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(54) Title: POLYPEPTIDES HAVING XYLANASE ACTIVITY WITH A HIGH CONVERSION RATE OF XYLOSE-CONTAINING POLYSACCHARIDES

(57) Abrégé/Abstract:
The present application provides novel polypeptides having xylanase activity and the respective nucleic acid sequences encoding those polypeptides as well as vectors comprising these nucleic acid sequences and host cells transformed by these vectors. In addition the present invention provides a method for producing these polypeptides and a composition comprising the inventive polypeptides.

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(54) Title: POLYPEPTIDES HAVING XYLANASE ACTIVITY WITH A HIGH CONVERSION RATE OF XYLOSE-CONTAINING POLYSACCHARIDES

(57) Abstract: The present application provides novel polypeptides having xylanase activity and the respective nucleic acid sequences encoding those polypeptides as well as vectors comprising these nucleic acid sequences and host cells transformed by these vectors. In addition the present invention provides a method for producing these polypeptides and a composition comprising the inventive polypeptides.

Polypeptides having xylanase activity with a high conversion rate of xylose-containing polysaccharides

The present application provides novel polypeptides having xylanase activity and the respective nucleic acid sequences encoding those polypeptides as well as vectors comprising these nucleic acid sequences and host cells transformed by these vectors. In addition the present application provides a method for producing these polypeptides and a composition comprising the inventive polypeptides.

Hemicelluloses and particularly xylan-containing polysaccharides are a valuable source for the production of monosaccharides which can be converted into biofuels, industrial platform chemicals, consumer products, food and feed additives. Due to the heterogeneous chemical structure of this material its degradation requires a series of physicochemical and/or enzymatic treatment steps. Processes enabling an effective complete or selective hydrolysis of pentose-containing polysaccharides are highly desirable.

An important source of pentoses from biomass is xylan. Xylan constitutes about 15 to 25 wt.-% of lignocellulosic biomass and up to 70 wt.-% of other feedstocks such as oat spelts. Xylans represent one of the major components of plant cell walls and constitute major parts of agricultural waste products, e.g. wheat straw, corn stover, corn cobs, and cotton seed. Xylans consist of xylose monomeric subunits linked by ß-1-4-glycosidic bonds in a complex polymer with various other components such as arabinose, glucuronic acid, methylglucuronic acid, and acetyl groups. In cereals, xylans frequently contain side chains of α-1,2- and/or α-1,3-linked L-arabinofuranoside. These substituted xylans are commonly referred to as arabinoxylans. Xylans that are substituted with glucose are referred to as glucoxylans. Also mixed forms of these xylans exist.

Xylanases (ß-1,3- or ß-1,4-xylan xylohydrolase; E.C. 3.2.1.8) are xylanolytic enzymes that depolymerize xylan, arabinoxylan, and/or other xylose-containing polysaccharides. Endo-xylanases (e.g. endo-ß-1,4-xylanase) hydrolyze the internal ß-glycosidic linkages in xylan, arabinoxylan, and/or other xylose-containing polysaccharides to produce smaller molecular weight xylo-oligomers or xylose monomers.

Major industrial applications of xylanases today are for example in the pulp and paper industry to improve the bleachability of pulps and the food industry to produce xylose as basis for the

sweetener xylitol. Furthermore, xylanases can be used in food and feed compositions which contain cereals (e.g. barley, wheat, maize, rye, triticale, or oats) or cereal by-products that are rich in xylans, arabinoxylans and/or glucoxylans. Addition of xylanases to animal feed or baking products improves the break-down of plant cell walls which leads to better utilization of plant nutrients and/or prolonged bread freshness, respectively. In feed compositions xylanase addition leads to improved animal growth rate and feed conversion. Additionally, the viscosity of feed compositions containing xylan can be reduced by xylanase leading to better acceptability and adsorption.

Despite the relatively high number of known fungal and bacterial xylanases, the number of xylanases which do not only serve the intended purpose but also industrially applicable (and thus commercially profitable) remains limited. This is mainly due to particular physical process conditions, such as high temperature and specific pH conditions, as well as lack of substrate and/or product selectivity and compatibility of the particular xylanase leading to low conversion rates. Such drawbacks limit the use of xylanases.

As transformation of biomass such as cellulose- and lignocellulose-containing biomass of various origins to valuable products is gaining more and more importance, there is an increasing need for xylanases which enable efficient and industrially applicable conversion. Within the EP 2 336 152 highly efficient xylanases with enhanced thermostability are disclosed. To even more increase efficiency of product generation and purification, further improvements are, however, mandatory.

The inventors of the present application have therefore set themselves the task to develop novel xylanases which allow efficient product generation due to high conversion rates also of recalcitrant substrates such as xylose-containing polysaccharides. In addition, a high substrate compatibility and ability of synergistic interaction with other enzymes should lead to further process intensification and further cost reduction. Finally, the xylanases should also show a high temperature and pH stability.

The inventors of the present application have now surprisingly found that this task can be solved by polypeptides having xylanase activity with a high conversion rate of xylose-containing polysaccharides, wherein the polypeptide comprises an amino acid sequence having at least 75% sequence identity to SEQ ID No: 2.

The term "xylanase activity" refers to all polypeptides which are capable of catalyzing the hydrolysis of ß-1,3- or ß-1,4-xylosidic linkages with the release of smaller molecular weight xylo-

oligomers or xylose monomers. The term "xylanase" is defined herein as a ß-1,3- or ß-1,4-xylan xylohydrolase (E.C. 3.2.1.8).

The term "xylose-containing polysaccharides" refers to any substrate containing xylose oligomers such as xylotetranose, xylopentanose, xylohexanose or xylose polymers such as xylan or hemicellulose. Examples for xylose-containing polysaccharides are wheat straw, wood, cereal straw and/or husks, corn stover, bagasse, oat hulls, switch grass, cellulose, raw paper pulp (obtained from pulp and paper production) and mixtures thereof and other kind of lignocellulosic plant material.

The nomenclature of amino acids, peptides, nucleotides and nucleic acids is done according to the suggestions of IUPAC. Generally amino acids are named within this document according to the one letter code.

The term "high conversion rate of xylose-containing polysaccharides" refers to the ability to convert at least 60 wt.-% of the xylose-containing polysaccharides of a certain substrate to xylose and/or xylose-containing oligosaccharides and glucose, preferably at least 70 wt.-%, more preferred at least 75 wt.-%, even more preferred at least 80 wt.-%, particularly preferred at least 85 wt.-% and most preferred at least 90 wt.-% when subjecting neutral steam-exploded wheat straw to the polypeptide at pH 5 and 50°C for 24 hours.

Within a further preferred embodiment, the polypeptides according to the present application convert xylose-containing polysaccharides to xylose and/or xylose-containing oligosaccharides and glucose monomers in a weight ratio of at least 5:1, preferably 7:1 and most preferred 10:1 when subjecting neutral steam-exploded wheat straw to the polypeptide at pH 5 and 50°C for 24 hours.

The polypeptide according to the present application comprises an amino acid sequence having at least 75% sequence identity, preferably at least 77%, further preferred at least 80%, particularly preferred at least 85%, even more preferred at least 90%, also preferred at least 95%, furthermore preferred at least 98% and most preferred at least 99% sequence identity to SEQ ID No: 2.

The polypeptide according to the present application preferably comprises a signal peptide which is cleaved off during secretion into the supernatant.

The polypeptide according to the present application preferably comprises a polypeptide chain of more than 250 amino acids. More preferably, the length is between 290 and 500 amino acids, even more preferably between 320 and 400 amino acids. Most preferably the polypeptide comprises between 379 and 390 amino acid residues.

The polypeptide according to the present application preferably has a molecular weight of more than 30 kD. More preferably, the size is between 32 and 45 kD, even more preferably between 34.5 and 42.5 kD. Most preferably the polypeptide has a size between 40 and 42 kD. A particularly suitable size is 41,9 kD of the unmodified polypeptide molecule.

Furthermore, it is particularly preferred that the amino acid sequence of the polypeptide has the sequence as defined by SEQ ID No: 2 or SEQ ID No: 4 wherein 1 to 30 amino acid residues are substituted, deleted, or inserted (all also referred to as "mutations").

Particularly preferred are variants of the protein of SEQ ID NO: 2. "Protein variants" are polypeptides whose amino acid sequence differs in one or more positions from this parental protein, whereby differences might be replacements of one amino acid by another, deletions of single or several amino acids, or insertion of additional amino acids or stretches of amino acids into the parental sequence. Per definition variants of the parental polypeptide shall be distinguished from other polypeptides by comparison of sequence identity (alignments) using the ClustalW Algorithm (Larkin M.A., Blackshields G., Brown N.P., Chenna R., McGettigan P.A., McWilliam H., Valentin F., Wallace I.M., Wilm A., Lopez R., Thompson J.D., Gibson T.J. and Higgins D.G. (2007) ClustalW and ClustalX version 2. Bioinformatics 2007 23(21): 2947-2948). Methods for the generation of such protein variants include random or site directed mutagenesis, site-saturation mutagenesis, PCR-based fragment assembly, DNA shuffling, homologous recombination in-vitro or in-vivo, and methods of gene-synthesis.

