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(54) Title: MANNANASE VARIANTS

(57) Abstract: The present description is related to variants of mannanase, compositions comprising said variants, to methods for their production and to methods of using said variants to degrade and modify mannan containing material.

MANNANASE VARIANTS

FIELD OF THE INVENTION

This invention relates to variants of mannanase enzyme. The variants are useful in industrial applications wherein degradation or modification of mannan is desired, such as in laundry and cleaning applications, in feed, food, pulp and paper and oil industry. The invention also provides useful mannanases enzymes, polynucleotides encoding these enzymes, enzyme compositions and methods for their production and use.

BACKGROUND

Mannans are mannose containing polysaccharides found in various plants. Mannans are poorly soluble in aqueous environment and their physicochemical properties give rise to viscous dispersions. Additionally, mannans have high water binding capacity. All of these characteristics cause problems in several industries including brewing, baking, animal nutrition, and laundry and cleaning applications.

In plant-based diets different β -mannans are present and depending on their amounts and properties they can compromise nutrient digestion, microbial colonisation and growth performance. Enzymatic degradation of mannans reduces digesta viscosity of high water soluble mannans and leads to production of manno-oligosaccharides that may form water-insoluble linear mannans present in leguminosae. Mannanase increases average daily gain, feed efficiency, weight uniformity and livability in all monogastric animals.

For animal feed applications, such as feed for monogastric animals with cereal diets, mannan is a contributing factor to viscosity of gut contents and it thereby adversely affects the feed digestibility and animal growth rate. For ruminants, mannan represents a substantial component of fiber intake and a more complete digestion of mannan would facilitate higher feed conversion efficiencies.

For laundry and cleaning applications enzyme compositions comprising mannanase can be used to degrade mannan. However, providing mannanases

that are stable in varying storage and use conditions while still showing good mannan degrading activity is difficult.

Stability of industrial enzymes is an important property because the enzymes are often used in conditions that are very different from the natural environment of the enzymes. It is often that a wild type enzyme showing good performance in initial tests is not suitable for production in industrial scale, or is unstable in typical application or storage conditions.

N-linked glycosylation of proteins is a type of post-translational modification where a sugar molecule oligosaccharide known as glycan is attached to an amide nitrogen group of an asparagine (Asn, N) residue of a protein. This type of linkage is important for both the structure and function of enzymes and other proteins.

It is an object of the present invention to provide variants of mannanase having improved stability and exhibiting mannanase activity when applied in different industrial processes, as well as enzyme compositions for mannan degradation or modification.

SUMMARY

According to the first aspect is provided a variant of mannanase comprising at least one substitution of an amino acid at a position corresponding to the position 123, 158, 180, 272, 307, or 316, wherein the variant has mannanase activity and is selected from the group consisting of:

1) a polypeptide having at least 85% sequence identity to residues 27-331 of SEQ ID NO: 2;

2) a variant encoded by a polynucleotide that hybridizes under high stringency conditions with

a) nucleotides 79 - 993 of SEQ ID NO: 1 (man7) or

b) the full-length complement of a); and

3) a variant encoded by a polynucleotide having at least 95% sequence identity to the SEQ ID NO: 1 or the genomic DNA sequence thereof;

and wherein the amino acid numbering corresponds to the amino acid numbering of SEQ ID NO: 2 (Man7) full-length amino acid sequence containing a signal sequence.

The present variant of mannanase is advantageous in having good stability and mannanase activity. The variant has improved stability in detergents and in particular at high temperature. Thus, the present variant of mannanase may provide improved yield in production and better performance in use. The stability of the variant is particularly good in detergents and at temperatures typically used in applications where mannan degradation is used, such as in laundry detergents.

In an embodiment the variant has at least two substitutions. This is advantageous to further improve stability in detergents and in use at high temperature. Alternatively, two substitutions can be selected such that in addition to stability, another property of the variant is improved.

According to the second aspect of the invention is provided an enzyme composition comprising the variant of mannanase of the first aspect and

a. at least one preservative selected for example from group consisting of organic acid, citric acid, ascorbic acid, benzoic acid and their salts and derivatives, sodium benzoate, benzoate, hydroxybenzoate and derivatives, sorbic acid, sodium sorbate, sorbate, salts, such as sodium chloride or potassium chloride, 1,2-Benzisothiazolin-3-one (BIT) or a combination thereof;

b. optionally at least one stabilizer selected from polyol, propylene glycol, polyethylene glycol, hexylene glycol, glycerol, a sugar, sugar alcohol, polysaccharide, lactic acid, boric acid, boric acid derivative, aromatic borate ester, 4-formylphenyl boronic acid, phenyl boronic acid derivative, peptide, surfactant or a combination thereof;

c. optionally at least one enzyme selected from proteases, amylases, cellulases, lipases, xylanases, mannanases, cutinases, esterases, phytases, DNAses, pectinases, pectinolytic enzymes, pectate lyases, carbohydrases, arabinases, galactanases, xanthanases, xyloglucanases,

laccases, peroxidases and oxidases with or without a mediator, or a combination thereof; and

d. optionally at least one filler selected from maltodextrin, flour, sodium chloride, sulfate, sodium sulfate, or a combination thereof.

- 5 As evidenced by the Examples, the variants comprised in the enzyme composition according to the invention have a structure and properties that allow production in recombinant host cells and make them useful in enzyme compositions for industrial applications. The enzyme composition is particularly good for detergent formulations because the variant of mannanase has good
- 10 stability, wash performance and specific activity when used to degrade mannan in laundry and washing use.

According to the third aspect there is provided a detergent composition comprising the variant of mannanase of the first aspect or the enzyme composition of the second aspect.

- 15 The present detergent composition is advantageous in that it is stable, efficient and economical in removing mannan containing stains.

According to another aspect is provided a use of, and a method of using, the present enzyme composition or the present variant of mannanase in a detergent.

- According to the fourth aspect is provided a recombinant host cell comprising
- 20 genetic elements that allow producing at least one recombinant polypeptide comprising the variant of mannanase of the first aspect.

According to the fifth aspect is provided a method for producing a recombinant polypeptide having mannanase activity and comprising:

- a. cultivating a recombinant host cell of the fourth aspect, wherein
- 25 the genetic elements comprise at least one control sequence which controls the production of the recombinant polypeptide in the recombinant host cell;
- the genetic elements optionally comprise at least one sequence encoding a signal sequence for transporting the recombinant
- 30 polypeptide outside the host cell; and

cultivating is carried out in conditions allowing production of the recombinant polypeptide; and

b. recovering the recombinant polypeptide.

The method provides an efficient way to produce a recombinant polypeptide comprising a variant of mannanase. Because the variant of mannanase is produced in a recombinant host cell, a production system is provided which can be optimized, tailored, and controlled in a desired manner. The variant of mannanase produced by the method may differ from natural mannanases at a structural and functional level.

According to another aspect is provided an enzyme preparation comprising a recombinant polypeptide having mannanase activity and obtainable by using the present host cell.

The enzyme preparation or the enzyme composition may further comprise other enzyme(s) selected from the group consisting of proteases, amylases, cellulases, lipases, xylanases, mannanases, cutinases, esterases, phytases, DNAses, pectinases, pectate lyases, pectinolytic enzymes, carbohydrases, arabinases, galactanases, xanthanases, xyloglucanases, laccases, peroxidases and oxidases with or without a mediator, as well as suitable additives selected from the group consisting of stabilizers, buffers, surfactants, bleaching agents, mediators, anti-corrosion agents, builders, anti-redeposition agents, optical brighteners, dyes, pigments, perfumes, caustics, abrasives and preservatives.

According to a sixth aspect is provided a method for degrading or modifying mannan containing material comprising treating said mannan containing material with an effective amount of the present enzyme composition or the present variant of mannanase.

According to the seventh aspect is provided an animal feed comprising the present enzyme composition or the present variant of mannanase, and at least one protein source of plant origin or a mannan containing product or by-product, and

a. Optionally at least one enzyme selected from protease, amylase, phytase, xylanase, endoglucanase, beta-glucanase, or a combination thereof; and

- b. Optionally at least one filler selected from maltodextrin, flour, salt, sodium chloride, sulfate, sodium sulfate, or a combination thereof.

According to the eighth aspect is provided a feed supplement comprising the present enzyme composition or the present variant of mannanase; and

- 5 a. Optionally at least one enzyme selected from protease, amylase, phytase, xylanase, endoglucanase, beta-glucanase, or a combination thereof; and
- b. Optionally at least one filler selected from maltodextrin, flour, salt, sodium chloride, sulfate, sodium sulfate, or a combination thereof.

The feed and the feed supplement improve nutritional value of feed compared to
10 a feed without the variant. The present enzyme composition comprises the variant of mannanase, which has improved stability. The present enzyme composition and the present variant degrade mannan present in the feed and thereby make it more easily digestible for the animal. In particular for soybean meal containing feeds mannan-oligosaccharides that result from enzymatic
15 digestion have a beneficial effects on the intestinal microbes, and consequently on the performance of the animals. The effect of the variants of mannanase can be enhanced by including xylanase to digest arabinoxylans present in corn soybean based diets. The present variants can also be used to modify rheological properties of wet feeds.

- 20 In an embodiment the feed may comprise animal protein, such as meat meal or bone meal.

According to another aspect is provided a use, and a method of using, the present animal feed or the present feed supplement in:

- a. feeding animals;
- 25 b. improving weight gain of animals.

In an embodiment the animal is a monogastric animal or a ruminant. In another embodiment the animal is a broiler chicken, egg-laying chicken, swine, turkey, or an aquaculture organism such as fish. In another embodiment the animal is a ruminant.

According to a ninth aspect is provided a use of, and a method of using, the present variant or the present enzyme composition in oil drilling or hydro-fracturing.

- 5 The present enzyme composition and the present variant are advantageous in modifying rheological properties of oil drilling fluids and hydro-fracturing fluids, and to improve oil recovery.

According to an tenth aspect is provided a use of, and a method of using, the present variant or the present enzyme composition in processing coffee extract, fruit juice, pineapple juice, or soya milk.

- 10 Using the present variant and the present enzyme composition is advantageous in processing coffee extract because it reduces viscosity of the coffee extract.

Using the present variant and the present enzyme composition is advantageous in processing and manufacturing fruit juice because it lowers viscosity and improves filtration rate, stability and helps to extract fruit components.

- 15 Using present variant and the present enzyme composition is advantageous in processing and manufacturing soya milk because it improves yield, colour, protein content and taste of soya milk.

In another aspect is provided a nucleic acid molecule encoding the present variant of mannanase.

- 20 In another aspect is provided a vector comprising the present nucleic acid molecule.

In another aspect is provided a functional fragment of the present variant of mannanase, and a polynucleotide encoding it.

- 25 In another aspect the disclosed sequence information herein relating to a polynucleotide sequence encoding a mannanase of the invention can be used as a tool to identify other homologous mannanases. For instance, polymerase chain reaction (PCR) can be used to amplify sequences encoding other homologous mannanases from a variety of biological sources. In addition, genome mining approaches can be used to identify sequences encoding other homologous mannanases from genome databases.
- 30

BRIEF DESCRIPTION OF THE FIGURES

Fig 1 shows schematic representation of vector pEV1 for replication in *Bacillus*.

Fig 2 schematically shows the expression cassettes used in the transformation of *Trichoderma reesei*.

Fig 3A-B describe the stain removal performance of variants and wild type Man7 (produced in *Trichoderma*) as an increase of lightness (sum of ΔL^* of 3 stains) in the presence of 4,4 g/l of Commercial heavy duty liquid detergent A at 40°C, 16 °dH, 60 min, pH approx. 8.3 and enzymes dosed as activity units (MNU) per wash liquor.

Fig 3A shows variants TBH1, TBH2, TBH3, TBH4, TBH5, TBH6, TBH7, TBH8, TBH9 and wild type.

Fig 3B shows variants TBH6, TBH10, TBH11 and wild type.

Fig 4A-B describe the stain removal performance of variants and wild type Man7 (produced in *Trichoderma*) as an increase of lightness (sum of ΔL^* of 3 stains) in the presence of 3.8 g/l of Commercial color detergent powder at 40°C, 16 °dH, 60 min, pH approx. 10 and enzymes dosed as activity units (MNU) per wash liquor.

Fig 4A shows variants TBH1, TBH2, TBH3, TBH4, TBH5, TBH6, TBH7, TBH8, TBH9 and wild type.

Fig 4B shows variants TBH6, TBH10, TBH11 and wild type.

Fig 5 describes the stain removal performance of variants TBH1, TBH2, TBH3, TBH4, TBH5, TBH6, TBH7, TBH8, TBH9 and wild type Man7 (produced in *Trichoderma*) as an increase of lightness (sum of ΔL^* of 3 stains) in the presence of 3.8 g/l of Commercial bleach detergent powder at 40°C, 16 °dH, 60 min, pH approx. 9.5 and enzymes dosed as activity units (MNU) per wash liquor.

Fig 6 describes the stain removal performance of variants BH18, BH21, BH23, BH24, BH25 and wild type Man7 (produced in *Bacillus*) as an increase of lightness (sum of ΔL^* of 3 stains) in the presence of 4,4 g/l of Commercial heavy duty liquid detergent A at 40°C, 16 °dH, 60 min, pH approx. 8.3 and enzymes dosed as activity units (MNU) per wash liquor.

Fig 7 describes the stain removal performance of variants BH18, BH21, BH23, BH24, BH25 and wild type Man7 (produced in *Bacillus*) as an increase of

lightness (sum of ΔL^* of 3 stains) in the presence of 3.8 g/l of Commercial color detergent powder at 40°C, 16 °dH, 60 min, pH approx. 10 and enzymes dosed as activity units (MNU) per wash liquor.

Fig 8 describes the stain removal performance of variants BH18, BH21, BH23, BH24, BH25 and wild type Man7 (produced in *Bacillus*) as an increase of lightness (sum of ΔL^* of 3 stains) in the presence of 3.8 g/l of Commercial bleach detergent powder at 40°C, 16 °dH, 60 min, pH approx. 9.5 and enzymes dosed as activity units (MNU) per wash liquor.

Fig 9 shows the stability of variant TBH6 in Commercial heavy duty liquid detergent at 37°C compared to wild type enzyme produced in *Trichoderma*.

Fig 10 shows the stability of variant BH25 in Commercial heavy duty liquid detergent at 37°C compared to wild type enzyme produced in *Bacillus*.

Fig 11A-B show the stability of variants and wild type produced in *Trichoderma* in Commercial heavy duty liquid detergent at 50°C and 7 days .

Fig 11A shows variants TBH1, TBH2, TBH3, TBH4, TBH5, TBH6, TBH7, TBH8, TBH9 and wild type.

Fig 11B shows variants TBH6, TBH10, TBH11 and wild type.

Fig 12 shows a flow chart of instant coffee production involving use of the mannanase variants of the invention.

Fig. 13 shows the stability of the combination variant TBH14 (combination of TBH5 and TBH6) compared to variants TBH5 and TBH6 in Commercial heavy duty liquid detergent at 50°C and for 7 days.

DEPOSITS

The following strain depositions according to the Budapest Treaty on the International Recognition of Deposit of Microorganisms for the Purposes of Patent Procedure were made:

The *E.coli* strain RF12379 including the plasmid pALK4434 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32425.

The *E.coli* strain RF12380 including the plasmid pALK4435 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32426.

- 5 The *E.coli* strain RF12381 including the plasmid pALK4436 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32427.

- 10 The *E.coli* strain RF12382 including the plasmid pALK4437 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32428.

- 15 The *E.coli* strain RF12383 including the plasmid pALK4438 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32429.

- 20 The *E.coli* strain RF12384 including the plasmid pALK4439 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32430.

The *E.coli* strain RF12385 including the plasmid pALK4440 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32431.

- 25 The *E.coli* strain RF12386 including the plasmid pALK4441 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32432.

The *E.coli* strain RF12387 including the plasmid pALK4442 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 2 March, 2017 and assigned accession number DSM 32433.

- 5 The *E.coli* strain RF12456 including the plasmid pALK4432 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 18 May, 2017 and assigned accession number DSM 32518.

- 10 The *E.coli* strain RF12457 including the plasmid pALK4433 was deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Inhoffenstrasse 7 b, D-38124 Braunschweig, Germany on 18 May, 2017 and assigned accession number DSM 32519.

SEQUENCE LISTINGS

SEQ ID NO: 1 DNA sequence of the *man7*

- 15 SEQ ID NO: 2 Full-length amino acid sequence of the Man7

SEQ ID NO: 3 Deduced amino acid sequence (mature) of the Man7

SEQ ID NO: 4 Core amino acid sequence of the Man7, no CMB

SEQ ID NO: 5 Synthetic gene sequence of the variant *tbh1*

SEQ ID NO: 6 Deduced amino acid sequence (mature) of the variant TBH1

- 20 SEQ ID NO: 7 Synthetic gene sequence of the variant *tbh2*

SEQ ID NO: 8 Deduced amino acid sequence (mature) of the variant TBH2

SEQ ID NO: 9 Synthetic gene sequence of the variant *tbh3*

SEQ ID NO: 10 Deduced amino acid sequence (mature) of the variant TBH3

SEQ ID NO: 11 Synthetic gene sequence of the variant *tbh4*

- 25 SEQ ID NO: 12 Deduced amino acid sequence (mature) of the variant TBH4

SEQ ID NO: 13 Synthetic gene sequence of the variant *tbh5*

SEQ ID NO: 14 Deduced amino acid sequence (mature) of the variant TBH5

SEQ ID NO: 15 Synthetic gene sequence of the variant *tbh6*

SEQ ID NO: 16 Deduced amino acid sequence (mature) of the variant TBH6

SEQ ID NO: 17 Synthetic gene sequence of the variant *tbh7*

SEQ ID NO: 18 Deduced amino acid sequence (mature) of the variant TBH7

5 SEQ ID NO: 19 Synthetic gene sequence of the variant *tbh8*

SEQ ID NO: 20 Deduced amino acid sequence (mature) of the variant TBH8

SEQ ID NO: 21 Synthetic gene sequence of the variant *tbh9*

SEQ ID NO: 22 Deduced amino acid sequence (mature) of the variant TBH9

SEQ ID NO: 23 Synthetic gene sequence of the variant *tbh10*

10 SEQ ID NO: 24 Deduced amino acid sequence (mature) of the variant TBH10

SEQ ID NO: 25 Synthetic gene sequence of the variant *tbh11*

SEQ ID NO: 26 Deduced amino acid sequence (mature) of the variant TBH11

SEQ ID NO: 27 DNA sequence of the variant *bh18*

SEQ ID NO: 28 Deduced amino acid sequence (mature) of the variant BH18

15 SEQ ID NO: 29 DNA sequence of the variant *bh21*

SEQ ID NO: 30 Deduced amino acid sequence (mature) of the variant BH21

SEQ ID NO: 31 DNA sequence of the variant *bh23*

SEQ ID NO: 32 Deduced amino acid sequence (mature) of the variant BH23

SEQ ID NO: 33 DNA sequence of the variant *bh24*

20 SEQ ID NO: 34 Deduced amino acid sequence (mature) of the variant BH24

SEQ ID NO: 35 DNA sequence of the variant *bh25*

SEQ ID NO: 36 Deduced amino acid sequence (mature) of the variant BH25

SEQ ID NO: 37 Sequence of the oligonucleotide primer Man7_Var1

SEQ ID NO: 38 Sequence of the oligonucleotide primer Man7_Var2

25 SEQ ID NO: 39 Sequence of the oligonucleotide primer Man7_Var3

SEQ ID NO: 40 Sequence of the oligonucleotide primer Man7_Var4

SEQ ID NO: 41 Sequence of the oligonucleotide primer Man7_Var5

SEQ ID NO: 42 Sequence of the oligonucleotide primer Man7_Var6

SEQ ID NO: 43 Sequence of the oligonucleotide primer Man7_Var7

SEQ ID NO: 44 Sequence of the oligonucleotide primer Man7_Var8

5 SEQ ID NO: 45 Sequence of the oligonucleotide primer Man7_Var9

SEQ ID NO: 46 Sequence of the oligonucleotide primer Man7_Var10

SEQ ID NO: 47 Sequence of the oligonucleotide primer Man7_Var11

SEQ ID NO: 48 Sequence of the oligonucleotide primer Man7_Var12

DETAILED DESCRIPTION

- 10 Mannan refers to polysaccharides consisting of a mannose backbone linked together by β -1,4-linkages with side-chains of galactose attached to the backbone by α -1,6-linkages. Mannans comprise plant-based material such as guar gum and locust bean gum. Glucomannans are polysaccharides having a backbone of more or less regularly alternating β -1,4 linked mannose and
- 15 glucose, galactomannans and galactoglucomannans are mannans and glucomannans with alpha-1,6 linked galactose side branches.

