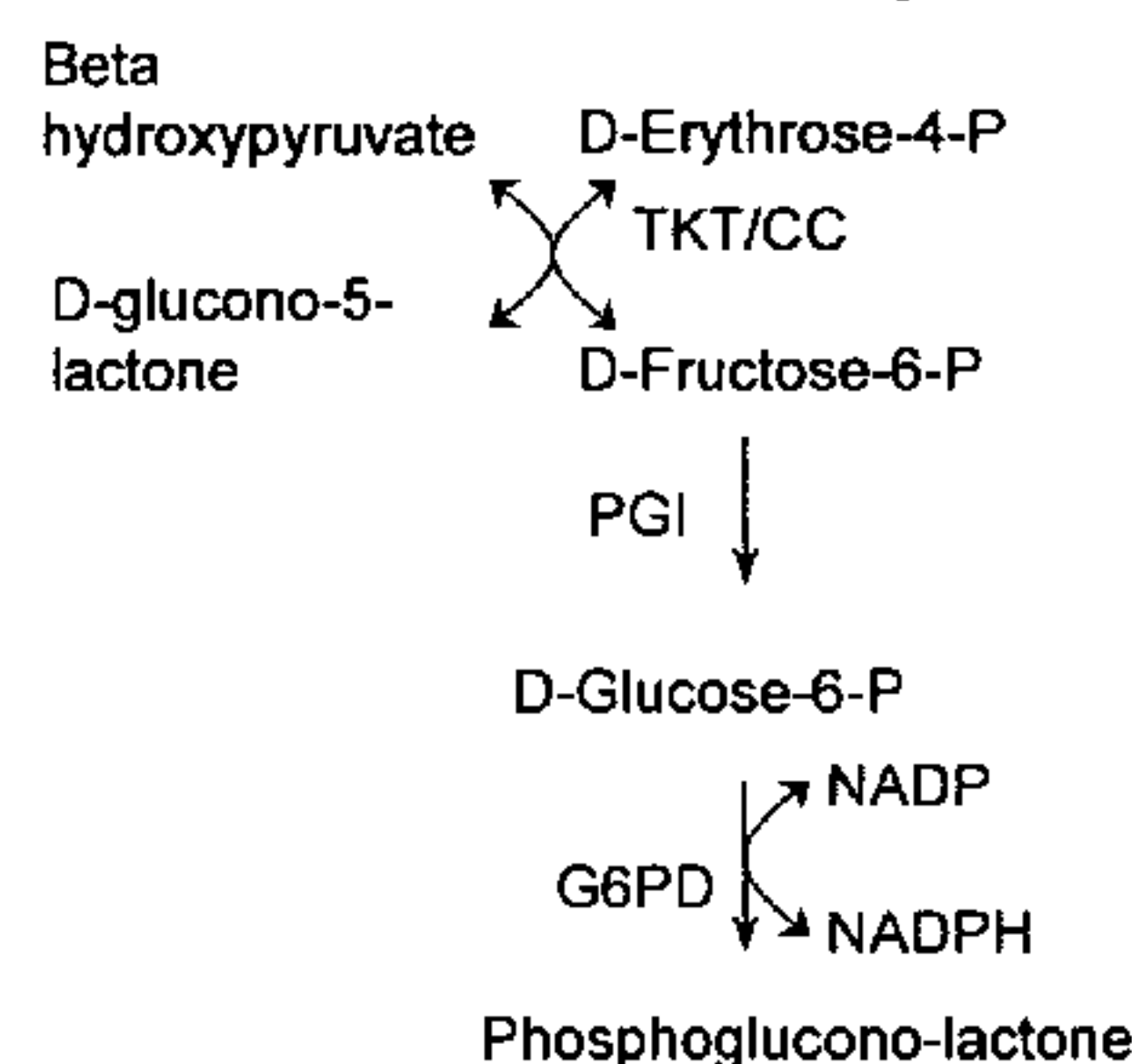
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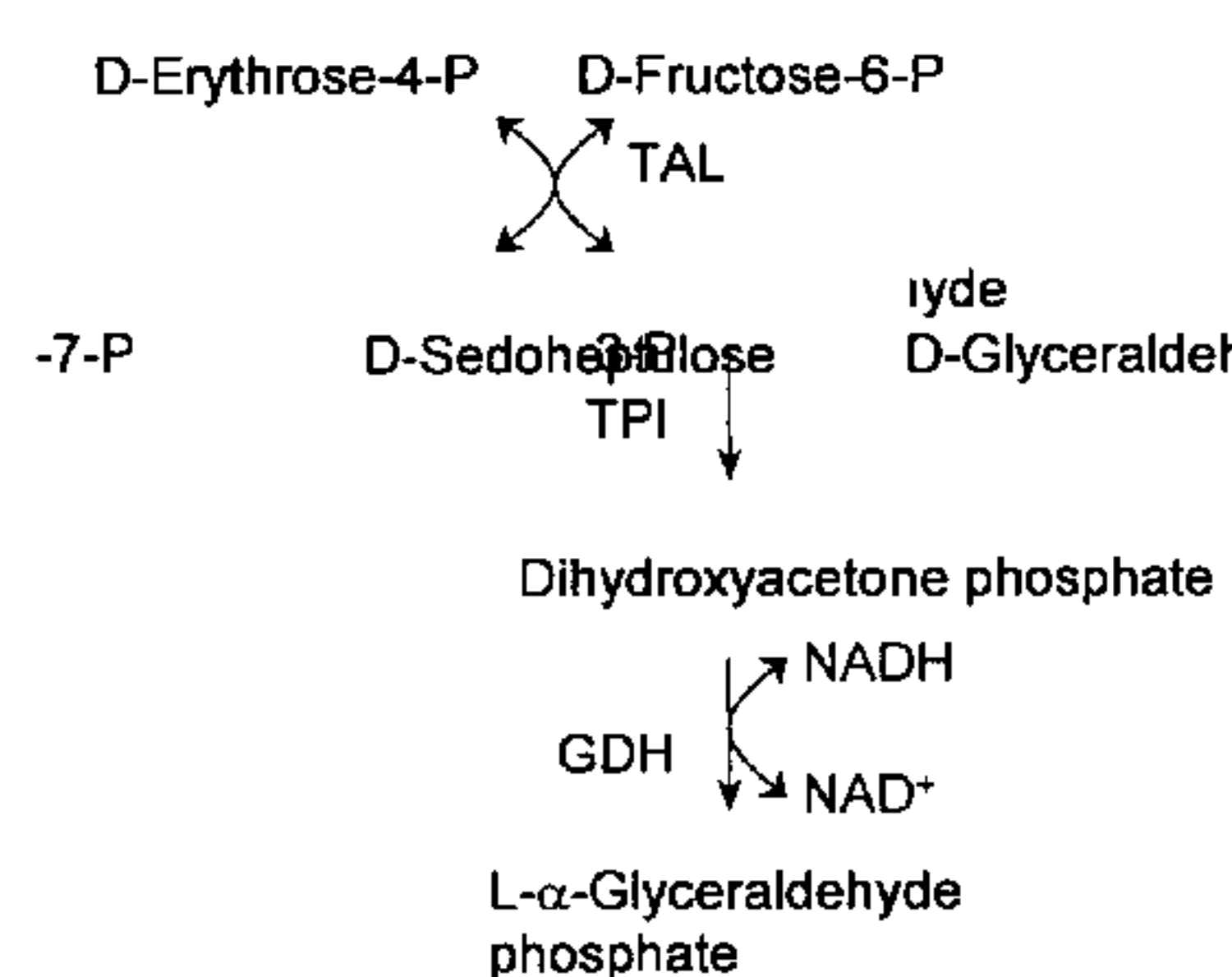
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(54) Title: IMPROVED ETHANOL PRODUCTION IN FERMENTATION OF MIXED SUGARS CONTAINING XYLOSE IN THE PRESENCE OF SUGAR ALCOHOLS

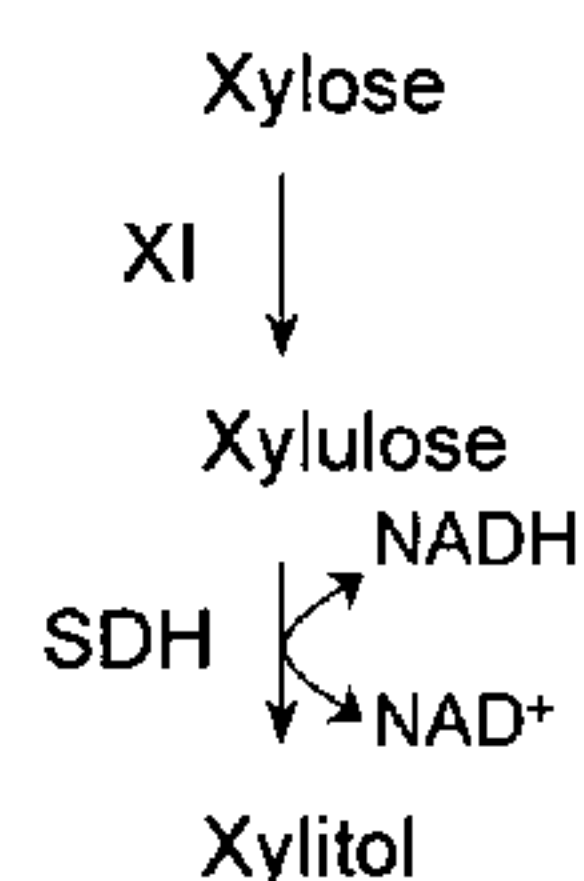
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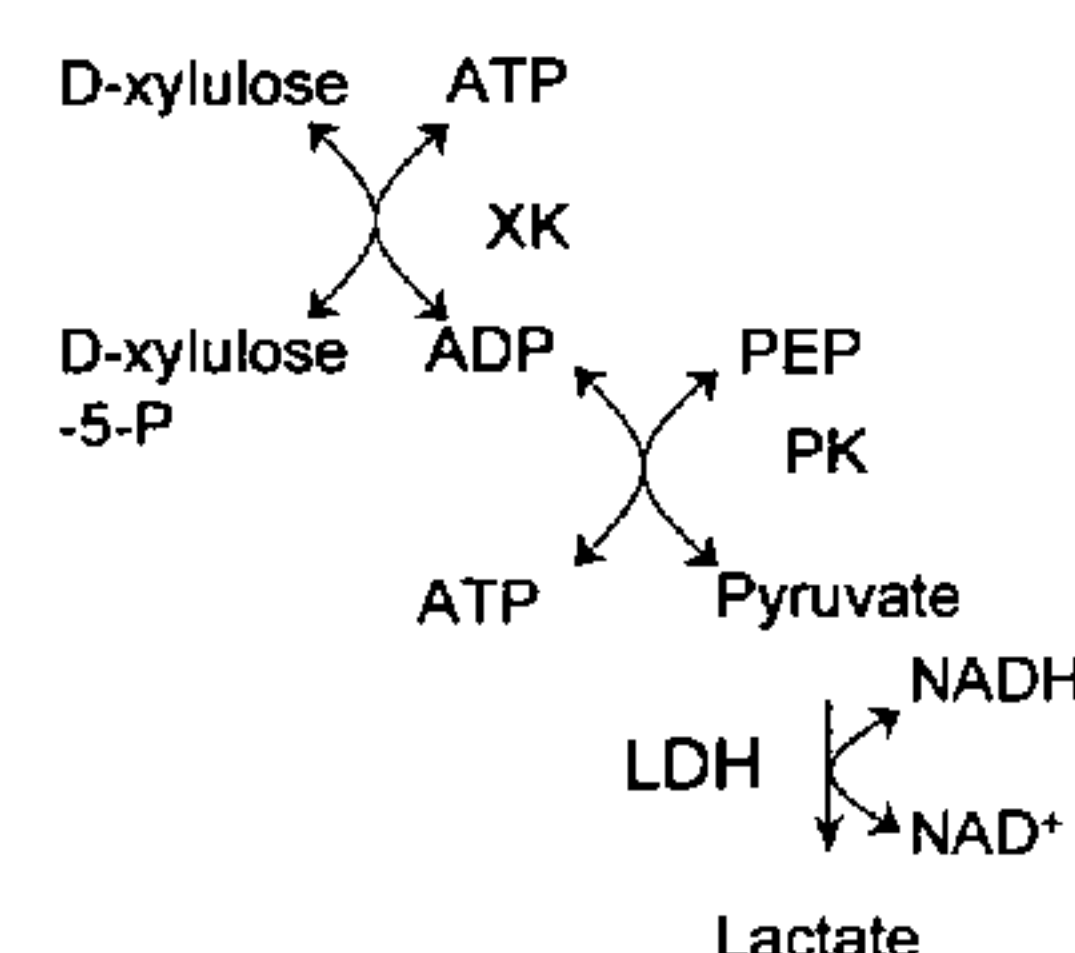
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C Xylose Isomerase Assay



D Xylok kinase Assay

(57) Abstract: Xylose-utilizing *Z. mobilis* strains were found to have improved ethanol production when grown in medium containing mixed sugars including xylose if sorbitol or mannitol was included in the medium. The effect was seen in concentrations of mixed sugars where no growth lag period occurs, as well as in higher sugars concentrations.

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TITLEIMPROVED ETHANOL PRODUCTION IN FERMENTATION OF MIXED
SUGARS CONTAINING XYLOSE

This application claims the benefit of U.S. Provisional Application No. 60/847997, filed September 28, 2006.

STATEMENT OF GOVERNMENT RIGHTS

This invention was made with United States government support under Contract Nos. 04-03-CA-70224 and DE-FC36-03GO13146 awarded by the Department of Energy. The government has certain rights in this invention.

FIELD OF INVENTION

The invention relates to the fields of microbiology and genetic engineering. More specifically, a method of improving ethanol production during fermentation of xylose-containing mixed sugars was developed.

BACKGROUND

Fuel ethanol produced from renewable resources is one of the long-term solutions to global fossil fuel shortages, rising energy costs, and global warming effects related to increased atmospheric carbon dioxide. Fuel ethanol from renewable resources is produced by fermentation of sugars. Currently in the United States, glucose derived from corn grain is the most abundant sugar source for ethanol production. Due to the demands for corn grain as a feed and food supply, methods of converting various types of cellulosic biomass (including hemicellulose) to fermentable sugars are being developed. Sugar derived from this biomass source is a mixture of hexoses and pentoses, primarily glucose and xylose. As a result of developments in cellulosic biomass processing, these sugars may be released in high concentrations and used in fermentation in high concentrations to produce ethanol, with reduced

water consumption and higher throughput. As such, conversion of biomass to ethanol poses great possibility for improving environmental impacts compared to fossil fuel ethanol production. Further, it provides a potentially economically viable alternative to fossil fuel ethanol production.

In addition to improvements in biomass processing, genetic engineering has been used to make improvements in microorganisms that are able to produce ethanol. In order to enhance the utilization of sugars from cellulosic biomass, the ethanologen *Zymomonas* (*i.e.*, *Z. mobilis*) has been made capable of utilizing xylose by engineering strains for expression of four enzymes: 1) xylose isomerase, which catalyses the conversion of xylose to xylulose; 2) xylulokinase, which phosphorylates xylulose to form xylulose 5-phosphate; 3) transketolase; and 4) transaldolase (US 5514583, US 6566107; Zhang et al. (1995) Science 267:240-243). Though these strains do metabolize xylose, at high xylose concentration the xylose is not fully utilized, so that the theoretical ethanol yield is not achieved. The ethanol yield also is limited due to synthesis of xylitol as a by-product of xylose metabolism (Feldmann et al. (1992) Appl Microbiol Biotechnol 38: 354-361; Kim et al. (2000) Applied and Environmental Microbiology 66:186-193). Xylitol is toxic to cells due to its phosphorylation to xylitol 5-phosphate, which is a compound that accumulates in the cell and inhibits growth. The yield of ethanol is also reduced due to the synthesis of xylitol, since xylose-utilizing recombinant strains of *Z. mobilis* cannot convert xylitol to ethanol. In addition, xylitol is a potent inhibitor of xylose isomerase, which catalyzes the first step of xylose utilization in the engineered xylose metabolism pathway. Therefore, fermentations in high sugar medium including xylose, with xylose-utilizing *Z. mobilis*, do not achieve maximal xylose usage and ethanol production.

Complete use of 8% xylose in a sugars mixture with 4% glucose, by xylose utilizing *Z. mobilis*, took 2-3 days (Lawford and Rousseau (1999) Appl Biochem and Biotech. 77-79: 235-249). Complete use of 65 g/L xylose in a mixture with 65 g/L glucose required 48 hours (Joachimsthal and Rogers (2000) Appl Biochem and Biotechnol 84-86: 343-356), and

using higher concentrations of sugars (75 g/L xylose and 75 g/L glucose) resulted in incomplete xylose utilization.

Sorbitol has been added as an osmoprotectant to enhance growth of non-engineered *Z. mobilis* in high concentrations of glucose (Loos et al. (1994) J Bacteriol 176:7688-7693). Sorbitol was accumulated intracellularly. In addition, sorbitol was shown to be produced by the cells and accumulated when growing on high sucrose. Any effects of sorbitol on production of ethanol by *Z. mobilis* strains that are engineered to utilize xylose, when grown in the presence of a sugar mixture including xylose, have not been determined previously.

There remains a need to develop fermentation conditions that enhance ethanol production in sugar media including xylose, allowing xylose-utilizing strains to reach their maximal ethanol production capacity with maximal xylose utilization in reduced time.

SUMMARY OF INVENTION

The present invention provides a method for improving the production of ethanol made by fermentation. In the instant method, *Zymomonas mobilis* cells that have been engineered to express genes involved in xylose utilization are grown in a medium containing mixed sugars, including xylose and at least one additional sugar. The medium also includes sorbitol, mannitol, galactitol, or ribitol (also called adonitol), which results in increased xylose utilization and increased ethanol production. Production of the by-product xylitol is also reduced. In one embodiment of the invention the method comprises:

- a) providing recombinant *Zymomonas* cells capable of converting xylose to ethanol;
- b) providing a suitable medium comprising (i) a mixed sugar composition comprising xylose and at least one additional sugar, and (ii) at least one sugar alcohol selected from the group consisting of sorbitol, mannitol, galactitol, and ribitol; and
- c) contacting (a) with (b) whereby the *Z. mobilis* cells produce ethanol.

In certain embodiments the contacting of step (c) above occurs for at least about 24 hours. The contacting above may occur at a temperature of about 25 ° C to about 40 ° C, or about 30°C to about 37° C. The contacting above may occur at a pH of about 4.5 to about 7.5, or a pH of about 5.0 to about 6.0.

In another embodiment, the contacting occurs by inoculating the cells of (a) into the medium of (b) using an inoculation ratio that is between about 0.01% and about 20% (v/v), or about 0.1% and about 20%.

In one embodiment the xylose and at least one additional sugar are produced from biomass that has been treated and/or saccharified.

In another embodiment the mixed sugar composition comprises at least about 10% xylose, or in an alternate embodiment the mixed sugar composition comprises about 40% to about 60% xylose.

In another embodiment the contacting of (a) and (b) occurs under fermentation conditions without supplying gases.

Another aspect of the invention is the xylose-utilizing *Z. mobilis* strain referred to herein as ZW658, ATCC Deposit No. PTA-7858, deposited September 12, 2006.

BRIEF DESCRIPTION OF THE FIGURES, SEQUENCE DESCRIPTIONS AND BIOLOGICAL DEPOSITS

The invention can be more fully understood from the following detailed description, the figures, and the accompanying sequence descriptions that form a part of this application.

Figure 1 shows the strategies for enzyme assays of transketolase (A), transaldolase (B), xylose isomerase (C), and xyulokinase (D).

Figure 2 shows a plasmid map of pMODPgapaltktCm.

Figure 3 shows a plasmid map of pMODPgapxylABCm.

Figure 4 shows a graph of xylose isomerase (XI) and xylulokinase (XK) activities in T2C, T3C, T4C, and T5C lines transformed with PgapxylAB.

Figure 5 shows a graph of transaldolase (TAL) and transketolase (TKT) activities in T2C, T3C, T4C, and T5C lines transformed with *PgapxylAB*.

Figure 6 shows a graph of % theoretical ethanol yield and % xylose utilization of selected adapted xylose-utilizing strain colonies.

Figure 7 shows a graph of growth of adapted xylose-utilizing strains at 70 hr on RM (rich medium) with 5% xylose (RMX5%) before and after growing 50 generations in RM with 5% glucose (RMG).

Figure 8 shows graphs of growth, glucose or xylose utilization, and ethanol and xylitol production for the selected strain, ZW658 in comparison to the control, 8b, in RM + 10% glucose (RMG10%) (A, B) and RM + 8% xylose (RMX8%) (C, D).

Figure 9 shows graphs of growth, glucose and xylose utilization, and ethanol and xylitol production for the selected strain, ZW658 in comparison to the control, 8b, in RM + 10% glucose and 8% xylose without acetate (A, B) or with 0.6% acetate (C, D).

Figure 10 shows graphs of the effects of sorbitol on growth (A) and ethanol production (B) on xylose-utilizing *Z. mobilis* grown on glucose alone or xylose alone.

Figure 11 shows a graph of the growth, glucose utilization, xylose utilization, ethanol production, acetic acid production and xylitol production of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the presence of sorbitol.

Figure 12 shows a graph of the growth, glucose utilization, xylose utilization, ethanol production, acetic acid concentration and xylitol production of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the absence of sorbitol.

Figure 13 shows a graph of the growth, glucose utilization, xylose utilization, ethanol production, acetic acid concentration and xylitol production of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the presence of acetate and sorbitol.

Figure 14 shows a graph of the growth, glucose utilization, xylose utilization, ethanol production, acetic acid concentration and xylitol

production of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the presence of acetate and absence of sorbitol.

Figure 15 shows a graph of the growth, glucose utilization, xylose utilization, ethanol production, acetic acid concentration and xylitol production of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the presence of glutamate.

Figure 16 shows a graph of the growth of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the presence of varying amounts of sorbitol between 0 and 10 mM.

Figure 17 shows graphs of the growth (A) and ethanol production (B) of xylose-utilizing *Z. mobilis* grown on glucose and xylose mixed sugar in the presence of varying amounts of sorbitol between 10 mM and 200 mM.

Figure 18 shows graphs of growth (A), xylose utilization (B), and ethanol production (C) in the presence of different polyols.

Figure 19 shows graphs of growth (A), xylose utilization (B), and ethanol production (C) in the presence of acetate and different polyols.

Figure 20 shows graphs of growth (A) and glucose utilization (B) in the presence of different sugar alcohols.

Figure 21 shows graphs of xylose utilization (A) and ethanol production (B) in the presence of different sugar alcohols.

Figure 22 shows graphs of growth (A) and glucose utilization (B) in the presence of different sugar alcohols.

Figure 23 shows graphs of xylose utilization (A) and ethanol production (B) in the presence of different sugar alcohols.

The following sequences conform with 37 C.F.R. 1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide

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and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

A Sequence Listing is provided herewith on Compact Disk.

The Compact Discs are submitted in duplicate and are identical to one another. The discs are labeled "Copy 1 – Sequence Listing" and "Copy 2 Sequence listing" The discs contain the following file: CL3425 seq list.ST25.

SEQ IDs NO:1 and 2 are the nucleotide sequences of primers for amplification of a DNA fragment containing the glyceraldehyde-3-phosphate dehydrogenase gene promoter (P_{gap}) from pZB4.

SEQ IDs NO:3 and 4 are the nucleotide sequences of primers for amplification of a DNA fragment containing a *tal* coding region from pZB4.

SEQ IDs NO:5 and 6 are the nucleotide sequences of primers for amplification of a DNA fragment containing $P_{gap}tal$ from the P_{gap} and *tal* fragments.

SEQ IDs NO:7 and 8 are the nucleotide sequences of primers for amplification of a DNA fragment containing *loxP::Cm* from pZB186.

SEQ ID NO:9 is the complete nucleotide sequence for the pMODP_{gap}*tal**tk**t*Cm plasmid.

SEQ IDs NO:10 and 11 are the nucleotide sequences of primers for amplification of a 3 kb DNA fragment containing *tal* and *tk**t* coding regions in transformants receiving pMODP_{gap}*tal**tk**t*Cm.

SEQ ID NO:12 is the complete nucleotide sequence for the pMODP_{gap}*xy*/ABCm plasmid.

SEQ ID NOs:13 and 14 are the nucleotide sequences of primers for amplification of a 1.6 kb *Pgapxy*/A DNA fragment from the T2C, T3C, T4C and T5C integrants with pMODP_{gap}*xy*/ABCm.

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SEQ ID NOs:15 and 16 are the nucleotide sequences of primers for amplification of a 1.3 kb xylB DNA fragment from the T2C, T3C, T4C and T5C integrants with pMODP_{gap}xyl/ABCm.