Exchanges or substitutions of single amino acids or are described by naming the single letter code of the original amino acid followed by its position number and the single letter code of the replacing amino acid, i.e. the change of glutamine at position one to a leucine at this position is described as "Q1L". For deletions of single positions from the sequence the symbol of the replacing amino acid is substituted by the three letter abbreviation "del" thus the deletion of glutamine at position 3 would be referred to as "Q3del". Inserted additional amino acids receive the number of the preceding position extended by a small letter in alphabetical order relative to their distance to their point of insertion. Thus, the insertion of two tryptophanes after position 3 is referred to as "3aW, 3bW". Introduction of untranslated codons TAA, TGA and TAG into the

nucleic acid sequence is indicated as "*" in the amino acid sequence, thus the introduction of a terminating codon at position 4 of the amino acid sequence is referred to as "T4*".

Multiple mutations are separated by a plus sign or a slash or a comma. For example, two mutations in positions 20 and 21 substituting alanine and glutamic acid for glycine and serine, respectively, are indicated as "V20G+S21T" or "V20G/S21T" "V20G,S21T".

When an amino acid residue at a given position is substituted with two or more alternative amino acid residues these residues are separated by a comma or a slash. For example, substitution of alanine at position 30 with either glycine or glutamic acid is indicated as "V20G,E" or "V20G/E", or "V20G, V20E".

When a position suitable for mutation is identified herein without any specific mutation being suggested, it is to be understood that any amino acid residue may be substituted for the amino acid residue present in the position. Thus, for instance, when a mutation of a valine in position 20 is mentioned but not specified, it is to be understood that the alanine may be deleted or substituted for any other amino acid residue (i.e. any one of R, N, D, C, Q, E, G, H, I, L, K, M, F, P, S, T, W, Y and V).

The terms "similar mutation" or e.g. "similar substitution" refer to an amino acid mutation that a person skilled in the art would consider similar to a first mutation. Similar in this context means an amino acid that has similar chemical characteristics. If, for example, a mutation at a specific position leads to a substitution of a non-aliphatic amino acid residue (e.g. Ser) with an aliphatic amino acid residue (e.g. Leu), then a substitution at the same position with a different aliphatic amino acid (e.g. Ile or Val) is referred to as a similar mutation. Further amino acid characteristics include size of the residue, hydrophobicity, polarity, charge, pK-value, and other amino acid characteristics known in the art. Accordingly, a similar mutation may include substitution such as basic for basic, acidic for acidic, polar for polar etc. The sets of amino acids thus derived are likely to be conserved for structural reasons. These sets can be described in the form of a Venn diagram. Similar substitutions may be made, for example, according to the following grouping of amino acids: Hydrophobic: F W Y H K M I L V A G; Aromatic: F W Y H; Aliphatic: I L V; Polar: W Y H K R E D C S T N; Charged H K R E D; Positively charged: H K R; Negatively charged: E D.

As convention for numbering of amino acids and designation of protein variants for the description of protein variants the first glutamine (Q) of the amino acid sequence QAQTWG within the parental protein sequence given in SEQ ID NO: 4 is referred to as position number 1 or Q1 or

glutamine 1. The numbering of all amino acids will be according to their position in the parental sequence given in SEQ ID No: 2 relative to this position number 1.

Within a particular preferred embodiment, the present application provides the novel polypeptide FfXyn1 (SEQ ID No: 2). Furthermore, the polypeptides mFfXyn1 (SEQ ID No: 4) comprising the respective mature protein as well as fusions with N-terminal signal peptides, exemplified by the coding nucleic acids of SEQ ID No: 4 and SEQ ID No: 5, are provided within particularly preferred embodiments.

The present application also provides fusion proteins of the polypeptide of the present application with other protein sequences. Such sequences can represent catalytically active proteins, binding proteins, proteins influencing aspects of the cellular expression or sequences influencing chemical, catalytic, biological or physical properties of the fused target protein, or being without particular effect. The fusions also include those containing only parts of the target sequence, wherein this part contributes to the enzymatic activity of the fusion protein. Of special interest among the fusions with catalytically active proteins are those with proteins selected from the group of carbohydrate-modifying enzymes. Of special interest among the fusions with binding proteins are those made with binding modules from carbohydrate-modifying enzymes. It is well known that such fusions can beneficially influence the enzymatic and physical properties of the fused parts, especially those of the target protein.

Within a particular preferred embodiment of the present application the polypeptide according to the present application is fused with a carbohydrate-binding module with special affinity to xylan or other polymeric sugars found in hemicellulose.

Within an even more preferred embodiment of the present application the fusion partners of the polypeptide according to the present application are selected from carbohydrate-binding module (CBM) sequences from the classes 13, 15, 22, 31, 35, 36 or 37 (CAZy database; Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res 37:D233-238 [PMID: 18838391]).

Further preferred CBM fusion partners of the polypeptide according to the present application are selected from class 13 are the xylan-binding modules of *Streptomyces lividans* (Blast Entry no. AAC26525.1) and *Aspergillus fumigatus* Af293 (Blast Entry no. EAL91233.1).

Further preferred fusion partners of the polypeptide according to the present application are the CBMs of *Thermobifida fusca* (Blast Entry no. AAZ55678.1) and *Teredinibacter turnerae* T7901 (Blast Entry no. ACS93528.1).

Further preferred CBM fusion partners for the polypeptide according to the present application from class 22 are the xylan-binding modules of *Paenibacillus barcinonensis* (Blast Entry no. CAA07173.1), *Thermoanaerobacterium saccharolyticum* (Blast Entry no. AAC43719.1) or *Xylanimicrobium pachnodae* (Blast Entry no. AAD54768.1), *Cellulomonas fimi* (Blast Entry no. CAA90745.1) or *Caldicellulosiruptor sp.* Rt69B.1 (Blast Entry no. AAB95326.1).

A further preferred CBM fusion partner of the polypeptide according to the present application selected from class 36 is the xylan-binding modules of *Clostridium phytofermentans* ISDg (Blast Entry no. ABX42059.1).

A further preferred CBM fusion partner of the polypeptide according to the present application selected from class 37 is the xylan-binding modules of *Ruminococcus albus* 8 (Blast Entry no. AAT48119.1).

A further preferred CBM fusion partner of the polypeptide according to the present application is the class 1 cellulose binding module of *Trichoderma reesei* cellobiohydrolase 1 (Blast Entry no. XP_006969224).

The polypeptides according to the present application are also characterized by high thermal process stability. Preferably, the polypeptide according to the present application maintains at least 80%, more preferably more than 85%, even more preferred at least 90%, particularly at least 95% and most preferred at least 99% of its xylanase activity after 4 hours incubation in 50 mM phosphate buffer at 50°C.

Activity at elevated temperatures of the polypeptide according to the present application is determined by measuring xylan hydrolysis at various temperatures for a certain amount of time under the following conditions: pH 5, 2% w/w dry weight substrate concentration, enzyme : xylan ratio (E/S) of 1% wt./wt. dry weight.

The polypeptide according to the present application preferably shows optimum xylanase activity in the temperature range of from 40 to 77°C. Most preferably, the polypeptide according to the present application shows xylanase activity in the temperature range of from 45 to 70°C and most preferred of from 50 to 65 °C. In this context, the term "optimum xylanase activity" is to be

understood as temperature which leads to the highest release of reducing sugar-ends when incubating the enzyme with xylan for 30 minutes at pH 5.

The polypeptide according to the present application is also characterized by a wide pH activity profile. Within a preferred embodiment, the polypeptide according to the present application is active over a pH range from 5.0 to 5.5, more preferred from 4.0 to 6.0 and most preferred from 3.5 to 8.5. The term "active at pH" is to be understood as a minimum of 10% remaining activity at the pH of the measurement compared to the maximum pH- activity when incubating the enzyme with xylan for 30 minutes at 50 °C.

The polypeptide according to the present application is also characterized by high protease stability. Within a preferred embodiment the polypeptide according to the present application maintains at least 80%, preferably at least 85%, particularly preferred at least 90% and most preferred at least 95%, of its xylanase activity after having been subjected to trypsin at pH of 7.8 and a temperature of 50°C for 1 hour.

Within a preferred embodiment the polypeptide according to the present application maintains at least 80%, preferably at least 85%, particularly preferred at least 90% and most preferred at least 95%, of its xylanase activity after having been subjected to pepsin at pH of 3 and a temperature of 37°C for 2 hours.