The term "functional fragment" or "effective fragment" means a fragment or portion of the SEQ ID NO: 2 variant that retains about the same enzymatic function or effect.

- 20 The term "mannanase variants" and "variant of mannanase" means any mannanase molecule obtained by site-directed or random mutagenesis, insertion, substitution, deletion, recombination and/or any other protein engineering method, which leads to mannanases that differ in their amino acid sequence from the parent mannanase, i.e. a wild-type mannanase. The terms "wild type mannanase", "wild type enzyme", "wild type", or "wt" in accordance with the disclosure describe a mannanase enzyme with an amino acid sequence found in nature or a fragment thereof.
- 25

- The term "catalytic activity" or "activity" describes quantitatively the conversion of a given substrate under defined reaction conditions. The term "residual activity" is
- 30 defined as the ratio of the catalytic activity of the enzyme under a certain set of

conditions to the catalytic activity under a different set of conditions. Therefore the residual activity a_i is given by $a_i = v_i/v_0$ where v denotes any measure of catalytic activity and $a_i * 100$ is the relative activity in percent. The term "specific activity" describes quantitatively the catalytic activity per amount of enzyme under defined reaction conditions.

The term "proteolytic stability" describes the property of a protein to withstand a limited exposure to proteases under conditions where the proteases are active, without losing activity under conditions where its activity can be measured.

As used herein, the term "mannanase" or "galactomannanase" denotes a mannanase enzyme defined according to that known in the art as mannan endo-1,4-beta-mannosidase and having the alternative names beta-mannanase and endo-1,4-mannanase and catalysing hydrolysis of 1,4-beta-D-mannosidic linkages in mannans, galactomannans, glucomannans, and galactoglucomannans. Mannanases are classified according to the Enzyme Nomenclature as EC 3.2.1.78.

As used herein, "isolated" means a substance in a form or environment that does not occur in nature. Non-limiting examples of isolated substances include (1) any non-naturally occurring substance, (2) any substance including any enzyme, variant, nucleic acid, protein, peptide or cofactor, that is at least partially removed from one or more or all of the naturally occurring constituents with which it is associated in nature; (3) any substance modified by the hand of man relative to that substance found in nature, such as a variant; or (4) any substance modified by increasing or decreasing the amount of the substance relative to other components with which it is naturally associated (e.g., recombinant production in a host cell; one or multiple copies of a gene encoding the substance; and use of an alternative promoter to the promoter naturally associated with the gene encoding the substance). In an embodiment a polypeptide, enzyme, variant, polynucleotide, host cell or composition of the invention is isolated.

As used herein, the term "comprising" includes the broader meanings of "including", "containing", and "comprehending", as well as the narrower expressions "consisting of" and "consisting only of".

As used herein, "variant" means a sequence or a fragment of a sequence (nucleotide or amino acid) inserted, substituted or deleted by one or more nucleotides/amino acids, or which is chemically modified. In an embodiment the term variant also includes a recombinant mannanase enzyme.

- 5 As used herein, "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. In an embodiment the conservative amino acids in the present description refer to the amino acids within following groupings: Hydrophobic (F W Y H K M I
10 L V A G C); Aromatic (F W Y H); Aliphatic (I L V); Polar (W Y H K R E D C S T N Q); Charged (H K R E D); Positively charged (H K R); Negatively charged (E D); Small (V C A G S P T N D); Tiny (A G S). Thus, a conservative substitution occurs when an amino acid is substituted with an amino acid in the same group.

- In an embodiment the substitution is a substitution with at least one amino acid
15 residue, such as with one amino acids, two amino acids, or three amino acids. In a further embodiment the at least one amino acid is Ala.

- As used herein, a "non-conservative amino acid substitution" is one in which an amino acid is substituted with an amino acid in a different group as defined above. The non-conservative substitution may result into a change of an amino
20 acid to another amino acid with different biochemical properties, such as charge, hydrophobicity and/or size. In an embodiment the non-conservative substitution changes at least one property of the variant, such as stability, glycosylation pattern, folding, structure, activity, or affinity.

- N-linked glycosylation process occurs in eukaryotes, but very rarely in bacteria.
25 The attachment of a glycan residue to a protein requires the recognition of a consensus sequence. N-linked glycans are almost always attached to an asparagine (Asn) side chain that is present as a part of Asn-X-Ser/Thr consensus sequence, wherein X is any amino acid except for proline (Pro). The inventors have also found that a non-glycosylated Asn side chain structurally close to the
30 active site of the mannanase is important for obtaining a variant with good mannan degrading performance. Without being bound to any theory, glycan sugars are polar molecules and when attached to an Asn, they locate on the

surface of the protein causing structural changes in the glycosylated Asn and in its vicinity. Site directed mutagenesis of Asn or Ser/Thr residues in the Asn-Xaa-Thr(Ser) consensus sequence can be used to prevent glycosylation of the desired N-linked glycosylation sites in the variant of the first aspect of the invention.

In an embodiment the variant has a substitution of an amino acid by a residue which prevents N-linked glycosylation of the residue 283 when expressed in a host cell capable of N-linked glycosylation.

In an embodiment the present variant comprises at least one Asn-X-Ser/Thr consensus sequence.

In an embodiment the present variant comprises Pro residue in the location X of the at least one Asn-X-Ser/Thr consensus sequence.

In an embodiment the substitution is either a conservative or a non-conservative substitution.

As used herein, a "peptide" and a "polypeptide" are amino acid sequences including a plurality of consecutive polymerized amino acid residues. For purpose of this invention, peptides are molecules including up to 20 amino acid residues, and polypeptides include more than 20 amino acid residues. The peptide or polypeptide may include modified amino acid residues, naturally occurring amino acid residues not encoded by a codon, and non-naturally occurring amino acid residues. As used herein, a "protein" may refer to a peptide or a polypeptide of any size. A protein may be an enzyme, a protein, an antibody, a membrane protein, a peptide hormone, regulator, or any other protein.

The term "polynucleotide" denotes a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules.

As used herein, "modification", "modified", and similar terms in the context of polynucleotides refer to modification in a coding or a non-coding region of the polynucleotide, such as a regulatory sequence, 5' untranslated region, 3'

untranslated region, up-regulating genetic element, down-regulating genetic element, enhancer, suppressor, promoter, exon, or intron region. The modification may in some embodiments be only structural, having no effect on the biological effect, action or function of the polynucleotide. In other
5 embodiments the modification is a structural modification, which provides a change in the biological effect, action or function of the polynucleotide. Such a modification may enhance, suppress or change the biological function of the polynucleotide.

As used herein, "identity" means the percentage of exact matches of amino acid
10 residues between two aligned sequences over the number of positions where there are residues present in both sequences. When one sequence has a residue with no corresponding residue in the other sequence, the alignment program allows a gap in the alignment, and that position is not counted in the denominator of the identity calculation. Identity is a value determined with the
15 Pairwise Sequence Alignment tool EMBOSS Needle at the EMBL-EBI website (www.ebi.ac.uk/Tools/psa/emboss_needle/).

As used herein, low stringency conditions mean for probes of at least 100 nucleotides in length conditions corresponding to hybridizing at prehybridisation and hybridisation at 55 °C in 5× SSC, 0.1 % N-lauroylsarcosine, 0.02 % SDS, 1%
20 blocking reagent (Roche 11 096 176 001), following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed two to three times each for 15 minutes using 2X SSC, 0.1% SDS at 55°C.

As used herein, high stringency conditions mean for probes of at least 100 nucleotides in length conditions corresponding to hybridizing at prehybridisation
25 and hybridization at 65°C in 5× SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent (Roche 11 096 176 001), following standard Southern blotting procedures for 12 to 24 hours. The carrier material is finally washed two to three times each for 15 minutes using 0.1X SSC, 0.1% SDS at 65°C.

As used herein, "host cell" means any cell type that is susceptible to
30 transformation, transfection, transduction, mating, crossing or the like with a nucleic acid construct or expression vector comprising a polynucleotide. The term "host cell" encompasses any progeny that is not identical due to mutations that

occur during replication. Non-limiting examples of a host cell are fungal cells, filamentous fungal cells from Division *Ascomycota*, Subdivision *Pezizomycotina*; preferably from the group consisting of members of the Class *Sordariomycetes*, Subclass *Hypocreomycetidae*, Orders *Hypocreales* and *Microascales* and

5 *Aspergillus*, *Chrysosporium*, *Myceliophthora* and *Humicola*; more preferably from the group consisting of Families *Hypocreaceae*, *Nectriaceae*, *Clavicipitaceae*, *Microascaceae*, and Genera *Trichoderma* (anamorph of *Hypocrea*), *Fusarium*, *Gibberella*, *Nectria*, *Stachybotrys*, *Claviceps*, *Metarhizium*, *Villosiclava*, *Ophiocordyceps*, *Cephalosporium*, and *Scedosporium*; more preferably from the

10 group consisting of *Trichoderma reesei* (*Hypocrea jecorina*), *T. citrinoviridae*, *T. longibrachiatum*, *T. virens*, *T. harzianum*, *T. asperellum*, *T. atroviridae*, *T. parareesei*, *Fusarium oxysporum*, *F. graminearum*, *F. pseudograminearum*, *F. venenatum*, *Gibberella fujikuroi*, *G. moniliformis*, *G. zeae*, *Nectria* (*Haematonectria*) *haematococca*, *Stachybotrys chartarum*, *S. chlorohalonata*,

15 *Claviceps purpurea*, *Metarhizium acridum*, *M. anisopliae*, *Villosiclava virens*, *Ophiocordyceps sinensis*, *Acremonium* (*Cephalosporium*) *chrysogenum*, and *Scedosporium apiospermum*, and *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus oryzae*, *Chrysosporium lucknowense*, *Myceliophthora thermophila*, *Humicola insolens*, and *Humicola grisea*, most preferably *Trichoderma reesei*.

20 Non-limiting examples of a host cell are bacterial cells, preferably gram positive Bacilli (e.g. *Bacillus subtilis*, *B. licheniformis*, *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*), gram-negative bacteria (e.g. *Escherichia coli*), actinomycetales (e.g. *Streptomyces* sp.) and yeasts (e.g. *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*).

25 In an embodiment the host cell is a fungal cell, preferably a filamentous fungal cell, such as *Trichoderma* or *Trichoderma reesei*. In an embodiment the host cell is a bacterial cell, preferably a gram positive *Bacillus* cell, such as *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*.

In an embodiment the host cell is capable of N-linked glycosylation.

30 A "recombinant cell" or "recombinant host cell" refers to a cell or host cell, which has been genetically modified or altered to comprise a nucleic acid sequence which is not native to said cell or host cell. The genetic modification may comprise integrating the polynucleotide in the genome of the host cell. The

polynucleotide may also be exogenous in the host cell. In an embodiment the present host cell is a recombinant host cell.

As used herein, "expression" includes any step involved in the production of a polypeptide in a host cell including, but not limited to, transcription, translation, post-translational modification, and secretion. Expression may be followed by harvesting, i.e. recovering, the host cells or the expressed product.

The term "expression vector" denotes a DNA molecule, linear or circular, that comprises a segment encoding a polypeptide of interest operably linked to additional segments that provide for its transcription. Such additional segments may include promoter and terminator sequences, and may optionally include one or more origins of replication, one or more selectable markers, an enhancer, a polyadenylation signal, carrier and the like. Expression vectors are generally derived from plasmid or viral DNA, or may contain elements of both. The expression vector may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which the vector is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector, which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated. In an embodiment the present vector is an expression vector.

The term "recombinant produced" or "recombinantly produced" used herein in connection with production of a polypeptide or protein is defined according to the standard definition in the art.

The term "obtained from" and "obtainable" as used herein in connection with a specific microbial source means that the polynucleotide is expressed by the specific source (homologous expression), or by a cell in which a gene from the source has been inserted (heterologous expression).

The term "enzyme composition" means either a conventional enzymatic fermentation product, possibly isolated and purified, from a single species of a microorganism, such preparation usually comprising a number of different

enzymatic activities; or a mixture of monocomponent enzymes, preferably enzymes derived from bacterial or fungal species by using conventional recombinant techniques, which enzymes have been fermented and possibly isolated and purified separately and which may originate from different species, preferably fungal or bacterial species or the fermentation product of a microorganism which acts as a host cell for production of a recombinant mannanase, but which microorganism simultaneously produces other enzymes.

The term "operably linked", when referring to DNA segments, denotes that the segments are arranged so that they function in concert for their intended purposes, e.g. transcription initiates in the promoter and proceeds through the coding segment to the terminator.

The term "promoter" denotes a portion of a gene containing DNA sequences that provide for the binding of RNA polymerase and initiation of transcription. Promoter sequences are commonly, but not always, found in the 5' non-coding regions of genes.

The term "secretory signal sequence" or "signal sequence" denotes a DNA sequence that encodes a polypeptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a host cell in which it is produced. The secretory signal sequence can be native or it can be replaced with secretory signal sequence or carrier sequence from another source. Depending on the host cell, the larger peptide may be cleaved to remove the secretory peptide during transit through the secretory pathway.

The term "core region" or "catalytic domain" denotes a domain of an enzyme, which may or may not have been modified or altered, but which has retained at least part of its original activity. The core region of a mannanase according to the invention corresponds to the amino acids aligned with the amino acids 27-331 of Man7, SEQ ID NO: 2.

By the term "linker" or "spacer" is meant a polypeptide comprising at least two amino acids which may be present between the domains of a multidomain protein, for example an enzyme comprising an enzyme core and a binding domain such as a carbohydrate binding module (CBM) or any other enzyme

hybrid, or between two proteins or polypeptides produced as a fusion polypeptide, for example a fusion protein comprising two core enzymes. For example, the fusion protein of an enzyme core with a CBM is provided by fusing a DNA sequence encoding the enzyme core, a DNA sequence encoding the linker and a DNA sequence encoding the CBM sequentially into one open reading frame and expressing this construct.

Efficient amount means an amount, which is sufficient to degrade mannose in the selected application.

The following abbreviations are used for amino acids:

10	A	Ala	Alanine
	C	Cys	Cysteine
	D	Asp	Aspartic acid
	E	Glu	Glutamic acid
	F	Phe	Phenylalanine
15	G	Gly	Glycine
	H	His	Histidine
	I	Ile	Isoleucine
	K	Lys	Lysine
	L	Leu	Leucine
20	M	Met	Methionine
	N	Asn	Asparagine
	P	Pro	Proline
	Q	Gln	Glutamine
	R	Arg	Arginine
25	S	Ser	Serine
	T	Thr	Threonine
	V	Val	Valine
	W	Trp	Tryptophan
	Y	Tyr	Tyrosine

30

Substitutions are described using of the following nomenclature: amino acid residue in the protein scaffold; position; substituted amino acid residue(s).

According to this nomenclature the substitution of, for instance, a serine residue for a glycine residue at position 20 is indicated as Ser20Gly or S20G.

The terms "detergent composition" and "detergent" include, unless otherwise indicated, solid, granular or powder-form all-purpose or heavy-duty washing agents, especially cleaning detergents; liquid, gel or paste-form all-purpose washing agents, especially the so-called heavy-duty liquid (HDL) types; liquid fine-fabric detergents; hand dishwashing agents or light duty dishwashing agents, especially those of the high-foaming type; machine dishwashing agents, including the various tablet, granular, liquid and rinse-aid types for household and institutional use; liquid cleaning and disinfecting agents, car or carpet shampoos, bathroom cleaners; metal cleaners; as well as cleaning auxiliaries such as bleach additives and "stain-stick" or pre-treat types. The terms "detergent", "detergent composition" and "detergent formulation" are used in reference to mixtures, which are intended for use in a wash medium for the cleaning of soiled objects. In some embodiments, the term is used in reference to laundering fabrics and/or garments (e.g., "laundry detergents"). In alternative embodiments, the term refers to other detergents, such as those used to clean dishes, cutlery, etc. (e.g., "dishwashing detergents"). It is not intended that the present invention be limited to any particular detergent formulation or composition. It is intended that in addition to the mannanases according to the invention, the term encompasses detergents that may contain e.g., surfactants, builders, chelators or chelating agents, bleach system or bleach components, polymers, fabric conditioners, foam boosters, suds suppressors, dyes, perfume, tannish inhibitors, optical brighteners, bactericides, fungicides, soil suspending agents, anticorrosion agents, hydrotropes, fabric hueing agents, dispersants, dye transfer inhibiting agents, fluorescent whitening agents, soil release polymers, anti-redepositions agents, anti-shrink agents, anti-wrinkling agents, bactericides, binders, carriers, dyes, enzyme stabilizers, fabric softeners, fillers, foam regulators, perfumes, pigments, sod suppressors, solvents, and structurants for liquid detergents, structure elasticizing agents, enzyme inhibitors or stabilizers, enzyme activators, transferase(s), hydrolytic enzymes, oxido reductases, bluing agents and fluorescent dyes, antioxidants, and solubilizers.

The term "textile" means any textile material including yarns, yarn intermediates, fibers, non-woven materials, natural materials, synthetic materials, and any other textile material, fabrics made of these materials and products made from fabrics (e.g., garments, linen and other articles). The textile or fabric may be in the form of knits, wovens, denims, non-wovens, felts, yarns, and towelling. The textile may be cellulose based, such as natural cellulose including cotton, flax/linen, jute, ramie, sisal or coir or manmade cellulose (e.g. originating from wood pulp) including viscose/rayon, ramie, cellulose acetate fibers (tricell), lyocell or blends thereof. The textile or fabric may also be non-cellulose based such as natural polyamides including wool, camel, cashmere, mohair, rabbit and silk or synthetic polymer such as nylon, aramid, polyester, acrylic, polypropylene and spandex/elastane, or blends thereof as well as blend of cellulose based and non-cellulose based fibers. Examples of blends are blends of cotton and/or rayon/viscose with one or more companion material such as wool, synthetic fibers (e.g. polyamide fibers, acrylic fibers, polyester fibers, polyvinyl alcohol fibers, polyvinyl chloride fibers, polyurethane fibers, polyurea fibers, aramid fibers), and cellulose-containing fibers (e.g. rayon/viscose, ramie, flax/linen, jute, cellulose acetate fibers, lyocell). Fabric may be conventional washable laundry, for example stained household laundry. When the term fabric or garment is used it is intended to include the broader term textiles as well.

The term "stability" includes storage stability and stability during use, e.g. during a wash process (in wash stability) and reflects the stability of the mannanase according to the invention as a function of time, e.g. how much activity is retained when the mannanase is kept in solution, in particular in a detergent solution. The stability is influenced by many factors, e.g. pH, temperature, detergent composition e.g. proteases, stabilizers, builders, surfactants etc. The mannanase stability may be measured using the 'activity assay' as described in examples.