Applicants made the following biological deposit under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure at the American Type Culture Collection (ATCC) 10801 University Boulevard, Manassas, VA 20110-2209:

Depositor Identification Reference	International Depository Designation	Date of Deposit
ZW658	ATCC # PTA-7858	Sept. 12, 2006

DETAILED DESCRIPTION

Further, when an amount, concentration, or other value or parameter is given as either a range, preferred range, or a list of upper preferable values and lower preferable values, this is to be understood as specifically disclosing all ranges formed from any pair of any upper range limit or preferred value and any lower range limit or preferred value, regardless of whether ranges are separately disclosed. Where a range of numerical values is recited herein, unless otherwise stated, the range is intended to include the endpoints thereof, and all integers and fractions within the range. It is not intended that the scope of the invention be limited to the specific values recited when defining a range.

The present invention provides a method for the production of ethanol using fermentation that involves the addition of sorbitol, mannitol, galactitol, or ribitol to a medium containing mixed sugars including xylose. The medium including sorbitol provides for growth of xylose-utilizing *Zymomonas mobilis* strains, thus increasing the ability of such strains to produce of ethanol from the mixed sugars.

In this disclosure, a number of terms are used. The following definitions are provided:

RM is rich medium.

RMG5% is RM + 5% glucose.

RMG10% is RM + 10% glucose.

RMX8% is RM + 8% xylose.

RMX2% is RM + 2% xylose.

RMX5% is RM + 5% xylose.

RMGX10%8% is RM + 10% glucose and 8% xylose.

RMGX5%8% is RM + 5% glucose and 8% xylose.

The term “fermentable sugar” refers to oligosaccharides and monosaccharides that can be used as a carbon source by a microorganism in a fermentation process.

As used herein “suitable medium” refers to a medium that supports growth of *Z. mobilis* under various conditions. The suitable medium includes xylose and at least one additional sugar, and one or more sugar alcohol that may be sorbitol, mannitol, galactitol, ribitol or mixtures thereof. In addition, if a sufficient concentration of the sugar alcohol is not present in the medium, the medium is one that while supporting growth, does not provide for optimal xylose utilization.

The term “lignocellulosic” refers to a composition comprising both lignin and cellulose. Lignocellulosic material may also comprise hemicellulose.

The term “cellulosic” refers to a composition comprising cellulose and additional components, including hemicellulose.

The term “saccharification” refers to the production of fermentable sugars from polysaccharides.

The term “pretreated biomass” means biomass that has been subjected to pretreatment prior to saccharification.

“Biomass” refers to any cellulosic or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or monosaccharides. Biomass may also comprise additional components, such as protein and/or lipid. Biomass may be derived from a single source, or biomass can comprise a mixture derived from more than one source; for example,

biomass could comprise a mixture of corn cobs and corn stover, or a mixture of grass and leaves. Biomass includes, but is not limited to, bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. Examples of biomass include, but are not limited to, corn grain, corn cobs, crop residues such as corn husks, corn stover, grasses, wheat, wheat straw, barley, barley straw, hay, rice straw, switchgrass, waste paper, sugar cane bagasse, sorghum, soy, components obtained from milling of grains, trees, branches, roots, leaves, wood chips, sawdust, shrubs and bushes, vegetables, fruits, flowers and animal manure.

"Biomass hydrolysate" refers to the product resulting from saccharification of biomass. The biomass may also be pretreated prior to saccharification.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" or "wild type gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes.

The term "genetic construct" refers to a nucleic acid fragment that encodes for expression of one or more specific proteins. In the gene construct the gene may be native, chimeric, or foreign in

nature. Typically a genetic construct will comprise a "coding sequence". A "coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence.

"Promoter" or "Initiation control regions" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters which cause a gene to be expressed in most cell types at most times are commonly referred to as "constitutive promoters".

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from a gene. Expression may also refer to translation of mRNA into a polypeptide.

The term "transformation" as used herein, refers to the transfer of a nucleic acid fragment into a host organism, resulting in genetically stable inheritance. The transferred nucleic acid may be in the form of a plasmid maintained in the host cell, or some transferred nucleic acid may be integrated into the genome of the host cell. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid" and "vector" as used herein, refer to an extra chromosomal element often carrying genes which are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA molecules. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a

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single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., that the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term "selectable marker" means an identifying factor, usually an antibiotic or chemical resistance gene, that is able to be selected for based upon the marker gene's effect, i.e., resistance to an antibiotic, wherein the effect is used to track the inheritance of a nucleic acid of interest and/or to identify a cell or organism that has inherited the nucleic acid of interest.

Xylose-utilizing *Zymomonas* strain

Any strain of *Z. mobilis* that has been engineered for xylose metabolism may be used in fermentations to produce ethanol according to the present method. Typically four genes are introduced into *Z. mobilis* for expression of four enzymes involved in xylose metabolism as described in US 5514583.

These include genes encoding xylose isomerase, which catalyses the conversion of xylose to xylulose, and xylulokinase, which phosphorylates xylulose to form xylulose 5-phosphate. In addition, transketolase and transaldolase, two enzymes of the pentose phosphate pathway, convert xylulose 5-phosphate to intermediates that couple pentose metabolism to the glycolytic Entner-Doudoroff pathway permitting the metabolism of xylose

to ethanol. DNA fragments with sequences encoding these enzymes may be obtained from any of numerous microorganisms that are able to metabolize xylose, such as enteric bacteria, and some yeasts and fungi. The coding regions of these genes are operably linked to promoters that are expressed in *Z. mobilis* cells, such as the promoter of *Z. mobilis* glyceraldehyde-3-phosphate dehydrogenase or *Z. mobilis* enolase, and other regulatory elements. These chimeric genes are typically constructed in vectors that are transformed into *Z. mobilis* cells to engineer a xylose utilizing strain. Xylose utilizing *Z. mobilis* strains that are known and new strains of *Z. mobilis* that are engineered for xylose utilization may be used in the present method. Xylose utilizing *Z. mobilis* strains include *Z. mobilis* ZM4(pZB5) (Joachimsthal and Rogers, *supra*), *Z. mobilis* CP4:pZB5 (Lawford et al., *supra*), *Z. mobilis* 8b (US 20030162271), ZW658 (described herein; ATCC # PTA-7858), and ZW800, ZW801-4 and ZW801-6 (described in co-owned and co-pending US patent application 60/847813).

Particularly useful are the strains *Z. mobilis* 8b, ZW658, ZW800, ZW801-4 and ZW801-6.

The xylose-utilizing *Z. mobilis* strain ZW658 was found to have distinct properties as compared to the *Z. mobilis* 8b strain, as shown in Example 2 herein. Under the same fermentation conditions ZW658 shows reduced xylitol production, increased xylose utilization, and increased ethanol production as compared to 8b. These properties are evident following the adaptation process, which did not include mutagenesis as in the case of the 8b strain adaptation.

The xylose-utilizing *Z. mobilis* strain ZW800 has an additional genetic modification that inactivates the gene encoding glucose-fructose oxidoreductase (GFOR) and results in reduced xylitol synthesis. Specifically, ZW800 has an insertion of a selection marker into the GFOR coding region that disrupts expression of the encoded protein, as described in detail in co-owned and co-pending US patent application 60/847813 .

The GFOR knockout mutant produced reduced amounts of xylitol when grown on xylose-containing sugar mixtures, consumed more xylose, and produced

higher concentrations and yields of ethanol when grown in high sugar mixtures in the presence of sorbitol than the parent strain that expressed GFOR. Described also in US patent application 60/847813 are strains ZW801-4 and ZW801-6 which were derived from ZW800 and have had the selection marker removed by Cre/lox mediated excision, resulting in a deletion and lox site footprint addition within the GFOR coding region that disrupts the GFOR coding region. Strains ZW801-4 and ZW801-6 have the same properties described above for ZW800.

Z. mobilis strains that are additionally engineered to utilize sugars other than xylose, which it does not naturally use, may be used in the present method. An example is a strain of *Z. mobilis* engineered for arabinose utilization as described in US 5843760.

Mixed Sugars

In the present method, cells of xylose-utilizing *Z. mobilis* strains are grown in medium containing mixed sugars. The mixed sugars referred to herein include xylose, which the *Z. mobilis* cells have been engineered to utilize, and at least one additional sugar. Any sugar that may provide an energy source for metabolism of the *Z. mobilis* cells, or any sugar that is present in a mixture containing xylose may be included. It is desirable to grow xylose-utilizing *Z. mobilis* cells on sugars that are produced from biomass saccharification. Typically biomass is pretreated, for example as described in Patent Application WO2004/081185 and in co-owned and co-pending US application 60/670437, and then treated with saccharification enzymes as reviewed in Lynd, L. R., *et al.* (Microbiol. Mol. Biol. Rev. (2002) 66:506-577). Biomass saccharification produces sugars that may typically include a mixture of xylose with glucose, fructose, sucrose, galactose, mannose, and/or arabinose.

The mixed sugars may be used in a high concentration in medium for growth of the *Z. mobilis* cells. This allows the direct use of biomass saccharification sugars, or use with little dilution, thereby reducing fermentation volumes, which is desirable for commercial scale ethanol

production. High sugars concentrations are used so that greater concentrations of ethanol may be produced. The mixed sugar concentration is typically at least about 120 g/L and up to about 300 g/L. In this range of mixed sugars concentration, a sugar alcohol may be added to the growth medium to provide the beneficial effect that is described below. Particularly useful is a mixed sugar concentration that is between about 150 g/L and about 235 g/L.

The ratio of different sugars may vary in the mixture, with xylose typically at least about 10% of the total amount of sugars. Preferably xylose is between about 40% and about 60%. Fructose is present in sugars produced by saccharification of some biomass such as sugar cane bagasse, and may replace a portion of xylose or glucose, such that xylose remains at least about 10% of the sugar mixture. In addition, arabinose is derived from hemicellulose and thus is a typical component of mixed sugars derived from saccharified biomass containing hemicellulose.

Improved Ethanol Production

In the present method, the production of ethanol by xylose-utilizing *Z. mobilis* is improved by including a sugar alcohol from the family of 6-carbon sugars, selected from sorbitol (including D-sorbitol or L-sorbitol), mannitol, galactitol, and a mixture thereof, in a medium containing mixed sugars including xylose. In addition to these effective sugar alcohols, the 5-carbon sugar alcohol ribitol (also called adonitol) is also effective and may be used. While addition of sorbitol to medium containing only xylose as the sugar had no effect on ethanol production, as demonstrated in Example 3 herein, it was surprisingly found that sorbitol had a beneficial effect on ethanol production by xylose-utilizing *Z. mobilis* when xylose was included in a mixed sugar composition in the medium. Applicants found that in the presence of sorbitol, ethanol production was increased significantly in the mixed sugar medium that included xylose. In one embodiment, the mixed sugar comprises xylose and one or more other sugars. In one embodiment the one or more other mixed sugar is glucose.

In general, production of ethanol varies depending on fermentation conditions used, including the composition of the medium. A medium composition that supports growth may not provide for optimal xylose utilization in xylose-utilizing *Z. mobilis* due to the presence of high sugars concentration, inhibitors, or other factors. Addition of sorbitol, or any of the other sugar alcohols listed above, to such a medium renders it a suitable medium in the present method. For example, media may include inhibitors such as acetate, as is present in saccharified biomass, which affect ethanol production levels. Addition of sorbitol to mixed sugar medium containing acetate was shown herein to improve ethanol production by xylose-utilizing *Z. mobilis*. In the presence of sorbitol, or other designated sugar alcohol, ethanol production may be increased by at least about 5%, though the exact amount varies depending upon the composition of the suitable medium in use. Comparisons are made at stationary phase of the fermentation culture, when maximal ethanol production is reached.

The increase in ethanol production in the presence of sorbitol or other designated sugar alcohol, may occur when xylose-utilizing *Z. mobilis* is grown in media containing mixed sugars at concentrations where no growth lag occurs. The concentration of mixed sugars in media that support growth with no lag varies depending on other components in the medium, such as inhibitors. For example, an obvious lag period typically does not occur when xylose-utilizing *Z. mobilis* is grown in media without acetate containing mixed sugars at concentrations below about 165 g/L. With no lag in growth occurring, the *Z. mobilis* cells are not characterized as undergoing osmotic stress, since the typical feature of osmotic stress is the growth lag. Thus, it is surprising that sorbitol or other sugar alcohol described herein increases ethanol production under the no-lag condition with mixed sugars, which is distinguished from the osmotic effect of sorbitol on wild type *Z. mobilis* growth observed by Loos et al, (*supra*) in high concentrations of glucose. At higher concentrations of mixed sugars, where typically a growth lag does occur, the same effect of sorbitol or other sugar alcohol described herein on ethanol production, characterized for no-lag conditions, continues to occur.

In the present method where sorbitol or other sugar alcohol described herein is included in the medium with mixed sugars, forming a suitable medium, xylose-utilizing *Z. mobilis* also showed increased utilization of xylose. As with ethanol production described above, in general xylose utilization varies depending on fermentation conditions, including the composition of the medium. For example, media may include inhibitors such as acetate, as is present in saccharified biomass, which can affect xylose utilization. Addition of sorbitol to the mixed sugar medium containing acetate improved xylose utilization by xylose-utilizing *Z. mobilis*. In the presence of sorbitol or other sugar alcohol described herein, xylose utilization may be increased by at least about 5% over the level of xylose utilized under the same conditions without sorbitol or other sugar alcohol described herein though the exact amount varies depending upon the composition of the suitable medium in use. For example, in Examples 4 and 5 herein, in the absence of acetate in the medium, 85 g/L of xylose, in a sugars mixture with 100 g/L glucose, was completely utilized within about 30 hours in the presence of sorbitol, while without sorbitol in the medium, about 20% of the xylose remained when the culture reached stationary phase at about 60 hours. Comparisons are made at stationary phase of the fermentation culture, when maximal xylose utilization is reached.

In addition, in accordance with the discovery herein, when xylose-utilizing *Z. mobilis* is grown on medium containing mixed sugars under conditions where xylitol is produced with no sorbitol present, the amount of xylitol produced is decreased when sorbitol is included in the medium. Reduction in xylitol production is at least about 6% in the presence of sorbitol, though the exact amount varies depending upon the composition of the suitable medium in use. For example, in Examples 4 and 5 herein, xylitol was reduced with addition of sorbitol by about 3-fold.

Without wishing to be bound by theory, it is thought that reduced xylitol production allows increased xylose utilization. The increased xylose utilization, along with the reduction in carbon flow to the by-product xylitol, together allow increased ethanol production. Therefore, the reduced xylitol

production, increased xylose utilization, and increased ethanol production are all manifestations of improved performance of xylose-utilizing *Z. mobilis* grown on the suitable medium described herein as containing mixed sugars, including xylose, and sorbitol or other sugar alcohol as described above.

Sugar alcohols such as erythritol, maltitol, and lactitol, as well as the frequently used osmoprotectant glutamate, do not increase ethanol production nor do they increase xylose utilization by xylose-utilizing *Z. mobilis* grown on suitable medium containing mixed sugars. Glutamate does act as a typical osmoprotectant, eliminating the lag period for initial growth when the xylose-utilizing *Z. mobilis* is grown under conditions where a lag occurs. However, in the presence of glutamate, xylitol production is not reduced, xylose utilization is not enhanced, and ethanol production is not improved. Thus, a compound that does act as an osmoprotectant does not improve performance as do sorbitol and mannitol, galactitol, or ribitol. This finding indicates that these sugar alcohols are performance enhancers for xylose-utilizing *Z. mobilis*.

Sorbitol, mannitol, galactitol, ribitol or mixtures thereof may be added in an amount that is between about 0.5 mM and about 200 mM. In addition, these sugar alcohols may be used together in any ratio in a total amount that is between about 0.5 mM and about 200 mM. Particularly useful is an amount that is between about 2 mM and about 100 mM, with 5 mM to 20 mM preferred.

For production of ethanol, recombinant xylose-utilizing *Z. mobilis* is brought in contact with a suitable medium that contains a mixed sugar including xylose, and either sorbitol, other designated sugar alcohol, or a mixture thereof. The xylose-utilizing *Z. mobilis* is typically inoculated into the suitable medium. An inoculation ratio that is between about 0.01% and about 20% (v/v) is desirable for initiating a fermentation culture. Typically, an inoculation ratio that is between about 0.1% and about 20% (v/v) is used, while more typically the ratio is between about 1% and about 20% (v/v). The *Z. mobilis* grows in the medium where fermentation occurs and ethanol is produced. The fermentation is run without supplying air,

oxygen, or other gases (which may include conditions such as anaerobic, microaerobic, or microaerophilic fermentation), for at least about 24 hours, and may be run for 30 or more hours. The timing to reach maximal ethanol production is variable, depending on the fermentation conditions.

Typically, if inhibitors are present in the medium, a longer fermentation period is required. The contacting, and continuing fermentation, are typically at temperatures that are between about 25 ° C and about 40 ° C, at a pH of about 4.5 to about 7.5. More suitable is contacting, and continuing fermentation, at temperatures between about 30°C and about 37° C. More suitable is contacting, and continuing fermentation, at pH of about 5.0 and 6.0.

Fermentation for Ethanol Production

In the present method xylose-utilizing *Z. mobilis* may be grown in a suitable medium containing mixed sugars including xylose in laboratory scale fermenters, and in scaled-up fermentation where commercial quantities of ethanol are produced. Where commercial production of ethanol is desired, a variety of culture methodologies may be applied. For example, large-scale production from xylose-utilizing *Z. mobilis* may be produced by both batch and continuous culture methodologies. A classical batch culturing method is a closed system where the composition of the medium is set at the beginning of the culture and not subjected to artificial alterations during the culturing process. Thus, at the beginning of the culturing process the medium is inoculated with the desired organism and growth or metabolic activity is permitted to occur adding nothing to the system. Typically, however, a "batch" culture is batch with respect to the addition of carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. In batch systems the metabolite and biomass compositions of the system change constantly up to the time the culture is terminated. Within batch cultures cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase will eventually die. Cells in log phase are often

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responsible for the bulk of production of end product or intermediate in some systems. Stationary or post-exponential phase production can be obtained in other systems.

A variation on the standard batch system is the Fed-Batch system. Fed-Batch culture processes are also suitable and comprise a typical batch system with the exception that the substrate is added in increments as the culture progresses. Fed-Batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the medium. Measurement of the actual substrate concentration in Fed-Batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH and the partial pressure of waste gases such as CO₂. Batch and Fed-Batch culturing methods are common and well known in the art and examples may be found in *Biotechnology: A Textbook of Industrial Microbiology*, Crueger, Crueger, and Brock, Second Edition (1989) Sinauer Associates, Inc., Sunderland, MA, or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.* 36, 227, (1992).

Commercial production of ethanol may also be accomplished with a continuous culture. Continuous cultures are open systems where a defined culture medium is added continuously to a bioreactor and an equal amount of conditioned medium is removed simultaneously for processing. Continuous cultures generally maintain the cells at a constant high liquid phase density where cells are primarily in log phase growth. Alternatively, continuous culture may be practiced with immobilized cells where carbon and nutrients are continuously added, and valuable products, by-products or waste products are continuously removed from the cell mass. Cell immobilization may be performed using a wide range of solid supports composed of natural and/or synthetic materials as is known to one skilled in the art.

Continuous or semi-continuous culture allows for the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one method will maintain a limiting nutrient

such as the carbon source or nitrogen level at a fixed rate and allow all other parameters to moderate. In other systems a number of factors affecting growth can be altered continuously while the cell concentration, measured by medium turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions and thus the cell loss due to medium being drawn off must be balanced against the cell growth rate in the culture. Methods of modulating nutrients and growth factors for continuous culture processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

Particularly suitable for ethanol production is a fermentation regime as follows. The desired xylose-utilizing *Z. mobilis* strain is grown in shake flasks in semi-complex medium at about 30 °C to about 37 °C with shaking at about 150 rpm in orbital shakers and then transferred to a 1-10 L seed fermentor containing similar medium. The seed culture is grown in the seed fermentor anaerobically until OD₆₀₀ is between 3 and 6, when it is transferred to the production fermentor where the fermentation parameters are optimized for ethanol production. Typical inoculum volumes transferred from the seed tank to the production tank range from about 2% to about 20% v/v. Typical fermentation medium contains minimal medium components such as potassium phosphate (1.0 – 10.0 g/l), ammonium sulfate (0- 2.0 g/l), magnesium sulfate (0 – 5.0 g/l), a complex nitrogen source such as yeast extract or soy based products (0 – 10 g/l). A final concentration of about 5-20 mM sorbitol or designated sugar alcohol is present in the medium. Mixed sugars including xylose and at least one additional sugar such as glucose (or sucrose), providing a carbon source, are continually added to the fermentation vessel on depletion of the initial batched carbon source (50 - 200 g/l) to maximize ethanol rate and titer. Carbon source feed rates are adjusted dynamically to ensure that the culture is not accumulating glucose in excess, which could lead to build up of toxic byproducts such as acetic acid. In order to maximize yield of ethanol produced from substrate utilized, biomass growth is restricted by the amount of phosphate that is either batched initially or that is fed during

the course of the fermentation. The fermentation is controlled at pH 5.0 – 6.0 using caustic solution (such as ammonium hydroxide, potassium hydroxide, or sodium hydroxide) and either sulfuric or phosphoric acid. The temperature of the fermentor is controlled at 30 °C - 37 °C. In order to minimize foaming, antifoam agents (any class- silicone based, organic based etc) are added to the vessel as needed. An antibiotic, for which there is an antibiotic resistant marker in the strain, such as kanamycin, may be used optionally to minimize contamination.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used here are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (hereinafter “Maniatis”); and by Silhavy, T. J., Bennis, M. L. and Enquist, L. W., *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and by Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987).

The meaning of abbreviations is as follows: “kb” means kilobase(s), “bp” means base pairs, “nt” means nucleotide(s), “hr” means hour(s), “min” means minute(s), “sec” means second(s), “d” means day(s),

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"L" means liter(s), "ml" means milliliter(s), "μL" means microliter(s), "μg" means microgram(s), "ng" means nanogram(s), "mM" means millimolar, "μM" means micromolar, "nm" means nanometer(s), "μmol" means micromole(s), "pmol" means picomole(s), "Cm" means chloramphenicol, "Cm^r" means chloramphenicol resistant, "Cm^s" means chloramphenicol sensitive, "Sp^r" means spectinomycin resistance, "Sp^s" means spectinomycin sensitive, "XI" is xylose isomerase, "XK" is xylulokinase, "TAL" is transaldolase, "TKT" is transketolase, "EFT" means elapsed fermentation time, "RM" means rich medium containing 10 g/L yeast extract plus 2 g/L KH₂PO₄, "MM" means mating medium containing 10 g/L yeast extract, 5 g/L tryptone, 2.5 g/L (NH₄)₂SO₄ and 0.2 g/L KH₂PO₄.

Measurement of Optical Density (OD)

Approximately 1 ml of well mixed sample was obtained from flask or fermentor. First the sample absorbance was measured directly with a spectrophotometer (Amersham Biosciences Ultrospec 3300pro, GE Healthcare, Piscataway, New Jersey, USA). If the reading was lower than 0.6 unit, no dilution was needed. If the reading was higher than 0.6 unit, the sample was diluted with culture medium until the actual reading was in the range of 0.1 to 0.6 unit. The original OD was calculated from the dilution ratio and the actual reading.

Enzyme Assays

Preparation of Cell-Free Extracts of *Zymomonas* for Enzymatic Assays

Cells were grown in 50 ml of RM + 2% glucose at 30°C overnight to an OD₆₀₀ of 1.0-1.2. Cells were harvested by centrifugation at 4500 rpm for 10 min at 4°C. The supernatant was discarded and the cell pellet washed with 25 ml ice-cold sonication buffer. (10 mM Tris, pH 7.6, 10 mM MgCl₂), followed by centrifugation at 4500 rpm for 10 min. The pellet was resuspended in 2.0-2.5 ml sonication buffer plus 1 mM dithiothreitol. A 500 μl aliquot was centrifuged for 1 min in an eppendorfTM centrifuge at 4°C. Most of supernatant was discarded, leaving ~10-20 μl behind to keep the pellet from drying out. The cells were frozen and stored at -80 °C until

assayed. Prior to assay, the cells were thawed and resuspended with 500 μ l of sonication buffer + 1 mM DTT. The mix was sonicated 2x for 45 seconds at 62% duty cycle and an output control of 2 using a Branson sonifier 450, letting samples cool ~ 3-5 min between sonications. Samples were centrifuged at 14,000 rpm for 60 min in a Beckman microfuge at 4°C. The supernatant was transferred to a new tube and kept at 4°C. The Pierce BCA assay was used for determining protein concentrations.