The polypeptide according to the present application is also characterized by high expression and high secretion rates from various microorganisms, in particular by secretion from fungal and/or yeast hosts. More preferably, the polypeptide according to the present application is expressed and secreted at a level of more than 100 mg/l, preferably at a level of more than 500 mg/l, particularly preferred at a level of more than 750 mg/l, even more preferred at a level of 1 g/l and most preferred at a level of 1,25 g/l into the supernatant after introduction of a promotor functionally linked to a nucleic acid encoding the polypeptide into a suitable expression host. Promotors disclosed within the present application are preferred.

A suitable expression host is preferably yeast, more preferably a yeast of the genus *Saccharomyces, Kluyveromyces, Schizosaccharomyces, Candida, Yarrowia, Komagataella, Pichia, Hansenula*; particularly selected from the group *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces marxianus, Yarrowina lipolytica, Hansenula polymorpha, Pichia angusta, Komagataella pastoris* and *Pichia pastoris*.

Another suitable expression host is a bacterium. Particularly suitable expression hosts are *Lactococcus lactis*, *Lactobacillus brevis, Bacillus subtilis*, *Bacillus megaterium, Bacillus lentus, Bacillus amyloliquefaciens, Bacillus licheniformis, Pseudomonas fluorescence, Klebsiella planticola* and *Escherichia coli*.

Another suitable expression host is a fungus, selected from the genus *Penicillium, Trichoderma, Hypocrea, Aspergillus, Cantharellu, Boletos, Agraicus, Pleurotus, Trametes, Phanerochaete, Myceliophthora, Chaetomium, Humicola, Chrysosporium, Talaromyces* and *Neurospora*. Particularly suitable expression hosts are *Aspergillus niger, Aspergillus oryzae, Aspergillus nidulans, Penicillium chrysogenum, Trichoderma reesei, Myceliophthora thermophila, Chrysosporium lucknowense, Trichoderma viridae, Trichoderma harzianum, Hypocaea pseudokonigii* and *Talaromyces emersonii*.

Methods of determining expressibility, i.e. yield of a secreted protein and/or enzyme in the supernatant of a culture are known to a person skilled in the art.

The present application further provides a nucleic acid encoding the polypeptide according to the present application having an amino acid sequence with at least 70% sequence identity to SEQ ID No: 1, No: 3, No: 5 or No: 6. In a preferred embodiment, a nucleic acid encoding a polypeptide having an amino acid sequence with at least 75% sequence identity, preferably at least 80%, further preferred at least 85%, particularly preferred at least 90%, even more preferred at least 92%, also preferred at least 95%, furthermore preferred at least 98% and most preferred at least 99% sequence identity to SEQ ID NO: 1 (encoding FfXyn1) in the original and the mature form (SEQ ID NO: 3) as well as fusions with signal peptides for the enhanced heterologous production in filamentous fungi like *Trichoderma reesei* (SEQ ID NO: 5) and yeasts such as *Saccharomyces cerevisiae* (SEQ ID NO: 6) are provided.

In a further preferred embodiment, the nucleic acid encodes a polypeptide according to the present application having the sequence as defined by SEQ ID No: 1, No: 3, No: 5 or No: 6, wherein 1 to 30 nucleic acids are substituted, deleted or inserted (all referred to as "mutations"). Mutations within the coding region of the amino acid sequence, the protein structure and/or the active center of the xylanase are particularly preferred.

The term "mutation" comprises any kind of nucleotide sequence modification including insertions, deletions, points mutations, inversions, or combinations thereof. The definitions regarding amino acid sequence modifications and mutations apply accordingly.

The present application further provides vectors comprising a nucleic acid of the present application. The definitions regarding the inventive nucleic acid sequences apply accordingly.

Examples for episomally maintained vectors are derivatives of bacterial plasmids, yeast plasmids, centromer based linear DNA, constructs of viral origin like SV40, phage DNA, fungal ARS based DNA-vehicles, baculovirus, vaccinia, adenovirus, fowl pox virus, and pseudorabies as well as vectors derived from combinations of plasmids and phage or viral DNA.

A suitable expression vector according to the present application may comprise one or more genetic elements representing promotor sequences, transcription initiation sites, elements for the initiation of translation, and functional elements for protein export that are translationally coupled to the nucleic acid according to the present application.

The vector according to the present application may encode more than one polypeptide including more than one xylanase or may encode a fusion polypeptide comprising the xylanase according to the application.

The vector according to the present application may be episomally maintained in the host cell or integrated into the chromosome of the host.

The present application further provides a host cell transformed with a vector according to the present application. The host cell according to the present application may be used for recombinant protein production or for metabolic transformation of xylose containing substrates to preferred metabolites.

The recombinant host cell according to the present application is preferably selected from bacteria, yeast, or fungal cells. In a particularly preferred embodiment, the host cell is selected from the group consisting of *Escherichia, Klebsiella, Pseudomonas, Lactobacillus, Bacillus, Streptomyces*; *Saccharomyces, Kluyveromyces, Schizosaccharomyces, Candida, Yarrowia, Komagataella, Pichia, Hansenula, Penicillium, Trichoderma, Hypocrea, Aspergillus, Cantharellu, Agraicus, Boletos, Pleurotus, Trametes, Phanerochaete, Myceliophthora. Chaetomium, Humicola, Chrysosporium, Talaromyces* and *Neurospora*.

Preferably, the host cell is selected from *Lactococcus lactis, Lactobacillus brevis, Bacillus subtilis, Bacillus megaterium, Bacillus lentus, Bacillus amyloliquefaciens, Bacillus licheniformis, Pseudomonas fluorescence, Klebsiella planticola, Escherichia coli, Streptomyces lividans , Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces marxianus, Yarrowina*

lipolytica, Hansenula polymorpha, Pichia angusta, Komagataella pastoris, Pichia pastoris, Aspergillus niger, Aspergillus oryzae, Trichoderma reesei and *Myceliophthora thermophile.*

The recombinant host cell according to the present application may comprise one or more vectors according to the present application.

A further aspect of the application includes expression cassettes allowing the expression of the polypeptide according to the present application, particularly of the FfXyn1 protein, *in vivo or in vitro.*

An expression cassette preferably comprises a promotor region upstream to the coding sequence of the gene encoding the polypeptide according to the present application, preferably the FfXyn1 gene, sites involved in the formation of the translation initiation complex, optional regulatory sequence elements such as repressor binding or enhancer sites and optional a transcription termination sequence. Promotors may contain sequences allowing the binding of protein factors required for the transcription of coding sequence or functional mRNA. Furthermore sequences of the promotor may influence the effectiveness of transcription under a given physiological or chemical condition. A promotor may comprise elements in close proximity to the coding region or situated in more distant regions acting as enhancers. Promotors may be of prokaryotic, eukaryotic, archeal or viral origin or synthetic in nature. Preferred promotors include bacterial promotors of beta galactosidase (lacZ) gene, the tryptophane operon promotor (trp), tetracycline resistance gene promotor (tet), the araBAD promotor, virus-derived promotors T7, T3, PL or PR. Preferred promotors for the expression in yeast include glyceraldehyde phosphate dehydrogenase (GAP) promotor, hexokinase promotor, alcohol dehydrogenase ADE2 promotor, GAL1, GAL10, TEF and promotors of the methanol metabolic pathway of methylotrophic yeasts such as AOXI, MOXI or FMDH, as well as the copper-inducible CUP1 promotor. Preferred promotors for the expression in filamentous fungi include those from the celluloytic enzymes, such as CBHI, CBHII, or EGI or II, □-amylase, glucoamylase, phosphoglycerate kinase (pgk), and any promotor of genes of the glycolytic pathway.

Expression levels of a gene encoding the polypeptide according to the present application can be increased by adjustment of the copy-number of the gene introduced into the host cells, preferably resulting in more than single copies of the gene. For optimized expression of the gene, the promotor can be regulated, either by induction following the addition of a chemical inductor by adjustment of a physical parameter. Examples for inducible systems include tetracycline repressor system, Lac repressor system or the temperature inducible phage lambda PL promotor.

Alternatively, de-repression of the promotor by reaching a suitable physiological state in the culture can be a useful strategy (Promotor of PhoA, Trp, Adh2, Fmdh, CBHI). Application of strong stationary promotors might be preferable in other situations (GAP, TEF).

A translational coupling of signal peptide sequences can be used for the directing of the expressed polypeptide according to the present application to cellular compartments, organelles or the export from the host cell. Signal sequences are well known in the art. Examples are leader sequences for the periplasmatic targeting from OmpA, OmpT, PelB, PhoA, glucanase or beta-lactamase. Signal peptides for secretion of the proteins can be found among naturally secreted carbohydrate modifying enzymes, namely leaders from coding sequences of celloiohydrolasel or II, endoglucanases, amyE or signal peptides of the S. cerevisiae Mfa or chicken egg lysozyme.

