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Patentinhaber
Proprietor(s) of the patent
Titulaire(s) du brevet

Stellenbosch University
Admin B
Victoria Street
Stellenbosch
7600 Western Cape Province/ZA

António Campinos

Präsident des Europäischen Patentamts
President of the European Patent Office
Président de l'Office européen des brevets

München, den
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(54) **A MODIFIED B-FRUCTOFURANOSIDASE FOR FRUCTOOLIGOSACCHARIDE PRODUCTION**

MODIFIZIERTE B-FRUCTOFURANOSIDASE ZUR HERSTELLUNG VON FRUCTOOLIGOSACCHARIDEN

B-FRUCTOFURANOSIDASE MODIFIÉE POUR LA PRODUCTION DE FRUCTO-OLIGOSACCHARIDE

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(73) Proprietor: **Stellenbosch University**
7600 Western Cape Province (ZA)

(72) Inventors:
- **TROLLOPE, Kim**
  7600 Stellenbosch (ZA)
- **VOLSCHENK, Heinrich**
  Strand 7140 (ZA)
- **GÖRGENS, Johann Ferdinand**
  7600 Stellenbosh (ZA)
- **COETZEE, Gerhardt**
  7140 Strand (ZA)

(74) Representative: **Potter Clarkson**
The Belgrave Centre
Talbot Street
Nottingham NG1 5GG (GB)

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EP 3 201 335 B1

Description

FIELD OF THE INVENTION

[0001] The invention related to modified β-fructofuranosidases which have improved enzyme activity, in particular fructosyltransferase activity, relative to the parent enzyme.

BACKGROUND TO THE INVENTION

[0002] The global demand for fructooligosaccharides (FOS) is growing due to human health benefits associated with their consumption. FOS are prebiotics that selectively stimulate the growth of bifidobacteria, thereby promoting colonic health [1,2]. Further claims as to the effect of FOS consumption relate to mineral absorption, lipid metabolism and the control of type II diabetes and have been extensively reviewed [2-4]. Further to their health benefits, FOS are used in the food industry as low calorie sweeteners. They are also added to food products to improve their organoleptic properties and their inclusion allows producers to label their products as 'functional foods' - a claim that resonates with health conscious consumers [2,3].

[0003] It is well known that some β-fructofuranosidases possess the ability to transform sucrose to FOS. β-fructofuranosidases are family 32 glycoside hydrolase (GH32) enzymes that act on sucrose and related β-D-fructofuranosides [5]. They are also known as invertases (EC 3.2.1.26) as they hydrolyse sucrose to produce invert sugar - an equimolar mixture of dextrorotatory D-glucose and levorotatory D-fructose [6]. Crystal structures for GH32 β-fructofuranosidases reveal that the enzymes display a bimodular arrangement of a N-terminal catalytic domain containing a five-bladed β-propeller fold linked to a C-terminal β-sandwich domain [7-10]. β-fructofuranosidases hydrolyse β-glycosidic bonds by a double displacement catalytic mechanism that retains the configuration of the fructose anomeric carbon [11]. Multiple sequence alignments (MSAs) identified a highly conserved aspartate close to the N terminus that serves as the catalytic nucleophile and a glutamate residue that acts as a general acid/base catalyst [12]. The β-fructofuranosidases which are capable of transforming sucrose to FOS possess fructosyltransferase activity whereby the sugar moiety is transferred from the enzyme-fructosyl intermediate to an acceptor other than water [7,13]. This reaction forms the basis of FOS synthesis from sucrose. Enzymes from *Aspergillus* spp. [14-16] and *Aureobasidium pullulans* [17] exhibit good propensities for the synthesis of inulin type FOS from sucrose, with β-(2 → 1) linkages between fructose units.

[0004] Synthesis of FOS (GF_n) from sucrose (GF) occurs via a disproportionation reaction with the reaction generalised as $GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1}$ [18,19]. In a batch reaction the initial products are glucose and 1-kestose (GF2), and as the reaction progresses, nystose (GF3) and β-fructofuranosyl nystose (GF4) levels increase. Reaction conditions influence the dominance of hydrolytic or transferase reactions with high substrate concentrations favouring the latter [14].