The transketolase (TKT) assay was usually performed first since this enzyme is more labile than the others. A diagram of the TKT assay is shown in Figure 1A. In a microplate assay, 20 μ l of cell free extract was added to each well in a reaction mix, at 30°C, that included the following final concentrations of components: 0.37 mM NADP, 50 mM TrisHCl pH 7.5, 8.4 mM Mg Cl₂, 0.1 mM TPP ((thiamine pyrophosphate chloride), 0.6 mM E4P (erythrose-4-phosphate), 4mM BHP (betahydroxypyruvate), 4U/ml PGI (phosphoglucose isomerase), and 4 U/ml G6PD (glucose-6-phosphate dehydrogenase). The A₃₄₀ was read on a plate reader for 3-5 min. TKT activity was calculated as follows:

1 unit corresponds to the formation of 1 μ mol of D-fructose 6-phosphate/min at 30°C.

$$U (\mu\text{mole/min}) = \text{slope (dA}_{340}/\text{min}) * \text{volume of reaction } (\mu\text{L}) / 6220 / 0.55 \text{ cm}$$

(moles of NADP→NADPH is 6220 A₃₄₀ per mole per L in a 1 cm cuvette)

(pathlength of 200 μ l per well in microplate=0.55 cm)

$$\text{Specific Activity } (\mu\text{mole/min-mg}) = \mu\text{mole/min} / \text{protein concentration (mg)}$$

The basis of the transaldolase (TAL) assay is shown in Figure 1B. In a microplate assay, 20 μ l of cell free extract was added to each well in a reaction mix, at 30°C, that included the following final concentrations of components: 0.38 mM NADH, 87 mM thiethanolamine, 17 mM EDTA, 33 mM F6P (fructose-6-phosphate), 1.2 mM E4P (erythrose-4-phosphate), 2.0 U/ml GDH (Glycerol-3-phosphate dehydrogenase), and 20 U/ml TPI

(Triose phosphate isomerase). The plate was incubated for 5 min., then the A_{340} was read for 3-5 min. TAL activity was calculated as follows:

1 unit corresponds to the formation of 1 μ mol of D-glyceraldehyde per minute at 30°C

$U (\mu\text{mole/min}) = \text{slope } (dA_{340}/\text{min}) * \text{volume of reaction } (\mu\text{L}) / 6220 / 0.55 \text{ cm}$

(moles of $\text{NADH} \rightarrow \text{NAD}$ is 6220 A_{340} per mole per L in a 1 cm cuvette)

(pathlength of 200 μ l per well in microplate=0.55 cm)

Specific Activity ($\mu\text{mole/min-mg}$) = $\mu\text{mole/min}$ / protein

The basis of the xylose isomerase (XI) assay is shown in Figure 1C.

In a microplate assay, 20 μ l of cell free extract was added to each well in a reaction mix, at 30°C, that included the following final concentrations of components: 0.256 mM NADH, 50 mM xylose, 10 mM MgSO_4 , 10 mM thiethanolamine, and 1U/ml SDH (sorbitol dehydrogenase). The A_{340} was read on a plate reader for 3-5 min. XI activity was calculated as follows:

1 unit of XI corresponds to the formation of 1 μ mol of D-xylulose per minute at 30°C

$U (\mu\text{mole/min}) = \text{slope } (dA_{340}/\text{min}) * \text{volume of reaction } (\mu\text{L}) / 6220 / 0.55 \text{ cm}$

(moles of $\text{NADHP} \rightarrow \text{NAD}$ is 6220 A_{340} per mole per L in a 1 cm cuvette)

(pathlength of 200 μ l per well in microplate=0.55 cm)

Specific Activity ($\mu\text{mole/min-mg}$) = $\mu\text{mole/min}$ / protein concentration (mg)

The basis of the xylulokinase (XK) assay is shown in Figure 1D. In a microplate assay, 20 μ l of cell free extract was added to each well in a reaction mix, at 30°C, that included the following final concentrations of components: 0.2 mM NADH, 50 mM Tris HCl pH 7.5, 2.0 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$,

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2.0 M ATP 0.2 M PEP (phosphoenolpyruvate), 8.5 mM D-xylulose, 5 U/ml PK (pyruvate kinase), and 5 U/ml LDH (lactate dehydrogenase). The A_{340} was read on a plate reader for 3-5 min. XI activity was calculated as follows:

1 unit corresponds to the formation of 1 μ mol of D-xylulose-5-phosphate per minute at 30°C

$U (\mu\text{mole/min}) = \text{slope } (dA_{340}/\text{min}) * \text{volume of reaction } (\mu\text{L}) / 6220 / 0.55 \text{ cm}$

(moles of $\text{NADH} \rightarrow \text{NAD}$ is 6220 A_{340} per mole per L in a 1 cm cuvette)

(pathlength of 200 μ l per well in microplate=0.55 cm)

Specific Activity ($\mu\text{mole/min-mg}$) = $\mu\text{mole/min} / \text{protein concentration (mg)}$

HPLC Method

The analysis of sugars, acetate, ethanol, and other by-products was done with an Agilent 1100 series HPLC and Agilent ChemStation software for LC 3D. The column was BioRad Aminex HPX-87H (HPLC Organic Analysis Column 125-0140) with BioRad Micro-Guard Cartridge Cation-H (125-0129). The operating conditions were:

Flow	0.6 ml/min
Solvent	0.01N H_2SO_4
Stop Time	25 min
Injection Volume	5 μ l
Auto Sampler	Temp Control @ 10°C or 4 °C
Column Temp	55°C
Detector	Refractive Index (40°C) with External Standard Calibration Curves

Seed Cultivation

Glycerol stock of seed frozen at -80°C was thawed, then inoculated into sterile culture tubes with mixture of 60 g/L glucose, 20 g/L xylose, 10 g/L yeast extract (YE), 10 g/L KH_2PO_4 , 2 g/L $(\text{NH}_4)_2\text{SO}_4$, and 1 g/L MgSO_4 . Initial pH was adjusted to 5.5 with 4 N KOH. This revival culture was grown statically overnight at 37°C. It was transferred into shake flasks

with 75 g/L glucose, 25 g/L xylose, 10 g/L yeast extract (YE), 10 g/L KH_2PO_4 , 2 g/L $(\text{NH}_4)_2\text{SO}_4$, and 1 g/L MgSO_4 . Initial pH was adjusted to 5.5 with 4 N KOH. Growth was monitored by measuring OD_{600} . When the OD reached approximately 5, a certain volume of the seed was withdrawn into a sterile syringe for inoculation.

Example 1

Construction of ZW658, a xylose-fermenting *Zymomonas mobilis* strain

ZW658 was constructed by integrating two operons, $P_{gap}xylAB$ and $P_{gap}talkt$, containing four xylose-utilizing genes encoding xylose isomerase, xylulokinase, transaldolase and transketolase, into the genome of ZW1 (ATCC #31821) via sequential transposition events, and followed by adaptation on selective media containing xylose. Previously, a xylose-fermenting *Zymomonas mobilis* strain called 8b was constructed, as described in United States Patent Application 20030162271, by integrating the two operons $P_{gap}xylAxyIB$ and $P_{eno}talkt$, along with selectable antibiotic markers, into the genome of *Zymomonas mobilis* 5C via a combination of homologous recombination and transposon approaches followed by adaptation and NTG mutagenesis. In the preparation of ZW658, transposition (Epicentre's EZ::Tn in vitro transposition system) was used, as opposed to site specific homologous recombination, because this approach offers the advantages of multiple choices of integration sites and relatively high insertion frequency. The four genes encoding the xylose utilization enzymes were arranged and cloned as two separate operons: $P_{gap}xylAB$ and $P_{gap}talkt$ for the integration. An antibiotic resistance marker, a chloramphenicol resistance (Cm^r) gene flanked by two P1 phage Cre-recombinase recognition sequences ($loxP$), was attached to each operon for the selection of integrants. The integration of the two operons was accomplished in a two-step, sequential manner: $P_{gap}talkt$ followed by $P_{gap}xylAB$. Cm resistance selection was used in both integration events, since it was removed by expressing a Cre recombinase on a plasmid followed by curing of the plasmid after each integration. This process allowed the use of the same antibiotic marker for

selection multiple times. More importantly, it allowed the removal of the antibiotic marker introduced for selection of the integration of the operons. This process eliminated the negative impact of antibiotic resistance gene(s) on the fermentation strain for commercial use.

Construction of pMODP_{gap}taltktCm for Transposition

As described in the US Patent Application 20030162271 (Example 9 therein), a 2.2 kb DNA fragment containing the transketolase (tkt) coding region from *E. coli* was isolated from pUCtaltkt (US Patent Application 20030162271) by BglII/XbaI digestion and cloned in a pMOD (Epicentre Biotechnologies, Madison, WI) vector digested with BamHI/XbaI, resulting in pMODtkt. A PCR fragment named P_{gap}tal was generated by fusing the promoter region of the *Zymomonas mobilis gap* (P_{gap}; glyceraldehyde-3-phosphate dehydrogenase) gene to the coding region of *E. coli* transaldolase (tal) as follows. A P_{gap} fragment was amplified from pZB4, the construction of which is described in US 5514583 (Example 3), using primers with SEQ ID NOs:1 and 2. pZB4 contains a P_{gap}-xylA/xylB operon and a P_{ENO}-tal/tkt operon. A tal coding region fragment was amplified from pZB4 using primers with SEQ ID NOs: 3 and 4. A P_{gap}tal fragment was amplified using the P_{gap} and tal fragments as template using primers with SEQ ID NOs:5 and 6. This fragment was digested with XbaI and cloned into the plasmid pMODtkt, upstream of the tkt coding region. A loxP::Cm fragment was generated by PCR using Cmlox(F,sfi) and Cmlox(R,sfi) primers (SEQ ID NOs:7 and 8) and pZB186 as the template. pZB186 is a combination of a native *Z. mobilis* plasmid and pACYC184, described in US514583 (Example 3) and Zhang et al. ((1995) Science 267:240-243). Finally, the loxP::Cm PCR fragment was inserted in the SfiI site of the plasmid containing P_{gap}taltkt to form the integrative plasmid pMODP_{gap}taltktCm (Figure 2). In this plasmid, the P_{gap}taltkt loxP::Cm fragment was inserted between two mosaic ends (transposase binding sites) in the pMOD vector. The complete nucleotide sequence for the pMODP_{gap}taltktCm plasmid is given as SEQ ID NO:9.

Transposition and transformation of pMODP_{gap}*tal**tkt*Cm in ZW1

Plasmid pMOD is a pUC-based vector, and therefore is a non-replicative vector in *Zymomonas*. Plasmid pMODP_{gap}*tal**tkt*Cm was treated with transposase in the presence of Mg²⁺ at room temperature for one hour and used to transform ZW1 cells by electroporation (using a BioRad Gene Pulser set at 200 ohms, 25 μ F and 16 kV/cm). Electroporated cells were incubated in a mating medium (MM), which consists of 10 g/L yeast extract, 5 g/L tryptone, 2.5 g/L (NH₄)₂SO₄, 0.2 g/L KH₂PO₄) supplemented with 50 g/L glucose and 1 mM MgSO₄ for 6 hours at 30° C. The transformation mixture was plated on agar plates containing 15 g/L BactoTM agar in MM supplemented with 50 g/L glucose and 120 μ g/mL chloramphenicol and incubated anaerobically at 30 °C. The transformants were visible after about 2 days. The transformation/transposition frequency was approx. 3×10^1 / μ g DNA.

A total of 39 Cm^r transformant colonies was obtained. Twenty-one colonies were picked and further analyzed by PCR and enzymatic activity assays. PCR using primers SEQ ID NOs:10 and 11 confirmed the presence of a 3 kb DNA fragment containing *tal* and *tkt* coding regions in the transformants. Back transformation with plasmid DNA from the 21 integrant colonies generated no back transformants in *E. coli* suggesting the *tal* and *tkt* were integrated in the genome of ZW1. These integrants were tested for transaldolase and transketolase activities using protocols modified for microplates (General Methods). The Pierce BCA proteinTM assay was used for the determination of protein concentrations. The transformants were grown up in RM containing 2% (w/v) glucose supplemented with 120 μ g/ml chloramphenicol in 50 ml conical centrifuge tubes at 30°C. The control strains 8b and ZW1 were grown up as well (RM + 2% glucose was used for ZW1) for enzymatic assays. Cells were harvested when the OD₆₀₀ reached 1.0. Cells were washed once and resuspended in sonication buffer (10 mM Tris-HCl, pH 7.6 and 10 mM MgCl₂). Enzymatic assays were conducted as described in US Patent Application, 20030162271. Units are given as μ mole/min-mg. All samples had transaldolase and transketolase activities except for one.

Southern hybridization was performed on genomic and plasmid DNA of selected integrants digested with PstI using a *tkt* probe. ZW1 DNA did not hybridize with the *tkt* probe. A common 1.5 kb band was visible in all integrant genomic DNA samples, which is the expected DNA fragment between a PstI site in *tkt* and a PstI site in *tal*. A second visible high molecular weight (6 kb or greater) band was unique between independent lines T2, T3, T4 and T5 indicating a separate genomic integration site in each line. Interestingly, both plasmid and genomic DNA of T5 hybridized with the *tkt* probe indicating it was likely that $P_{gap}tal/tkt$ was also integrated in T5 on the native plasmid. These four strains (T2, T3, T4 and T5) were selected for further Cre treatment to remove the Cm^r marker.

Cre treatment to remove Cm^r marker from *tal/tkt* integrants

To remove the Cm^r marker, T2, T3, T4 and T5 were transformed with a derivative of the *Zymomonas-E.coli* shuttle vector pZB188 [Zhang et al. (1995) Science 267:240-243; US 5514583] carrying a Cre expression cassette with a spectinomycin resistance marker (pZB188aadACreF). The transformants were selected on MM agar plates supplemented with 2% glucose and 200 μ g/ml spectinomycin). Sp^r resistant colonies were picked onto RM agar plates supplemented with 2% glucose and 200 μ g/ml spectinomycin and RM agar plates supplemented with 2% glucose and 120 μ g/mL Cm . One hundred percent of the colonies picked were Cm^s indicating the high efficiency excision of Cm^r by Cre. Sp^rCm^s transformants were cultured in RM + 2% glucose at 37°C for 2 to 5 daily transfers to cure pZB188aadACreF. At each transfer, cells were diluted and plated on RM + 2% glucose agar plates for picking onto additional plates of the same medium with or without 200 μ g/mL Sp . Sp^s colonies were analyzed by PCR to confirm the loss of pZB188aadACreF. The plasmid-cured descendents of the integrants were named T2C, T3C, T4C and T5C. To examine whether these transposition integrants were stable, these 4 strains were grown in RM + 2% glucose and then transferred to 10 ml of the same medium and grown at 37°C in duplicate test tubes. Cells

were transferred daily for ten days, or approximately 100 generations. Colonies were diluted and plated onto RMG plates for colony isolation after the 1st and 10th transfers. Twelve colonies from each transfer of each strain tested positive for the presence of $P_{gap}tal tkt$ by colony PCR using 5' P_{gap} and 3' tkt primers (SEQ ID NOs 1 and 11). Transaldolase and transketolase activities were also measured for isolates after the 1st and 10th transfers (as described in General Methods). All 4 integrants had similar levels of both TAL and TKT activities after 100 generations on the non-selective medium, suggesting these integrants were genetically stable.

Construction of pMODP_{gap}xyIABcm for Transposition

The next step was to further integrate the $P_{gap}xyIAB loxP::Cm$ operon into the $ZW1::P_{gap}tal tkt$ integrants (T2C, T3C, T4C and T5C). The integrative plasmid pMODP_{gap}xyIABcm (Figure 3) was constructed based on the plasmid pMODP_{gap}tal tktCm (Figure 2). The $P_{gap}tal tkt$ DNA fragment was removed by SacI/SfiI digestion. An adaptor fragment containing SacI, NotI, and SfiI restriction sites was introduced by ligation. A NotI fragment of $P_{gap}xyIAB$, that was isolated from pZB4 (US 5514583), was then cloned in the NotI site of the adaptor. Xylose isomerase (XI) is encoded by *xyI*A and xylulokinase (XK) is encoded by *xyI*B. The complete nucleotide sequence for the pMODP_{gap}xyIABcm plasmid is given as SEQ ID NO: 12.

Transposition and transformation of pMODP_{gap}xyIABcm in T2C, T3C, T4C and T5C

Using a similar approach to the integration of $P_{gap}tal tktCm$, T2C, T3C, T4C and T5C were transformed/transposed with pMODP_{gap}xyIABcm (described above) treated with transposase. Six integrants (T3CCmX1, T3CCmX2, T3CCmX3, T4CCmX1, T5CCmX1, T5CCmX2) were obtained in 2 transformation/transposition experiments following Cm selection. All were confirmed for the presence of *xyIAB* by PCR using two sets of primers: SEQ ID NOs:13, and 14, and SEQ ID NOs:15 and 16 except for

T2CcmX1 and T2CcmX6 from which no PCR fragment was detected using the primers SEQ ID NOs:13 and 14.

The integrants, including the 2 PCR negative lines, were assayed for XI, XK, TAL and TKT activities (General Methods). The results shown in Figures 4 and 5 indicated that the six *xylAB* integrants T3CCmX1, T3CCmX2, T3CCmX3, T4CCmX1, T5CCmX1, and T5CCmX2 all had XI, XK, TAL and TKT activities. XI and XK activities were newly acquired as compared to the negative parental controls (Figure 4). TAL and TKT activities were maintained as in the parental controls. All results indicated that the proteins were made and functional. Enzyme activity levels varied, with TI and XK activities similar to those of ZW1 integrants transformed/transposed with the same plasmid. The levels of activities of XI, XK, TAL and TKT were lower than those in strain 8b.

The integration of the *xylAB* operon was confirmed by Southern hybridization. Both genomic and plasmid DNA of the 6 lines were digested with *SphI* and hybridized to a digoxenin labeled *xylB* probe. A common band of about 3 kb, which is generated from an *SphI* site in *xylB* and another *SphI* site in the adjacent cloning sites on the pMOD vector, was present in all genomic DNA samples, and in addition, higher molecular weight hybridizing bands in the genomic DNA samples indicated that there were four sites of integration for the $P_{gap}xylAB$ operon in the chromosome. T3CCmX1 and T3CCmX2 appear to have the same integration site, T3CCmX3 and T4CCmX1 may have the same integration site, and T5CCmX1 and T5CCmX2 each have a separate integration site. Digestion of the same DNA with *PstI* followed by Southern hybridization with the *tkl* probe demonstrated that each integrant had the same hybridization pattern as its respective parental strain.

Adaptation of the ZW1:: $P_{gap}tal/tkt$ $P_{gap}xylAB$ Cm integrants on xylose media

Despite the presence of all four enzymatic activities for xylose utilization, our previous observations (US Patent Application 20030162271) indicated that the integrants may not grow on xylose immediately. Growth on xylose may occur after prolonged incubation on

xylose medium (either in test tubes or on plates), a process called adaptation.

The strains were adapted as follows. ZW1::P_{gap}*talkt*P_{gap}*xy*/ABCm integrant strains were inoculated into test tubes and plates containing RMX (containing 10 g/l yeast extract, 2 g/l KH₂PO₄, 20 g/l or 2% (w/v) xylose as well as RMGX (RM with 0.025% (w/v) glucose, 4% (w/v) xylose). The low level of glucose was used to support initial growth to increase the chance of mutation during adaptation. One of at least five attempts at adaptation on xylose in both cultures and plates was successful. After 10 days of anaerobic incubation at 30°C, 17 and 19 colonies were visible on MMGX plated with T3CCmX1 and T3CCmX2 cells, respectively. The colonies were small and looked unhealthy (transparent) on the plates. Twelve colonies (four from T3CCmX1 plating: T3CCmX11, T3CCmX12, T3CCmX13 and T3CCmX110; eight from T3CCmX2 plating: T3CCmX24, T3CCmX25, T3CCmX26, T3CCmX27, T3CCmX28, T3CCmX29, T3CCmX211 and T3CCmX212) were inoculated in RMGCm120 and transferred into 3 ml RMX for further adaptation to obtain lines that were able to grow faster on xylose.

Adaptation of integrants in test tubes containing 3 ml RMX was conducted at 30°C. OD₆₀₀ was constantly monitored in a Spectronic 601 spectrophotometer. When the growth reached mid-log phase, the cultures were transferred into fresh tubes of RMX. This process was continued for 7 transfers. The growth rates and final ODs (non-linear readings) were improved over the transfers.

At the 6th transfer, the cultures were streaked out on RMX plates to isolate single colonies. Three integrants grew faster than others on RMX streaked plates: T3CCmX13, T3CCmX26 and T3CCmX27, which are referred to as X13, X26 and X27 in the tables and discussion below. To screen for the best xylose growers, we selected four large (L1-4) and four small (S1-4) colonies each for TX13, X26 and X27 and grew these in RMX test tubes so we could monitor growth, sugar utilization, and ethanol production. Colonies were grown overnight at 30°C followed by inoculation of OD₆₀₀=0.05 into 3 ml of RMX in test tubes in duplicates.

X27 grew more slowly in RMG than the other cultures and was inoculated again 6.5 hrs later. After 69 hrs (62.5 hrs for X27), samples were taken for HPLC analysis (General Methods). Figure 6 charts the average ethanol yield (% of theoretical yield) and xylose utilization (%) for cultures at 69 hours (62.5 hr for all X27 cultures). There was no significant difference between the large and small colonies. Although the performance of X27 was better as compared to X26 on xylose, it showed slower growth on glucose. Therefore, the top performers, large colonies of X13 (X13L3) and X26 (X26L1), were chosen for further evaluation in pH-controlled fermentations. The fermentations were conducted in RMG(6% glucose), RMX(6% xylose) and RMGX(8%:4%; glucose:xylose) at 37°C for strains X13L3 and X26L1, as well as the control strain 8b. Fermentation of glucose by X13L3 and X26L1 grown in RMG(6%) and RMGX(8%:4%) proceeded rather quickly. The fermentation of xylose in the RMGX(8%:4%) was slower for both X13L3 and X26L1 as compared to that of strain 8b. In addition, growth on RMX(6%) at 37°C occurred after a long lag for both X13L3 and X26L1. Several isolates, X13b, X13c and X13FL, were recovered from RMX(6%) fermentations. These isolates along with the original strains X13a (an isolate of X13L3) and X26 were subjected to Cre treatment, as described previously in this Example, to remove the Cm^r marker from ZW1::P_{gap}taltktP_{gap}xylABCm strains. The resulting Cre treated, Cm^r-free integrants were named: X13aC, X13bC, X13cC, X13FLC and X26C.

Adaptation of integrants in xylose medium by serial transfers in RMX(5%) at 37°C

As described earlier, adaptation of the initial ZW1::P_{gap}taltktP_{gap}xylABCm strains on RMX at 30 °C greatly improved the growth of strains in these conditions. However, the adapted strains suffered a long lag during growth and fermentation in RMX(6%) at 37 °C. To further improve the integrants for xylose fermentation at preferred process conditions including higher sugar concentration and temperature, the evolutionary or adaptation process was continued in RMX(5%) at 37

°C. Serial transfers were conducted and the best growers were selected. Integrants used in this process included X13aC, X13bC, X13cC, X26C and X13FLC. These 5 strains were grown in RMX at 30°C for 6 transfers before being transferred to RMX(5%) at 37°C for another 5 to 16 transfers. During and after all the transfers cultures were streaked on RMXplates and incubated at 37°C to isolate single colonies. Large colonies were further streaked on RMX plates and incubated at 37°C for 3 to 4 times to purify the colonies. Final large colonies were selected for growth testing in RMX(5%) at 37°C.

Evaluation of strains from adaptation in RMX(5%) medium at 37°C

Eighteen colonies isolated after adaptation with serial transfers were tested in RMX(5%) test tubes at 37°C initially. Twelve strains were selected for a 2nd test tube evaluation. Strain 8b was included in all the evaluations for comparison. The 18 colonies were grown up in RMG at 37°C overnight, centrifuged and the cells were inoculated into 4 ml of RMX(5%) at 37°C, statically in test tubes for the 1st evaluation. Based on the growth (OD₆₀₀, non-linear) and end point HPLC results (low residual xylose and high ethanol), 12 strains were selected for the 2nd evaluation.

One of the purposes of the 2nd evaluation was to test the stability of improved growth on xylose and xylose utilization capability of the strains. All 12 strains were subjected to a stability study to see whether the adapted strains were stable after being exposed to a non-selective medium in which they were serially transferred in at 37°C for 50 generations. Cultures before and after RMG(5%) transfers were inoculated in RMX(5%) test tubes and grown at 37°C for evaluation. The non-linear ODs were monitored by direct reading of test tubes in a Spectronic 601 spectrophotometer. The ODs at the 70th hour of growth in RMX(5%) before and after 50 generations of growth in RMG are plotted in Figure 7. The results indicated that most strains were stable after 50 generations in RMG at 37°C. The endpoint (at stationary phase) supernatants were also analyzed by HPLC as described in General Methods for xylose and ethanol concentrations. The low residual xylose

and high ethanol concentrations in these cultures supported the fact that the strain grew and fermented xylose well.