"Mannanase activity" as used herein refers to the mannan degrading activity of a polypeptide. Degrading or modifying as used herein means that mannose units are hydrolyzed from the mannan polysaccharide by the mannanase. The mannan degrading activity of the polypeptides according to present invention can be tested according to standard test procedures known in the art. Example 5 provides an example of a standard method for determining mannanase activity.

In an embodiment the present variant comprises at least one substitution at the position 123, 158, 180, 272, 285, or 307 or a combination thereof.

In an embodiment the present variant comprises at least one substitution at the position M123, A158, F180, G272, T285, or T307 or a combination thereof.

- 5 In an embodiment the present variant comprises at least one further substitution at the position M123, A158, F180, G272, T307, or L316, or a combination thereof. In an embodiment the variant comprises one further substitution, two further substitutions, three further substitutions or four further substitutions.

10 In an embodiment the position 316 is not substituted. This is advantageous to preserve folding and interaction of residues near the site surrounding position 316. Further, Fig. 8 shows that in a commercial detergent similar performance may be obtained with lower amount of enzyme when using a variant substituted or unsubstituted with respect to 316.

15 In an embodiment the variant of mannanase comprises a set of substitutions listed in Table 1.

In an embodiment the substitution comprises a substitution at said position to an amino acid selected from Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

20 In an embodiment the variant comprises at least one substitution or a set of substitutions at the position(s):

a single substitution of M123, A158, F180, G272; T307, or L316; or

M123 and G272; or

A158 and T307; or

L316; or

25 T307; or

M123, A158 and T307; or

M123 and L316;

A158, T307, and L316;

F180 and L316; or

M123, A158, G272; T307, and L316;

or a combination thereof.

In an embodiment the substitution is a substitution resulting into an improved stability of the variant.

- 5 In an embodiment the variant comprises at least one additional substitution selected from:

a substitution which prevents glycosylation of N283;

a substitution of 283, or 285; or

a substitution of N283, T285, or S285; or

- 10 a substitution of T285 or S285 to a residue other than T or S

a substitution of T285 or S285 to the residue A.

- 15 In an embodiment the additional substitution results into an altered glycosylation of the variant when produced in a host cell capable of N-linked glycosylation. In an embodiment the altered glycosylation is decreased degree of glycosylation of the variant.

- 20 In an embodiment the variant has mannanase activity and comprises at least one further substitution of an amino acid at a position corresponding to the position 123, 158, 180, 272, 307, or 316, wherein the variant has mannanase activity and is selected from the group consisting of 1) a variant having at least 85% sequence identity to residues 27-331 of SEQ ID NO: 2; 2) a variant encoded by a polynucleotide that hybridizes under high stringency conditions with a) nucleotides 79 – 993 of SEQ ID NO: 1 (man7) b) the full-length complement of a); and 3) a variant encoded by a polynucleotide having at least 95% sequence identity to the SEQ ID NO: 1 or the genomic DNA sequence thereof; and wherein
25 the amino acid numbering corresponds to the amino acid numbering of SEQ ID NO: 2 (Man7).

In an embodiment the variant comprises a P residue in the position 284 and/or 286. A substitution to a P residue in any or both of the above positions may cause a structural change in the variant, which prevents N-linked glycosylation.

In an embodiment in the present variant the position T285 or S285 is substituted to a residue other than T or S.

In an embodiment in the present variant the position T285 or S285 is substituted to an alanine.

- 5 In an embodiment the variant has at least 85% sequence identity to residues 27-331 of SEQ ID NO: 2, and a non-glycosylated Asn residue at position 283 when produced in a host cell capable of N-linked glycosylation.

In an embodiment the present variant comprises at least one additional glycosylated N site, wherein the position corresponding to the N283 is not
10 glycosylated. Such a variant is obtainable by producing the variant in a host cell which is capable of N-glycosylation, resulting into at least partial glycosylation of the other N-glycosylation sites, but wherein the N-glycosylation of N283 is inhibited.

In a further embodiment the present variant does not comprise any glycosylated
15 N site. Such a variant is obtainable by producing the variant in a host cell which is capable of N-glycosylation, resulting into inhibited glycosylation of the variant.

In an embodiment the variant has a predicted molecular weight between 50000 and 51000, preferably between 50800 and 50970, without including the signal sequence.

- 20 In an embodiment the variant has a predicted pI between 4.5 and 4.8, preferably between 4.6 and 4.75.

Prediction of pI or molecular weight of a variant can be carried out as described in the Table 3.

In a further embodiment of the invention the variant has mannanase activity. In
25 an embodiment the variant has a non-glycosylated Asn residues at position 283 when produced in a host cell capable of N-linked glycosylation, and increased specific activity compared to a wild type mannanase when produced in a eukaryotic host cell.

In one embodiment of the present enzyme composition, the enzyme composition
30 further comprises one or more additional enzymes selected from the group consisting of protease, lipase, cutinase, amylase, carbohydrase, cellulase,

pectinase, pectatelyase, pectinolytic enzyme, esterase, phytase, mannanase, arabinase, galactanase, xylanase, oxidase, xanthanase, xyloglucanase, DNase, laccase, and/or peroxidase, preferably selected from the group consisting of proteases, amylases, cellulases and lipases.

- 5 The present enzyme composition comprising mannanase and an additional enzyme is advantageous in providing synergistic effect. Such additional enzymes are desired when the present enzyme composition comprising mannanase is used in detergents e.g. when washing stains. Particularly advantageous synergistic enzymes that work with mannanase in detergents are amylases, proteases and cellulases, or a combination thereof, such as a composition
10 comprising mannanase, amylase and protease.

In one embodiment the present enzyme composition is provided in the form of a liquid composition or a solid composition such as solution, dispersion, paste, powder, granule, granulate, coated granulate, tablet, cake, crystal, crystal slurry,
15 gel, or pellet.

In an embodiment the present detergent composition is in the form of a liquid detergent or a solid detergent, preferably in the form of a bar, a homogenous tablet, a tablet having two or more layers, a pouch having one or more compartments, a regular or compact powder, a granule, granulate, a paste, a gel,
20 or a regular, compact or concentrated liquid.

In one embodiment the present detergent composition further comprises one or more additional enzymes selected from the group consisting of protease, lipase, cutinase, amylase, carbohydrase, cellulase, pectinase, pectatelyase, pectinolytic enzyme, esterase, mannanase, arabinase, galactanase, xylanase, oxidase, xanthanase, xyloglucanase, laccase, DNase and/or peroxidase, preferably
25 selected from the group consisting of proteases, amylases, cellulases and lipases.

The present enzyme composition can also be used in cleaning agents or boosters that are added on top of the detergent during or before the wash and that are for example in the form of liquid, gel, powder, granules or tablets.
30 Enzyme composition and detergent components may also be soaked in a carrier like textiles.

The present invention furthermore relates to the use of, and a method of using, the enzyme composition or the detergent composition as herein disclosed for degrading mannan.

In a further embodiment the present invention relates to the use of, and a method
5 of using, the enzyme composition or the detergent composition as herein disclosed in a laundry process.

The present invention furthermore relates to a method for removing a stain from a surface, comprising contacting the surface with the enzyme composition or the detergent composition as herein disclosed.

10 The present invention also relates to a method for degrading mannan comprising applying the enzyme composition or the detergent composition as herein disclosed to mannan, preferably wherein the mannan is on a surface of a textile, or at least partially embedded in a textile.

In general the properties of the selected enzyme(s) should be compatible with the
15 selected detergent, (*i.e.*, pH-optimum, compatibility with other enzymatic and non-enzymatic ingredients, etc.), and the enzyme(s) should be present in effective amounts.

A composition for use in solid laundry detergent, for example, may include
20 0.000001% - 5%, such as 0.000005-2%, such as 0.00001%-1%, such as 0.00001%-0.1% of enzyme protein by weight of the composition.

A composition for use in laundry liquid, for example, may include 0.000001%-3%, such as 0.000005%-1%, such as 0.00001%-0.1% of enzyme protein by weight of the composition.

A composition for use in automatic dishwash, for example, may include
25 0.000001%-5%, such as 0.000005%-2%, such as 0.00001%-1%, such as 0.00001%-0.1% of enzyme protein by weight of the composition.

The additional components a-d of the second aspect of the invention provide improved properties for the present enzyme composition. The enzyme composition is compatible with the additional components and improves
30 applicability of the enzyme composition in various uses.

Salts, such as sodium chloride and sodium sulfate function as drying aids.

The present invention furthermore relates to different uses of the present enzyme composition, such as for degrading mannan and for use in a laundry process.

In an embodiment the variant of mannanase comprises a core region.

In an embodiment the variant of mannanase comprises a CBM.

- 5 Providing mannanases that retain activity in temperatures above ambient temperature is advantageous for applications wherein mannan degradation is required in such conditions. Further, the mannanases according to invention may have good stability and activity in alkaline conditions, which is advantageous in detergent use and in biomass processing.

- 10 In an embodiment the variant of mannanase has an amino acid sequence with at least, or about, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO:2.

In an embodiment the variant of mannanase has an amino acid sequence with at least 90% sequence identity to SEQ ID NO:2.

- 15 In an embodiment the mannanase enzyme has an amino acid sequence which is not 100% identical to SEQ ID NO: 2 [Man7].

In an embodiment of the third aspect the host cell is selected from the group consisting of:

fungus cells,

- 20 filamentous fungus cells from Division *Ascomycota*, Subdivision *Pezizomycotina*; preferably from the group consisting of members of the Class *Sordariomycetes*, Subclass *Hypocreomycetidae*, Orders *Hypocreales* and *Microascales* and *Aspergillus*, *Chrysosporium*, *Myceliophthora* and *Humicola*;

- 25 more preferably from the group consisting of Families *Hypocreaceae*, *Nectriaceae*, *Clavicipitaceae*, *Microascaceae*, and Genera *Trichoderma* (anamorph of *Hypocrea*), *Fusarium*, *Gibberella*, *Nectria*, *Stachybotrys*, *Claviceps*, *Metarhizium*, *Villosiclava*, *Ophiocordyceps*, *Cephalosporium*, and *Scedosporium*;

more preferably from the group consisting of *Trichoderma reesei* (*Hypocrea jecorina*), *T. citrinoviridae*, *T. longibrachiatum*, *T. virens*, *T. harzianum*, *T. asperellum*, *T. atroviridae*, *T. parareesei*, *Fusarium oxysporum*, *F. graminearum*, *F. pseudograminearum*, *F. venenatum*,
 5 *Gibberella fujikuroi*, *G. moniliformis*, *G. zeae*, *Nectria* (*Haematonectria*) *haematococca*, *Stachybotrys chartarum*, *S. chlorohalonata*, *Claviceps purpurea*, *Metarhizium acridum*, *M. anisopliae*, *Villosiclava virens*, *Ophiocordyceps sinensis*, *Acremonium* (*Cephalosporium*) *chrysogenum*, and *Scedosporium apiospermum*, and *Aspergillus niger*, *Aspergillus*
 10 *awamori*, *Aspergillus oryzae*, *Chrysosporium lucknowense*, *Myceliophthora thermophila*, *Humicola insolens*, and *Humicola grisea*,
 bacterial cells, preferably gram positive Bacilli such as *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*, gram negative bacteria such as *Escherichia coli*, actinomycetales such as
 15 *Streptomyces* sp., and
 yeasts, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*,
 most preferably *Trichoderma reesei* or *Bacillus*.

In an embodiment the host cell is a eukaryotic host cell capable of N-linked
 20 glycosylation. In a preferred embodiment the host cell is *Trichoderma reesei*.

The recombinant host cell can be used to produce the variant of mannanase and to carry a polynucleotide encoding it. The recombinant host cell is useful also in preparation of variants of mannanase with different properties. For example, a
 25 host cell can be selected, which provides post-translational modifications beneficial for stability or activity, or which facilitates post-processing and formulation of the variant of mannanase produced in the host cell.

In an embodiment the present enzyme composition comprises the recombinant host cell of the second aspect.

In an embodiment the present variant of mannanase is a recombinant
 30 polypeptide, which is a fusion protein.

In an embodiment the present recombinant polypeptide is a fusion protein, which further comprises at least one of:

an amino acid sequence providing a secretory signal sequence;

an amino acid sequence which facilitates purification, such as an affinity tag, His-tag;

an amino acid sequence which enhances production, such as an amino acid sequence which is a carrier, such as CBM;

an amino acid sequence having an enzyme activity; and

an amino acid sequence providing for the fusion protein with binding affinity, such as a carbohydrate binding moiety.

The CBM, carbohydrate binding moiety, as a carrier is advantageous e.g. in *Trichoderma* production.

In an embodiment the host cell is non-pathogenic. This is particularly advantageous for using the host cell in feed, and in detergent applications such as in home laundry detergents.

In an embodiment of the fifth aspect the mannan containing material is selected from plant based material, textile, waste water, sewage, oil or a combination thereof.

In an embodiment the mannan containing material is textile material or fabric.

In another embodiment the mannan containing material is recycled waste paper; mechanical pulp, chemical pulp, semi chemical pulp, Kraft or other paper-making pulps; fibres subjected to a retting process; or guar gum or locust bean gum containing material.

In another embodiment degradation or modifying is carried out in an aqueous environment wherein mannanase shows activity.

In a preferred embodiment the mannan containing material, which is degraded or modified in the method, is on a textile or a fabric optionally with mannan stains. By degrading mannan attached to the textile or fabric, dirt or soil bound to mannan is released and not capable of binding again to the mannan or mannan stains. The textile or fabric can be of any material, for example cotton, flax/linen,

jute, ramie, sisal or coir or manmade cellulose (e.g. originating from wood pulp) including viscose/rayon, modal, cellulose acetate fibers (tricell), lyocell, cupro or blends thereof.

5 In an embodiment the present feed comprises or consists of maize and soybean meal.

In an embodiment the protein source of plant origin comprises or consist of soy, cereal such as barley, wheat, rye, oats, or maize.

In an embodiment the mannan containing product or by-product comprises or consists of palm kernel, guar meal or copra meal.

10 In an embodiment the present animal feed or the present feed supplement is formulated in the form of a wet composition or a dry composition.

In an embodiment the composition comprising at least one variant of mannanase enzyme is used in pulp and paper industry, biobleaching, fiber modification, drainage improvement and in the oil industry, i.e. in oil drilling or oil-servicing
15 industry for hydro-fracturing or controlling the viscosity of drilling fluids.

In an embodiment the composition comprising at least one variant of mannanase is used in textile and detergent industry, biomass processing and biomass hydrolysis, preferably in biofuel, starch, pulp and paper, food, baking, feed or beverage industries.

20 In an embodiment the variant of mannanase hydrolyses endo-beta-1,4-mannosidic linkages randomly.

In an embodiment the variant of mannanase, or the nucleotide sequence encoding the corresponding wild type mannanase, is obtainable or derivable from a bacterial source.

25 In an embodiment the variant of mannanase is fused with at least one further polypeptide, thus forming a fusion polypeptide. The fusion polypeptide or the further polypeptide may have other catalytic or binding activities in addition to those of mannanase. In an embodiment the further polypeptide comprises or consists of carbohydrate binding module, which is optionally a fragment of
30 another protein or enzyme derived from the same or different organism as the mannanase.

In an embodiment the variant of mannanase is connected to the further polypeptide with a linker.

In an embodiment is provided a process for machine treatment of fabrics which process comprises treating fabric during a washing cycle of a machine washing process with a washing solution containing the variant of mannanase of the first aspect or the enzyme composition of the second aspect.

In an embodiment is provided a use of the enzyme composition of the second aspect or the variant of mannanase of the first aspect, together with an enzyme selected from protease, amylase, cellulase, lipase, xylanase, mannanase, cutinase, esterase, phytase, DNase, pectinase, pectinolytic enzyme, pectate lyase, carbohydrase, arabinase, galactanase, xanthanase, xyloglucanase, laccase, peroxidase and oxidase with or without a mediator in a cleaning composition for fabric cleaning and/or fabric stain removal.

In an embodiment is provided a use of the variant of mannanase of the first aspect, or the enzyme composition of the second aspect, together with an enzyme selected from protease, amylase, cellulase, lipase, xylanase, mannanase, cutinase, esterase, phytase, DNase, pectinase, pectinolytic enzyme, pectate lyase, carbohydrase, arabinase, galactanase, xanthanase, xyloglucanase, laccase, peroxidase and oxidase with or without a mediator in a cleaning composition for cleaning hard surfaces such as floors, walls, bathroom tile and the like.

In an embodiment is provided a use of the variant of mannanase of the first aspect, or the enzyme composition of the second aspect, together with an enzyme selected from protease, amylase, cellulase, lipase, xylanase, mannanase, cutinase, esterase, phytase, DNase, pectinase, pectinolytic enzyme, pectate lyase, carbohydrase, arabinase, galactanase, xanthanase, xyloglucanase, laccase, peroxidase and oxidase with or without a mediator in a cleaning composition for hand and machine dishwashing.

EXAMPLES

The following examples are provided to illustrate various aspects of the present invention. They are not intended to limit the invention, which is defined by the accompanying claims.

Example 1. Variant design

To improve the stability and specific activity of the wild type Man7 mannanase, variant sets were designed based on structural analysis of wild type enzyme. The structural model of Man7 was created using Bioluminate software (Schrödinger LCC) with coordinates from *Bacillus* sp. Endo-Beta-D- 1,4-Mannanase (1WKY). The design included two or more specific mutations per variant. Table 1 shows list of variants. The amino acid numbering corresponds to the amino acid numbering of SEQ ID NO: 2 (Man7) full length amino acid sequence containing a signal sequence.

Table 1. List of Man7 variants

Variant	Mutations
TBH1:	M123I, S229A, G272Q, T285A
TBH2:	A158S, S229A, T285A, T307R
TBH3:	S229A, T285A, L316K
TBH4:	M123I, A158S, S229A, G272Q, T285A, T307R
TBH5:	M123I, S229A, G272Q, T285A, L316K
TBH6:	A158S, S229A, T285A, T307R, L316K
TBH7:	F180L, S229A, T285A, L316K
TBH8:	S229A, T285A
TBH9:	S229A, T285A, N300Q, N340Q, S400A, N419A, S433A, N446Q
TBH10:	A158S, S229A, T307R, L316K
TBH11:	A158S, T285A, T307R, L316K
TBH14:	M123I, S229A, G272Q, T285A, L316K, A158S, T307R
BH18:	M123I, G272Q
BH21:	A158S, T307R
BH23:	M123I, A158S, G272Q, T307R
BH24:	M123I, G272Q, L316K
BH25:	A158S, T307R, L316K

Example 2. Cloning of synthetic mannanase variant genes

Standard molecular biology methods were used in the isolation and enzyme treatments of DNA (e.g. isolation of plasmid DNA, digestion of DNA to produce

DNA fragments), in *E. coli* transformations, sequencing etc. The basic methods used were as described by the enzyme, reagent or kit manufacturer.