The expression cassette may be placed in a vector or a vector construct according to the present application which can be episomally maintained in the host cell or integrated into the chromosome of the host. Examples for known vectors are derivatives of bacterial plasmids, yeast plasmids, centromer based linear DNA, constructs of viral origin like SV40, phage DNA, baculovirus, vaccinia, adenovirus, fowl pox virus, and pseudorabies as well as vectors derived from combinations of plasmids and phage or viral DNA. Integration of the expression cassette can be achieved by homologous recombination, transposition or by application of viral integration systems. Additionally the use of episomally maintained constructs as basis for the integration of the expression cassette copies into the chromosomal DNA is possible. Finally, any system leading to the replication of the expression cassette in the host cells is suitable as a vector or vector-construct.

In an embodiment of the application the transferred DNA comprises further open reading frames coding for enzymes, wherein such further reading frames can be physically connected in continuous DNA strands or as a mixture of individual DNA strands. In a preferred embodiment these additional open reading frames are functionally connected to promotor elements or regulatory DNA elements themselves, thus leading to individually or co-regulated expression of the additional open reading frames in the transformed host cell. In a preferred embodiment, the additional open reading frames comprise at least one sequence selected from those coding for endo-xylanases, xyloglucanases, xylosidases, acetylxylan esterases, feruolic acid esterases, end-cellulases, exo-cellulases, arabinofuranosidases, galactanases, phytases, polysaccharide monooxygenases or arabinases.

The present application further provides an isolated polypeptide having xylanase activity, wherein the polypeptide comprises an amino acid sequence having at least 80% sequence identity over the full length to SEQ ID No: 2, SEQ ID No: 4, or SEQ ID No: 7.

The present application further provides an isolated polypeptide having xylanase activity, wherein the polypeptide comprises an amino acid sequence having at least 95% sequence identity over the full length to SEQ ID No: 2 and wherein the polypeptide converts at least 60 wt.-% of xylose-containing polysaccharides of neutral steam-exploded wheat straw to xylose and/or xylose-containing oligosaccharides, and glucose under conditions of pH 5 and 50°C for 24 hours.

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Preferred methods for the introduction of the expression cassette constructs into the host cell include transformation, transfection, conjugation and/or interbreeding. The transformation can be achieved by DNA transfer via electroporation, protoplast fusion, lipofection, ballistic bombardment, chemical transformation based on lithium acetate, calcium chloride, PEG or manganese chloride. Further strategies include the application of viral particles. A further alternative is the application of naturally competent organisms as host cells.

Methods for further increasing the yield of the expressed protein include the co-expression of helper proteins involved in translation, trafficking of proteins, folding of proteins (e.g. Chaperones hsp70-family proteins, protein disulfide isomerase) or correct processing of the polypeptide (*Kex*-protease, *Ste*-proteases) and other events contributing to the cellular production of the protein.

After transformation of the host strain with a vector of the present application and growth to an appropriate cell density, the selected inducible promotor is induced by temperature shift or chemical induction and cells cultured to yield the recombinant enzyme. Preferably, the polypeptide according to the present application is produced with a signal peptide that directs the recombinant protein to be secreted from the host cell. Cells are then removed by centrifugation or filtration and the polypeptide-containing supernatant is retained.

The application also provides methods of preparing the polypeptide according to the present application, comprising the steps:

a) obtaining a host cell, which has been transformed with a vector comprising the nucleic acid as defined within the present application;

b) cultivation of the host cell under conditions under which the polypeptide is expressed; and

c) recovery of the polypeptide.

All definitions within the present application, particularly the definitions regarding the polypeptide, host cell, vector and nucleic acid, apply accordingly.

Cultivation of the host cell of the present application is carried out according to methods and conditions well known to a person skilled in the art. Preferably the host cells can be cultivated on cultivation substrate from agricultural waste and/or residue streams. Agricultural waste and residues are obtained and recovered from farming and forestry and comprise parts of the harvest that cannot be converted to

the main product for physical, chemical, economical or political reasons. Examples for such cultivation substrate are wheat straw, bagasse, sugar cane leaves, sugar beet pulp, low quality paper pulp, waste paper, saw dust or other residues from lumber mills. In a particular preferred embodiment the cultivation substrate has been subjected to the inventive protein prior to the cultivation or alternatively is split into two streams, whereas the other stream not used for cultivation is treated with at least the inventive protein. In a particularly preferred embodiment the inductor of the transformed host cell is released from the cultivation substrate during the treatment of the cultivation substrate with at least the inventive protein. Examples of such inducers are xylose, glucose, arabinose, rhamnose and oligomers comprising such sugar moieties.

Recovery of the polypeptide according to the present application is carried out according to methods and conditions well known to a person skilled in the art. Within a preferred embodiment the enzyme is recovered and purified from the supernatant by methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Protein refolding steps maybe used within particularly preferred embodiments.

Within a preferred embodiment, the host cell is a yeast cell and the xylanase protein has a sequence as defined by SEQ ID No: 2 or No: 4, wherein - a particularly preferred embodiment - 1 to 30 amino acid residues are substituted, deleted, or inserted, is expressed. In a particular preferred embodiment the xylanase is equipped with an affinity tag, for example a 6x-His TAG.

The present application further provides a composition comprising the polypeptide according to the present application. The composition preferably contains from 0.01 to 50 wt.-% (refers to the total protein content of the composition) of the polypeptide according to the present application, further preferred from 1 to 35 wt.-% of the polypeptide according to the present application, particularly preferred from 5 to 20 wt.-% and most preferred from 8 to 12.5 wt.-%.

Within a preferred embodiment, the composition also comprises at least one cellulase. The at least one cellulase is preferably selected from cellulase mixtures obtained from respective cellulase secreting microbial cultures. In a preferred embodiment microbial cultures for this purpose are selected from cultures *of Trichoderma reesei, Myceliophthora thermophila, Talaromyces emersonii, Trichoderma viride, Penicillium verruculosum, Talaromyces stripitatus, Humicula grisaea, Chaethomium thermophilum, Humicola inculens, Clostridium thermocellum,Thermobifida fusca, Caldicellulosiruptor owenensis, Aspergillus fumigatus, Aspergillus niger, Neurospora crassa* and mixtures thereof.

Within a further preferred embodiment the composition comprises one or more enzyme activities selected from cellulases, GH61 pyranose monooxygenases (also referred to as "GH61 protein"), endo-xylanases, xyloglucanases, xylosidases, acetylxylan esterases, feruolic acid esterases, beta-glucosidases, arabinofuranosidases, galactanases and arabinases.

Within a particularly preferred embodiment, the composition of the present application is embedded in a cellulase mixture, which is enhanced by this particular addition with respect to the hydrolysis performance on cellulose, xylan and other hemicellulose material. Within a most preferred embodiment, the cellulose mixture further comprises increased levels of one or more activities selected from the group of GH61 protein, xylosidase and beta-glucosidase by 5% or more with respect to the specific activity level in the cellulase mixture.

Particularly preferred compositions comprise the polypeptide according to the present application as defined before, at least one cellulase as defined before as well as at least one endoglucanase IV wherein it is particularly preferred that the fraction of the polypeptide according to the present application and the endoglucanase IV are from 5 % (wt. /wt. determined after Bradford *versus* BSA) polypeptide to 40% endoglucanase IV, preferably from 8% polypeptide to 25% endoglucanase IV, whereas the percentage relates to the overall amount of protein of the composition which consists preferably of cellulase. It is also preferred that the polypeptide and the endoglucanase IV are contained in the same amount.

Within a further particularly preferred embodiment, the composition of the present application comprises the polypeptide according to the present application as defined before, at least one cellulase as well as at least one xylosidase and/or at least one GH61 protein. Within a preferred embodiment of the present application, the composition comprises from 30 to 99% (wt. /wt. determined after Bradford *versus* BSA), preferably from 40 to 90%, more preferred from 50 to 80% cellulase; from 1 to 70 % (wt. /wt. determined after Bradford *versus* BSA), preferably from 5 to 50%, even more preferred from 10 to 30% and most preferred from 15 to 20% of the polypeptide according to the present application; and xylosidase and/or GH61 protein from 1 to 25 % (wt. /wt. determined after Bradford *versus* BSA), preferably from 5 to 20% and most preferred from 10 to 15 %.

The application also provides the use of the polypeptide according to the present application and of the composition according to the present application for the enzymatic degradation of lignocellulosic biomass.

The application also relates the use of the polypeptide according to the present application in processes for the production of biofuels, pulp, paper and cellulose fibers, platform chemicals and food and feed products from complex substrates such as "xylose-containing polysaccharides".

Examples and Figures

In the following, the present application describes examples and figures. The examples and figures are considered for illustrative purpose only and do not limit the scope of the present application and claims in any respect.