Based on the results from the above test tube evaluation (low residual xylose, high ethanol concentration and higher OD) and a subsequent microtiter plate growth screening with high concentrations of glucose and/or xylose (up to 20%) and mixtures of glucose and xylose with acetate to select better growers in high sugars and in the presence of acetate, focus was directed to strain #26, designated as ZW658, which exhibited the best overall performance.

Example 2

Fermentation evaluation of top improved xylose-utilization strains at 37°C

The following example illustrates the fermentation performance of the improved xylose-utilizing *Zymomonas* strain ZW658 under fermentation conditions that mimic the sugar concentrations and the acetic acid level expected in a biomass hydrolysate. Strain ZW658 was inoculated into fermentors containing RM medium supplemented with 10% glucose (RMG10%), 8% xylose (RMX8%), 10% glucose+8% xylose (RMGX10%8%) and 10% glucose+8% xylose+0.6% acetic acid (RMGXAc10%8%0.6%), respectively. All fermentations were conducted in Sixfors with 300 ml media at 150 rpm, pH5.5 and 37°C. Nitrogen was purged through the media in the fermentors overnight and stopped right before inoculation. No nitrogen was purged during the fermentation. Inocula for the fermentation were prepared with RMGX(10%,4%) at 37°C in shake flasks (150 rpm) after reviving of the working stocks in RMG5%. Strain 8b was used as a control under the same conditions. Samples were taken periodically for OD₆₀₀ measurement, as well as for HPLC analysis (as described in General Methods) for glucose, xylose, xylitol, and ethanol. As shown in Figure 8, ZW658 grew more slowly on RMG10% as compared to 8b (A and B), and grew at a similar rate to 8b on RMX8% (C and D). Despite the slower growth rate, Figure 8 shows that the ethanol yield of ZW658 (93%) was similar to that of 8b at the end of fermentation in glucose medium. In RMX8% medium, the ethanol yield was higher for

ZW658 (0.46 g ethanol/g sugar) as compared to 8b (0.44 g ethanol/g sugar). ZW658 produced about 4 g/l more ethanol as compared to 8b in RMX8%. Interestingly, ZW658 did not produce any xylitol while 8b produced a low level of xylitol (0.7 g/l) at the end of the fermentation in RMX8%. Data shown in Figure 9 shows that ZW658 performed better as compared to 8b in fermenting 10% glucose+8% xylose with (C, D) or without (A, B) acetate, indicated by more glucose and xylose consumption, less xylitol production, and more ethanol production. Most of the glucose was used and substantial residual xylose remained at the end of the fermentation for both strains in RMG10%X8%, at 37°C and pH5.5, although ZW658 used about 8 g/l more xylose than 8b. Xylitol production (4.9 g/l) in ZW658 in RMG10%X8% at 37°C and pH5.5 at the end of the fermentation was significantly lower than that of 8b (8.2 g/l). In the presence of acetate (6 g/l), the cell growth of both strains was reduced significantly resulting in poor fermentation performance of both glucose and xylose, although ZW658 showed slightly better fermentation performance in terms of more glucose and xylose consumption, less xylitol production and more ethanol production. Unlike in the RMX8%, both strains produced the by-product xylitol in RMG10%X8% with or without acetate, although less xylitol was produced by ZW658 as compared to 8b. The fermentation performance of the two strains is summarized in Table 1. Overall, ZW658 performed better than 8b in pure sugar fermentations. As described in Example 1, ZW658 is free of antibiotic selection markers, which is a valuable property for fermentation organisms in commercial applications.

Table 1. Summary of fermentation performance of ZW658 and 8b for ethanol production.

	Ethanol Yield g ethanol/g sugar	Vol. Prod. g/l/h	Ethanol g/l	CPI g/g
8b (Glu)	0.47	5.15	52	21
ZW658 (Glu)	0.48	4.13	52	15
8b (Xyl)	0.44	1.66	37	24
ZW658 (Xyl)	0.46	1.83	41	23
8b (Glu Xyl)	0.43	1.80	58	52
ZW658 (Glu Xyl)	0.45	2.03	65	35
8b (Glu Xyl Ac)	0.46	0.67	48	136
ZW658 (Glu Xyl Ac)	0.47	1.04	50	90

CPI is Cell Productivity Index: g ethanol/g dry cell weight

Example 3

Ethanol production by strain ZW658 grown on high concentration glucose or xylose in the presence and absence of sorbitol

In four sterilized 125 ml Erlenmeyer culture flasks (Cat. No. 30180-036, VWR International, USA), 10 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 was inoculated into each flask with 100 ml of aqueous solution containing 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, 4 g/L KHCO₃. Before inoculation, the first flask also contained 200 g/L glucose and 20 mM sorbitol, comparing to the second flask with 200 g/L glucose only. The third flask contained 200 g/L xylose and 20 mM sorbitol, comparing to the fourth flask with 200 g/L xylose only. After inoculation, sugar concentrations were diluted to about 180 g/L. Initial pH was adjusted to 5.5 with 4 N H₃PO₄ solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, ethanol, and xylitol. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 10A shows the OD₆₀₀ at time points between 0 and 48 hr. After 48 hours, the OD₆₀₀ were 9.94, 6.46, 5.37 and 5.45, respectively, for the first, second, third, and fourth flask. These results show that sorbitol significantly

stimulated growth in high concentration glucose culture, but showed almost no effect on growth in high concentration xylose culture.

Results in Figure 10B show that ethanol production was more rapid in glucose with sorbitol, but ethanol in cultures with and without sorbitol reached the same level after about 20 hr. In the xylose only cultures, ethanol production was at the same rate with and without sorbitol, with the sorbitol sample producing less ethanol at 48 hr than the no sorbitol sample. No xylitol was produced in any of these cultures.

Example 4

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of sorbitol

In a sterilized 1-liter fermentor (BIOSTAT® B-DCU system, Sartorius BBI System Inc., Bethlehem, Pennsylvania, USA), 50 ml of ZW658 strain seed culture at Optical Density of approximately 5 (measured at 600 nm) was inoculated into 450 ml of aqueous solution containing sugars and medium. The final mixed broth contained 100 g/L glucose, 85.1 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH_2PO_4 , and 10 mM sorbitol. pH was adjusted to 5.5 with 4 N KOH solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH 5.5. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, ethanol, and acetate. Samples were also diluted with medium (as described in General Methods) and the OD_{600} was measured to monitor cell growth. Figure 11 shows the amounts of glucose, xylose, xylitol, ethanol and acetic acid present, as well as the OD_{600} at time points between 0 and 65 hr. After 29 hours, the glucose and xylose concentrations were 0 and 2.08 g/L, respectively, and the ethanol concentration reached 83.9 g/L. Cells grew to an OD_{600} of 13. The by-product xylitol concentration was 0.67 g/L at 29 hr, and increased to 0.95 g/L at 64 hr.

Example 5

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the absence of sorbitol

In a sterilized 1-liter fermentor (BIOSTAT® B-DCU system, Sartorius BBI System Inc., Bethlehem, Pennsylvania, USA), 50 ml of ZW658 strain seed culture at Optical Density of approximately 5 (measured at 600 nm) was inoculated into 450 ml of aqueous solution containing sugars and medium. The final mixed broth contained 103 g/L glucose, 85.0 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH_2PO_4 . pH was adjusted to 5.5 with 4 N KOH solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH 5.5. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, xylitol, ethanol, and acetate. Samples were also diluted with medium (as described in General Methods) and the OD_{600} was measured to monitor cell growth. Figure 12 shows the amounts of glucose, xylose, ethanol and acetic acid present, as well as the OD_{600} at time points between 0 and 70 hr. The glucose concentration did not reach 0 until 40 hours and the xylose concentration never was reduced to 2 g/L, as it was when sorbitol was present in the Example 3 experiment. At the end of the run, after 67 hr, xylose was only reduced to 17.8 g/L. Xylitol production was about 0.21 g/L at 29 hr, and increased to 3.08 g/L after 64 hr, an over 3-fold higher amount than was produced in the Example 1 experiment. After 67 hours, the ethanol concentration reached 77.2 g/L, and cells grew to an OD_{600} of 6.7.

Comparing Examples 3 and 4, the result showed that adding sorbitol significantly reduced the lag time and improved sugar utilization (or uptake) rates, final ethanol concentration (i.e. yield), and doubled the growth. Less xylitol was produced.

Example 6

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of sorbitol and acetate

In a sterilized 1-liter fermentor (BIOSTAT® B-DCU system, Sartorius BBI System Inc., Bethlehem, Pennsylvania, USA), 50 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 was inoculated into 450 ml of aqueous solution containing sugars and medium. The final mixed broth contained 99.0 g/L glucose, 82.7 g/L xylose, 5.8 g/L acetate (in the form of potassium acetate), 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, and 10 mM sorbitol. pH was adjusted to 5.5 with 4 N KOH solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH 5.5. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, ethanol, and acetate. Samples were also diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 13 shows the amounts of glucose, xylose, xylitol, ethanol and acetic acid present, as well as the OD₆₀₀ at time points between 0 and 64 hr. After 63.75 hours, the glucose and xylose concentrations were 0 and 29.6 g/L, respectively, and the ethanol concentration reached 70.0 g/L; cells grew to an OD₆₀₀ of 6.4; by product xylitol concentration was 3.55 g/L.

Example 7

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of acetate without sorbitol

In a sterilized 1-liter fermentor (BIOSTAT® B-DCU system, Sartorius BBI System Inc., Bethlehem, Pennsylvania, USA), 80 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 was inoculated into 720 ml of aqueous solution containing sugars and medium. The final mixed broth contained 97.2 g/L glucose, 81.0 g/L xylose, 5.7 g/L acetate (in the form of potassium acetate), 5 g/L yeast extract (YE), 2 g/L KH₂PO₄, 2 g/L (NH₄)₂SO₄ and 1 g/L MgSO₄·7H₂O. pH was adjusted to 5.5 with 4 N

KOH solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH 5.5. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, xylitol, ethanol, and acetic acid. Samples were also diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 14 shows the amounts of glucose, xylose, ethanol and acetic acid present, as well as the OD₆₀₀ at time points between 0 and 140 hr. After 63 hours, the glucose and xylose concentrations were 0 and 50 g/L, respectively, and the ethanol concentration reached 61.8 g/L. Cells grew to an OD₆₀₀ of 3.3. After 135 hours, the xylose concentration was reduced to 38 g/L, and the ethanol concentration reached 64.4 g/L. The by-product xylitol concentration was 1.3 g/L at 63 hr, and increased to 4.4 g/L after 135 hr. When the 4.4 g/L of xylitol produced at stationary phase of the fermentation culture with no sorbitol was compared to the 3.55 g/L of xylitol produced at stationary phase of the fermentation culture with sorbitol (Example 6), the results showed that adding sorbitol reduced the amount of xylitol produced in the presence of acetate.

Example 8

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of glutamate

In a sterilized 1-liter fermentor (BIOSTAT® B-DCU system, Sartorius BBI System Inc., Bethlehem, Pennsylvania, USA), 50 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 was inoculated into 450 ml of aqueous solution containing sugars and medium. The final mixed broth contained 98.0 g/L glucose, 81.2 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, and 10 mM potassium glutamate. pH was adjusted to 5.5 with 4 N KOH solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH 5.5. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for

compounds including glucose, xylose, xylitol, ethanol, and acetic acid. Samples were also diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 15 shows the amounts of glucose, xylose, ethanol and acetic acid present, as well as the OD₆₀₀ at time points between 0 and 63.75 hr. Glutamate reduced the lag period and the glucose concentration reached 0 at 24 hr. However, xylose was not well-utilized, cell growth and ethanol production were reduced as compared to that in the presence of sorbitol (Example 4), and xylitol production increased. After 63.75 hours, the glucose and xylose concentrations were 0 and 21.2 g/L, respectively, and the ethanol concentration reached 72.3 g/L; cells grew to an OD₆₀₀ of 6.9; by-product xylitol concentration was 4.38 g/L. The results indicated that glutamate reduced the lag time and improved glucose utilization (or uptake) rates, but had less beneficial effect than sorbitol on xylose usage, cell growth, and production of the xylitol by-product.

Example 9

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of sorbitol at various concentrations

In five sterilized 125 ml Erlenmeyer culture flasks (Cat. No. 30180-036, VWR International, USA), 10 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 (measured at 600 nm) was inoculated into each flask with 100 ml of aqueous solution containing 100 g/L glucose, 80 g/L xylose, 10 g/L yeast extract (YE), 10 g/L KH₂PO₄, 2 g/L (NH₄)₂SO₄, 1 g/L MgSO₄·7H₂O, and various concentrations of sorbitol (0, 0.5, 1, 2, and 10 mM). After inoculation, the total sugar concentration was about 160 g/L due to dilution by the seed culture. Initial pH was adjusted to 5.5 with 4 N KOH solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 16 shows the OD₆₀₀ at time points between 0 and 28 hr. Cultures with higher concentration of sorbitol, in this range, grew faster and better. After 14.25

hours, the OD₆₀₀ were 4.19, 7.10, 8.08, 8.62, and 10.1, respectively, for sorbitol concentrations of 0, 0.5, 1, 2, and 10 mM.

Example 10

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of sorbitol at various concentrations up to 200 mM

In five sterilized 125 ml Erlenmeyer culture flasks (Cat. No. 30180-036, VWR International, USA), 10 ml of ZW658 strain seed culture at Optical Density of approximately 5 (measured at 600 nm; OD₆₀₀) was inoculated into each flask with 100 ml of aqueous solution containing 110 g/L glucose, 90 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, 4 g/L KHCO₃, and various concentrations of sorbitol (10, 20, 50, 100, and 200 mM). Initial pH was adjusted to 5.5 with 4 N H₃PO₄ solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for ethanol. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 17 shows the OD₆₀₀ (A) and ethanol (B) produced at time points between 0 and 48 hr. After 24 hours, the OD₆₀₀ were 7.74, 7.86, 8.04, 7.82, and 7.12, respectively, for sorbitol concentrations of 10, 20, 50, 100, and 200 mM. After 48 hours, the OD₆₀₀ were 8.30, 8.26, 8.84, 8.12, and 7.72, respectively, for sorbitol concentrations of 10, 20, 50, 100, and 200 mM. These results showed that cultures with 10-100 mM sorbitol grew similarly, while the culture with 200 mM sorbitol grew slightly slower. Ethanol production was slightly slower with 100 or 200 mM sorbitol than with 10, 20, or 50 mM sorbitol, although all cultures reached about the same final amount of ethanol produced. No xylitol was detected in any of the samples. Thus 10 mM sorbitol is adequate to provide maximal ethanol production under these conditions.

Example 11

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of various polyols

In five sterilized 125 ml Erlenmeyer culture flasks (Cat. No. 30180-036, VWR International, USA), 10 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 was inoculated into each flask with 100 ml of aqueous solution containing 100 g/L glucose, 80 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, 1 g/L MgSO₄·7H₂O, and 4 g/L KHCO₃, and 10 mM of one of the following polyols: erythritol, sorbitol, mannitol, maltitol, or lactitol. After inoculation, the total sugar concentration was about 160 g/L due to dilution by the seed culture. Initial pH was adjusted to 5.5 with 4 N H₃PO₄ solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, ethanol, xylitol, and acetic acid. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 18 shows the OD₆₀₀ (A), xylose utilization (B), and ethanol production (C) at time points between 0 and 32 hr. Cultures with sorbitol or mannitol grew faster and better. After 32 hours, the OD₆₀₀ were 13.94 and 13.92, respectively, for cultures supplemented with sorbitol or mannitol; meanwhile, the OD₆₀₀ were 9.48, 9.02, and 9.24, respectively, for cultures supplemented with erythritol, maltitol, or lactitol. More xylose was utilized, and ethanol production was also better with sorbitol or mannitol in the medium. No xylitol was detected in any of the cultures. The presence of sorbitol or mannitol allowed more xylose utilization, without production of xylitol.

This example shows that mannitol is as effective on improving cell growth, xylose utilization, reducing xylitol, and increasing ethanol production as sorbitol at the same concentration in high concentration sugars.

Example 12

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of acetate and various polyols

In five sterilized 125 ml Erlenmeyer culture flasks (Cat. No. 30180-036, VWR International, USA), 10 ml of ZW658 strain seed culture at OD₆₀₀ of approximately 5 was inoculated into each flask with 100 ml of aqueous solution containing 100 g/L glucose, 80 g/L xylose, 3 g/L acetate, 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, 1 g/L MgSO₄·7H₂O, and 4 g/L KHCO₃, and 10 mM of the following polyols: erythritol, sorbitol, mannitol, maltitol, or lactitol; or 5 mM sorbitol plus 5 mM maltitol, or 5 mM sorbitol plus 5 mM lactitol. After inoculation, the total sugar concentration was about 160 g/L due to dilution by the seed culture. Initial pH was adjusted to 5.5 with 4 N H₃PO₄ solution. Mixing speed was set at 150 rpm. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for compounds including glucose, xylose, ethanol, and acetate. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Figure 19 shows the OD₆₀₀ (A), xylose utilization (B), and ethanol production (C) at time points between 0 and 32 hr. Cultures with sorbitol or mannitol grew faster and better than cultures without either of these components. After 32 hours, the OD₆₀₀ were 7.88, 7.40, 7.62 and 7.78, respectively, for cultures supplemented with 10 mM sorbitol, 10 mM mannitol, 5 mM sorbitol plus 5 mM maltitol, and 5 mM sorbitol plus 5 mM lactitol; meanwhile, the OD₆₀₀ were 5.20, 5.20, and 5.30, respectively, for cultures supplemented with erythritol, maltitol, or lactitol. More xylose was utilized, and ethanol production was also better with sorbitol or mannitol in the medium. No xylitol was detected in any of the cultures. The presence of sorbitol or mannitol allowed more xylose utilization, without production of xylitol.

This example shows that mannitol is as effective in improving cell growth, xylose utilization, reducing xylitol, and increasing ethanol production as sorbitol at the same concentration in high concentration

sugars with acetate. Combining sorbitol with polyols of higher molecular weight (maltitol or lactitol) did not show synergistic improvement on fermentation.

Example 13

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of various sugar alcohols

In eight sterilized 50 ml screw-cap centrifuge tubes (Cat. No. 21008-178, VWR International, USA), 200 μ l of ZW658 strain glycerol stock at OD₆₀₀ of approximately 10 was inoculated into each flask with 25 ml of aqueous solution containing 92 g/L glucose, 82 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, 1 g/L MgSO₄·7H₂O, and either 10 mM of sorbitol, arabitol, adonitol (also called ribitol), or galactitol; or 50 mM of arabitol, adonitol, or galactitol. The control had no sugar alcohol added. Initial pH was adjusted to 5.5 with 4 N H₃PO₄ solution. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically between 0 and 40 hr. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for glucose, xylose, xylitol, and ethanol. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Results of growth and glucose utilization are given in Figure 20A and 20B, respectively. Results of xylose utilization and ethanol production are given in Figure 21A and 21B, respectively. Cultures with sorbitol or galactitol grew much faster and better than the control. Cultures with adonitol also grew better. After 40 hours, the OD₆₀₀ were 7 to 8, for cultures supplemented with sorbitol or galactitol; the OD₆₀₀ were about 5, for cultures supplemented with adonitol; while the OD₆₀₀ were less than 1 for other cultures. More xylose was utilized, and ethanol production was also better with sorbitol or galactitol or adonitol in the medium. No xylitol was detected in any of the cultures. The presence of sorbitol, galactitol, or adonitol allowed more xylose utilization, without production of xylitol.

This example shows that galactitol is as effective on improving cell growth, xylose utilization, reducing xylitol, and increasing ethanol production as sorbitol at the same concentration in high concentration sugars. Adonitol is less effective than sorbitol.

Example 14

Ethanol production by strain ZW658 grown on high concentration glucose and xylose in the presence of various sugar alcohols

In six sterilized 50 ml screw-cap centrifuge tubes (Cat. No. 21008-178, VWR International, USA), 200 μ l of ZW658 strain glycerol stock at OD₆₀₀ of approximately 10 was inoculated into each flask with 25 ml of aqueous solution containing 92 g/L glucose, 82 g/L xylose, 10 g/L yeast extract (YE), 2 g/L KH₂PO₄, 1 g/L MgSO₄·7H₂O, and 10 mM of D-sorbitol, L-sorbitol, D-threitol, myo-inositol, or xylitol. The control had no addition of sugar alcohol. Initial pH was adjusted to 5.5 with 4 N H₃PO₄ solution. Fermentation was conducted at 33°C and pH was not controlled. Samples were taken periodically between 0 and 48 hr. Samples were filtered through 0.22 micron filters, and the filtrates were analyzed by HPLC as described in General Methods for glucose, xylose, xylitol, and ethanol. Samples were diluted with medium (as described in General Methods) and the OD₆₀₀ was measured to monitor cell growth. Results of growth and glucose utilization are given in Figure 22A and 22B, respectively. Results of xylose utilization and ethanol production are given in Figure 23A and 23B, respectively. Cultures with D-sorbitol or L-sorbitol grew faster and better than the control. After 48 hours, the OD₆₀₀ were 6.2 and 5.2, respectively, for cultures supplemented with D-sorbitol or L-sorbitol; while the OD₆₀₀ were less than 1 for other cultures. More xylose was utilized, and ethanol production was also better with D-sorbitol or L-sorbitol in the medium. No xylitol was detected in any of the cultures. The presence of D-sorbitol or L-sorbitol allowed more xylose utilization, without production of xylitol. D-sorbitol was more effective than L-sorbitol.

CLAIMS

What is claimed is

1. A method for increasing ethanol production comprising:
 - (a) providing recombinant *Zymomonas* cells which convert xylose to ethanol comprising at least one gene encoding each of the enzymes:
 - i) xylose isomerase;
 - ii) xylulokinase;
 - iii) transketolase; and
 - iv) transaldolase;
 - (b) providing a suitable medium comprising (i) a mixed sugar composition comprising xylose and at least one additional sugar, and (ii) at least one sugar alcohol, wherein the sugar alcohol is sorbitol, mannitol, galactitol, or ribitol; and
 - (c) contacting (a) with (b) whereby the *Zymomonas* cells produce ethanol.
2. The method of Claim 1, wherein the rate of the conversion of xylose to ethanol of the recombinant *Zymomonas* is increased by the presence of the sugar alcohol of Claim 1(b).
3. The method of Claim 2 whereby xylitol concentration is decreased in the presence of the sugar alcohol of Claim 1(b).
4. The method of Claim 1 wherein the sugar alcohol of Claim 1(b) is present in the medium in an amount of about 0.5 mM to about 200 mM, about 2 mM to about 100 mM, or about 5 mM to about 20 mM.
5. The xylose-utilizing *Zymomonas mobilis* strain ZW658 having the ATCC designation PTA-7858.