Variants *tbh1* - *tbh11* were ordered from GenScript as synthetic constructs without their own signal peptide encoding sequences and with codon optimization for *Trichoderma reesei*. Plasmid DNAs obtained from GenScript including the genes *tbh1* - *tbh11* were resuspended in sterile water, digested with *NruI* and *BamHI* restriction enzymes (Thermo Fisher Scientific) according to manufacturer's instructions and cloned into an expression vector cleaved with *NruI* and *BamHI*. Ligation mixtures were transformed into *Escherichia coli* XL1-Blue or XL10-Gold cells (AH Diagnostics) and plated on LB (Luria-Bertani) plates containing 50-100 µg/ml ampicillin. Several *E. coli* colonies were collected from the plates and DNA was isolated with GenJet Plasmid Miniprep Kit (Thermo Fisher Scientific). Positive clones were screened using restriction digestions and they were shown to contain inserts of expected sizes. The fusion sites to the expression plasmid of *tbh1*- *tbh11* mannanase genes were sequenced and the plasmids were named pALK4415 - pALK4423, pALK4430 and pALK4431, respectively (For details see Example 4). The plasmid DNAs including the *thb* genes delivered by GenScript were also transformed into XL10-Gold *E. coli* cells (Agilent) and deposited into DSMZ strain collection. The relevant information on the genes and the deduced amino acid sequences (SEQ ID NOs: 5-36) are summarized in Table 2 and Table 3, respectively. The *E. coli* strains RF12379 - RF12387, RF12456 and RF12457 including the plasmids pALK4434 - pALK4442, pALK4432 and pALK4433, respectively were deposited to the DSMZ collection under the accession numbers DSM 32425, DSM 32426, DSM 32427, DSM 32428, DSM 32429, DSM 32430, DSM 32431, DSM 32432, DSM32433, DSM 32518 and DSM 32519, respectively.

Table 2. The summary on the mannanase variant encoding synthetic genes *tbh1* - *tbh11*

Gene	Length (bp) ^(a)	SEQ ID NO
<i>tbh1</i>	1395	5
<i>tbh2</i>	1395	7
<i>tbh3</i>	1395	9
<i>tbh4</i>	1395	11
<i>tbh5</i>	1395	13
<i>tbh6</i>	1395	15
<i>tbh7</i>	1395	17
<i>tbh8</i>	1395	19
<i>tbh9</i>	1395	21
<i>tbh10</i>	1395	23
<i>tbh11</i>	1395	25

^(a) The STOP codon is included

5 **Table 3. The summary of the amino acid sequences deduced from the mannanase variant encoding gene sequences.**

Man protein	No of aas ^(b)	Length of ss ^(a)	CBM	Core (aa-aa)	Predicted (Da), ss not included ^(b)	Predicted pI, ss not included ^(b)	SEQ ID NO
TBH1	464	26	Yes	27-331	50886	4.62	6
TBH2	464	26	Yes	27-331	50904	4.67	8
TBH3	464	26	Yes	27-331	50848	4.67	10
TBH4	464	26	Yes	27-331	50957	4.67	12
TBH5	464	26	Yes	27-331	50901	4.67	14
TBH6	464	26	Yes	27-331	50919	4.72	16
TBH7	464	26	Yes	27-331	50814	4.67	18
TBH8	464	26	Yes	27-331	50833	4.62	20
TBH9	464	26	Yes	27-331	50800	4.62	22
TBH10	464	26	Yes	27-331	50949	4.72	24
TBH11	464	26	Yes	27-331	50935	4.72	26

^(a) The prediction on the signal sequence was made using the program SignalP v3.0, NN/HMM (Nielsen *et al.*, 1997; Nielsen & Krogh, 1998; Bendtsen *et al.*, 2004).

10 ^(b) The predicted signal sequence was not included. The prediction was made using Clone Manager Professional version 9 for Windows, Sci-Ed Software.

Example 3. Site directed mutagenesis of *man7* gene

Unless otherwise stated, the standard molecular biological methods including DNA manipulations and transformations were used. Mutations were introduced by site directed mutagenesis as described in Kunkel 1985. Mutations generated are listed in Table 4. The amino acid numbering corresponds to the amino acid numbering of SEQ ID NO: 2 (Man7).

Table 4. List of mutations introduced in Man7 by site directed mutagenesis

Variant Nr.	Mutation	SEQ ID NO (nt)	SEQ ID NO (aa)
BH18	M123I, G272Q	27	28
BH21	A158S, T307R	29	30
BH23	M123I, A158S, G272Q, T307R	31	32
BH24	M123I, G272Q, L316K	33	34
BH25	A158S, T307R, L316K	25	36

For amplification Pfx Accu Prime Polymerase (Invitrogen) was used. PCRs were performed according to manufacturer's instructions. Following PCR conditions were used for construction of the expression plasmids: 120 sec initial denaturation at 94 °C, followed by 35 cycles of 15 sec at 94 °C, 30 sec annealing at one of the following 50/55 °C, 110/290 sec extension at 68 °C and the final extension at 68 °C for 10 min. The pEV1 *man7* was used as template for PCR. Sequences of primers used for cloning are shown in Table 5. Overhangs for hybridization are underlined.

Table 5. List of primers used for generation of *man7* variants

Template	Primer	Length	Sequence	Seq ID No
pEV1 <i>man7</i>	Man7_Var1	30	<u>GATGCAACAGGATCTAATTCTATTGCTGAC</u>	37
pEV1 <i>man7</i>	Man7_Var2	34	<u>GCTAGCCAACCGACTTGTCTTTGTTGCGAGT</u> AGC	38
pEV1 <i>man7</i>	Man7_Var3	21	<u>GATCCTGTTGCATCGTGAACC</u>	39
pEV1 <i>man7</i>	Man7_Var4	20	<u>GTCGGTTGGCTAGCTTGGTC</u>	40
pEV1 <i>man7</i> / pEV1 BH18	Man7_Var5	25	<u>ATGAATGGTATGGATCATGGGACGG</u>	41
pEV1 <i>man7</i> / pEV1 BH18	Man7_Var6	25	<u>GGCCCGTGGACAATTCTATTTCCCC</u>	42
pEV1 <i>man7</i> / pEV1 BH18	Man7_Var7	22	<u>TCCATACCATTCAATGGCAATG</u>	43
pEV1 <i>man7</i> / pEV1 BH18	Man7_Var8	20	<u>ATTGTCCACGGGCCTAATGG</u>	44
pEV1 Var1/ pEV1 BH21	Man7_Var9	21	<u>GAAACATCCATTCCAAGCTCG</u>	45
pEV1 Var1/ pEV1 BH21	Man7_Var10	31	<u>AATGGATGTTTCTTTTAATCCATTAGGCCCG</u>	46
pEV1 Var1/ pEV1 BH21	Man7_Var11	36	<u>CGGTATATCTCTGTCTTATTGGATTGTTACAT</u> GATC	47
pEV1 Var1/ pEV1 BH21	Man7_Var12	21	<u>GACAGAGATATACCGACAGTG</u>	48

For cloning purposes NEBuilder®Hifi DNA Assembly Master Mix (NEB, Frankfurt) was used according to kit manufacturer's instructions. Generated expression plasmids (Fig. 1) were transformed by induced competence in *Bacillus subtilis* SCK6 as described in Zhang & Zhang 2011. The transformed cells were plated onto LB (Luria-Bertani) plates supplemented with 10 mg/l Kanamycin. Plates were incubated for 20h at 37°C. Growing colonies were picked and plasmid was isolated using QiaPrep MiniPrep Kit (Qiagen, Germany). The isolation procedure was carried out according to the manufacturer's recommendations for Gram positives plasmid preparations. Inserts were sequenced via Sanger sequencing (GATC, Germany) and revealed the DNA sequences corresponding to the mature parts of the variants 1 to 5. Sequence comparisons were done using ClustalW sequence alignment (Thompson et al 1994). Finally, expression plasmids were transformed in a *Bacillus* production strain via electroporation. *Bacillus* production strain was grown in electroporation medium containing 20 g/l Trypton, 10g/l yeast extract, 10 g NaCl and 2 M sucrose and 10 ml were harvested at an OD(600nm) of 0.4. Cells were washed with electroporation buffer

containing 0,272 M sucrose, 1 mM MgCl₂ and 7 mM KH₂PO₄ and finally resuspended in 250 µl electroporation buffer. Electroporation was performed using following conditions: 1,2 kV, 150 Ω, 50 µF. 1ml electroporation medium was added afterwards and cells were incubated for 3h at 37°C. Cells were plated on LB plates supplemented with 20 mg/l kanamycin and incubated for 18h at 37°C. Clones were verified as described above and used for generation of material for analytic tests. Therefore, strains were inoculated in a standard expression under protein inducing conditions and incubated for 30h at 37°C. Supernatants were harvested and used for analytical and application tests.

Example 4. Production of recombinant mannanase variant proteins in *Trichoderma reesei*

Expression plasmids were constructed for production of recombinant mannanase TBH1 – TBH11 (SEQ ID NO: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26) proteins in *Trichoderma reesei*. The expression plasmids constructed are listed in Table 6.

The recombinant mannanase genes without their own signal sequences were fused to the *T. reesei cel7A/cbh1* promoter with *T. reesei cel6A/cbh2* CBM carrier and linker followed by Kex2 protease recognition site. The transcription termination was ensured by the *T. reesei cel7A/cbh1* terminator and the *A. nidulans amdS* marker gene was used for selection of the transformants as described in Paloheimo *et al.* (2003). The linear expression cassettes (Fig. 2) were isolated from the vector backbones after *NotI* digestions and were transformed into *T. reesei* protoplasts. The host strains used, do not produce any of the four major *T. reesei* cellulases (CBHI, CBHII, EGI, EGII). The transformations were performed as in Penttilä *et al.* (1987) with the modifications described in Karhunen *et al.* (1993), selecting acetamidase as a sole nitrogen source (*amdS* marker gene). The transformants were purified on selection plates through single conidia prior to sporulating them on PD.

Table 6. The expression cassettes constructed to produce TBH1 – TBH11 (SEQ ID NO: 6, 8, 10, 12, 14, 16, 18, 20, 22, 24 and 26), recombinant proteins in *Trichoderma reesei*. The overall structure of the expression cassettes was as described in Fig. 2.

Mannanase protein	Expression plasmid	Expression cassette ^(a)
TBH1	pALK4415	7.5 kb <i>NotI</i>
TBH2	pALK4416	7.5 kb <i>NotI</i>
TBH3	pALK4417	7.5 kb <i>NotI</i>
TBH4	pALK4418	7.5 kb <i>NotI</i>
TBH5	pALK4419	7.5 kb <i>NotI</i>
TBH6	pALK4420	7.5 kb <i>NotI</i>
TBH7	pALK4421	7.5 kb <i>NotI</i>
TBH8	pALK4422	7.5 kb <i>NotI</i>
TBH9	pALK4423	7.5 kb <i>NotI</i>
TBH10	pALK4430	7.5 kb <i>NotI</i>
TBH11	pALK4431	7.5 kb <i>NotI</i>
TBH14	pALK4668	7.5 kb <i>NotI</i>

- 5 ^(a) The expression cassette for *T. reesei* transformation was isolated from vector backbone by using *NotI* digestions.

The mannanase production of the transformants was analyzed from the culture supernatants of the shake flask cultivations. The transformants were inoculated from the PD slants to shake flasks containing 50 ml of complex lactose-based cellulase inducing medium (Joutsjoki *et al.* 1993) buffered with 5% KH₂PO₄. The mannanase protein production of the transformants was analyzed from culture supernatants after growing them for 7 days at 30 °C, 250 rpm. Heterologous production of recombinant proteins was analyzed by SDS-PAGE with subsequent Coomassie staining. The supernatants were recovered also for application tests by centrifugation.

The best producing transformants were chosen to be cultivated in laboratory scale bioreactors. The transformants were cultivated in bioreactors either on batch or by additional feeding type of process under protein inducing conditions at a typical mesophilic fungal cultivation temperature and slightly acidic conditions. The cultivation was continued until depletion of the medium sugars or

until suitable yield was reached. The supernatants were recovered for application tests by centrifugation or by filtration.

Example 5. Assay of galactomannanase activity by DNS -method

Mannanase activity (MNU) was measured as the release of reducing sugars from galactomannan (0.3 w/w-%) at 50°C and pH 7.0 in 5 min. The amount of released reducing carbohydrates was determined spectrophotometrically using dinitrosalicylic acid.

Substrate (0.3 w/w-%) used in the assay was prepared as follows: 0.6 g of locust bean gum (Sigma G-0753) was in 50 mM sodium citrate buffer pH 7 (or citrate phosphate buffer pH 7) at about 80 °C using a heating magnetic stirrer and heated up to boiling point. The solution was cooled and let to dissolve overnight in a cold room (2 - 8 °C) with continuous stirring and insoluble residues were removed by centrifugation. After that solution was filled up to 200 ml by buffer. Substrate was stored as frozen and melted by heating in a boiling water bath to about 80 °C, cooled to room temperature and mixed carefully before use.

DNS reagent used in the assay was prepared by dissolving 50 g of 3,5-dinitrosalicylic acid (Sigma D-550) in about 4 liter of water. With continuous magnetic stirring 80.0 g of NaOH was gradually added and let to dissolve. An amount of 1500 g of Rochelle Salt (K-Na-tartrate, Merck 8087) was added in small portions with continuous stirring. The solution that was cautiously warmed to a maximum temperature of 45 °C., was cooled to room temperature and filled up to 5000 ml. After that it was filtered through Whatman 1 filter paper and stored in a dark bottle at room temperature.

The reaction was first started by adding 1.8 ml of substrate solution to each of the two test tubes and let to equilibrate at 50 °C for 5 minutes, after which 200 µl of suitably diluted enzyme solution was added to one of the tubes, mixed well with vortex mixer and incubated exactly for 5 min at 50 °C. Enzyme blanks didn't need to be equilibrated or incubated. The reaction was stopped by adding 3.0 ml of DNS reagent into both tubes and mixed. 200 µl of sample solution was added to the enzyme blank tubes. Both tubes were placed in a boiling water bath. After boiling for exactly 5 minutes, the tubes were placed in a cooling water bath and allow them to cool to room temperature. The absorbance of sample was

measured against the enzyme blank at 540 nm and activity was read from the calibration curve and multiplied by the dilution factor. A suitable diluted sample yielded an absorbance difference of 0.15 - 0.4.

Standard curve was prepared 20 mM from mannose stock solution by dissolving 360 mg of mannose (SigmaM-6020, stored in a desiccator) in assay buffer and diluted to solutions containing 3, 6, 10 and 14 $\mu\text{mol/ml}$ of mannose. Standards were handled like the samples except for incubating at 50°C. The absorbances were measured against the reagent blank (containing buffer instead of standard dilution of mannose) at 540 nm. Calibration curve was constructed for every series of assays.

One mannanase unit (MNU) was defined as the amount of enzyme that produces reductive carbohydrates having a reductive power corresponding to one nmol of mannose from galactomannan in one second under the assay conditions (1 MNU = 1 nkat).

Example 6. Stain removal performance of mannanase variants produced in *Trichoderma* with commercial detergents

Cultivation samples of TBH1 –TBH11 variants produced in *Trichoderma* (as described in Example 4) were tested for their ability to remove mannanase sensitive standard stains at 40 °C and water hardness of 16°dH with commercial detergents and compared to wild type enzyme. The following artificially soiled test cloths from Center for testmaterial B.V. (the Netherlands) were used: Chocolate pudding mannanase sensitive on cotton (E-165), Locust bean gum, with pigment on cotton (C-S-73) and Guar gum with carbon black on cotton (C-S-43). The fabric was cut in 6 cm x 6 cm swatches and 2 pieces of each were used in test.

Commercial heavy duty liquid detergent A containing all other enzymes except mannanase was used at concentration of 4.4 g per liter of wash liquor, Commercial Color detergent powder without enzymes was used at 3.8 g/l and Commercial bleach detergent powder without enzymes was used at 4.2 g/l. Detergent containing wash liquors we prepared in synthetic tap water with hardness of 16 °dH. Protease Savinase® 16 L (0.5 w/w %) and amylase Stainzyme® 12 L (0.4 w/w %) was added into hard water used with commercial commercial color and bleach detergent powders, the liquid detergent already contained amylase and

protease. pH of the wash liquor of liquid detergent was approximately 8.3, with color detergent powder approx. 10 and with the bleach detergent approx. 9.5.

Mannanases were dosed as 0.025 and/or 0.05 MNU activity per ml of wash liquor. Activity was measured as described in Example 5. Control sample contained the detergent solution without mannanase.

For synthetic tap water with hardness of 16 °dH the following stock solutions were prepared in deionized water (Milli-Q or equivalent):

Stock solution with 1000 °d Calcium-hardness: $\text{CaCl}_2 \times 2 \text{ H}_2\text{O}$ (1.02382.1000, Merck KGaA, Germany) 26,22 g/l

Stock solution with 200 °d Magnesium-hardness: $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$ (1.05886.1000, Merck KGaA, Germany) 8,79 g/l H_2O

NaHCO_3 stock solution: NaHCO_3 (1.06329.1000 Merck KGaA, Germany) 29,6 g/l

13,3 ml CaCl_2 solution, 13,3 ml MgSO_4 solution and 10,0 ml of freshly made NaHCO_3 solution were added in volumetric flask in the given order, made up to 1 liter with deionized water and mixed. The hardness of water was determined by complexometric titration and found correct.

Stain removal treatments were performed in Atlas LP-2 Launder-Ometer as follows. Launder-Ometer was first preheated to 40°C. Then detergent, 250 ml synthetic tap water with hardness of 16 °dH and diluted enzyme (<1,0 ml) were added into 1,2 liter containers. Stains were added and the Launder-Ometer was run at 40°C for 60 min with a rotation speed of 42 rpm. After that the swatches were carefully rinsed under running water and dried overnight at indoor air, on a grid protected against daylight.

The stain removal effect was evaluated by measuring the colour as reflectance values with Konica Minolta CM-3610A spectrophotometer using $L^*a^*b^*$ color space coordinates (illuminant D65/10°, 420 nm cut). Fading of the stains, indicating mannanase performance (stain removal efficiency) was calculated as ΔL^* (delta L^*), which means lightness value L^* of enzyme treated fabric minus lightness value L^* of fabric treated with washing liquor without mannanase (control).

Final results (total stain removal effect) were shown as sum of ΔL^* of each 3 stains. Color values of each stains were average of 2 swatches.

The results obtained with commercial liquid detergent are shown in Fig 3, the results with commercial color detergent powder in Fig 4 and the results with commercial bleach detergent in Fig 5. All variants (TBH1-TBH11) showed excellent stain removal performance with commercial detergents of different types. Performance of variants was similar to the wild type.

Example 7. Stain removal performance of mannanase variants produced in *Bacillus* with commercial detergent

Cultivation supernatants of variants BH18, BH21, BH23, BH24 and BH25 produced in *Bacillus* (as described in Example 3) were tested for their ability to remove mannanase sensitive standard stains at 40 °C and water hardness of 16°dH with commercial detergents and compared to wild type enzyme. Similar test system than described in Example 6 was used.

The results obtained with commercial liquid detergent are shown in Fig 6, the results with commercial color detergent powder in Figs 7 and the results with commercial bleach detergent in Fig 8. All variants (BH18, BH21, BH23, BH24, BH25) showed excellent stain removal performance with commercial detergents of different types. Performance of variants was similar to wild the type.

Example 8. Stability of mannanase variants in commercial liquid detergent at 37°C

The stability of cultivation supernatants of several mannanase variants produced in *Trichoderma* (as described in Example 4) or *Bacillus* (as described in Example 3) was tested in commercial liquid heavy duty detergent A containing protease and all other enzymes except mannanase and compared to the wild type enzymes. Mannanase preparations were added 4 w/w-% in detergent and samples were incubated in plastic tubes with caps at 37°C for about 16 or 24 weeks. The activity was measured at certain intervals by activity assay described in Example 5 except using 30 min incubation time. Results were calculated as residual activity (%), which was obtained by dividing the activity of a sample taken at certain time point by initial activity of the sample.