Example 1: Temperature optimum and pH-range

Characterization of affinity of purified *t*FfXyn1 polypeptide SEQ ID NO: 7 was done with respect to the pH for optimal activity between pH 4 to 7 and the residual activity after incubation at temperatures between 40 to 65 °C pre-incubation time. The activity level was determined with the substrate p-nitrophenyl-β-D-xylopyranoside (pNP-X) at 2 mg/ml substrate concentration. 50 °C and 1 hour incubation time was applied. Residual activity determination after the temperature pre-incubation step was done at pH 5. For the determination of optimal pH, the buffers as shown within table 1 were used. Release of p-nitro phenol was determined by absorbance measurement at 405 nm. Protein quantification was done using Bradford *versus* BSA standards. 2 mg/ml solutions of the purified *t*FfXyn1 were diluted in the respective application buffers.

Table1: Buffer compositions for pH-Optimum determination

pH	**mM**	
4	100	Lactic acid
4,5	100	Acetate
5	100	Acetate
5,5	100	Acetate
6	100	MES buffer
6,5	100	Phosphate

7	100	Phosphate

The purified *t*FfXyn1 polypeptide shows excellent activity from pH 4 to 6 with a maximum at pH 5 and from 50 to 65 °C with a maximum around 58 °C. The results are shown in Fig. 1A&B.

Example 2: Comparison performance of different Xylanase polypeptides on neutral straw

Hydrolysis reactions with steam exploded wheat straw (neutral conditions) were set up using 100% and 80% (wt. /wt. determined after Bradford *versus* BSA) fractions of SCFMX0375 (*Trichoderma reesei* with increased beta-glucosidase levels) cellulase enzyme loads for reference under conditions of 50 °C and pH5 for 24 hours.

The substitution of 20% (wt. /wt. determined after Bradford *versus* BSA) cellulase enzyme by the tFfXyn1 (SEQ ID NO: 7) polypeptide leads to an increase in glucose and xylose yields, whereas the substitution by the same amount of different xylanases from *Thermomyces lanuginosus* (TlXyn1_GH11; SEQ ID NO: 8) and *Trichoderma reesei* (TrXyn2_GH11 SEQ ID NO: 10, TrXyn1_GH11 SEQ ID NO: 9, TrXyn4_GH30 SEQ ID NO: 11) did not lead to such drastic improvement of the hydrolysis reaction.

The 80% reaction setup was duplicated and supplemented with 20% (wt.-%/wt.-% determined after Bradford *versus* BSA) of the *t*FfXyn1 polypeptide. Degree of saccharification was determined after 24h of saccharification under conditions of 50 °C and pH5 followed by sugar quantification on HPLC. As can be seen, the dosage of *t*FfXyn1 leads to the highest total sugar release. Results are shown in Fig. 2.

Example 3: Performance of *t*FfXyn1 on neutral straw in the presence of GH61 protein

The synergy of *t*FfXyn1 (SEQ ID NO: 7) with TrEGIV_GH61 (*T. reesei* EGIV SEQ ID NO: 14) was tested by addition of various ratios (enzyme weight determined after Bradford *versus* BSA per dry matter substrate) of the purified *t*fFxyn1 and TrEGIV_GH61 as a 25% (wt. /wt. determined after Bradford *versus* BSA) aliquote to SCFMX0375. A local maximum of 8% (wt. /wt. determined after Bradford *versus* BSA) *t*FfXyn1 was demonstrated to release a maximum glucose amount from pre-treated (neutral steam-exploded) wheat-straw, when 0,5 % enzyme to substrate ratios (enzyme weight determined after Bradford *versus* BSA per dry matter substrate) were applied

and saccharification is carried out at 50°C and pH5 for 24 hours on neutral steam exploded wheat straw. Results are shown in Fig. 3.

Example 4: Performance of *t*FfXyn1 on neutral straw in the presence of beta glucosidase (TeBgl_GH3 SEQ ID NO: 13) and endoglucanase (TrEGIV_GH61 SEQ ID NO: 14)

Effects of increased levels of beta glucosidase TeBgl_GH3 SEQ ID NO: 13 activity in the presence of *t*FfXyn1 and SCFMX375 with respect to glucose and xylose yields from neutral steam exploded wheat straw were evaluated. Increased levels of beta-glucosidases were found to further improve the enzymatic release of glucose from the samples in the presence of *t*FfXyn1 and TrEGIV_GH61. Saccharification was carried out at 50°C and pH5 for 24 hours. The results are shown in Fig. 4.

Example 5: Co-Action of *t*FfXyn1 with Xylosidase

Effects of increased levels of xylosidase from *Trichoderma reseei* (TrXyl_GH3 SEQ ID NO: 12) activity in the presence of *t*FfXyn1 and SCFMX375 with respect to glucose and xylose yields from neutral steam exploded wheat straw were evaluated. Saccharification was carried out at 50°C and pH5 for 24 hours. The results are shown in Fig. 5.

Brief description of the figures

Fig. 1Ashows the pH- stability of purified *t*FfXyn1

Fig. 1B shows the temperature-stability of purified *t*FfXyn1

Fig. 2 shows enhanced glucose and xylose yields from saccharification reactions of neutral steam exploded wheat straw by a combination of SCFMX0375 Cellulose and *t*FfXyn1 in comparison to other xylanases

Fig. 3 shows the synergy in glucose liberation from lignocellulosic substrate between *t*FfXyn1 and GH61 protein

Fig. 4 shows the synergy of TeBgl_GH3 beta-glucosidase with *t*FfXyn1 for the monomeric sugar release from lignocellulosic substrate

Fig. 5 shows that dosing of xylosidase significantly increases the xylose yield in the presence of *t*FfXyn1

SEQUENCE LISTING IN ELECTRONIC FORM

This application contains a sequence listing in electronic form in ASCII text format. A copy of the sequence listing in electronic form is available from the Canadian Intellectual Property Office.

Sequence Listing Description:

SEQ ID NO: 1 DNA sequence FfXyn1 xylanase

SEQ ID NO: 2 protein sequence FfXyn1 xylanase with signal peptide

SEQ ID NO: 3 DNA sequence mFfXyn1 xylanase

SEQ ID NO: 4 protein sequence mFfXyn1 xylanase mature protein

SEQ ID NO: 5 artificial DNA sequence coding tFfXyn1 xylanase mature protein fusion with the *Trichoderma reesei* CBHI signal peptide and C-terminal 6x-His-TAG

SEQ ID NO: 6 artificial DNA sequence coding yFfXyn1 xylanase mature protein fusion with the *Saccharomyces cerevisiae* MFalpha signal peptide and C-terminal 6x-His-TAG)

SEQ ID NO: 7 protein sequence tFfXyn1 xylanase mature protein fusion with the *Trichoderma reesei* CBHI signal peptide and C-terminal 6x-HIS-TAG

SEQ ID NO: 8 protein sequence TlXyn1_GH11 *Thermomyces lanuginosus* xylanase mature protein

SEQ ID NO: 9 protein sequence TrXyn1_GH11 *Trichoderma reesei* xylanase 1 mature protein with 6xHis-TAG)

SEQ ID NO: 10 protein sequence TrXyn2_GH11 *Trichoderma reesei* xylanase 2 mature protein with 6xHis-TAG)

SEQ ID NO: 11 protein sequence TrXyn4_GH30 *Trichoderma reesei* xylanase 4 mature protein with 6xHis-TAG

SEQ ID NO: 12 protein sequence TrXyl_GH3 *Trichoderma reesei* xylosidase mature protein with 6xHis-TAG

SEQ ID NO: 13 protein sequence TeBgl_GH3 *Talaromyces emersonii* beta glucosidase mature protein with 6xHis-TAG

SEQ ID NO: 14 protein sequence TrEGIV_GH61 *Trichoderma reesei* endoglucanase 4 mature protein with 6xHis-TAG

Claims

1. An isolated polypeptide having xylanase activity, wherein the polypeptide comprises an amino acid sequence having at least 95% sequence identity over the full length to SEQ ID No: 2 and wherein the polypeptide converts at least 60 wt.-% of xylose-containing polysaccharides of neutral steam-exploded wheat straw to xylose and/or xylose-containing oligosaccharides, and glucose under conditions of pH 5 and 50°C for 24 hours.

2. The isolated polypeptide according to claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 4 wherein 1 to 30 amino acid residues are substituted, deleted or inserted, and retains the same biological activity as the polypeptide comprising the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 4.

3. The polypeptide according to claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 4.

4. The polypeptide according to claim 1, wherein the polypeptide comprises the amino acid sequence of SEQ ID No: 7.

5. An isolated nucleic acid molecule encoding the polypeptide according to any one of claims 1 to 4.

6. A vector comprising the nucleic acid molecule of claim 5.

7. A host cell transformed with the vector of claim 6.

8. The host cell of claim 7, wherein the host cell is derived from the group consisting of *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Pichia pastoris, Pichia angusta, Hansenula polymorpha, Aspergillus niger, Trichoderma reesei, Penicillium sp.* and *Myceliophthora thermophile.*

9. The host cell according to claim 7 or 8 which is capable of over-expressing one or more activities selected from the group consisting of GH61 pyranose monooxygenase activity, xylosidase activity, xylanase activity and beta-glucosidase activity.