Figure 1

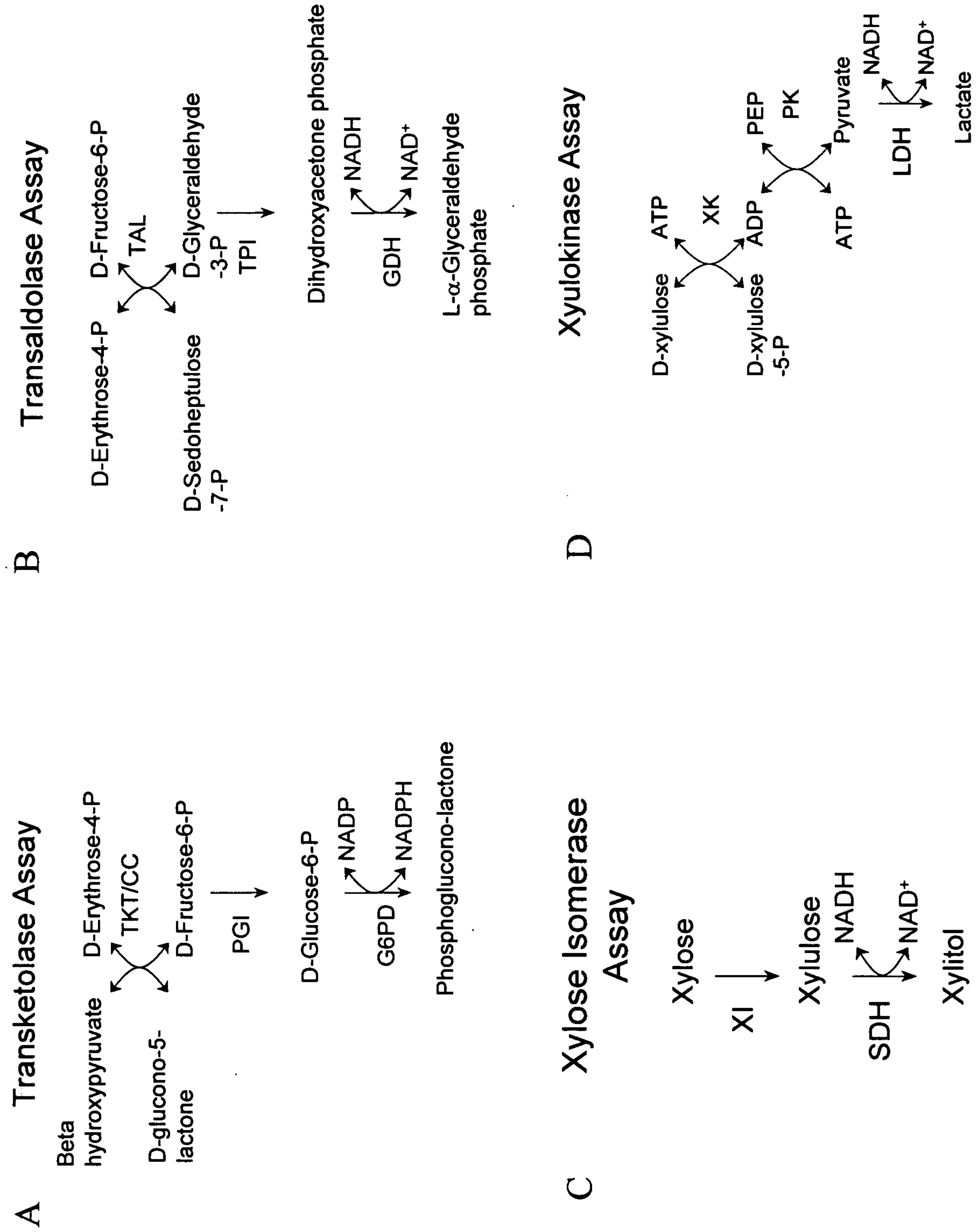
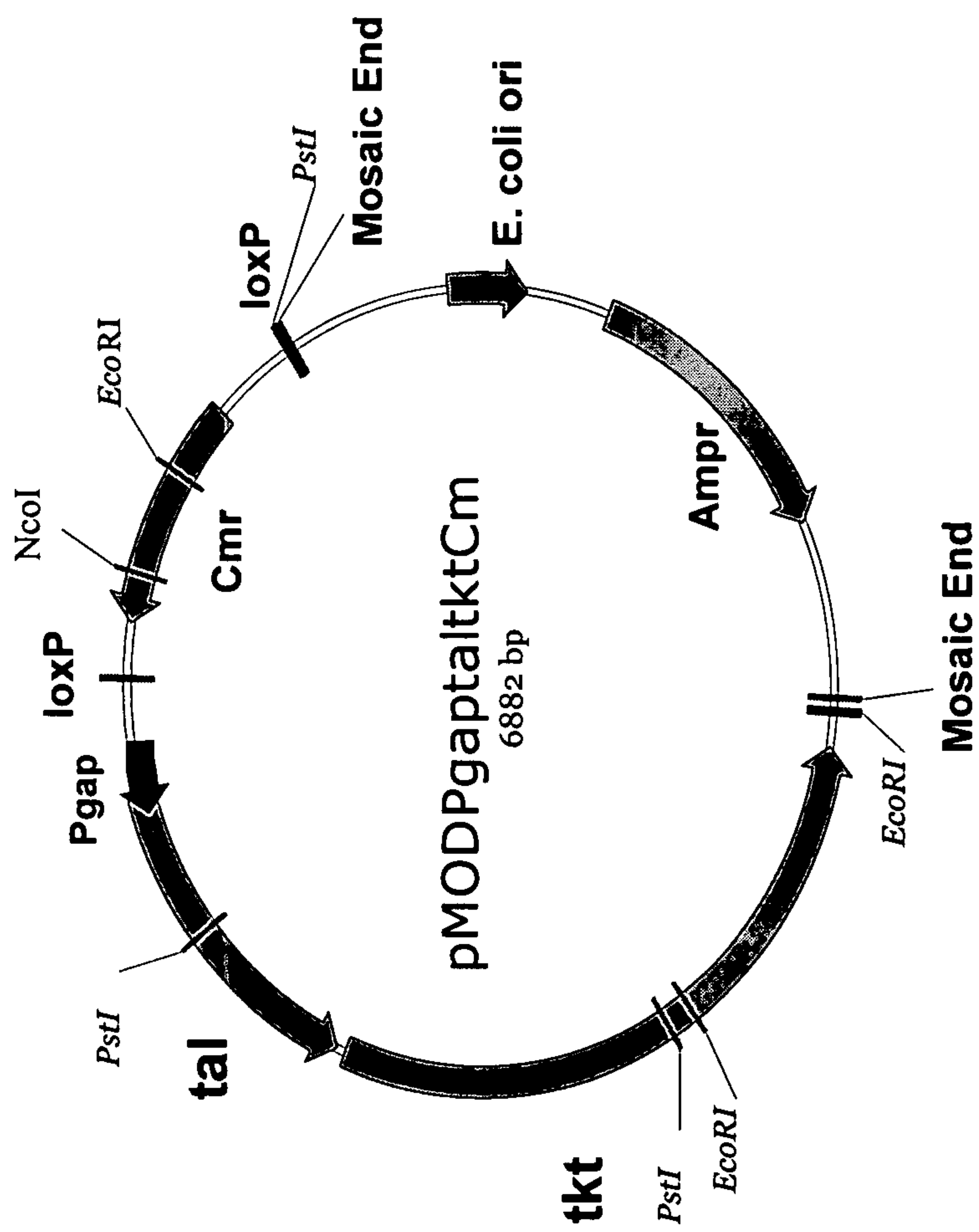


Figure 2



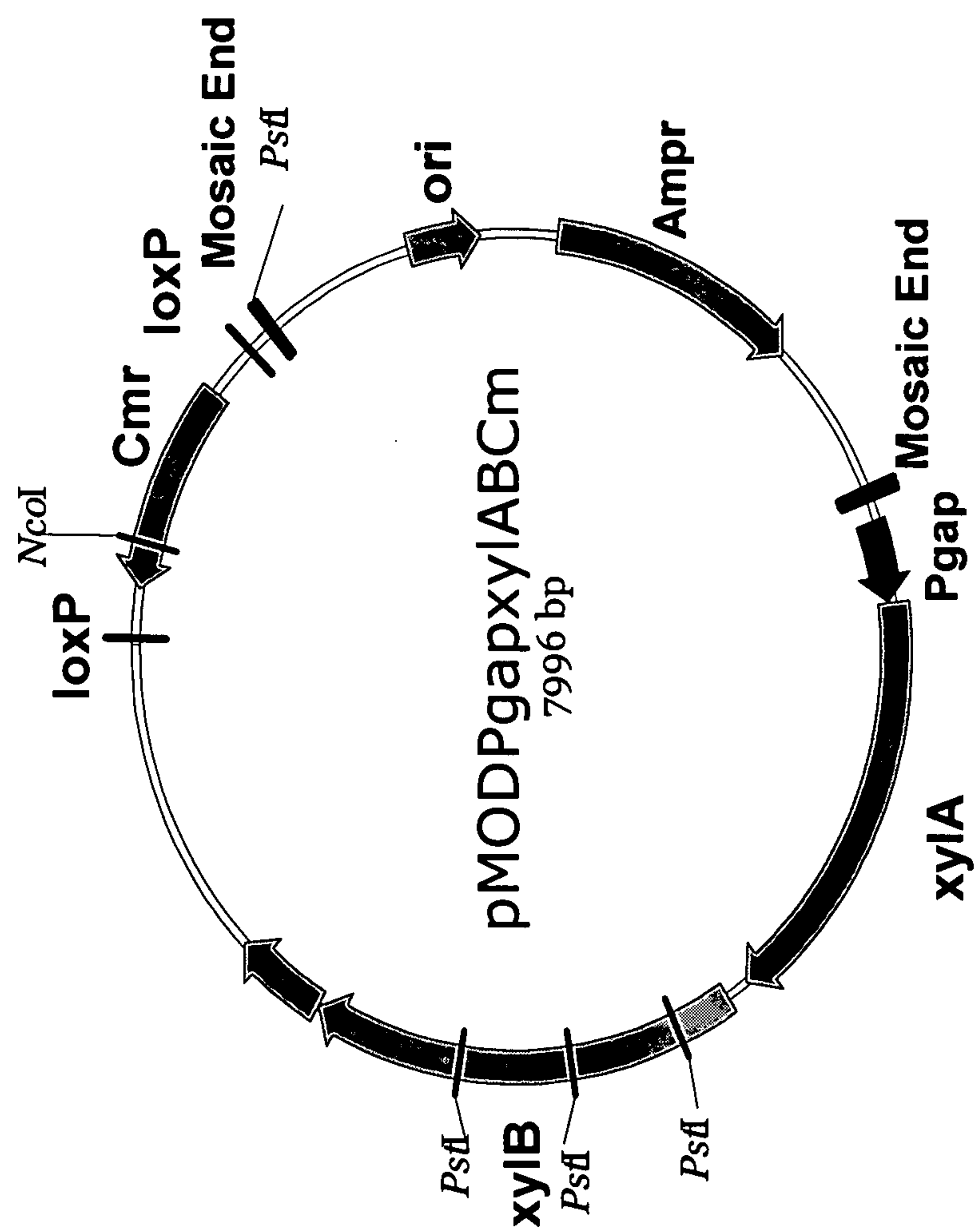


Figure 3

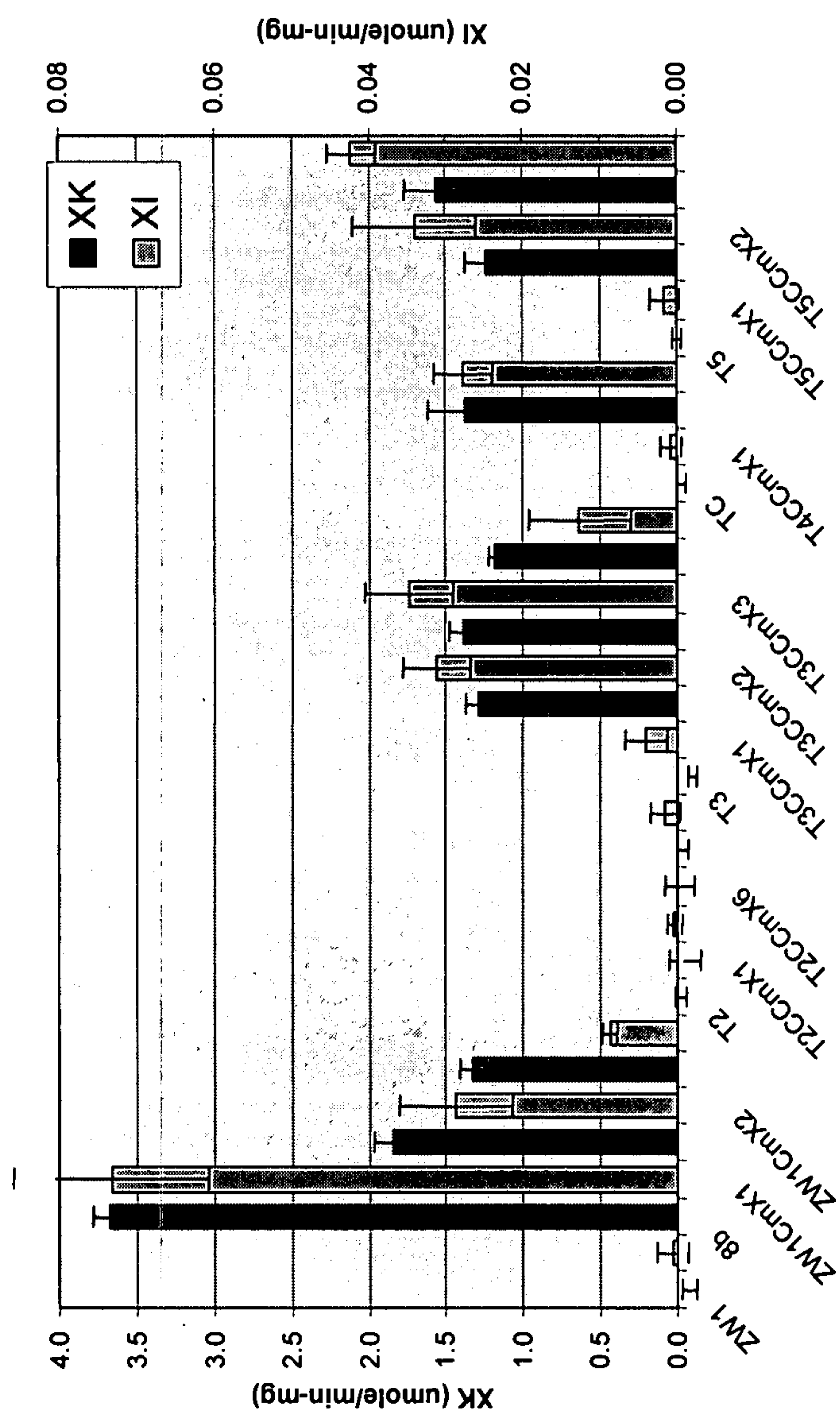


Figure 4

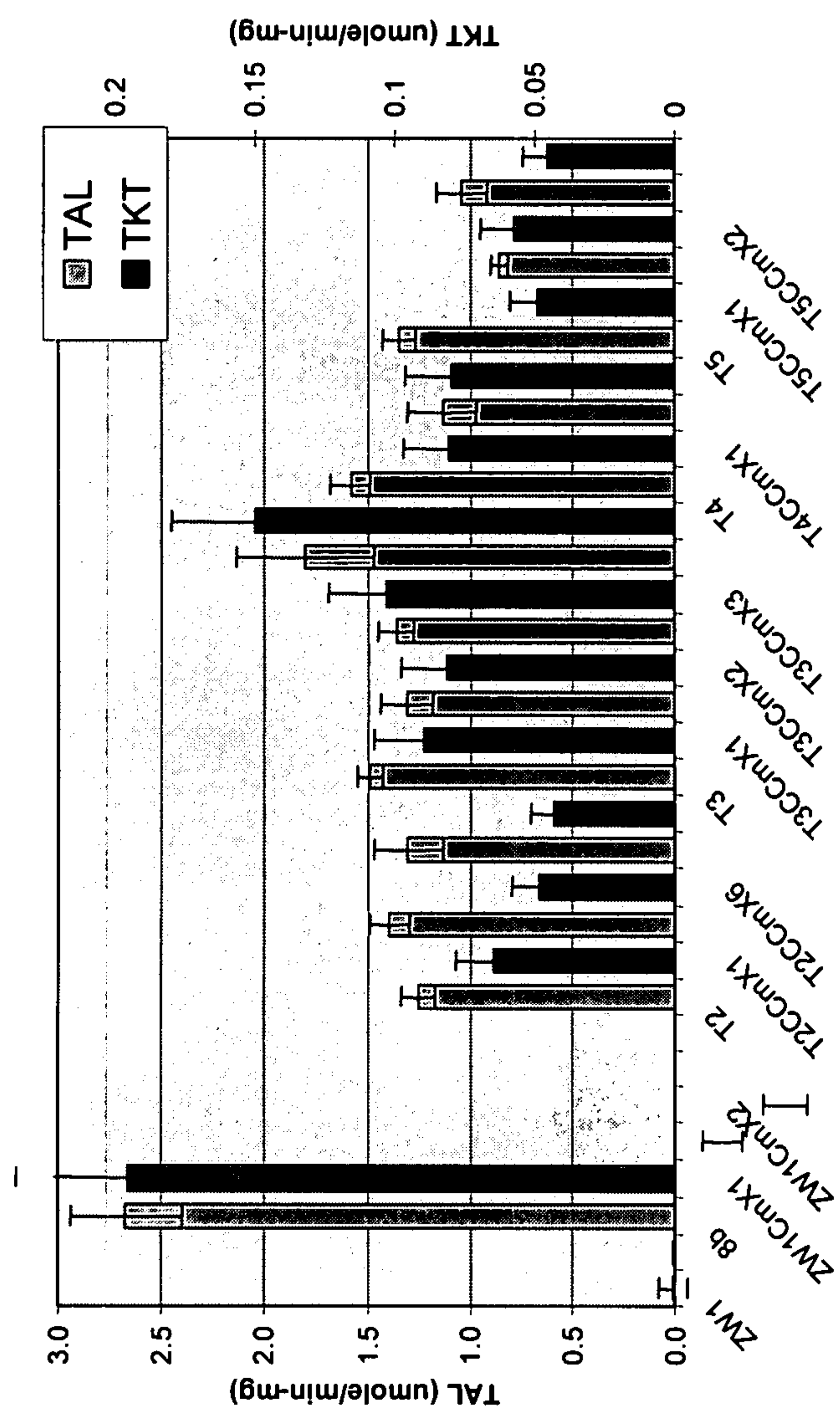
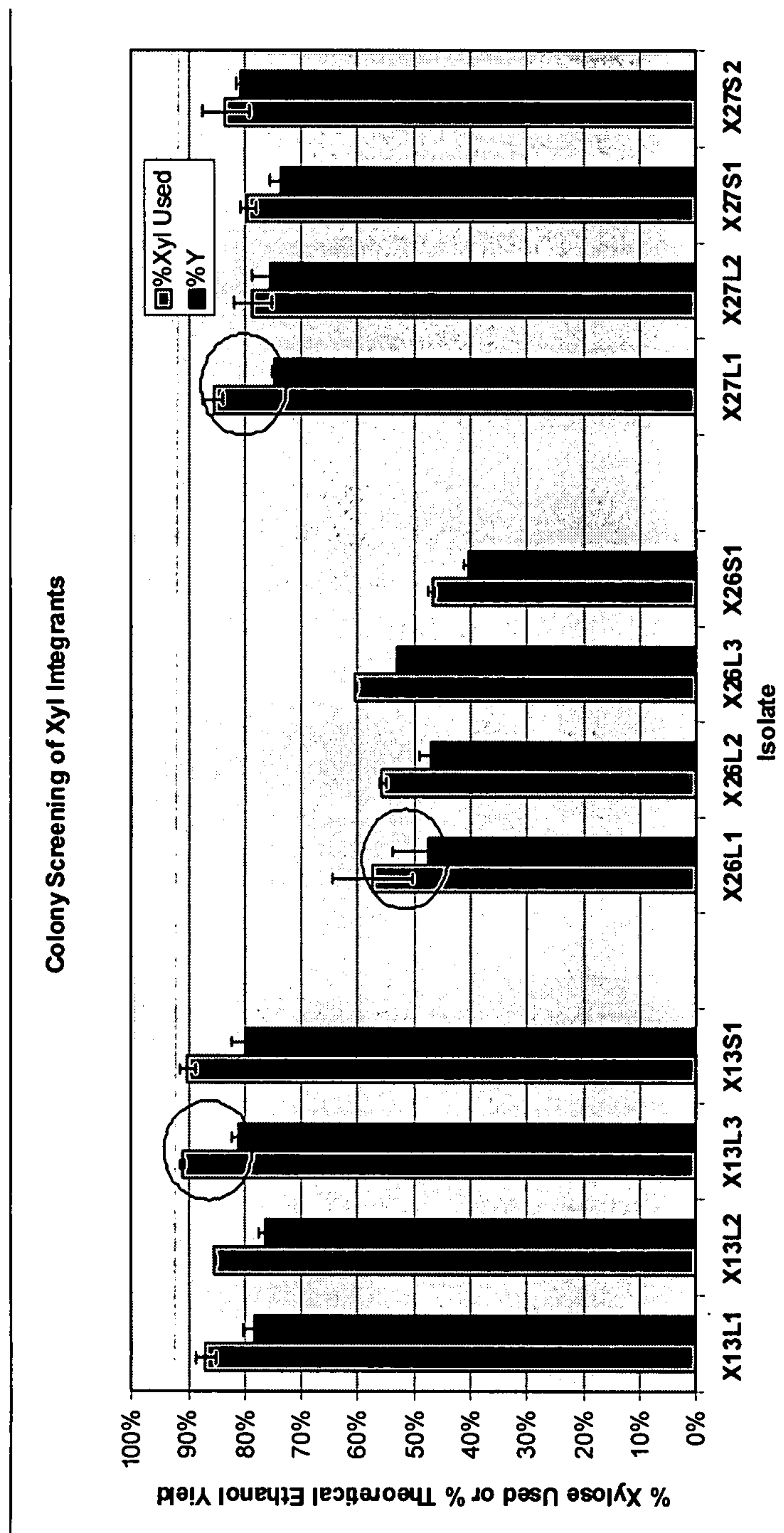