The stability of variants TBH2, TBH3, TBH4, TBH5 and especially TBH6 was improved compared to the wild type enzyme of Man7 produced in *Trichoderma*, when stored at high temperature like 37°C for 24 weeks. Results of stability tests with TBH6 compared to wild type are shown in Fig. 9.

- 5 The stability of variants BH21, BH23, BH24 and especially BH25 was improved compared to the wild type enzyme of Man7 produced in *Bacillus* when stored at high temperature like 37°C for several weeks. Results of stability tests with BH25 compared to the wild type are shown in Fig. 10.

Example 9. Stability of mannanase variants in commercial liquid detergent at 50°C

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The stability of cultivation supernatants of TBH1 – TBH11 variants produced in *Trichoderma* (as described in Example 4) was tested in commercial liquid heavy duty detergent A containing protease but no mannanase and compared to the wild type enzyme at extreme conditions. Mannanase preparations were added 4 w/w-% in detergent and samples were incubated in plastic tubes with caps at 50°C for 7 days. The activity was measured by activity assay described in Example X except using 30 min incubation time. Results were calculated as residual activity (%), which was obtained by dividing the activity of a sample taken at certain time point by initial activity of the sample.

15

- 20 Based on the results shown in Fig 11, the stability of all variant enzymes was better compared to the wild type at 50°C for 7 days. The stability at extreme conditions was considerably improved especially with TBH1, TBH2, TBH4, TBH5 TBH6, TBH10 and TBH11 compared to the wild type enzyme.

25

Some of the best producing variants were cultivated in laboratory scale bioreactors and the stability was retested using similar test system as described above except the amount of mannanase preparation was 1 w/w-% in detergent. The stability of mannanase variants TBH5 and TBH6 was considerably better than the wild type also when laboratory scale cultivation material was used (data not shown).

30

The results show that mannanase variants have excellent stain removal performance and considerably improved stability compared to the wild type enzyme

especially at elevated temperatures. They can also be produced more economically due to their higher specific activity and production yield (data not shown).

Example 10. Combination of variants TBH5 and TBH6

To further improve the mannanase properties, the mutations in TBH5 and TBH6 variants were combined (M123I, A158S, S229A, G272Q, T285A, T307R and L316K). The combination variant TBH14 was prepared as synthetic construct, cloned into the expression vector and produced in *Trichoderma reesei* as described in Examples 2 and 4. The stability of the combination variant TBH14 was tested at 50°C for 7 days as described in Example 9. Variants TBH6 and TBH5 were used for comparison. Results are shown in Figure 13. The stability of the combination variant TBH14 was at least as good or better than that of variants TBH6 or TBH5, that have showed improved stability compared to wild type in Example 9, i.e. also the stability of TBH14 was improved compared to the wild type enzyme produced in *Trichoderma* when stored in high temperatures like 50°C for several days. The combination variant TBH14 showed also excellent stain removal performance (similar to the wild type) with commercial detergents.

Example 10b. Efficiency study with mannanase alone and in combination with a non-starch polysaccharide (NSP) degrading enzyme in broilers

Effects of recombinant mannanase variants of the invention are studied on growth in broilers. Ultrafiltrate of the fermentation broth including the recombinant mannanase is dried and target levels applied to a pelleted broiler diet alone or in combination with a commercial available xylanase based product.

A control diet based on corn and dehulled solvent extracted soybean meal is fed without enzyme or added by different levels of the recombinant mannanase of the invention alone or in combination with a standard dose of a commercial xylanase.

Initial weight of the broilers is between 30 g and 50 g. The trial lasts between three and five weeks. Each treatment consists at minimum of six replicates with 10 broilers each. In each case the diet is analysed for moisture, crude protein, crude fibre, fat, ash, and enzyme protein.

Five diets are prepared:

- 1) unsupplemented control (BD)
- 2) BD + mannanase 1 – 500 mg/kg
- 3) BD + mannanase 1 – 1000 mg/kg
- 4) BD + mannanase 1 – 500 mg/kg + xylanase 1 – 10 mg/kg
- 5) BD + xylanase 1 – 10 mg/kg

Health status and mortality of the animals is checked daily by visual inspection. At days 0, 14, and 35 body weight gain (BW), feed intake (FI), and feed-conversion ratio (FCR) are measured. FCR is calculated as the total feed consumed divided by the weight gain during the same period. Determination of the effect of the recombinant mannanase variants is based on the comparison to those animals fed the same diet or the same diet but added by xylanase.

Example 11. Instant coffee production

Pure mannan is the main storage polysaccharide component of coffee endosperms and is responsible for their high viscosity, which negatively affects the technological processing of instant coffee and increases energy consumption during drying. Those effects are attributed to mannan forming hard, insoluble crystalline structures. β -mannanase, often together with other enzymes such as pectinase and cellulase, is added during the concentration step of instant coffee production to reduce viscosity in coffee extracts. Mannanase is also employed for hydrolyzing galactomannans present in a liquid coffee extract in order to inhibit gel formation during freeze drying of instant coffee. Furthermore, due to the use of enzymatic treatment the coffee bean extracts can be concentrated by a low cost procedure such as evaporation.

The test is performed according the following flow-chart of Fig. 12 at temperatures of 10°C and an enzyme dosage of 0.15% d.s.

Mannanase variants of the invention are tested in mixture composed of different enzymes, such as pectinases and cellulases.

The viscosity of the coffee extract increases significantly over time under standard process conditions. However, the viscosity is significantly reduced using the enzyme mixture containing the mannanase variants of the invention resulting an improved downstream processing such as spray- or freeze drying.

Example 12. Pineapple processing

In particular, mannanase is useful for pineapple mill juice extraction and clarification, as pineapple contains a large fraction of mannans, including glucomannans and galactomannans.

- 5 Mannanase helps to improve extraction of valuable fruit components, lower the viscosity of fruit juice prior to concentration, and increase filtration rate and stability of the final product.

The pineapples are crushed in a meat grinder and fill 500 g mash in a 1000 ml beaker. The enzyme is applied at 21 °C with a reaction time of 60 minutes. The
 10 mash is then pressed with a small Hafico press according to the press protocol: 0 bar 2 min - 50 bar 2 min - 100 bar 2 min - 150 bar 2 min - 200 bar 1 min - 300 bar 1 min - 400 bar 1min. The obtained juice is then centrifuged at 4500 rpm for 5 minutes and analyzed for turbidity and viscosity.

Mannanase variants of the invention are tested in enzyme mixtures A, B and C
 15 (Table 7).

The enzymes are first diluted with tap water before being added to the pineapple mash

Table 7. Enzyme mixtures

	Enzyme activity	Dosage [ppm]	5 ml of [% enzyme solution]
blank			5 ml H ₂ O
Mixture A	Pectinase	50	0.50%
Mixture B	Pectinase + Arabanase	50	0.50%
Mixture C	Pectinase + Mannanase	50	0.50%

- 20 Applying mannanase variants of the invention leads to increased yield and lower turbidity of juice in pineapple processing.

Example 13. Mannanase treatment of soya beans for soya milk production

For the enzymatic treatment of soya beans to get soya milk the “hot process” is commonly used. For the hot soya milk process the dried soya beans were mixed

and crushed in a mixer with boiling tap water in a ratio of 1:7 (soaked beans: water). The whole soya slurry is cooled down to 50-55 °C before enzyme addition. The pH level for the soya slurry should be around pH 6.5 and can be adjusted with NaHCO₃. The mannanase enzyme is dosed at 1 kg/t of dried soya beans into the slurry and stirred for 30 min. After completion of the reaction time, the slurry is pressed using a laboratory press to obtain the final product: soya milk. In order to ensure the same pressing profile, the pressure as well as the corresponding pressing time is specified, as shown in Table 8. Besides the sample for enzymatic reaction, a control sample without any enzyme is prepared, in which the enzyme solution was replaced with water.

Table 8. Press scheme

pressure [bar]	0	50	100	300
time [min]	2	2	2	1

After pressing the soya milk is heated in a microwave until boiling to stop the enzyme reaction. Analysis of the soya milk:

- Yield in gram/time
- °Brix, which gives a direct correlation of the amount of sugar in the soy milk, is determined with a refractometer
- The turbidity of the juice is measured with a NTU-photometer, which measures the nephelometric turbidity.
- The brightness will be measured with a LAB-measurement
- Protein content is determined with a CN-Analyser (combustion method)
- Flavour

Soya milk treated with the mannanase variants of the invention shows a increased yield, brighter colour, increased °Brix, a lower turbidity, a higher protein content and a better taste (off flavour removal).

Without limiting the scope and interpretation of the patent claims, certain technical effects of one or more of the aspects or embodiments disclosed herein are listed in the following: A technical effect is degradation or modification of mannan. Another technical effect is provision of mannanase which has good storage stability.

The foregoing description has provided by way of non-limiting examples of particular implementations and embodiments of the invention a full and informative description of the best mode presently contemplated by the inventors for carrying out the invention. It is however clear to a person skilled in the art that the invention is not restricted to details of the embodiments presented above, but that it can be implemented in other embodiments using equivalent means without deviating from the characteristics of the invention.

Furthermore, some of the features of the above-disclosed aspects and embodiments of this invention may be used to advantage without the corresponding use of other features. As such, the foregoing description should be considered as merely illustrative of the principles of the present invention, and not in limitation thereof. Hence, the scope of the invention is only restricted by the appended patent claims.

In an embodiment at least one component of the compositions of the invention has a different chemical, structural or physical characteristic compared to the corresponding natural component from which the at least one component is derived from. In an embodiment said characteristic is at least one of uniform size, homogeneous dispersion, different isoform, different codon degeneracy, different post-translational modification, different methylation, different tertiary or quaternary structure, different enzyme activity, different affinity, different binding activity, and different immunogenicity.

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Claims

1. A variant of mannanase comprising at least one substitution of an amino acid at a position corresponding to the position 123, 158, 180, 272, 307, or 316, wherein the variant has mannanase activity and is selected from the group consisting of:

1) a polypeptide having at least 85% sequence identity to residues 27-331 of SEQ ID NO: 2;

2) a variant encoded by a polynucleotide that hybridizes under high stringency conditions with

a) nucleotides 79 - 993 of SEQ ID NO: 1 (*man7*) or

b) the full-length complement of a); and

3) a variant encoded by a polynucleotide having at least 95% sequence identity to the SEQ ID NO: 1 or the genomic DNA sequence thereof;

and wherein the amino acid numbering corresponds to the amino acid numbering of SEQ ID NO: 2 (Man7) full length amino acid sequence containing a signal sequence.

2. The variant of claim 1 comprising at least one further substitution at the position M123, A158, F180, G272, T307, or L316, or a combination thereof.

3. The variant of claim 1 or 2 comprising at least one set of substitutions at the position(s):

M123 and G272; or

A158 and T307; or

L316; or

T307; or

M123, A158 and T307; or

M123 and L316;

A158, T307, and L316;

F180 and L316; or

M123, A158, G272; T307, and L316;

or a combination thereof.

4. The variant of claims 1-3 wherein the substitution results into an improved stability of the variant.

5 5. The variant of claims 1-4 comprising at least one additional substitution selected from:

a substitution which prevents glycosylation of N283;

a substitution of 283, or 285; or

a substitution of N283, T285, or S285; or

10 a substitution of T285 or S285 to a residue other than T or S

a substitution of T285 or S285 to the residue A.

6. The variant of claim 5 wherein the additional substitution results into an altered glycosylation of the variant when produced in a host cell capable of N-linked glycosylation.

15 7. An enzyme composition comprising the variant of any one of claims 1-6 and

a. at least one preservative selected for example from group consisting of organic acid, citric acid, ascorbic acid, benzoic acid and their salts and derivatives, sodium benzoate, benzoate, hydroxybenzoate and derivatives, sorbic acid, sodium sorbate, sorbate, salts, such as sodium chloride or
20 potassium chloride, 1,2-Benzisothiazolin-3-one (BIT) or a combination thereof;

b. optionally at least one stabilizer selected from polyol, propylene glycol, polyethylene glycol, hexylene glycol, glycerol, a sugar, sugar alcohol, polysaccharide, lactic acid, boric acid, boric acid derivative, aromatic borate
25 ester, 4-formylphenyl boronic acid, phenyl boronic acid derivative, peptide, surfactant or a combination thereof;

c. optionally at least one enzyme selected from proteases, amylases, cellulases, lipases, xylanases, mannanases, cutinases, esterases, phytases, DNAses, pectinases, pectinolytic enzymes, pectate lyases,
30 carbohydrases, arabinases, galactanases, xanthanases, xyloglucanases,

laccases, peroxidases and oxidases with or without a mediator, or a combination thereof; and

d. optionally at least one filler selected from maltodextrin, flour, sodium chloride, sulfate, sodium sulfate, or a combination thereof.

5 8. The enzyme composition of claim 7 in the form of a liquid composition or a solid composition such as solution, dispersion, paste, powder, granule, granulate, coated granulate, tablet, cake, crystal, crystal slurry, gel, or pellet.

9. A detergent composition comprising the variant of mannanase of any one of claims 1-6 or the enzyme composition of claims 7-8.

10 10. The detergent composition of claim 9 in the form of a liquid detergent or a solid detergent, preferably in the form of a bar, a homogenous tablet, a tablet having two or more layers, a pouch having one or more compartments, a regular or compact powder, a granule, a granulate, a paste, a gel, or a regular, compact or concentrated liquid.

15 11. The detergent composition of claim 9 or 10 further comprising one or more additional enzymes selected from the group consisting of protease, lipase, cutinase, amylase, carbohydrase, cellulase, pectinase, pectatylase, pectinolytic enzyme, esterase, mannanase, arabinase, galactanase, xylanase, oxidase, xanthanase, xyloglucanase, laccase, DNase and/or peroxidase, preferably
20 selected from the group consisting of proteases, amylases, cellulases and lipases.

12. A recombinant host cell comprising genetic elements that allow producing at least one recombinant polypeptide comprising the variant of claims 1-6.

13. The recombinant host cell of 12, wherein the host cell is selected from the
25 group consisting of:

fungal cells,

filamentous fungal cells from Division *Ascomycota*, Subdivision *Pezizomycotina*; preferably from the group consisting of members of the Class *Sordariomycetes*, Subclass *Hypocreomycetidae*, Orders *Hypocreales*
30 and *Microascales* and *Aspergillus*, *Chrysosporium*, *Myceliophthora* and *Humicola*;

more preferably from the group consisting of Families *Hypocreaceae*, *Nectriaceae*, *Clavicipitaceae*, *Microascaceae*, and Genera *Trichoderma* (anamorph of *Hypocrea*), *Fusarium*, *Gibberella*, *Nectria*, *Stachybotrys*, *Claviceps*, *Metarhizium*, *Villosiclava*, *Ophiocordyceps*, *Cephalosporium*, and *Scedosporium*;

more preferably from the group consisting of *Trichoderma reesei* (*Hypocrea jecorina*), *T. citrinoviridae*, *T. longibrachiatum*, *T. virens*, *T. harzianum*, *T. asperellum*, *T. atroviridae*, *T. parareesei*, *Fusarium oxysporum*, *F. graminearum*, *F. pseudograminearum*, *F. venenatum*, *Gibberella fujikuroi*, *G. moniliformis*, *G. zeaeae*, *Nectria* (*Haematonectria*) *haematococca*, *Stachybotrys chartarum*, *S. chlorohalonata*, *Claviceps purpurea*, *Metarhizium acridum*, *M. anisopliae*, *Villosiclava virens*, *Ophiocordyceps sinensis*, *Acremonium* (*Cephalosporium*) *chrysogenum*, and *Scedosporium apiospermum*, and *Aspergillus niger*, *Aspergillus awamori*, *Aspergillus oryzae*, *Chrysosporium lucknowense*, *Myceliophthora thermophila*, *Humicola insolens*, and *Humicola grisea*,

bacterial cells, preferably gram positive Bacilli such as *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. amyloliquefaciens*, *B. pumilus*, gram negative bacteria such as *Escherichia coli*, actinomycetales such as *Streptomyces* sp., and

yeasts, such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Yarrowia lipolytica*,

most preferably *Trichoderma reesei* or *Bacillus*.

14. The recombinant host cell of claims 12-13, wherein the recombinant polypeptide is a fusion protein which, in addition to having the amino acid sequence having mannanase activity, comprises at least one of:

an amino acid sequence providing a secretory signal sequence;

an amino acid sequence which facilitates purification, such as an affinity tag or His-tag;

an amino acid sequence which enhances production, such as an amino acid sequence which is a carrier, such as CBM;

an amino acid sequence having an enzyme activity; and

an amino acid sequence providing for the fusion protein with binding affinity, such as a carbohydrate binding moiety.

15. A method for producing a recombinant polypeptide comprising a variant of
mannanase comprising:

a. cultivating a recombinant host cell of claims 12-14, wherein

the genetic elements comprise at least one control sequence which controls the production of the recombinant polypeptide in the recombinant host cell;

the genetic elements optionally comprise at least one sequence encoding a signal sequence for transporting the recombinant polypeptide outside the host cell; and

cultivating is carried out in conditions allowing production of the recombinant polypeptide; and

b. recovering the recombinant polypeptide.

16. A method for degrading or modifying mannan containing material comprising treating said mannan containing material with an effective amount of the enzyme composition of claims 7-8 or the variant of claims 1-6.

17. The method of claim 16 wherein the mannan containing material is plant based material, textile, waste water, sewage, oil, or a combination thereof.

18. An animal feed comprising the enzyme composition of claims 7-8 or the variant of mannanase of any one of claims 1-6, and at least one protein source of plant origin or a mannan containing product or by-product, and

a. Optionally at least one enzyme selected from protease, amylase, phytase, xylanase, endoglucanase, beta-glucanase, or a combination thereof; and

b. Optionally at least one filler selected from maltodextrin, flour, salt, sodium chloride, sulfate, sodium sulfate, or a combination thereof.

19. A feed supplement comprising the enzyme composition of claims 7-8 or the variant of mannanase of any one of claims 1-6; and

a. Optionally at least one enzyme selected from protease, amylase, phytase, xylanase, endoglucanase, beta-glucanase, or a combination thereof; and

b. Optionally at least one filler selected from maltodextrin, flour, salt, sodium chloride, sulfate, sodium sulfate or a combination thereof.

20. A use of the variant of claims 1-6 or the enzyme composition of claims 7-8 in oil drilling or hydro-fracturing.

21. A use of the variant of claims 1-6 or the enzyme composition of claims 7-8 in processing coffee extract, fruit juice, pineapple juice, or soya milk.