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10. A method of producing a polypeptide having xylanase activity, comprising the steps:

a) obtaining a host cell, which has been transformed with a vector comprising the nucleic acid molecule as defined in claim 5;

b) cultivating the host cell under conditions under which the polypeptide is expressed; and

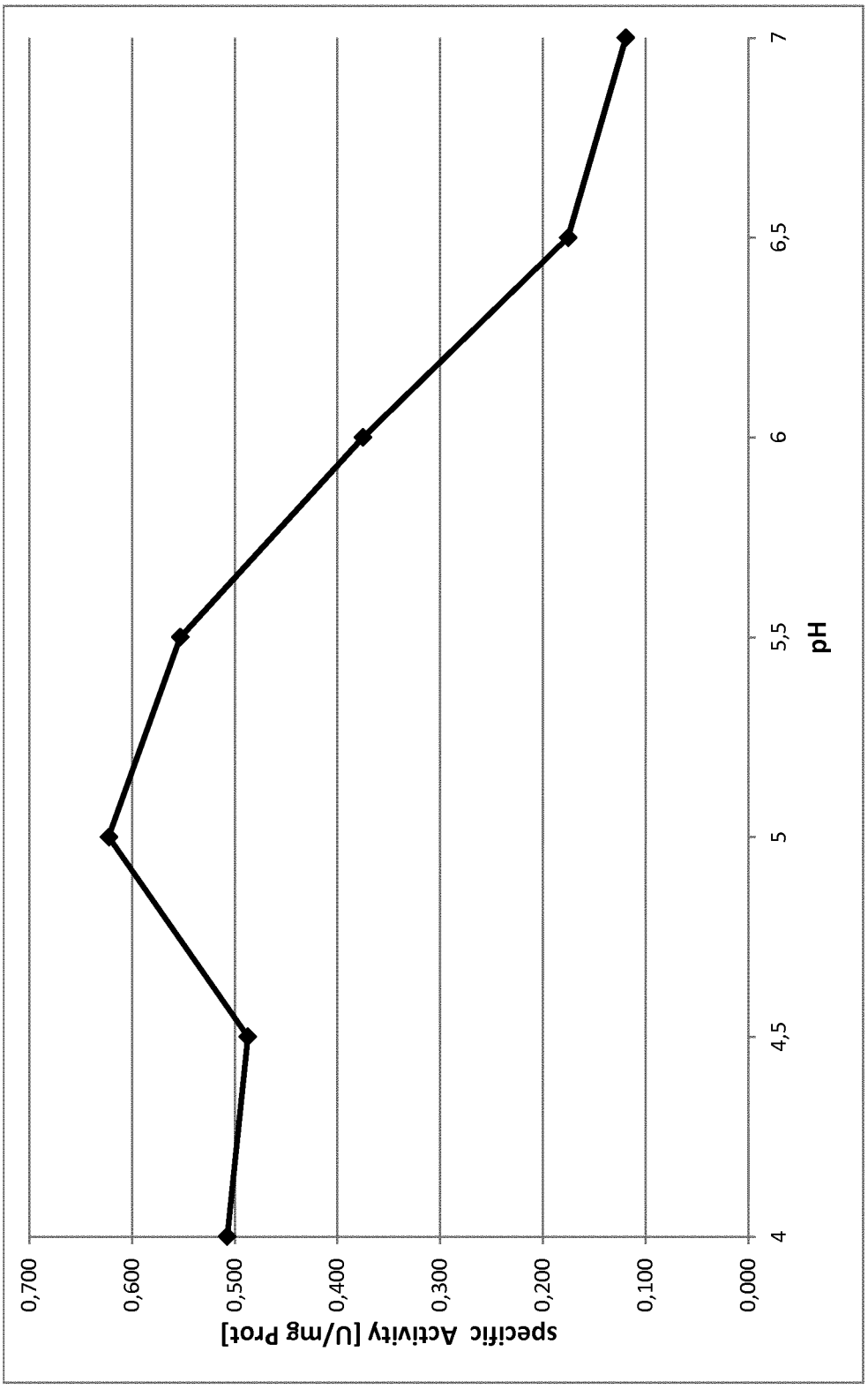
c) recovering the polypeptide.

11. The method of claim 10, wherein the host cell is a yeast cell and the polypeptide comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 4, wherein 1 to 30 amino acid residues are substituted, deleted or inserted, and retains the same biological activity as the polypeptide comprising the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 4.

12. A composition comprising the polypeptide of any one of claims 1 to 4 and one or more enzyme selected from cellulases, GH61 protein, endo-xylanases, xyloglucanases, xylosidases, acetylxylan esterases, feruolic acid esterases, beta-glucosidases, arabinofuranosidases, galactanases and arabinases.

13. Use of the polypeptide according to any one of claims 1 to 4 or of the composition of claim 12 for the enzymatic degradation of lignocellulosic biomass.

Fig. 1A


specific Activity [U/mg Prot]
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0,600
0,500
0,400
0,300
0,200
0,100
0,000
4
4,5
5
5,5
6
6,5
7
pH

Fig. 1B

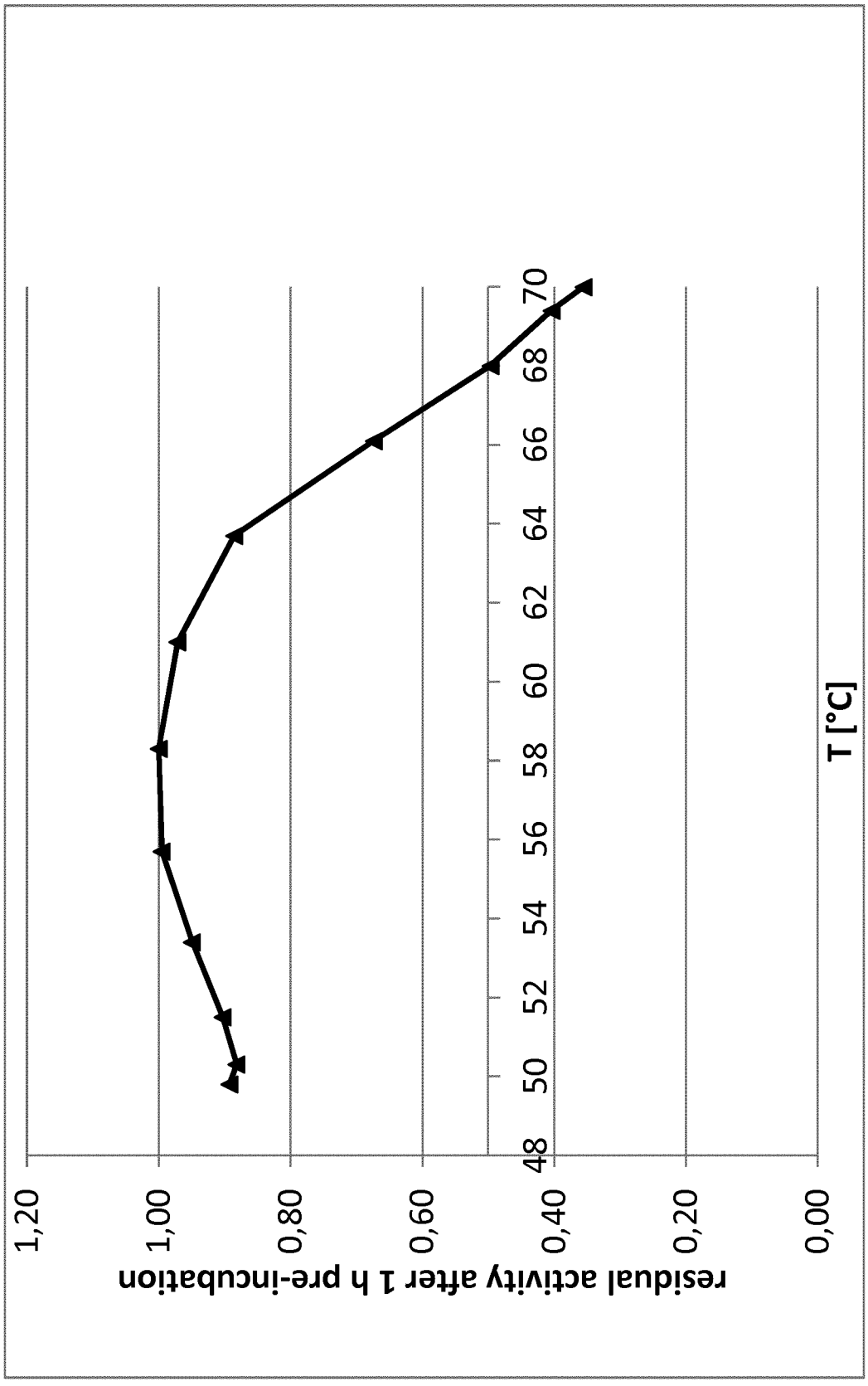

residual activity after 1 h pre-incubation
1,20
1,00
0,80
0,60
0,40
0,20
0,00
48
50
52
54
56
58
60
62
64
66
68
70
T [°C]