Figure 5

Figure 6



7

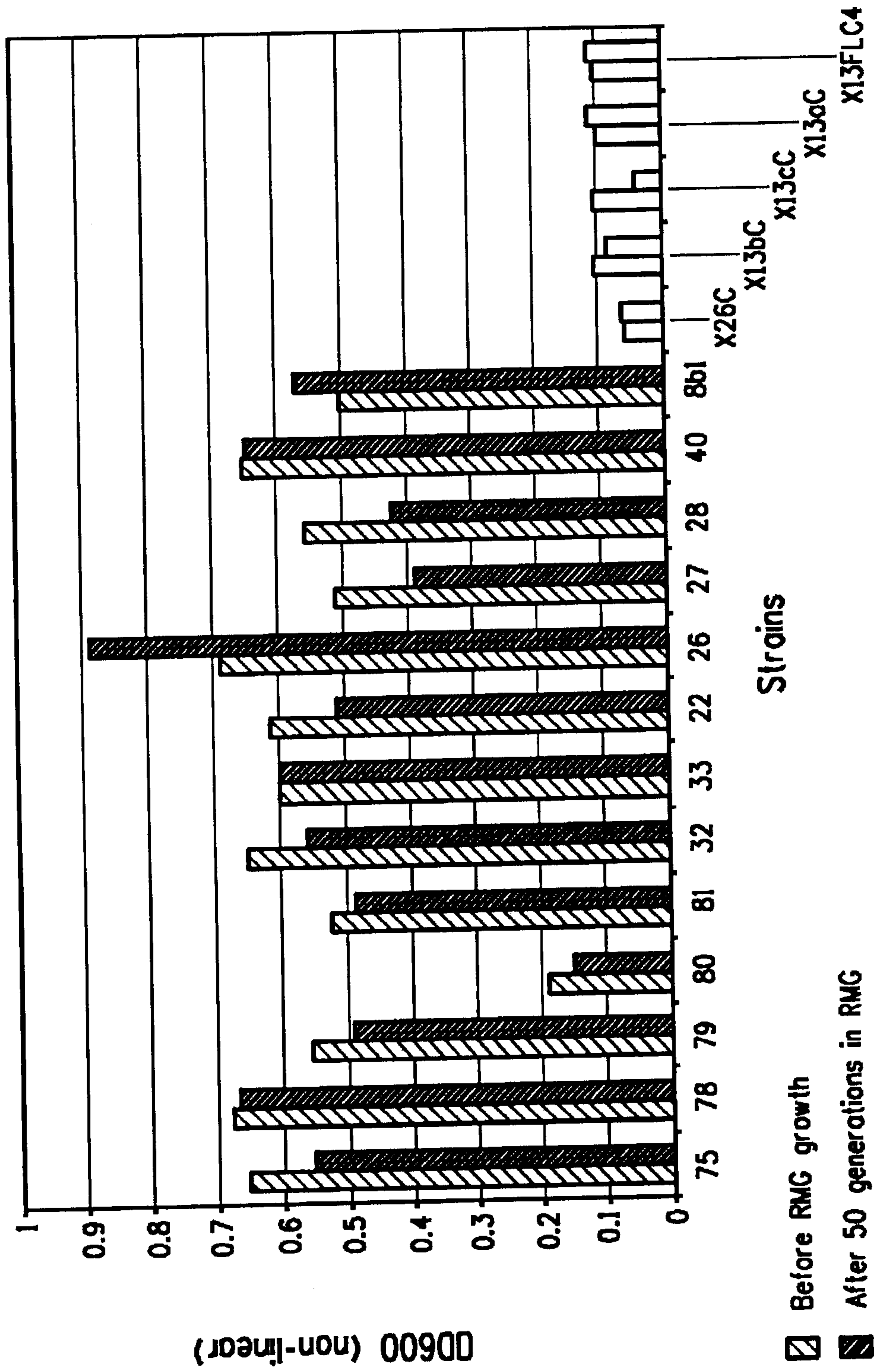


FIG. 7

Figure 8

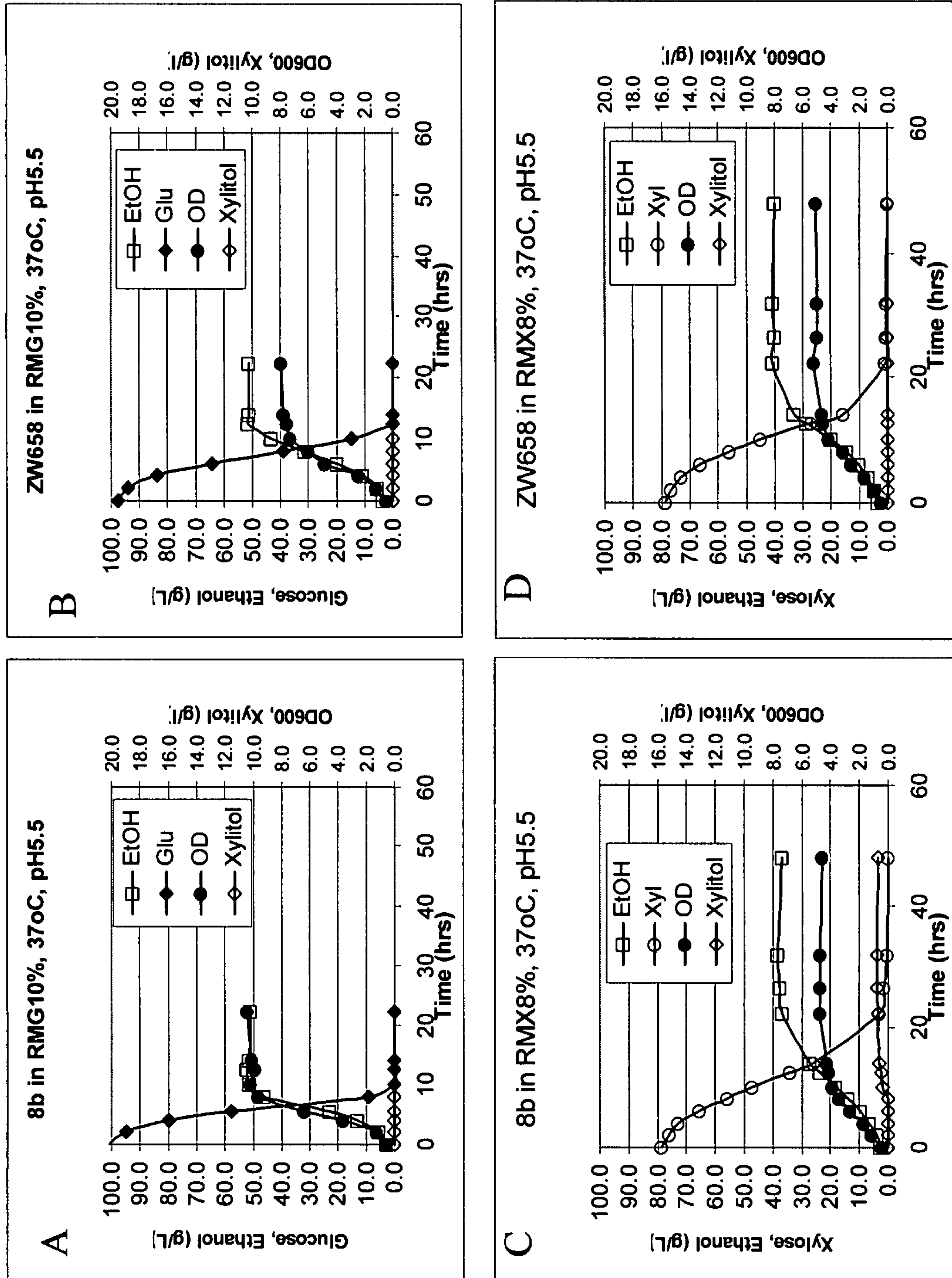
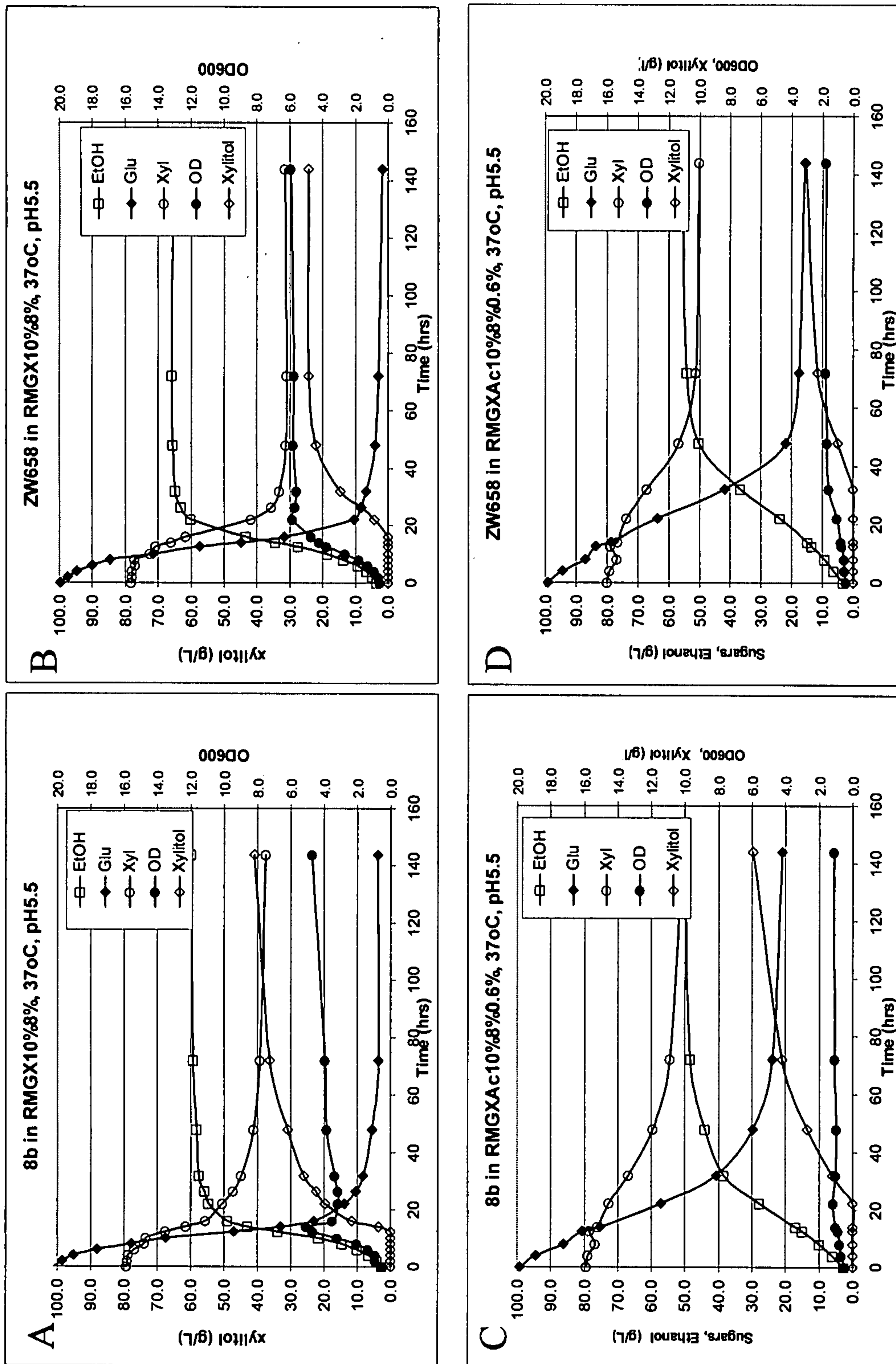


Figure 9



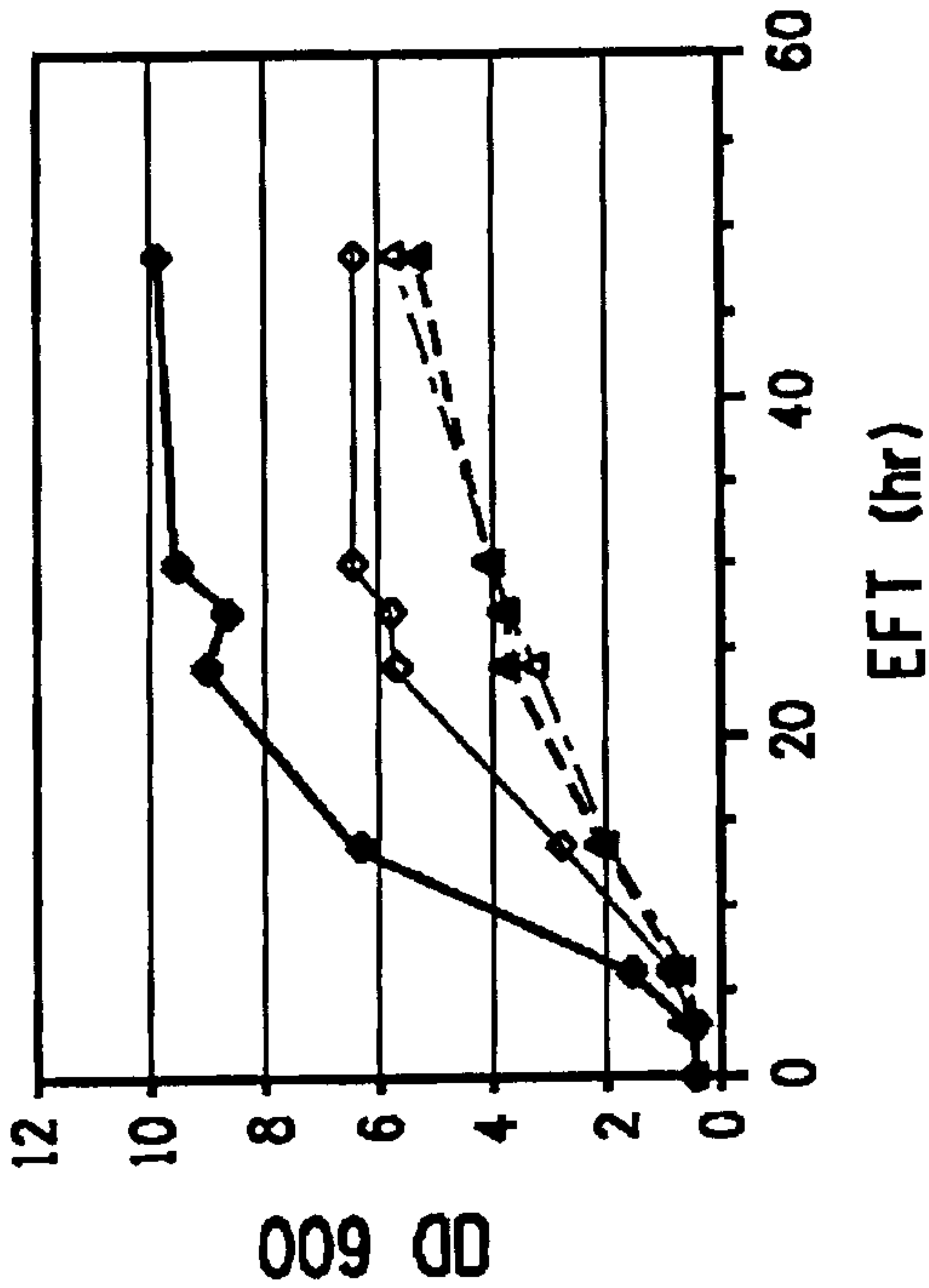


FIG. 10A

- 180 g/L glucose + 20 mM sorbitol
- 180 g/L glucose only
- -●- - 180 g/L xylose + 20 mM sorbitol
- -△- - 180 g/L xylose only

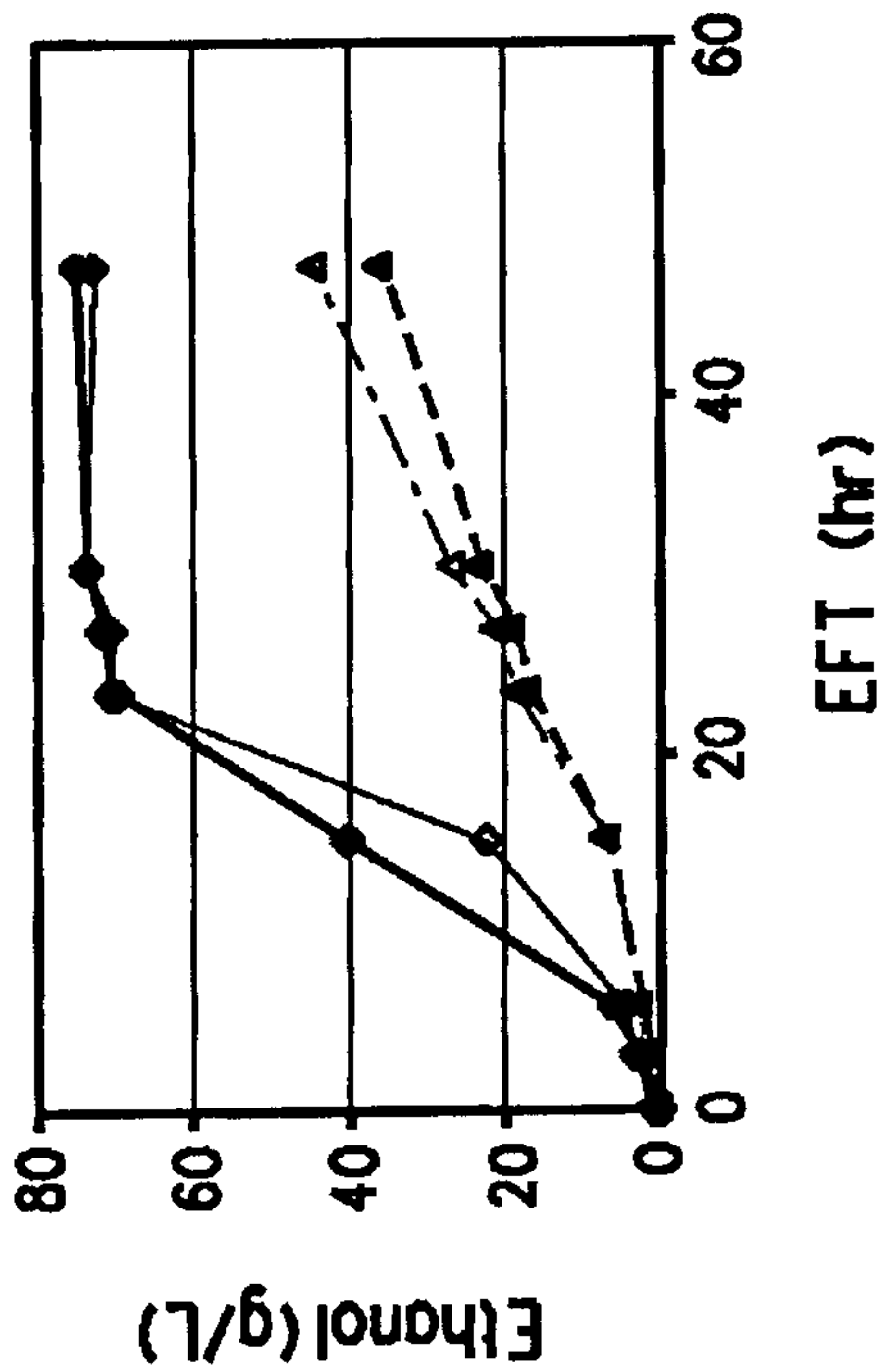


FIG. 10B

- 180 g/L glucose + 20 mM sorbitol
- 180 g/L glucose only
- -●- - 180 g/L xylose + 20 mM sorbitol
- -△- - 180 g/L xylose only