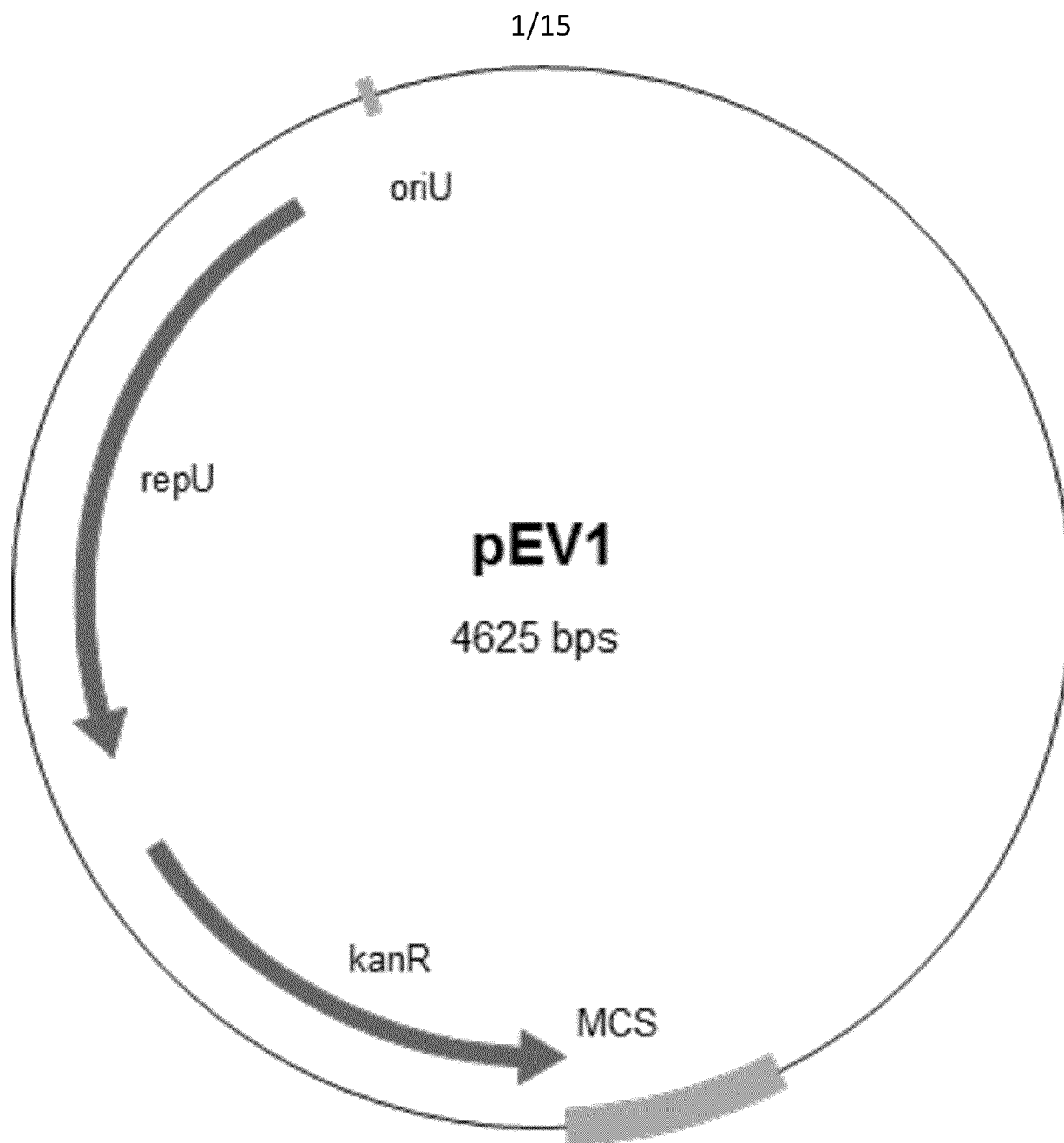


Figure 1.

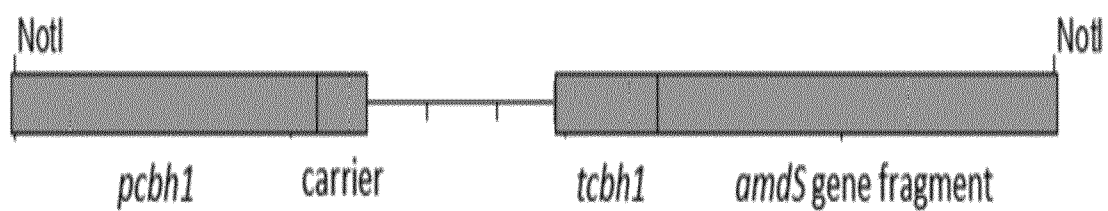


Figure 2.

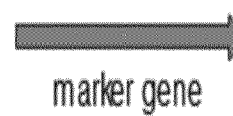
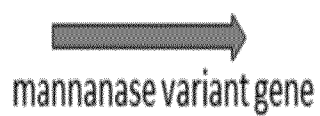
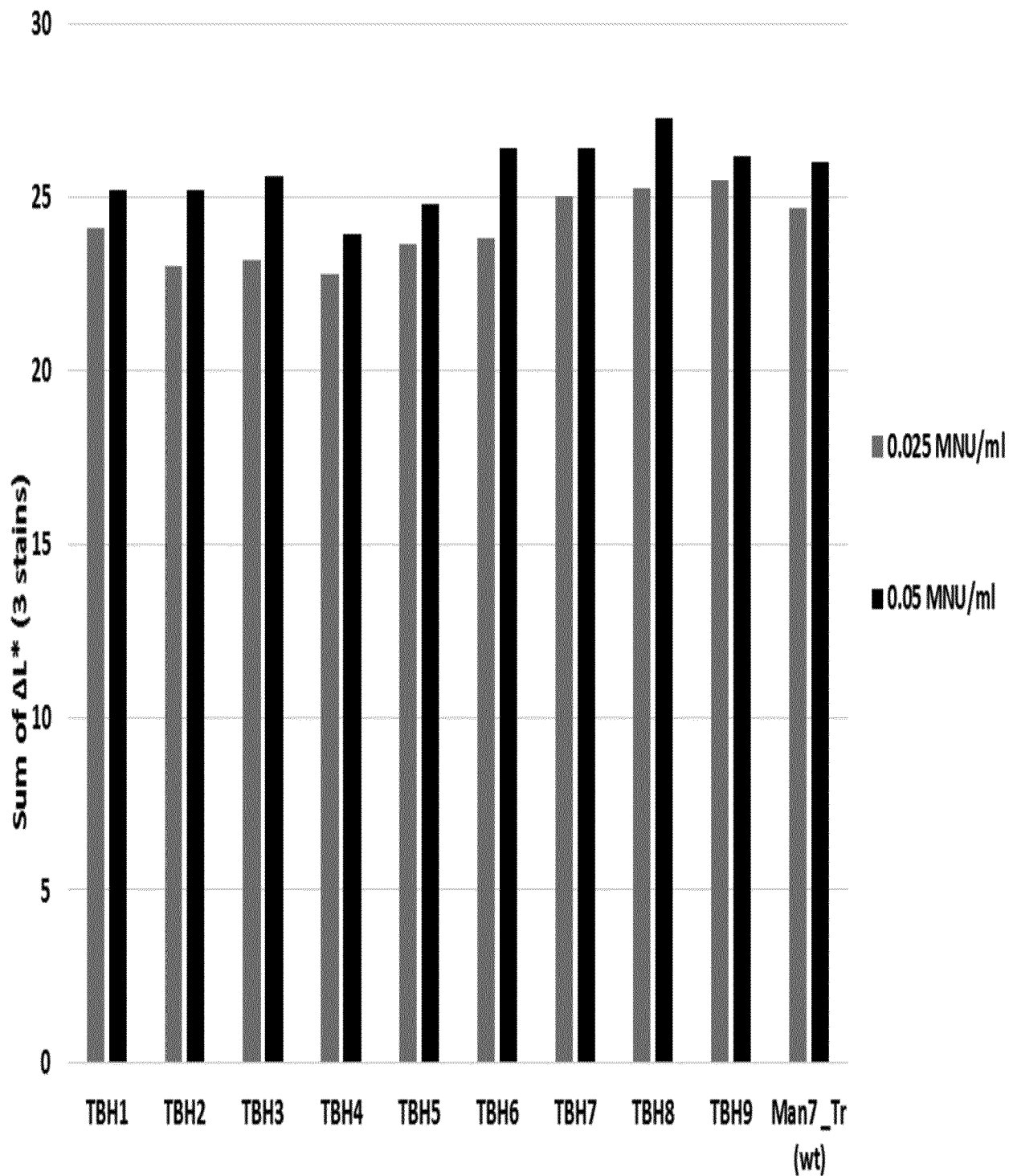


Figure 3A.



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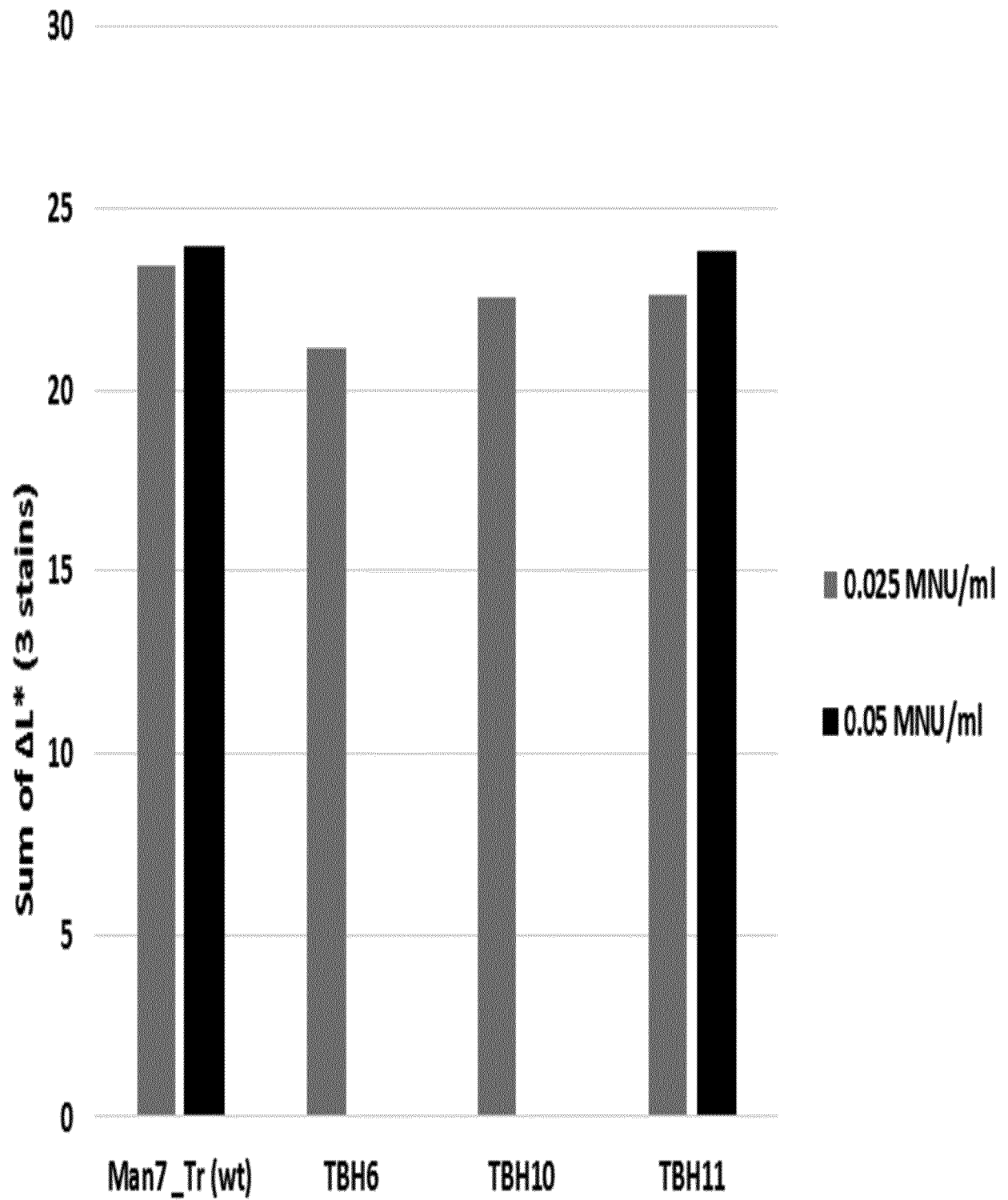


Figure 3B.

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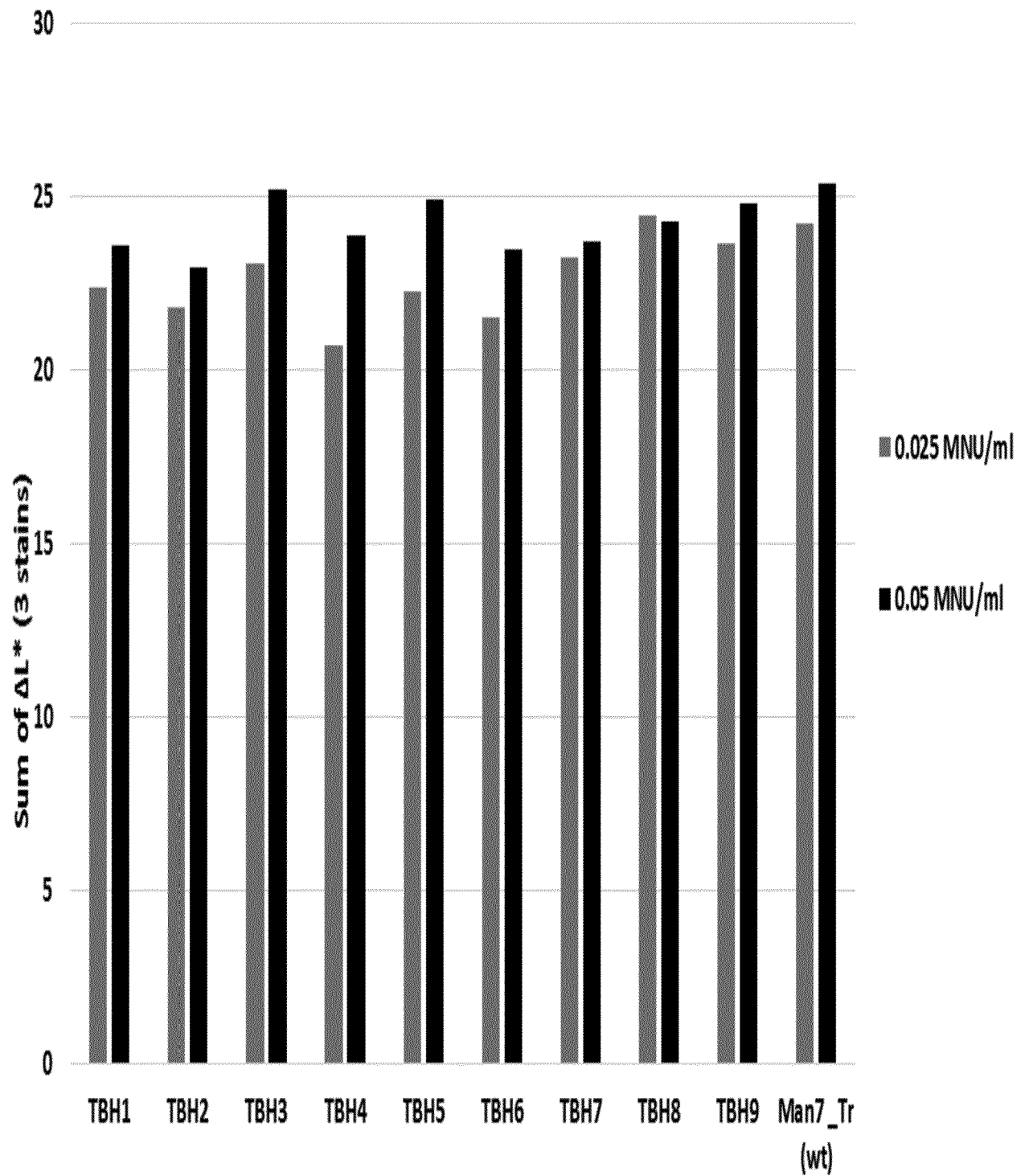


Figure 4A.

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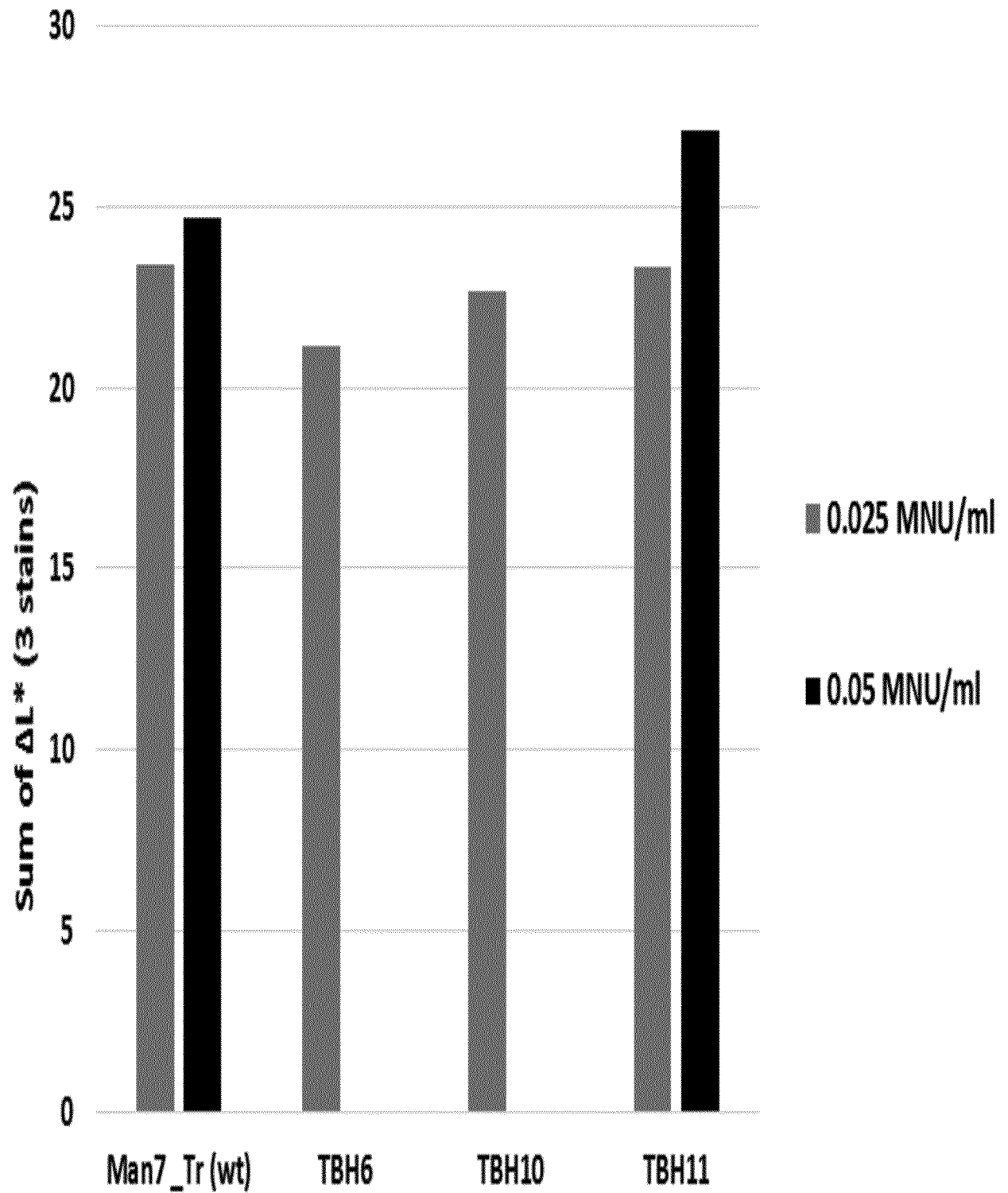


Figure 4B.