[0005] Industrial biotransformation of sucrose to FOS is currently conducted in a batch system using the β-fructofuranosidase from *A. niger* ATCC 20611 (subsequently classified as *A. japonicus*). The enzyme is added to a buffered 50 - 60% (wt/vol) sucrose solution with the reaction proceeding at 50 - 60°C for up to 20 hours [19]. These severe industrial conditions impose limitations on activity. The fructosyltransferase activity of the enzyme has been shown to be non-competitively inhibited by the glucose product, limiting complete sucrose conversion [19]. Furthermore, long-term enzyme stability is severely compromised at temperatures above 50°C despite immobilisation efforts [20].

[0006] There is thus still a need for alternative enzymes which are able to efficiently convert sucrose to FOS on an industrial scale.

[0007] EP 1 726 655 A1 describes a β-fructofuranosidase variant whose reaction specificity is improved to be suitable for the production of fructooligosaccharides. There is provided β-fructofuranosidase variant consisting of a mutated amino acid sequence of SEQ ID NO: 2 or a mutated homologue thereof, which has at least one mutation at positions 62, 122, 128, 165, 221, 395 and 550.

[0008] Chuankhayan *et al.* (2010) describes the crystal structures of recombinant fructosyltransferase (AjFT) from Aspergillus japonicus CB05 and its mutant D191A complexes with various donor/acceptor substrates, including sucrose, 1-kestose, nystose, and raffinose.

[0009] EP 0 889 134 A1 describes a beta-fructofuranosidase gene and a beta-fructofuranosidase encoded by the gene, a process for isolating a beta-fructofuranosidase gene using the beta-fructofuranosidase gene, and a beta -fructofuranosidase obtained by this isolation process. A mold fungus having no beta-fructofuranosidase activity suitable for the production of beta-fructofuranosidase, and a system for producing a recombinant beta-fructofuranosidase using the mold fungus as a host is disclosed. Further, a beta-fructofuranosidase variant which selectively and efficiently produces a specific fructooligosaccharide such as l-kestose from sucrose is disclosed.

Trollope *et al.* (2013) describes the development of a screening method displaying potential high-throughput capacity for the evaluation of β-fructofuranosidase libraries using Fourier transform mid-infrared attenuated total reflectance (FT-MIR ATR) spectroscopy and multivariate analysis, and highlights the application of FT-MIR ATR spectroscopy to a variant discovery pipeline in the directed evolution of a β-fructofuranosidase for enhanced short chain FOS production

van Wyk *et al.* (2013) identifies a β-fructofuranosidase gene (CmINV) from a Ceratocystis moniliformis genome sequence using protein homology and phylogenetic analysis.

SUMMARY OF THE INVENTION

[0010] This invention provides a modified polypeptide having specific fructofuranosidase activity more particularly as defined in the accompanying claims.

[0011] According to a first embodiment of the invention, there is provided a modified polypeptide having fructofuranosidase activity, wherein the amino acid sequence of the polypeptide is at least 90% or 95% identical to SEQ ID NO: 3 and has at least one of the following amino acid substitutions:

the phenylalanine (F) at amino acid position 121 is substituted by tyrosine (Y);
the alanine (A) at amino acid position 159 is substituted by proline (P) or serine (S);
the glycine (G) at amino acid position 302 is substituted by asparagine (N), aspartic acid (D), tyrosine (Y) or glutamic acid (E); and/or
the glutamine (Q) at amino acid position 471 is substituted by serine (S), lysine (K) or asparagine (N).

[0012] The polypeptide may differ only from SEQ ID NO: 3 at one or more of positions 121, 159, 302 and/or 471.

[0013] The polypeptide may include a secretion signal peptide at its 5' end, the secretion signal having an amino acid sequence which is at least 90% or 95% identical to SEQ ID NO: 25. The modified polypeptide with secretion signal may have an amino acid sequence which is at least 90% or 95% identical to SEQ ID NO: 26 and which has at least one amino acid substitution at position 154, 192, 335 and/or 504 of SEQ ID NO: 26.

[0014] The polypeptide may include any two of the above substitutions, such as at amino acid positions 121 and 302 of SEQ ID NO: 3.