Figure 11

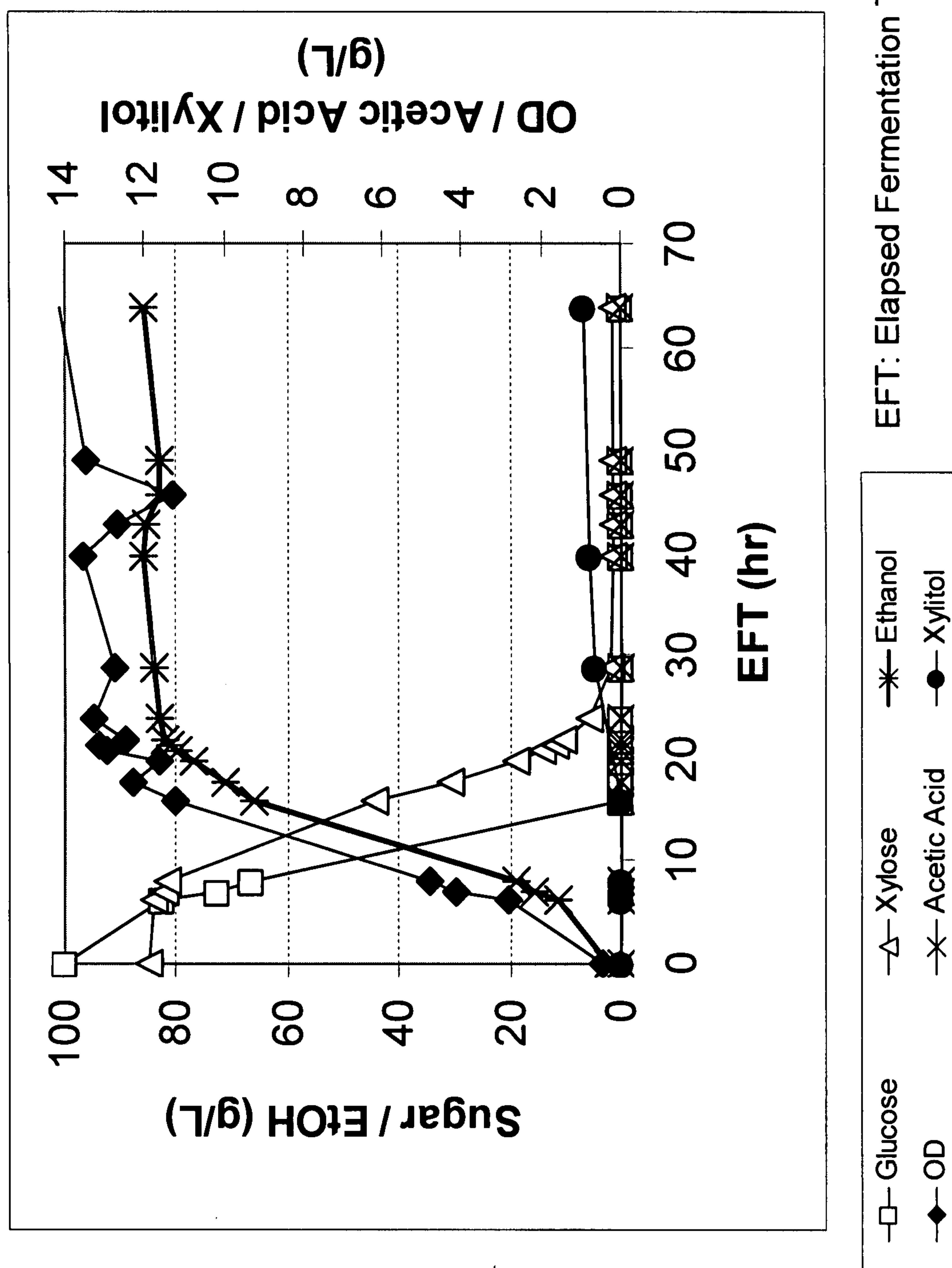


Figure 12

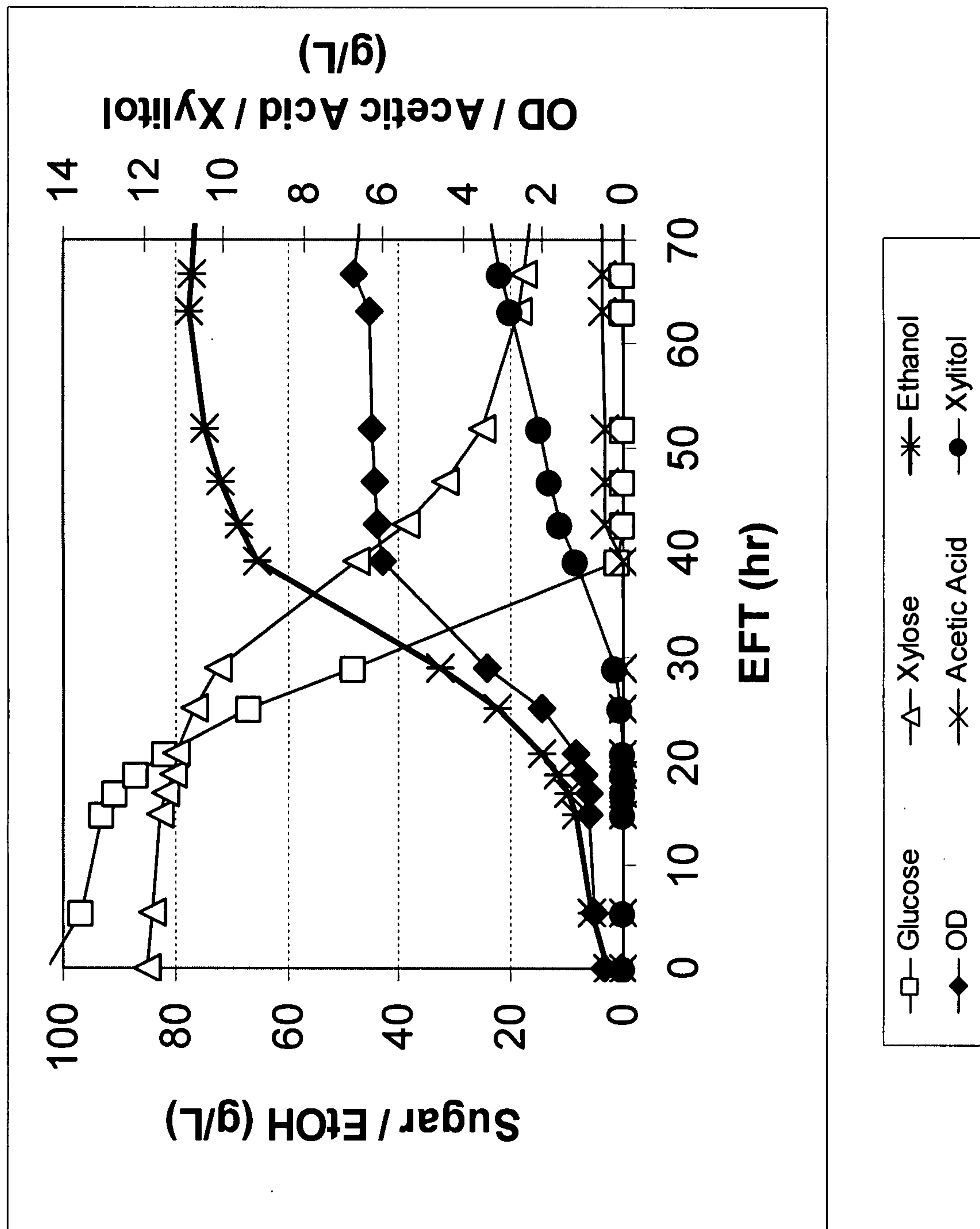


Figure 13

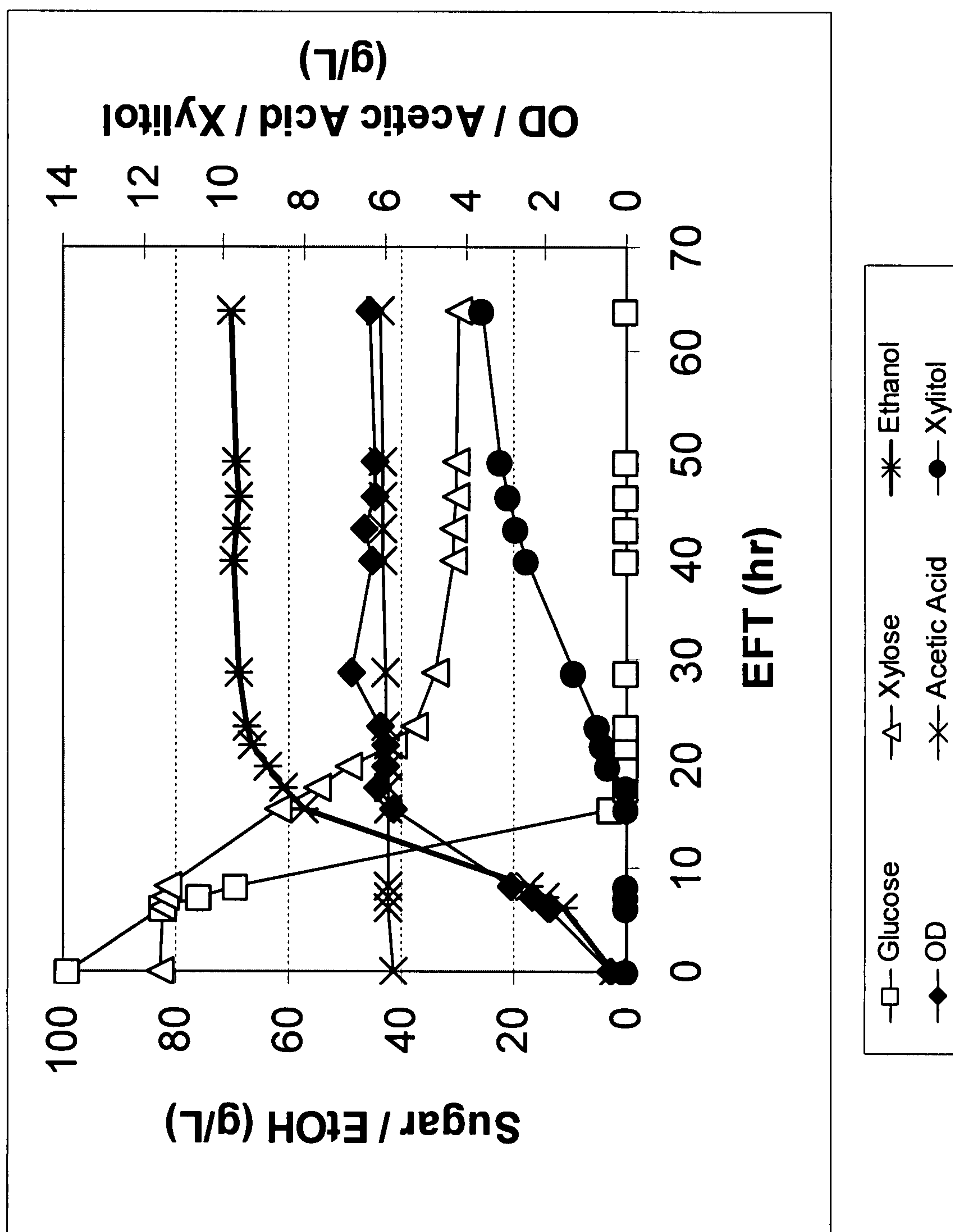


Figure 14

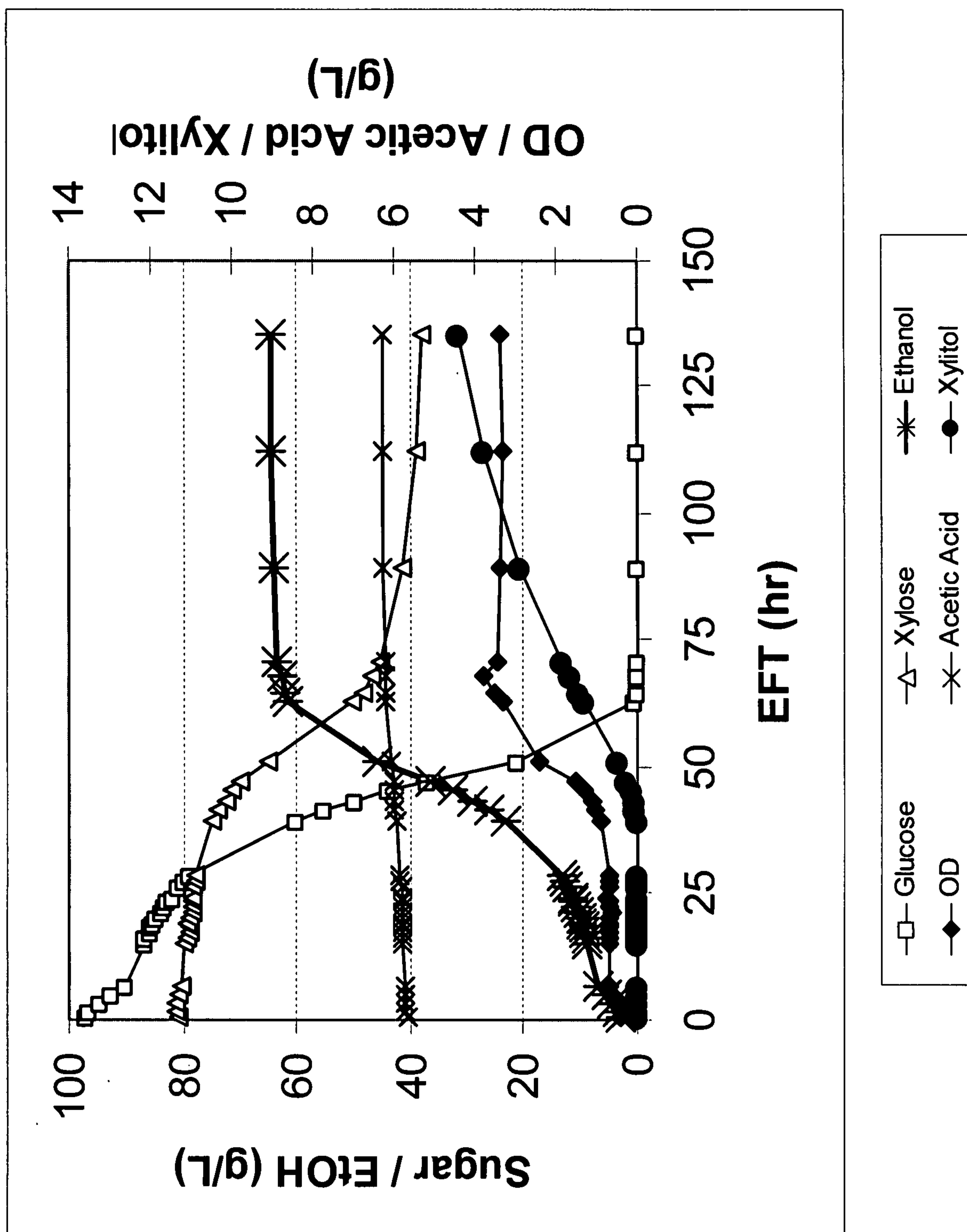


Figure 15

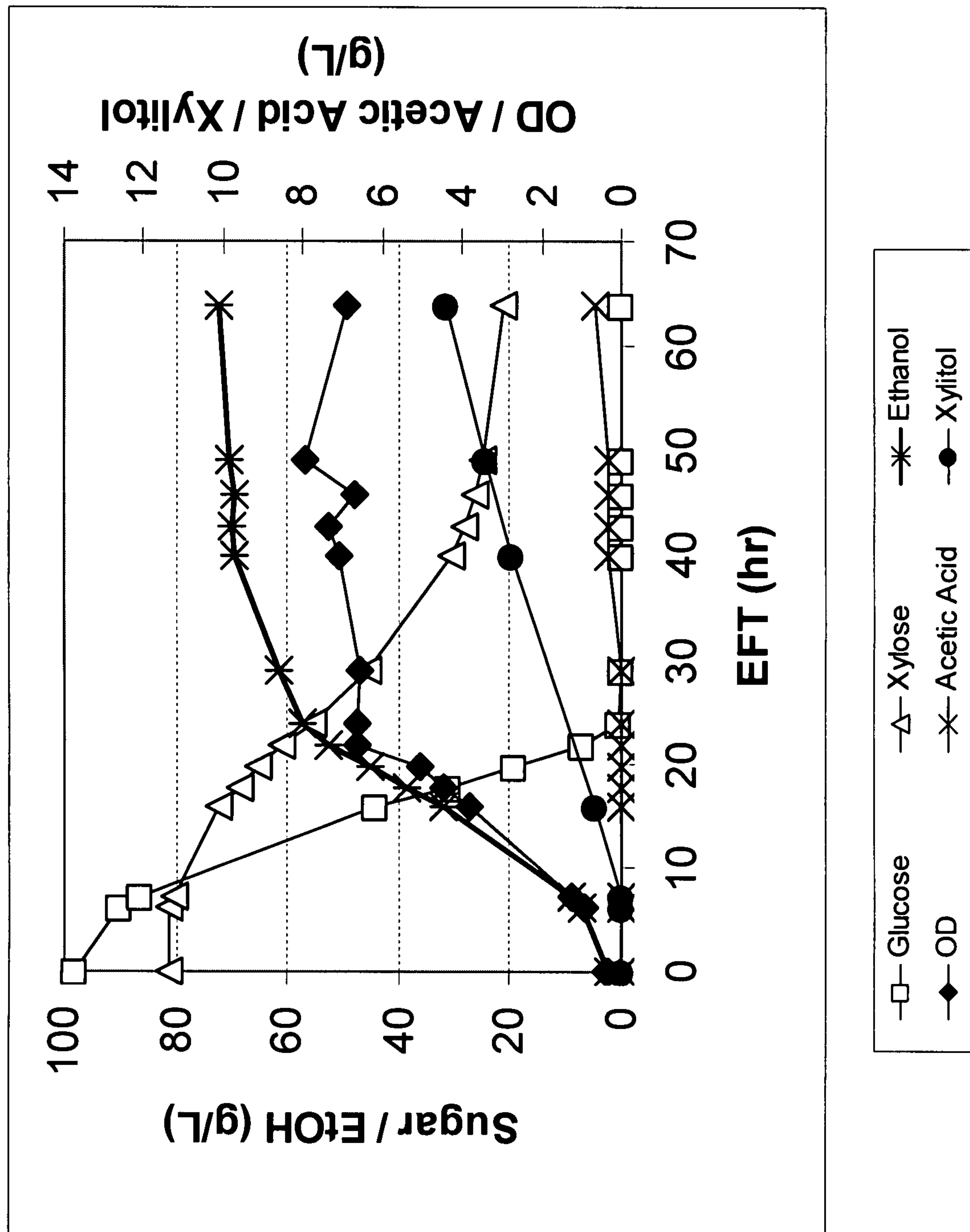


Figure 16

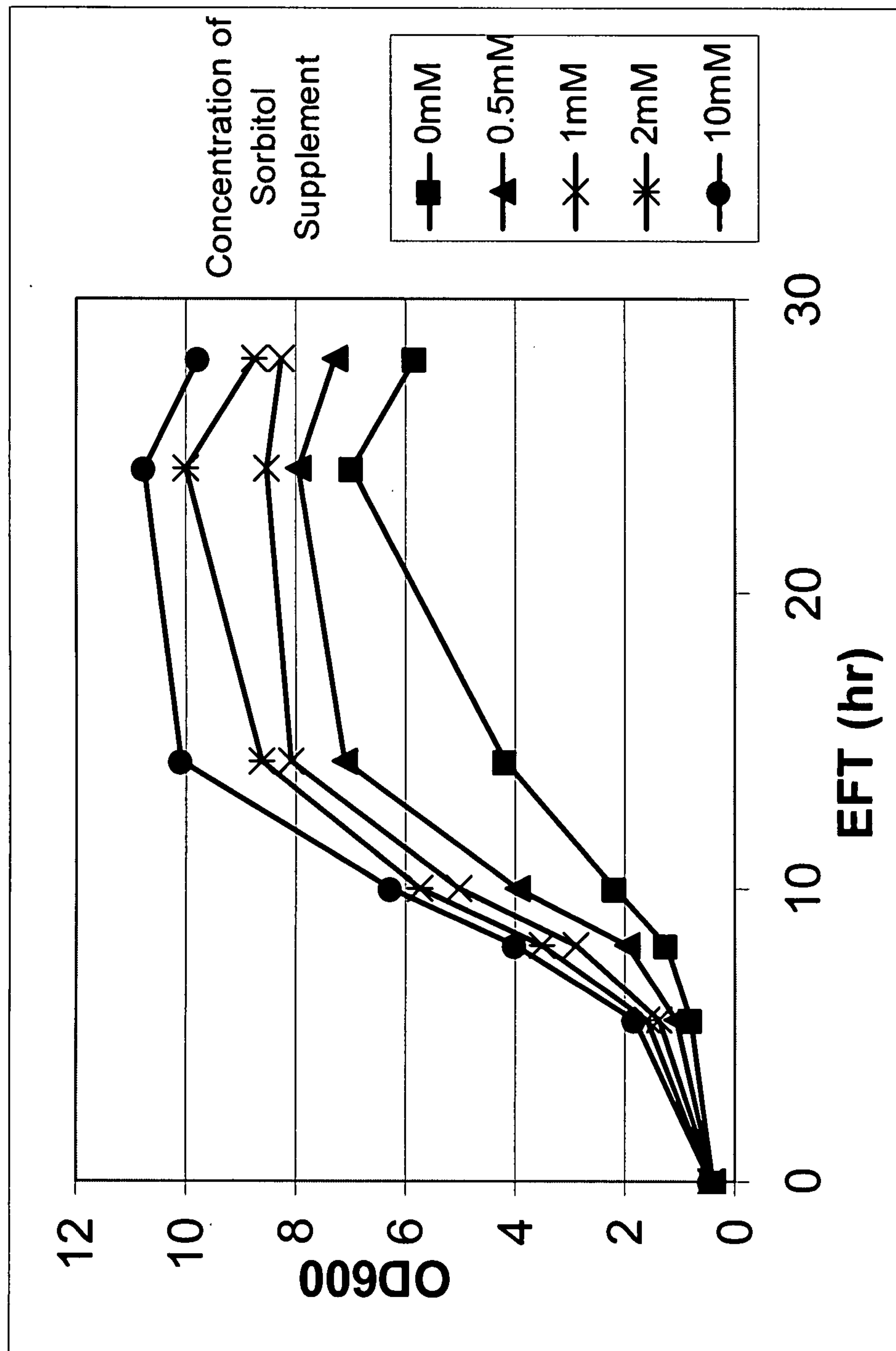


Figure 17

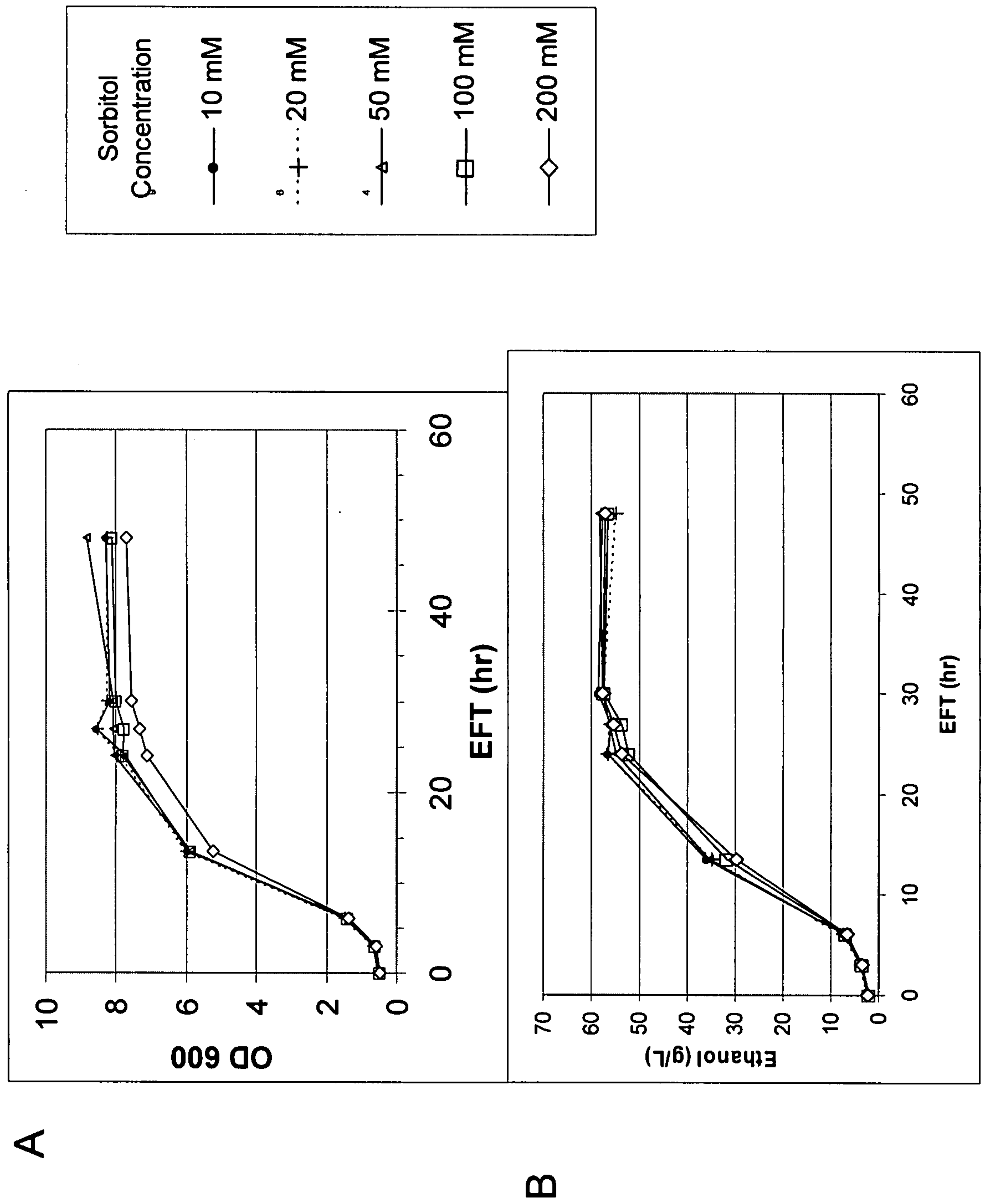


Figure 18

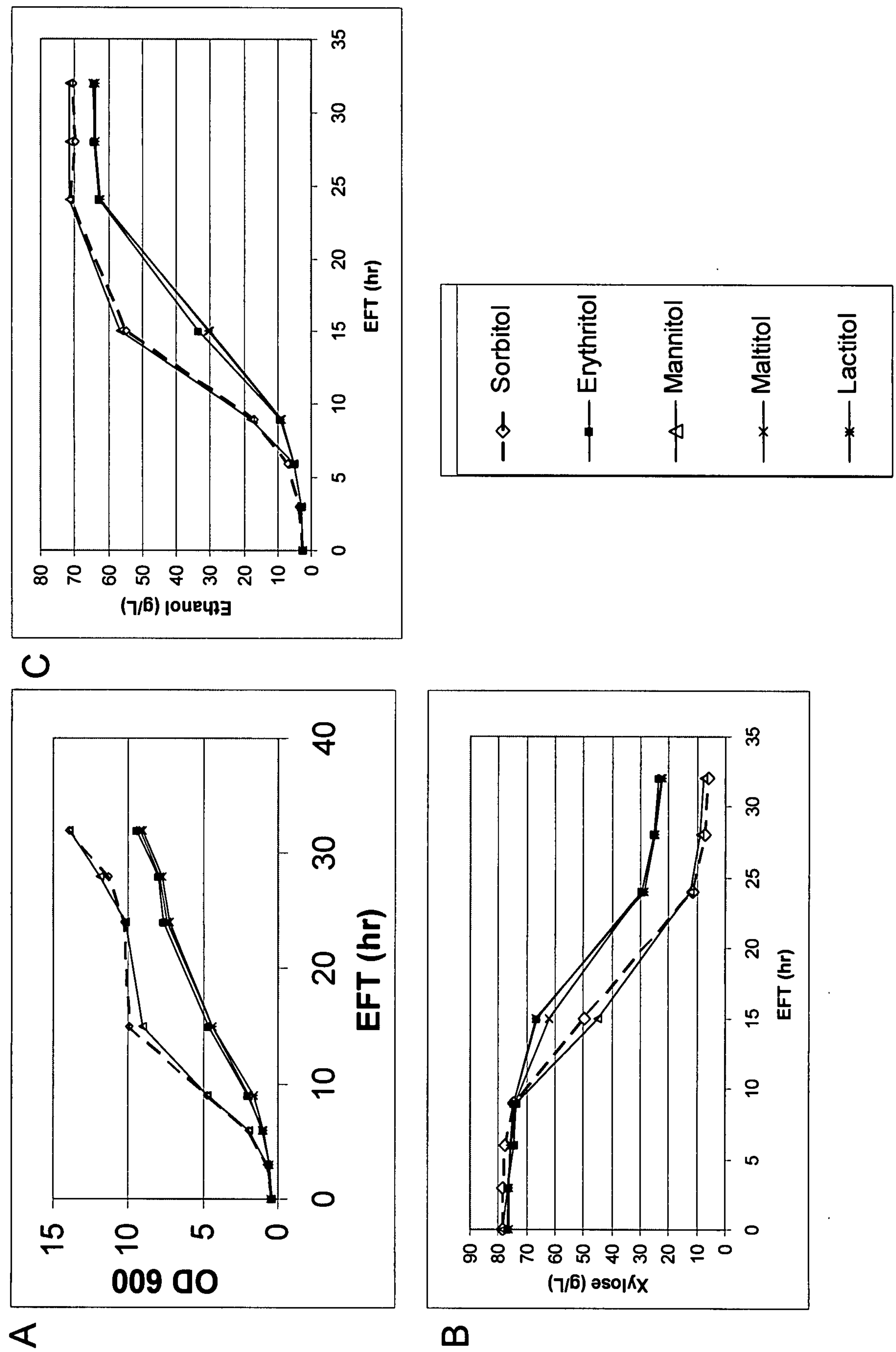


Figure 19