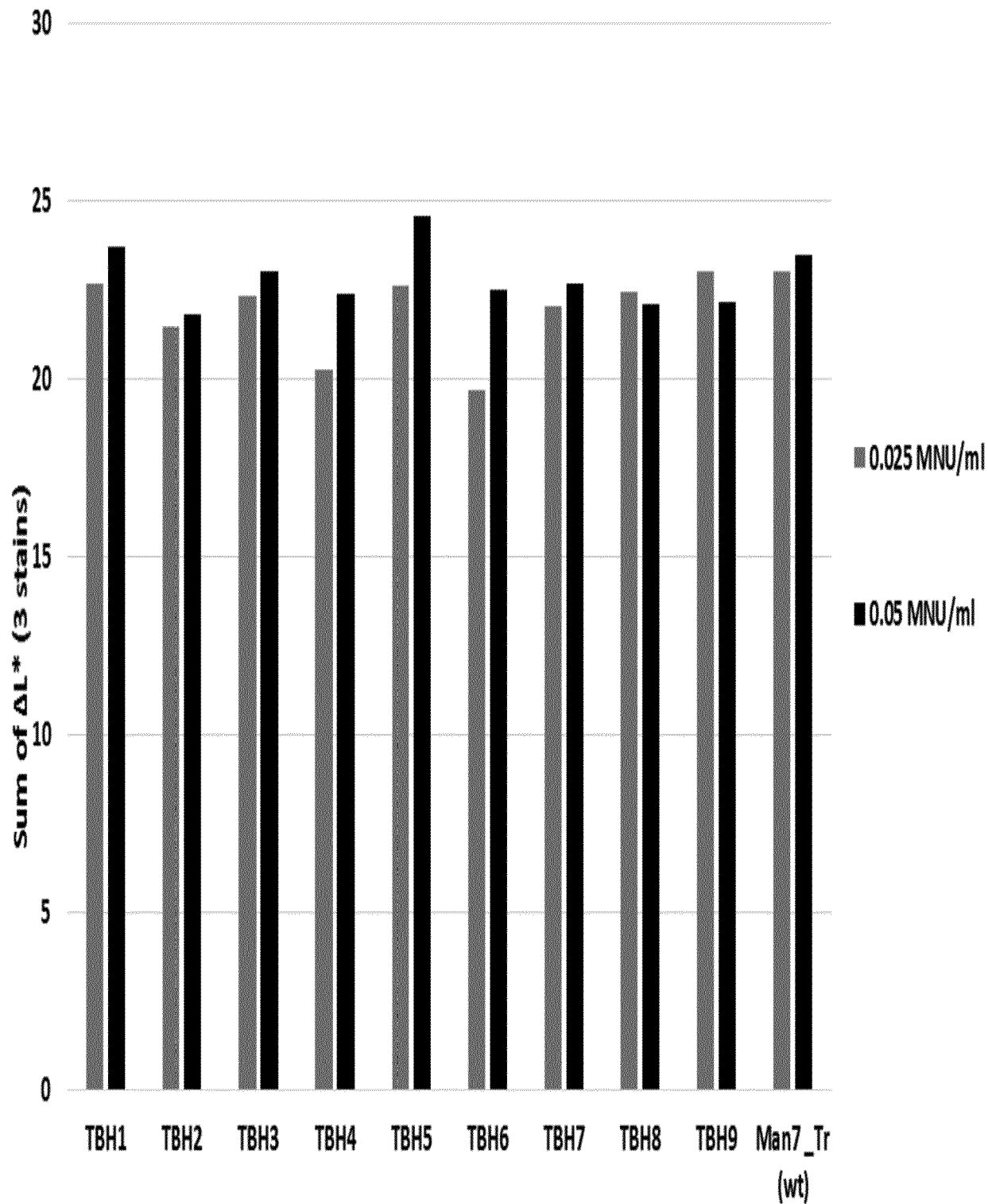


Figure 5.

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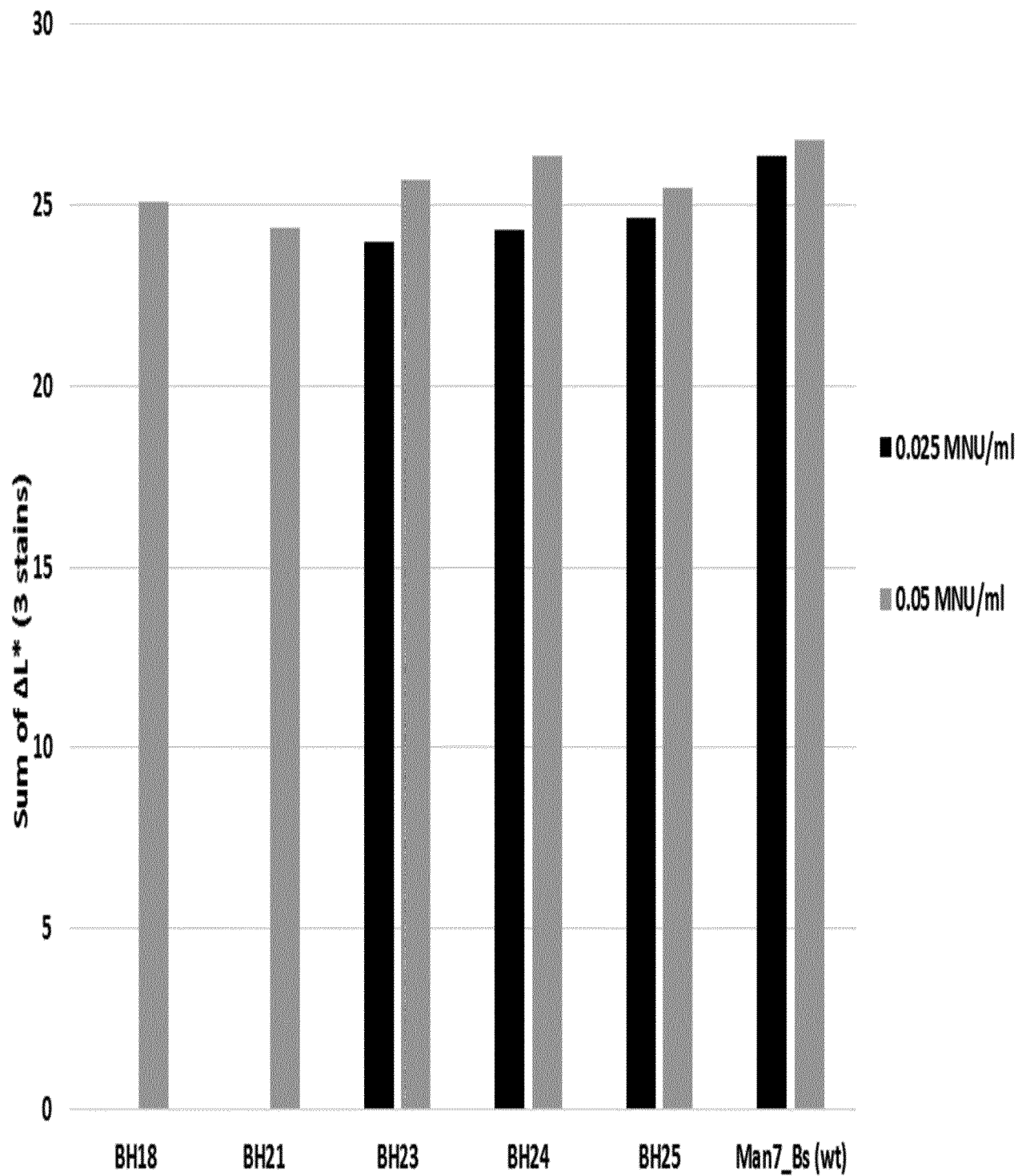


Figure 6.

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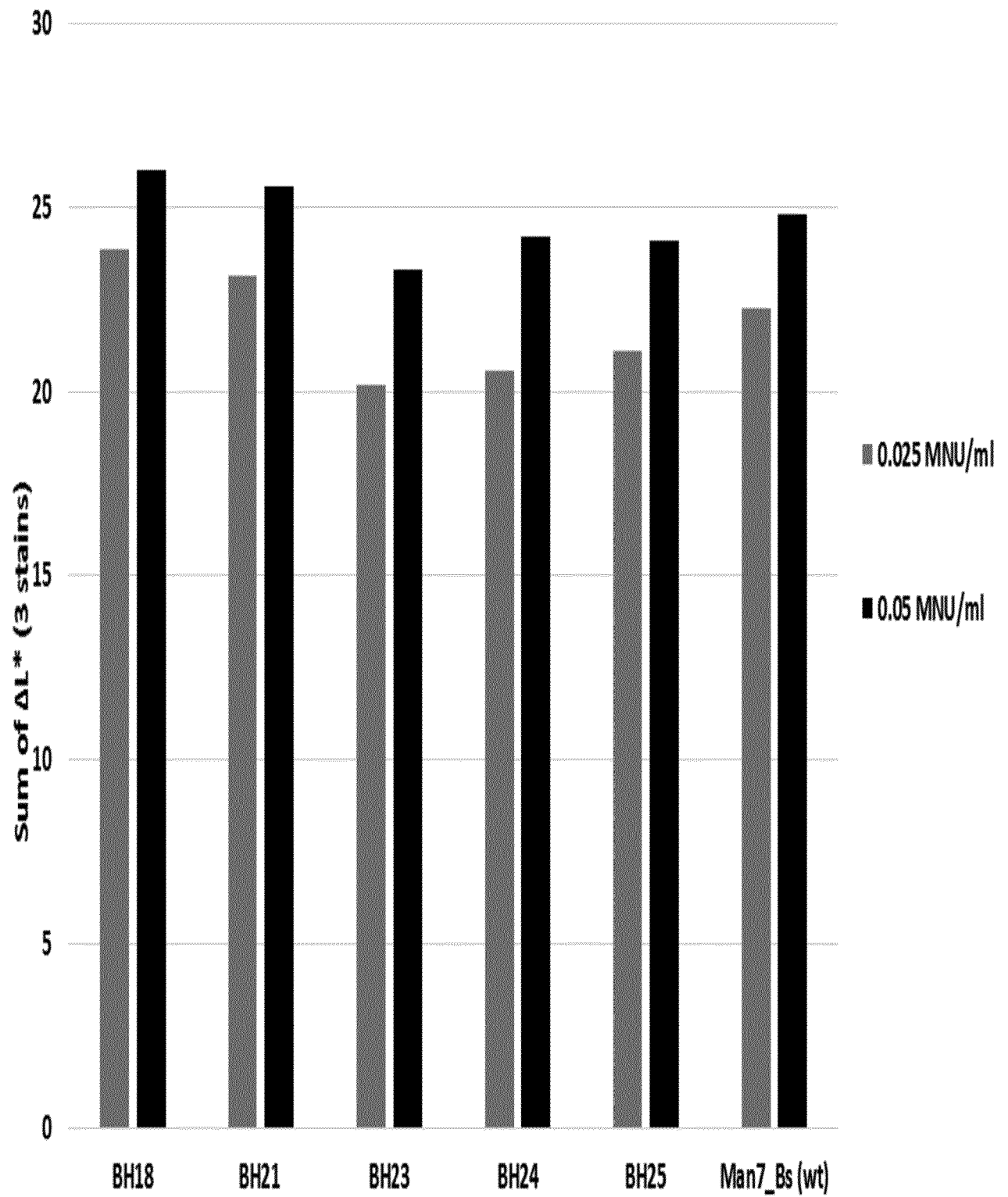


Figure 7.

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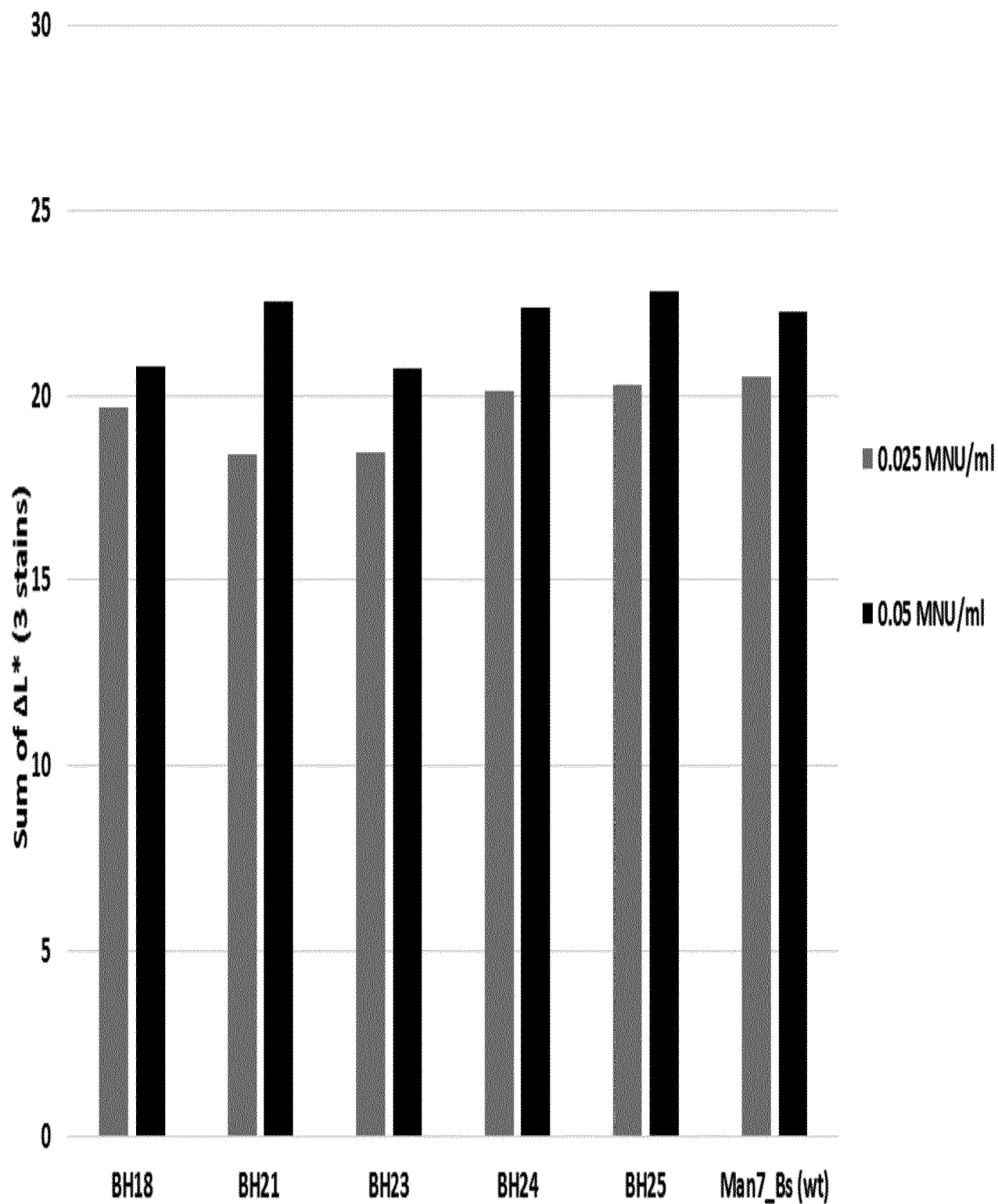


Figure 8.

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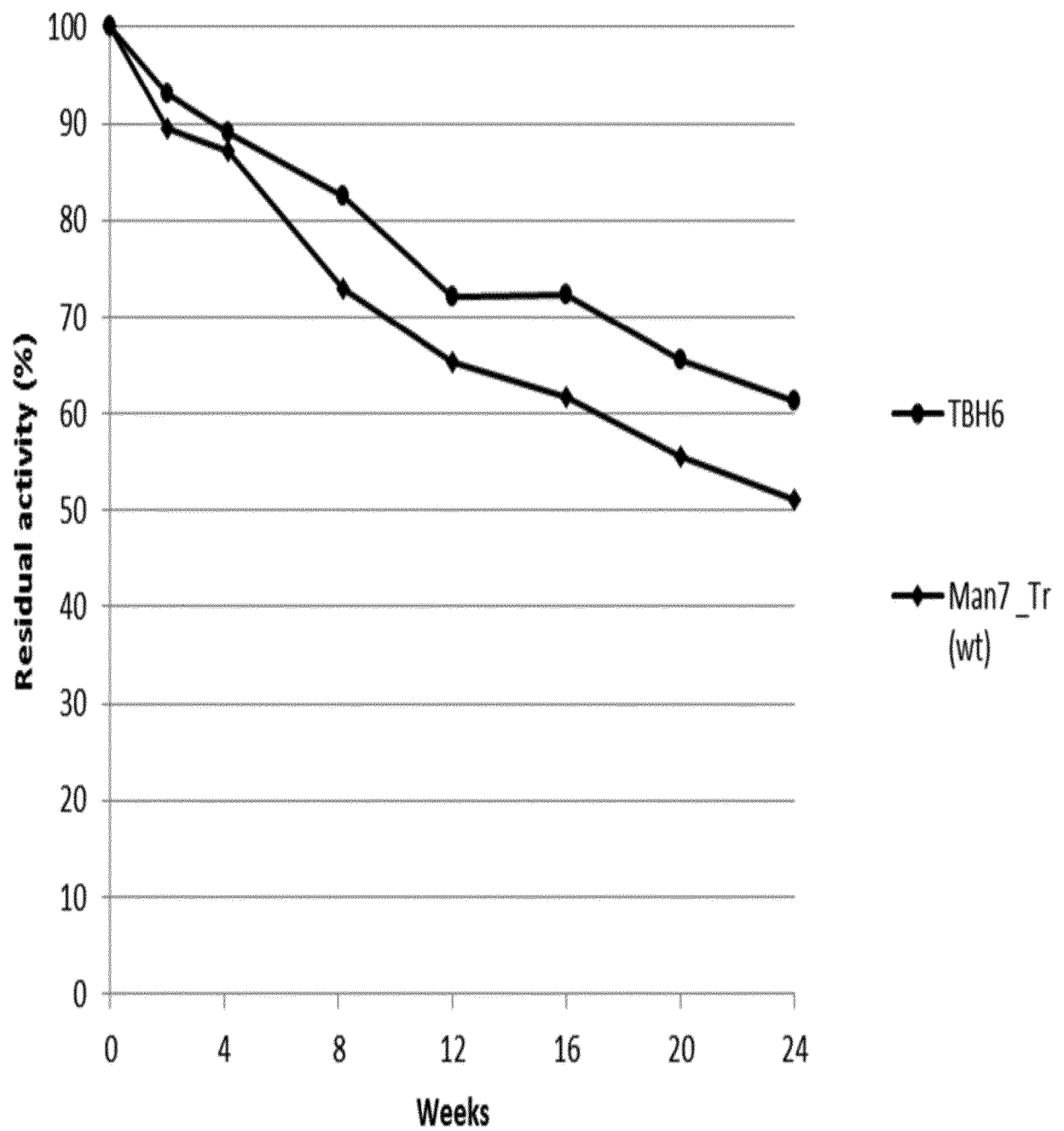


Figure 9.