Fig. 2

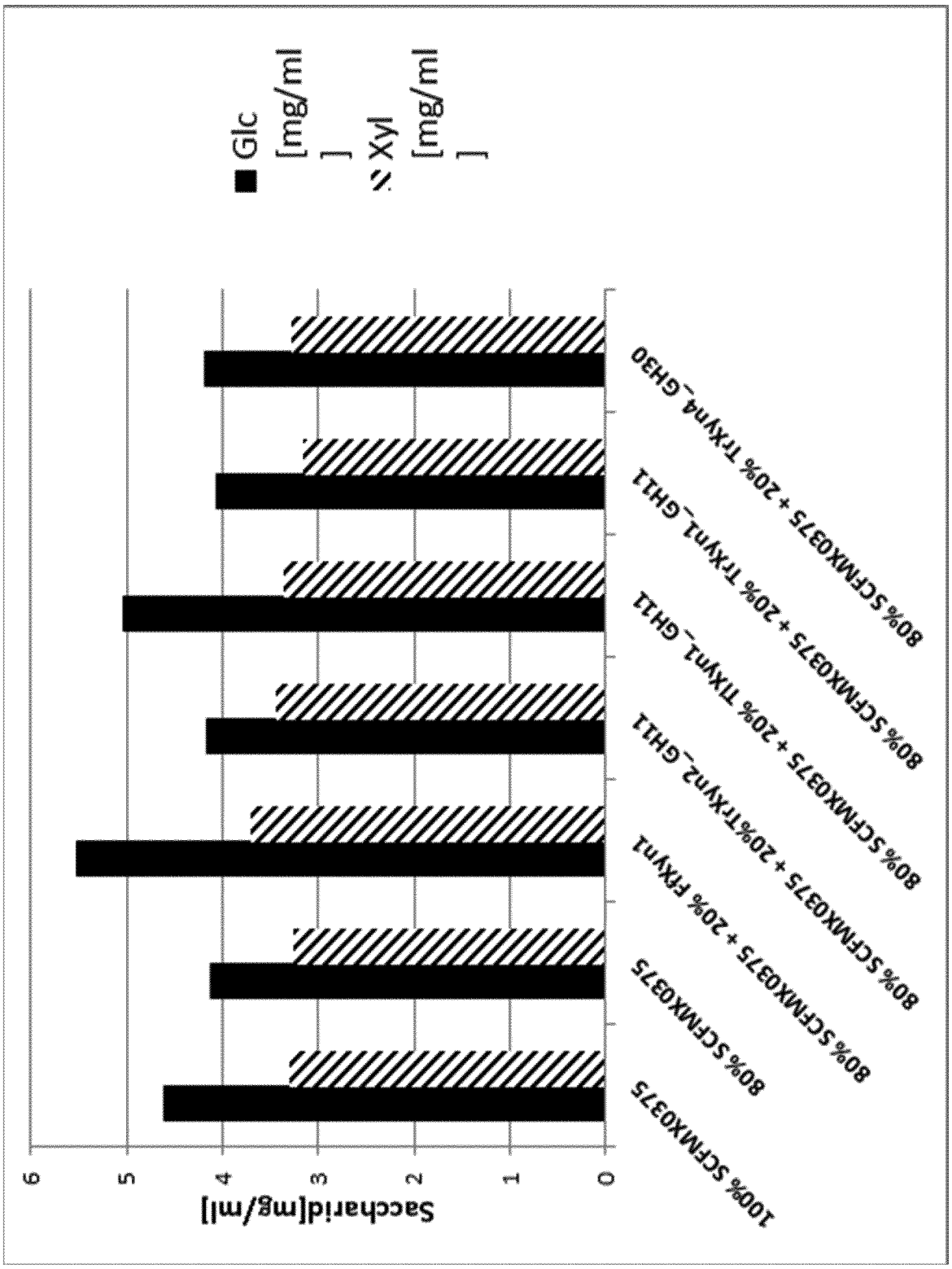
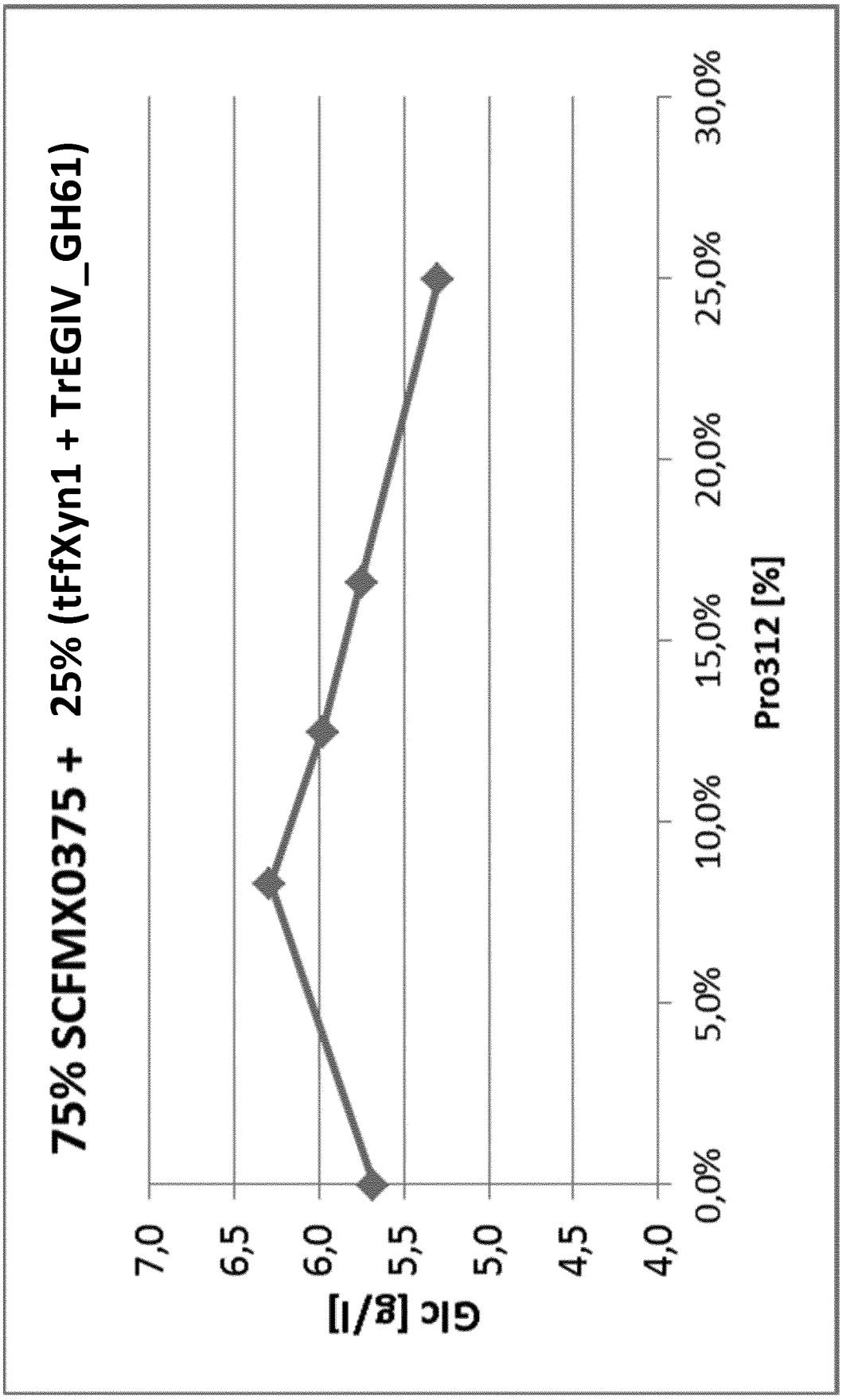

Glc [mg/ml]
Xyl [mg/ml]
Saccharid[mg/ml]
0
1
2
3
4
5
6
100% SCFMX0375
80% SCFMX0375
80% SCFMX0375 + 20% FXyn1
80% SCFMX0375 + 20%TrXyn2_GH11
80% SCFMX0375 + 20% TXyn1_GH11
80% SCFMX0375 + 20% TrXyn1_GH11
80% SCFMX0375 + 20% TrXyn4_GH30