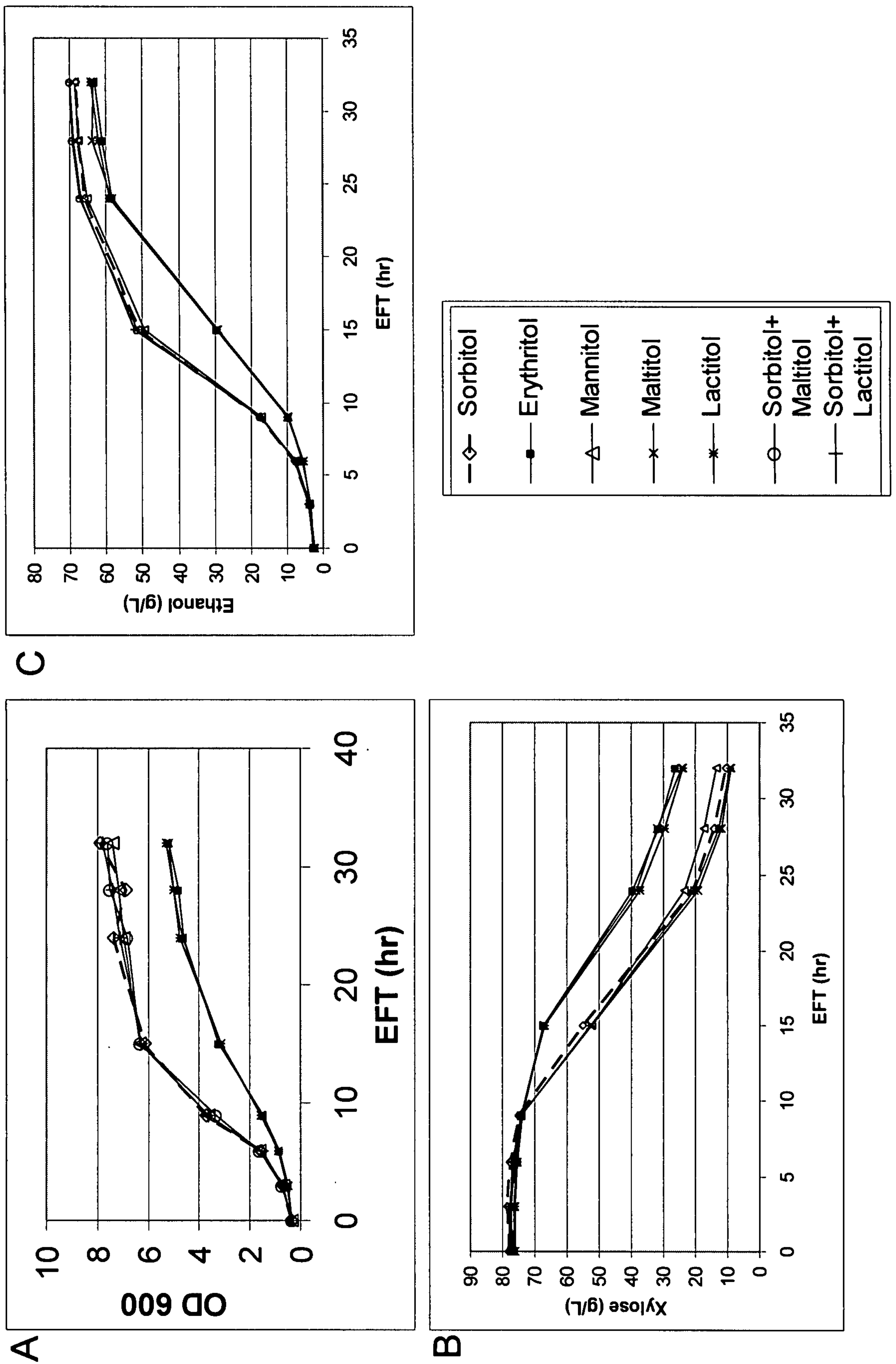


Figure 20

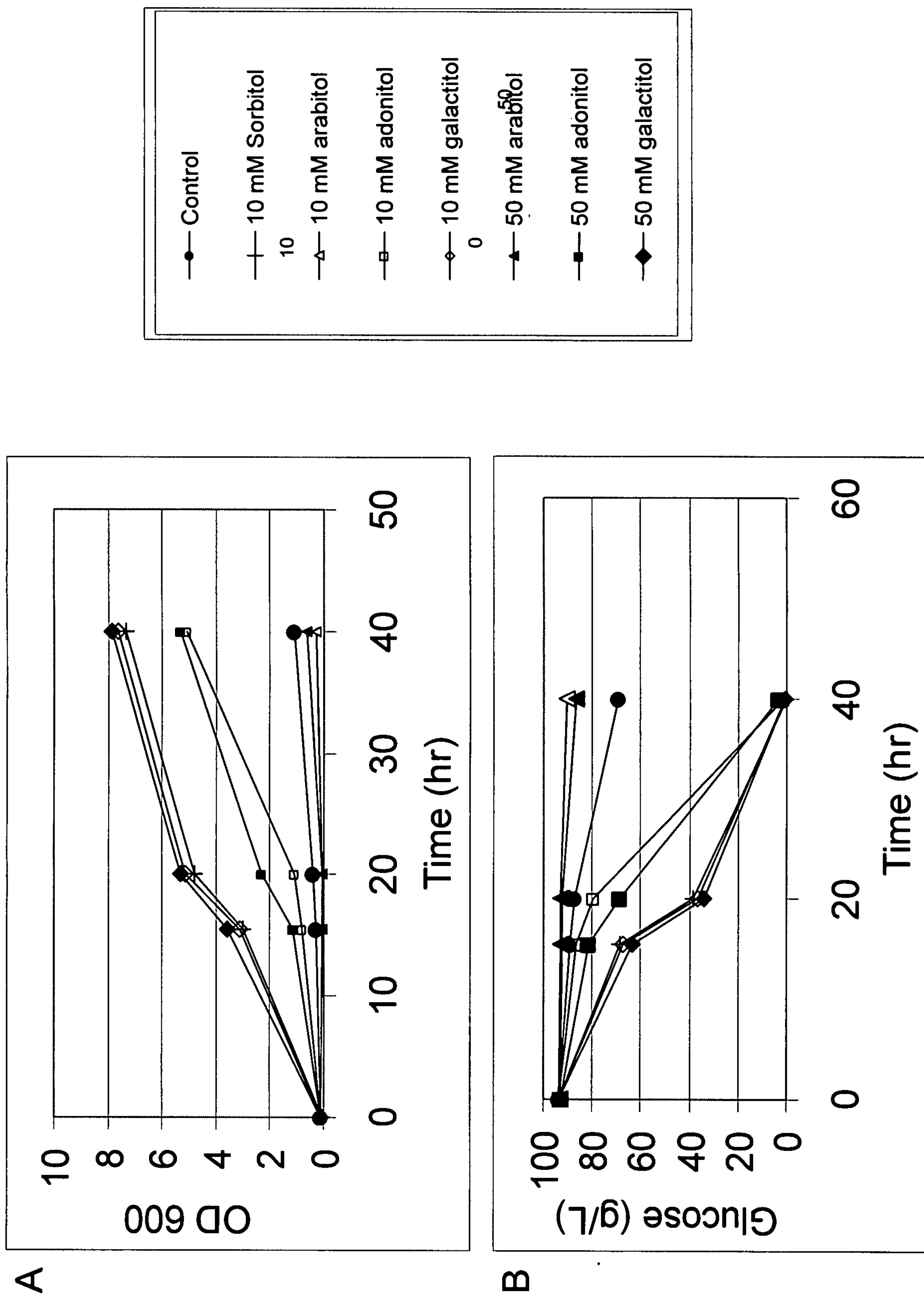


Figure 21

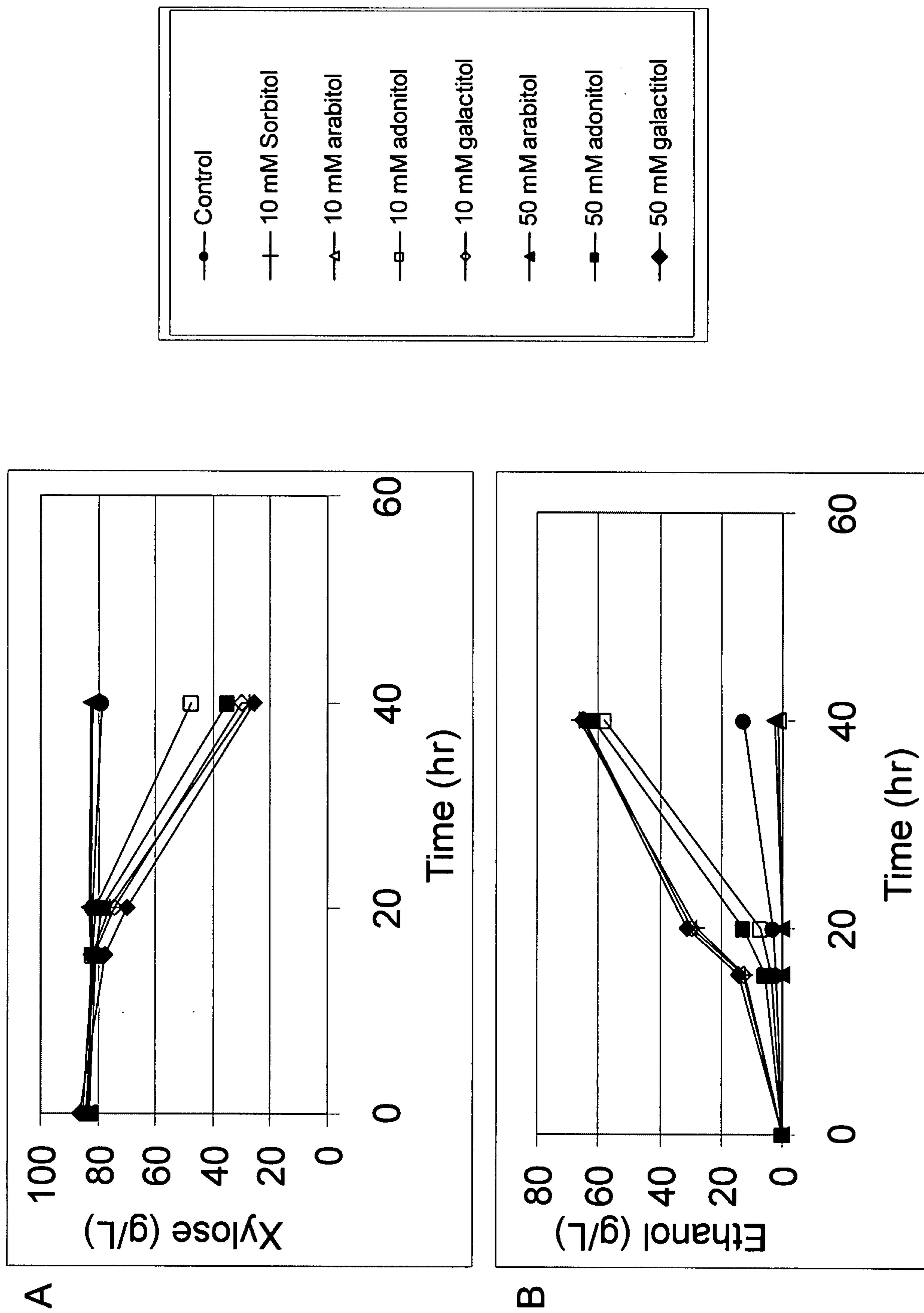


Figure 22

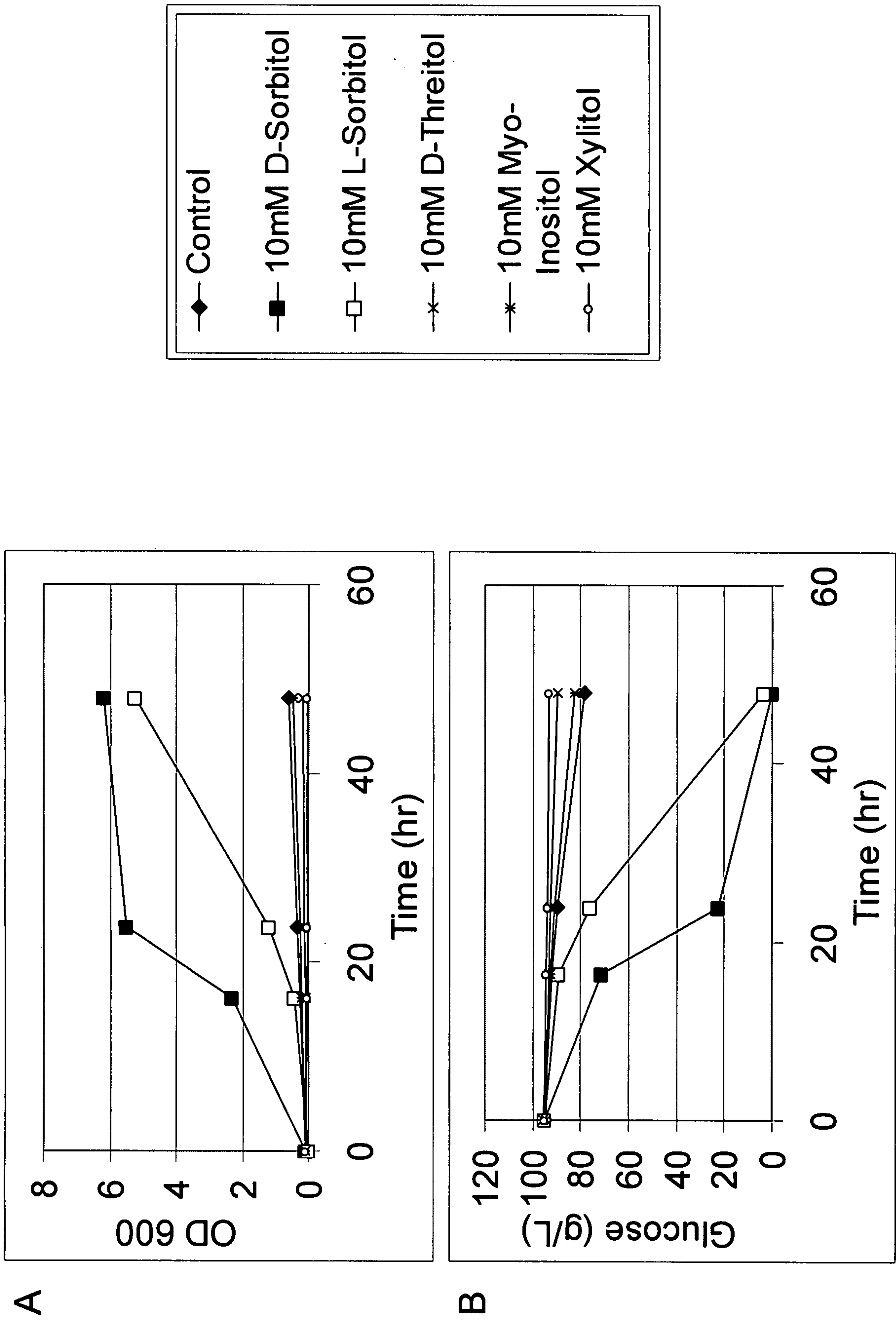
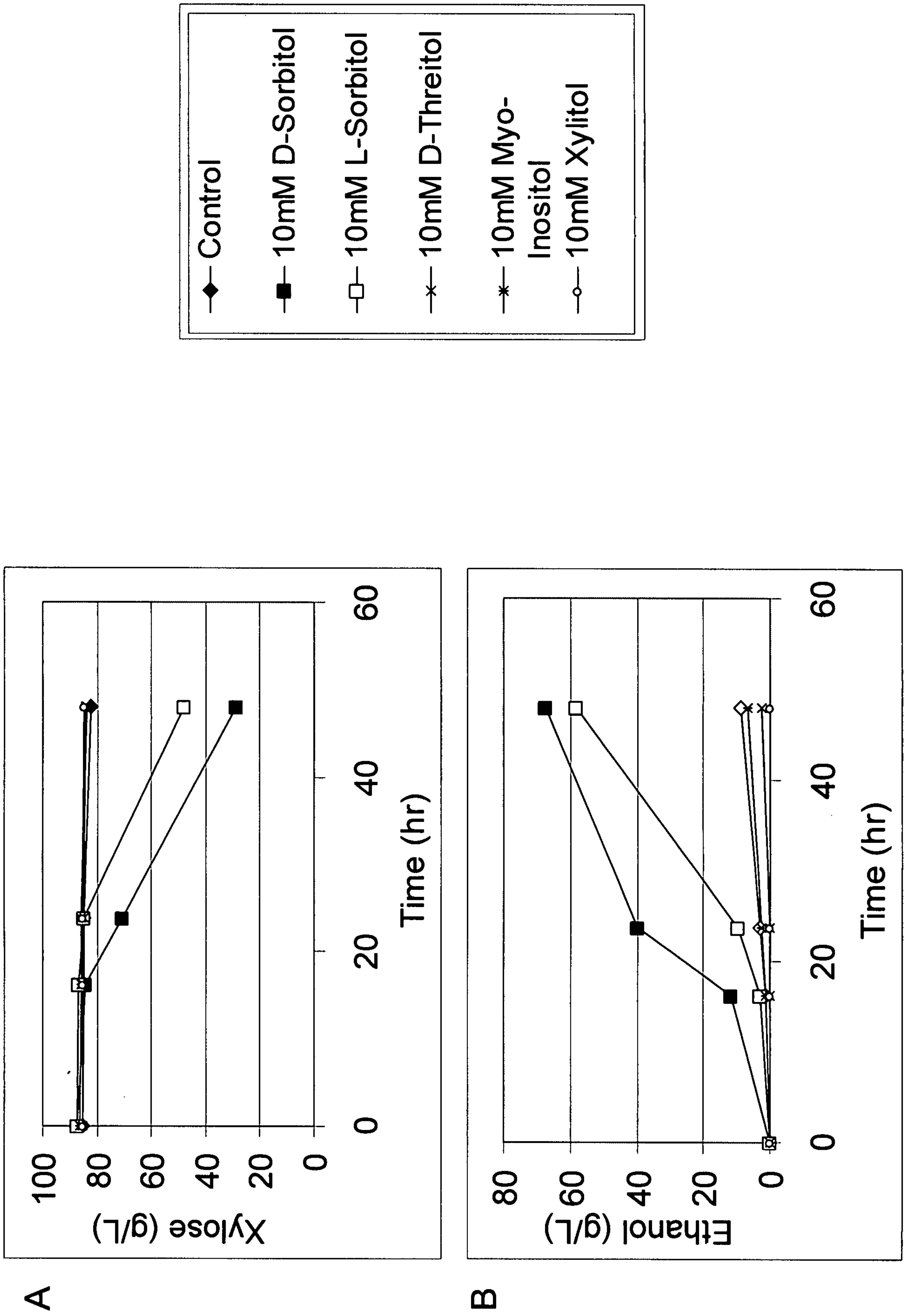
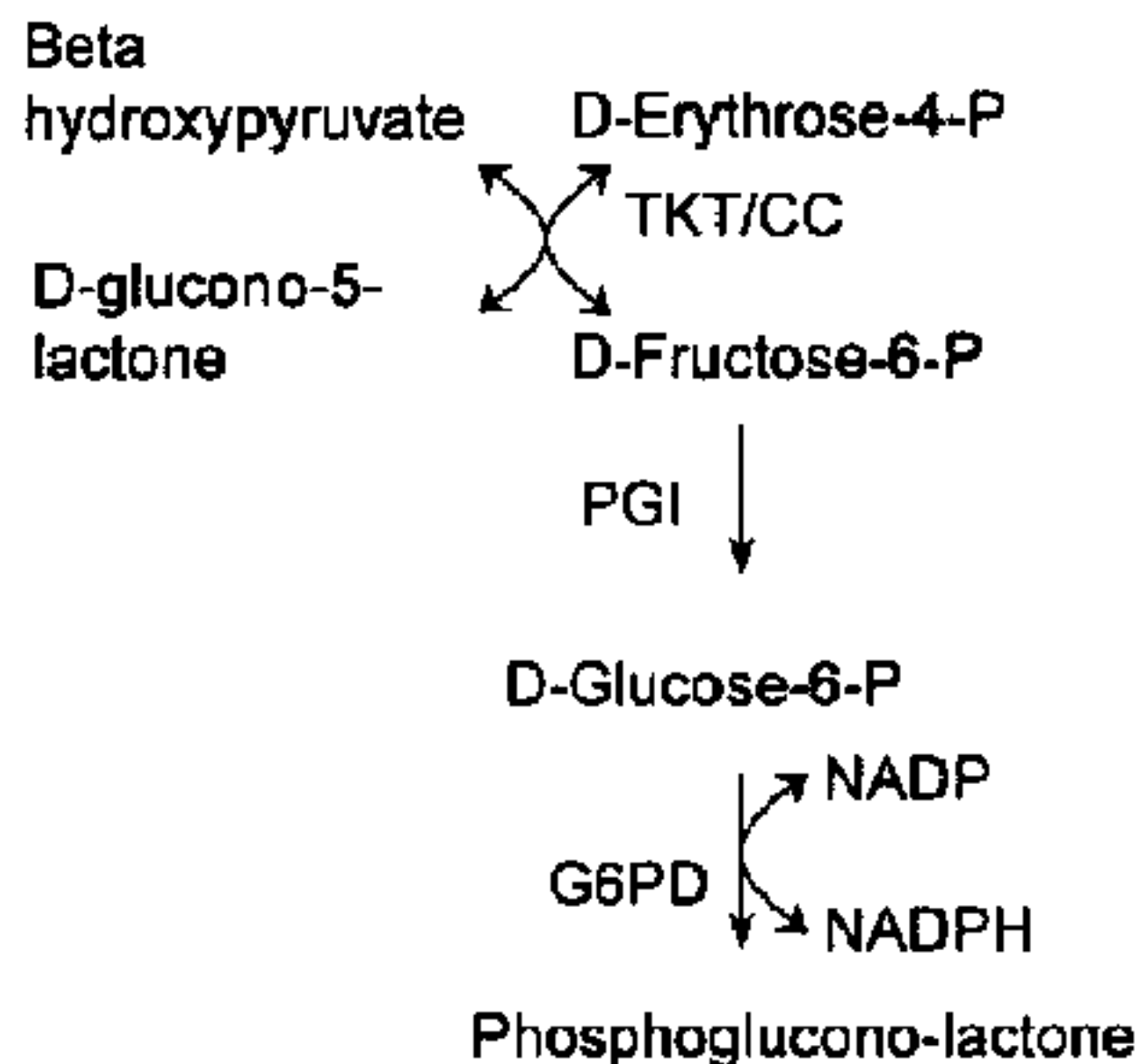


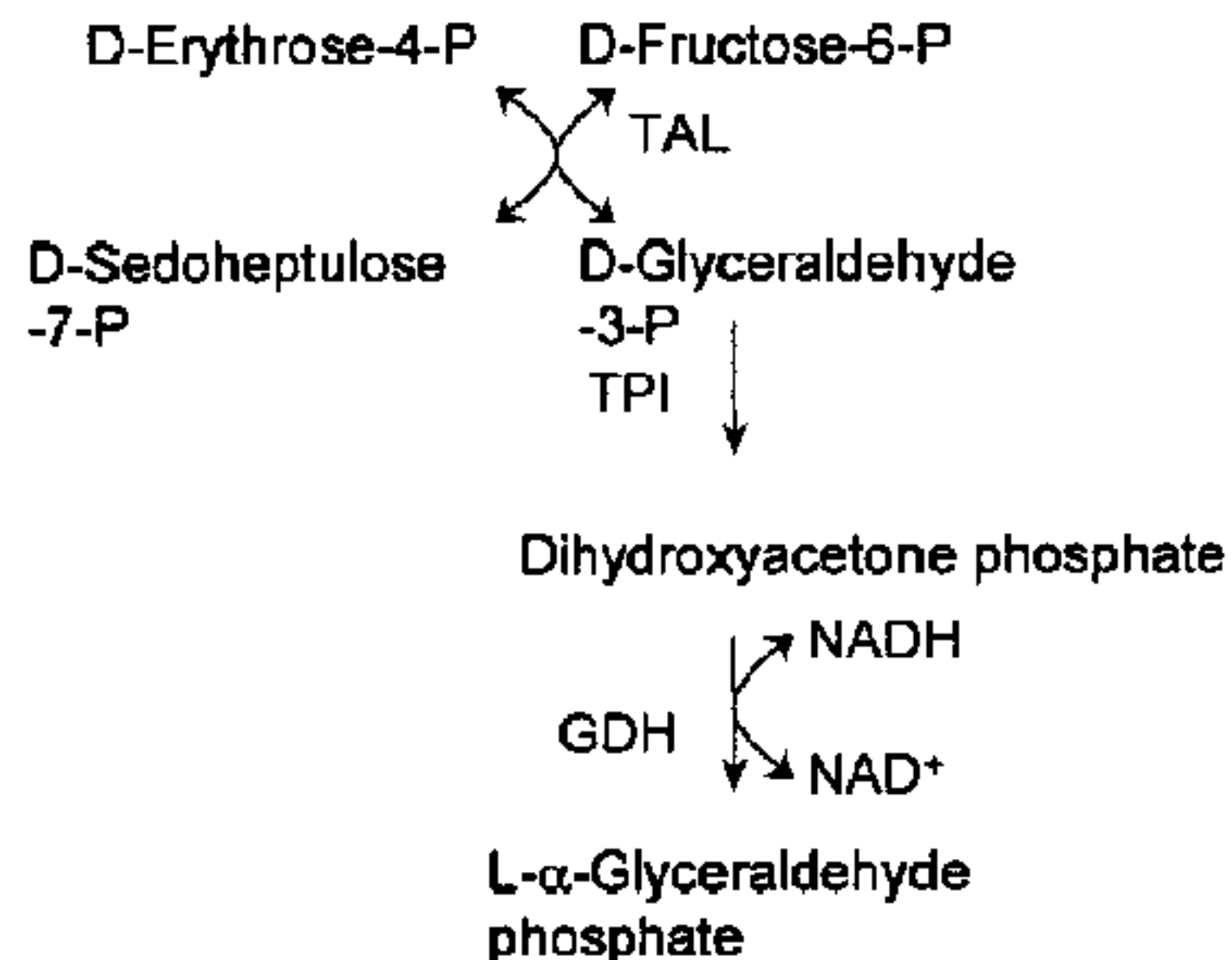
Figure 23



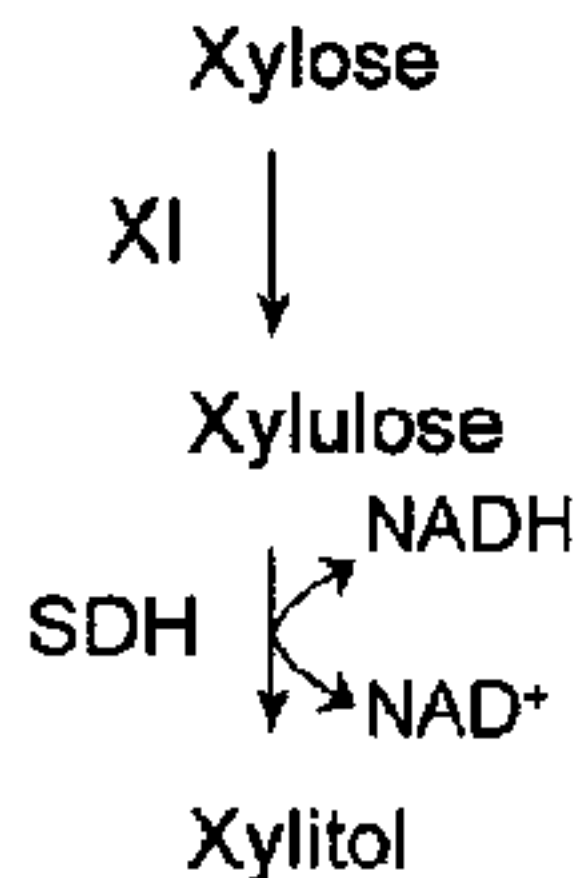
A Transketolase Assay



B Transaldolase Assay



C Xylose Isomerase Assay



D Xyulokinase Assay