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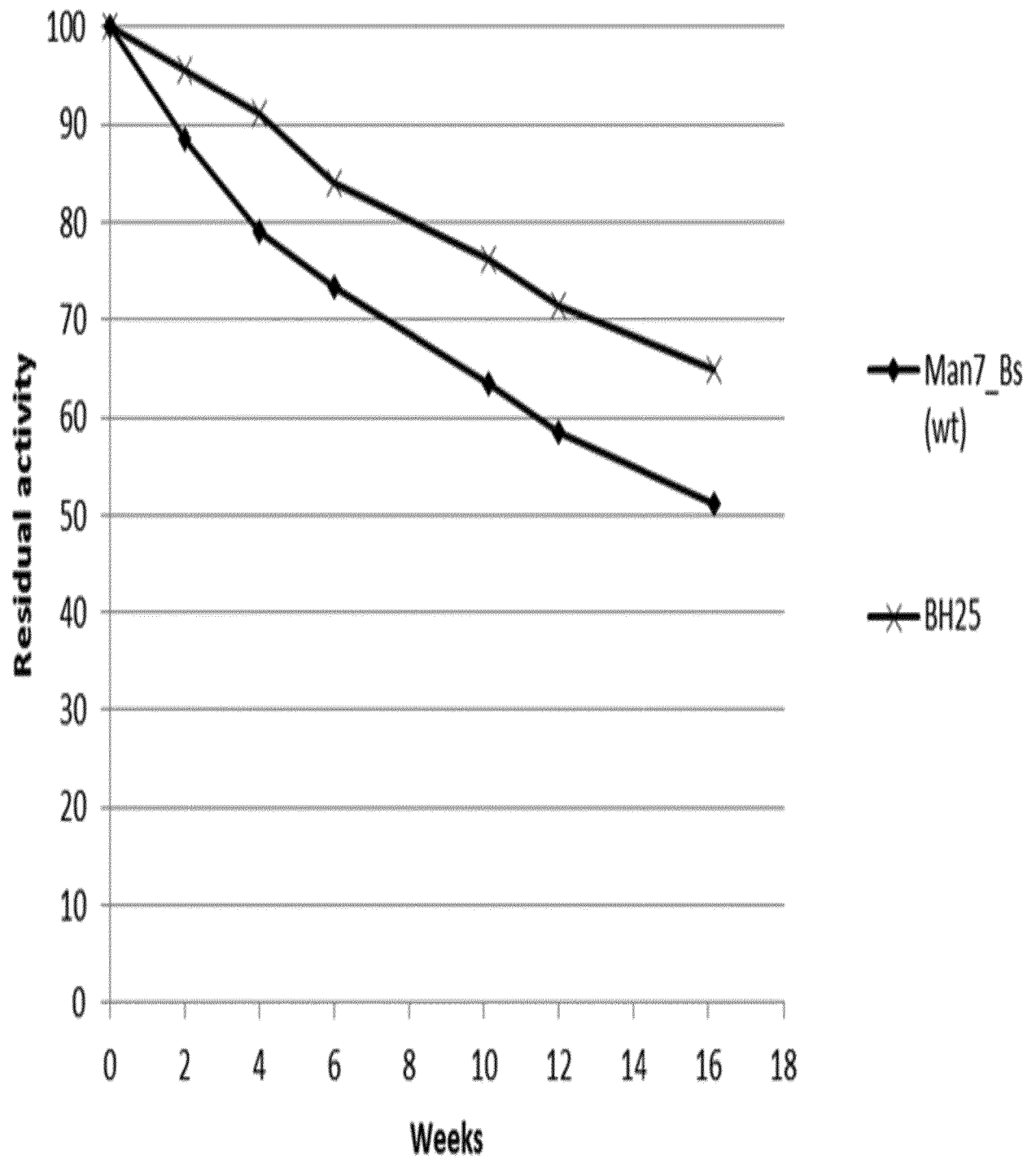


Figure 10.

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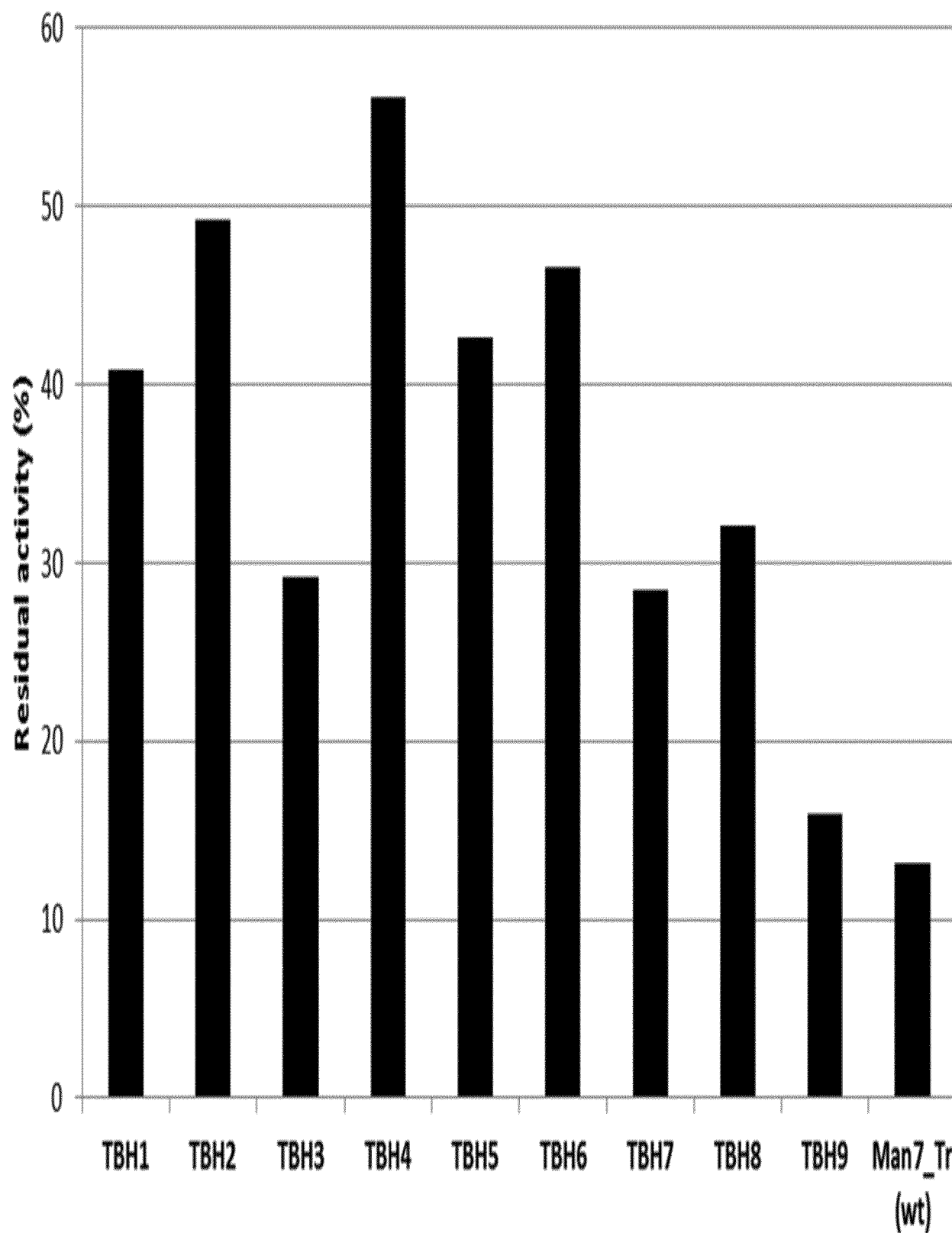


Figure 11A.

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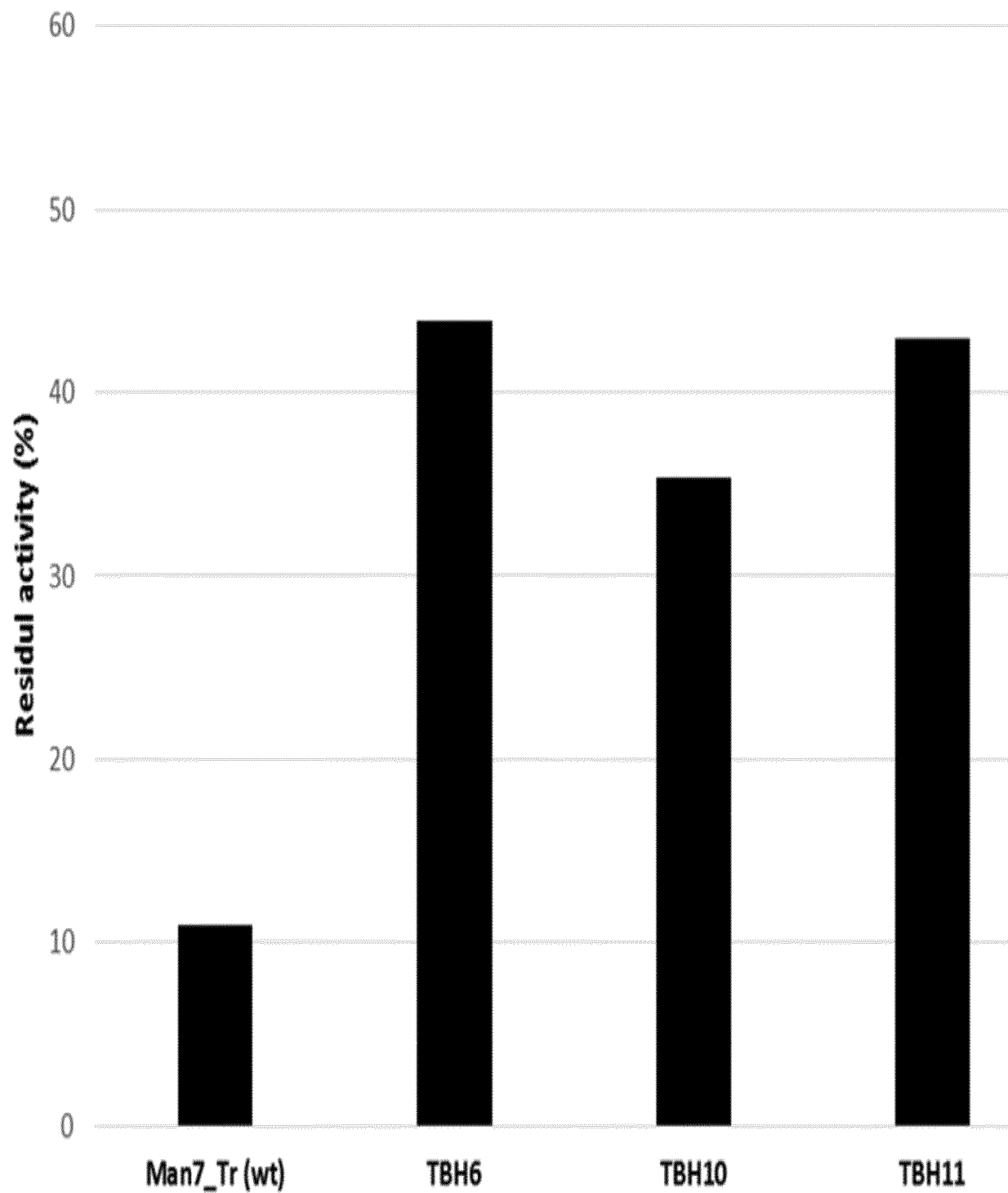


Figure 11B.

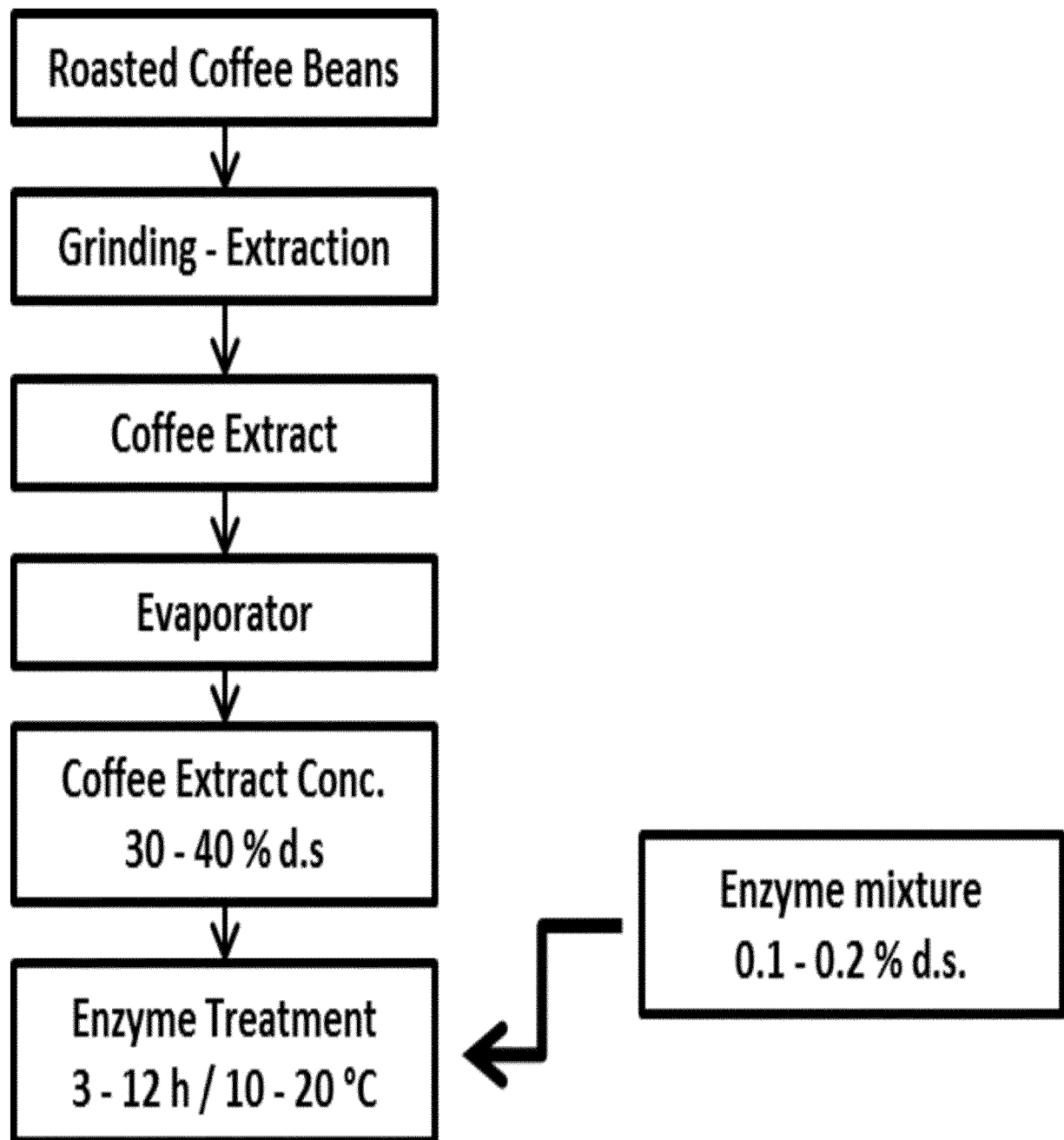


Figure 12.

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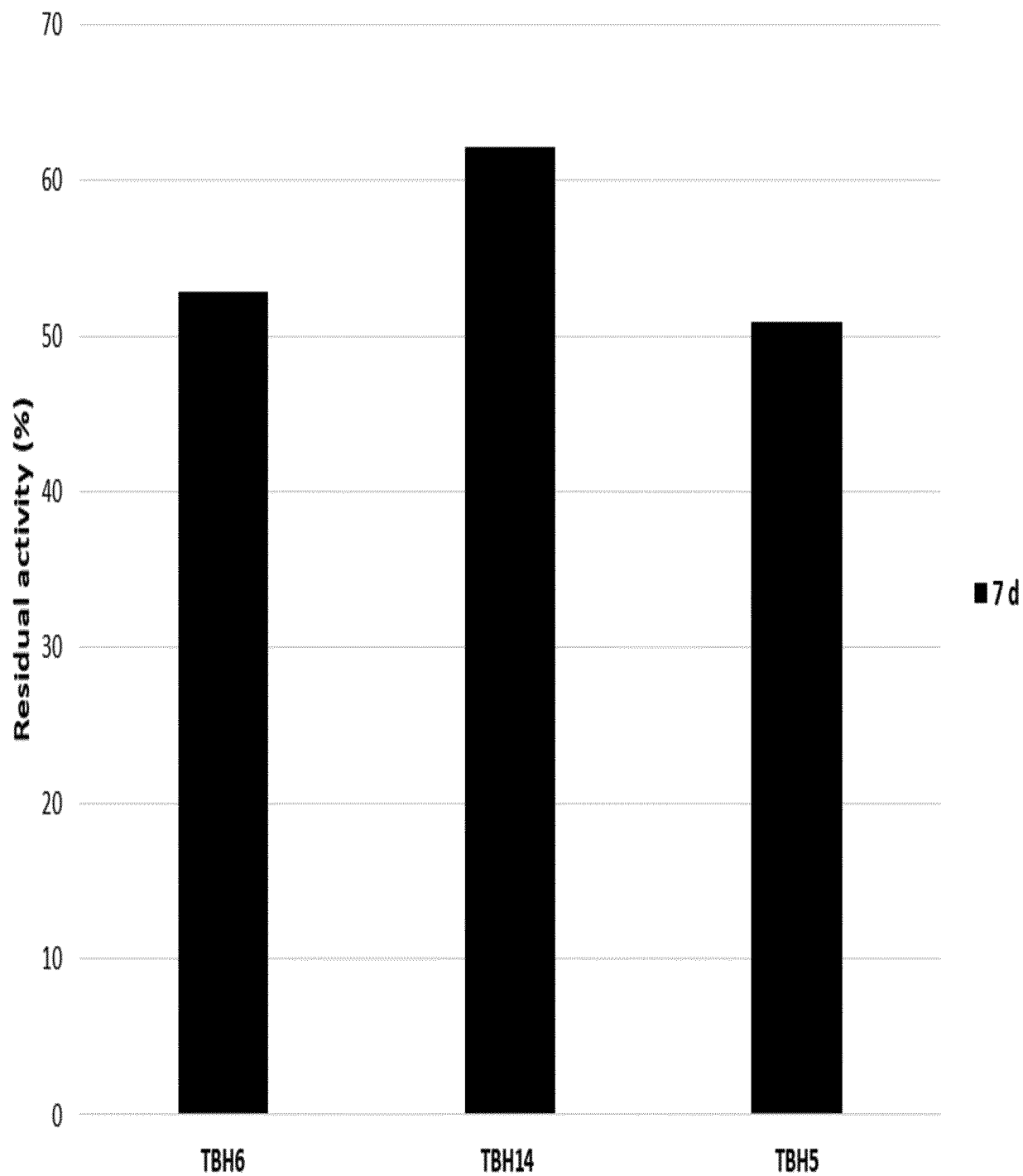


Figure 13

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2018/050402

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/24 C11D3/386
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C11D A23K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, COMPENDEX, FSTA, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE UniProt [Online] 19 March 2014 (2014-03-19), "SubName: Full=Mannanase {ECO:0000313 EMBL:GAE32229.1}";, XP002773470, retrieved from EBI accession no. UNIPROT:W4QLJ5 Database accession no. W4QLJ5 sequence -----	1-21
Y	US 2008/064064 A1 (KENSCH OLIVER [DE] ET AL) 13 March 2008 (2008-03-13) paragraphs [0078] - [0084] -----	1-21
A	WO 2014/088940 A1 (DANISCO US INC [US]) 12 June 2014 (2014-06-12) sequence 3 ----- -/--	1-21



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

27 June 2018

Date of mailing of the international search report

05/07/2018

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Authorized officer

Götz, Michael

INTERNATIONAL SEARCH REPORT

International application No
PCT/FI2018/050402

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 8 844 629 B2 (ARMSTRONG CHARLES DAVID [US]; BAKER HUGHES INC [US]) 30 September 2014 (2014-09-30) sequence 2 -----	1-21
A	WO 2017/079756 A1 (DANISCO US INC [US]; BAO KAI [US]; FIORESI CAROL MARIE [US]; FINAN DIN) 11 May 2017 (2017-05-11) sequence 18 -----	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/FI2018/050402

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2008064064	A1	13-03-2008	NONE
WO 2014088940	A1	12-06-2014	BR 112015012968 A2 12-09-2017
		CA 2892054 A1 12-06-2014	
		CN 104870637 A 26-08-2015	
		EP 2929021 A1 14-10-2015	
		US 2016115465 A1 28-04-2016	
		US 2018148704 A1 31-05-2018	
		WO 2014088940 A1 12-06-2014	
US 8844629	B2	30-09-2014	NONE
WO 2017079756	A1	11-05-2017	NONE