[0015] The polypeptide may include any three of the above substitutions, such as at amino acid positions 121, 159 and 302, or at amino acid positions 159, 302 and 471, or at amino acid positions 121, 159 and 471 of SEQ ID NO: 3.

[0016] The polypeptide may include any four of the above substitutions, such as at amino acid positions 121, 159, 302 and 471 of SEQ ID NO: 3.

[0017] Also disclosed herein, the phenylalanine (F) at amino acid position 154 may be substituted by tyrosine (Y), the alanine (A) at amino acid position 192 may be substituted by proline (P) or serine (S), the glycine (G) at amino acid position 335 may be substituted by asparagine (N), aspartic acid (D), tyrosine (Y) or glutamic acid (E), and/or the glutamine (Q) at amino acid position 504 may be substituted by serine (S), lysine (K) or asparagine (N).

[0018] Also disclosed herein, more preferably, the substitution at amino acid position 154 is tyrosine (Y), the substitution at amino acid position 192 is proline (P), the substitution at amino acid position 335 is asparagine (N), and/or the substitution at amino acid position 504 is serine (S).

[0019] Even more preferably, the polypeptide may include the following modifications:

F121Y-A159P-G302N-Q471S;
F121Y-A159P-G302N;
A159P-G302N-Q471S;
F121Y-A159P-Q471S; or
A159P-G302N.

[0020] Even more preferably, the polypeptide may include the following four modifications: F121Y, A159P, G302N and Q471S.

[0021] Also disclosed herein, the polypeptide may comprise an unsubstituted amino acid residue at positions 62, 122, 128, 165, 221, 395 and/or 550 of SEQ ID NO: 3

The polypeptide may comprise an amino acid sequence of any one of SEQ ID NOS: 4 - 21, such as SEQ ID NO: 4.

[0022] Also disclosed herein, the polypeptide may have an improved thermal stability, catalytic rate and lower glucose feedback inhibition levels relative to a polypeptide which has not been modified as described above.

[0023] According to a second embodiment of the invention, there is provided a polynucleotide which encodes a modified polypeptide as described above.

[0024] The polynucleotide may have a nucleotide sequence which is at least 90% identical to the sequence of nucleotides 100 to 2007 of SEQ ID NO: 1 or SEQ ID NO: 2, or a complement thereof, wherein SEQ ID NO: 1 or SEQ ID NO: 2 has been modified so that the polynucleotide encodes a modified polypeptide as described above.

[0025] The polynucleotide may include a *T. reesei* endoxylanase 2 *(xln2)* secretion signal. The secretion signal may be encoded by nucleotides 1-99 of SEQ ID NO: 1 or 2.

[0026] According to a third embodiment of the invention, there is provided a vector comprising a polynucleotide encoding

a modified fructofuranosidase polypeptide as described above.

[0027] According to a fourth embodiment of the invention, there is provided a host cell comprising the vector described above.

[0028] The host cell may be a microbial cell, such as from a yeast, bacterium or fungus.

[0029] Also disclosed herein, according to a fifth embodiment, there is provided a yeast, fungus or bacterium comprising at least one copy of an exogenous gene coding for a modified polypeptide as described above.

[0030] According to a sixth embodiment of the invention, there is provided a process for producing a modified fructofuranosidase polypeptide as described above, the process comprising the steps of transforming a host cell with the polynucleotide described above and causing the polypeptide to be expressed.

[0031] The process may also comprise the step of recovering the polypeptide, such as from the transformed host cell or a supernatant into which the polypeptide has been secreted.

[0032] The host cell may be a yeast cell, fungal cell or bacterium.

[0033] Also disclosed herein, the polypeptide may be expressed under the control of a constitutive or inducible promoter, such as the *S. cerevisiae* phosphoglycerate kinase 1 (PGK1), glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter or the alcohol oxidase (AOX1) promoter in *Pichia pastoris.*

[0034] According to a seventh embodiment of the invention, there is provided a process for producing fructooligosaccharides (FOS), the process comprising the steps of contacting sucrose with a modified polypeptide described above under conditions which cause the sucrose to be converted into fructooligosaccharides. The fructooligosaccharides may be short chain fructooligosaccharides.