Fig. 3


75% SCFMX0375 + 25% (tFfXyn1 + TrEGIV_GH61)
Glc [g/l]
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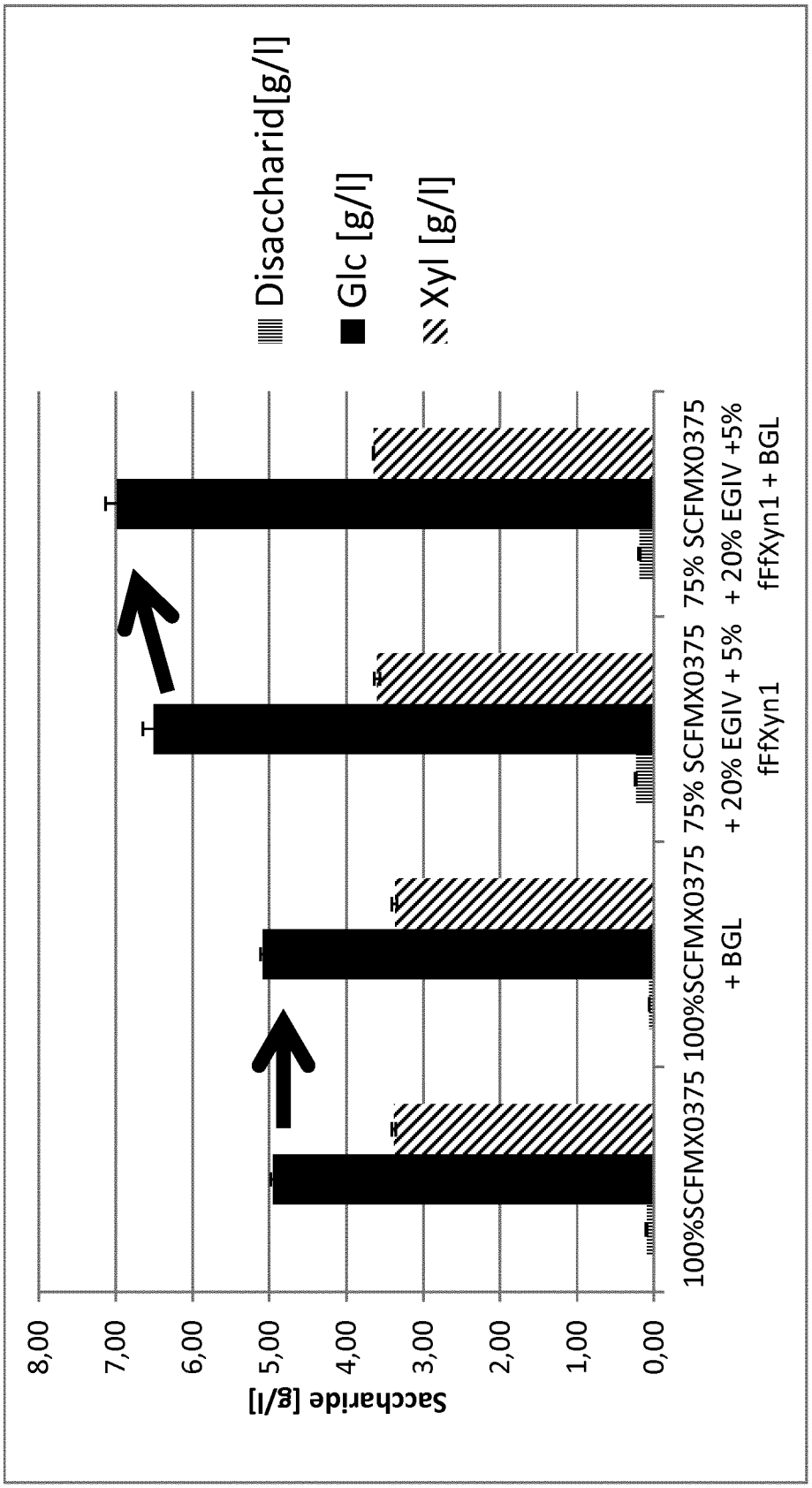
6,5
6,0
5,5
5,0
4,5
4,0
0,0%
5,0%
10,0%
15,0%
20,0%
25,0%
30,0%
Pro312 [%]

Fig. 4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Glc [g/l]
Xyl [g/l]
Saccharide [g/l]
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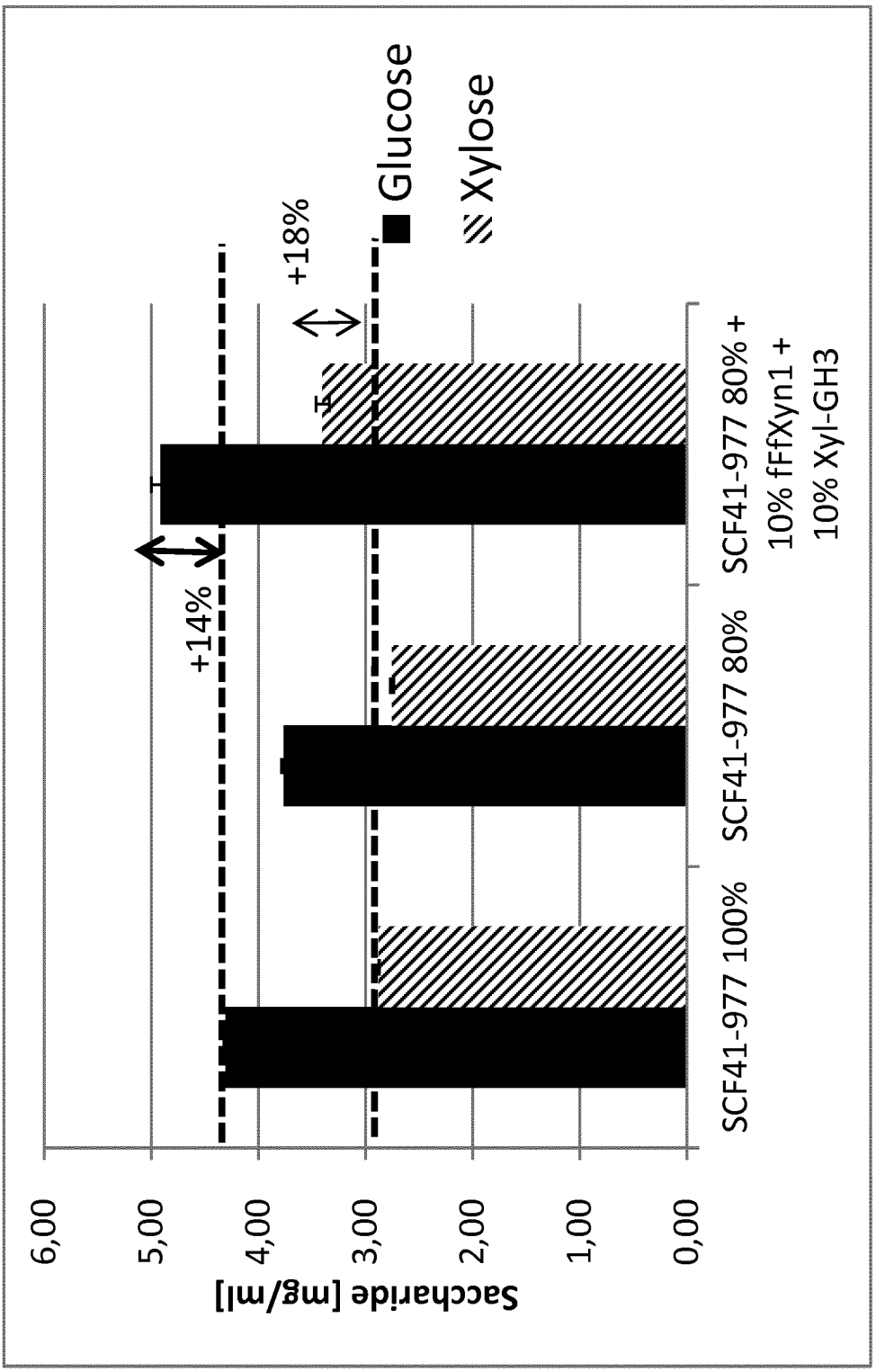
7,00
6,00
5,00
4,00
3,00
2,00
1,00
0,00
100%SCFMX0375 + BGL
100%SCFMX0375 + 20% EGIV + 5% fFfXyn1
75% SCFMX0375 + 20% EGIV + 5% fFfXyn1
75% SCFMX0375 + 20% EGIV +5% fFfXyn1 + BGL

Fig. 5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4,00
3,00
2,00
1,00
0,00
Glucose
Xylose
+14%
+18%
SCF41-977 100%
SCF41-977 80%
SCF41-977 80% + 10% fFfXyn1 + 10% Xyl-GH3