[0035] The transformed host cell described above may be added to a solution containing sucrose and be caused to express the polypeptide into the solution, or alternatively a purified, partially purified or crude extract of polypeptide may be added directly to the solution containing sucrose.

[0036] The fructooligosaccharides may include 1-kestose (GF2), nystose (GF3) and/or β-fructofuranosyl nystose (GF4).

[0037] Also disclosed herein, the process may require lower amounts of the modified polypeptide to produce the FOS or may be performed over a shortened reaction time, relative to an unmodified process.

BRIEF DESCRIPTION OF THE FIGURES

[0038]

Figure 1: Amino acid sequence of FopA (SEQ ID NO: 3) (numbered according to crystal structure) showing substitutions which were selected for generating a first round of FopA variants.

Figure 2: Screening data for the active first round variants harbouring single amino acid substitutions. The parent enzyme is indicated by the filled circle. Error bars denote standard error (n = 3). Nystose data were generated by HPLC from assays performed under glucose inhibiting conditions. Sucrose consumption under normal and glucose inhibiting conditions was quantified by Fourier transform mid-infrared spectroscopy. Relative inhibition was expressed as the difference in sucrose consumption between the two conditions divided by uninhibited activity. Data were normalised to the parental activity. The most improved variants in terms of nystose production and/or relative inhibition are labelled.

Figure 3: SDS-PAGE gels of purified (A) and crude (B, C) parent and five most improved combination variant β-fructofuranosidases. All gels were 8% and silver stained. B shows crude supernatants of *S. cerevisiae* NI-C-D4[fopA]. Lane 2 is a reference as yeast was transformed with empty vector pJC1. Lanes 3 to 8 show the parent and the five most improved variants. A shows the IMAC purified enzymes. Lanes 2 to 7 show the parent and variant enzymes. C shows the result of PNGase treatment of the crude supernatant (parent). Lane 2 control, lane 3 untreated and lane 4 treated sample. The Spectra multicolor high range protein ladder (Thermo Scientific) was loaded in lane 1 of all the gels and served as molecular weight marker.

Figure 4: Specific activity data for the purified parent and five most improved combination variants. Values above each bar indicate the average 1-kestose units per milligram purified enzyme. Error bars denote standard error (n = 3).

Figure 5: Isothermal denaturation data for the parent and five most improved combination variants. Purified proteins were incubated with SYPRO orange at 55°C and fluorescence was monitored for 10 hours. Increased fluorescence indicates thermal denaturation of the protein as SYPRO orange binds to newly exposed hydrophobic amino acids. Error bars denote standard error (n = 5).

Figure 6: Differential scanning fluorimetry-derived melting temperatures for purified parent and five most improved combination β-fructofuranosidase variants at pHs ranging from 4 - 7. Error bars denote 0.95 confidence intervals (n = 3).

Figure 7: ΔT_m values for the parent and 5 most improved combination variants in the presence of substrates sucrose (A), 1-kestose (B) and nystose (C). Differences between the given concentrations and the zero substrate T_ms are shown. Error bars denote 0.95 confidence intervals (n = 3).

Figure 8: Time course FOS synthesis by the purified parent and most improved variant (V1) enzymes. The enzyme dosage was 10 KU per gram sucrose with a starting concentration of 600 g/l sucrose. The reaction was conducted at 62 °C, pH 5.5 with shaking at 120 rpm. Error bars denote standard error (n = 3).

Figure 9: Response surface plots of GF4 percentage of total scFOS as a function of temperature (A) and enzyme dosage (B) for the enzyme fopA_V1 after 6 hrs.

Figure 10: Response surface plots of GF4 percentage of total scFOS as a function of temperature (A) and enzyme dosage (B) for the enzyme fopA_V1 after 8 hrs.

Figure 11: Response surface plots of GF4 percentage of total scFOS as a function of temperature (A) and enzyme dosage (B) for the enzyme fopA after 8 hrs.

DETAILED DESCRIPTION OF THE INVENTION

[0039] Modifications to the *Aspergillus japonicus* β-fructofuranosidase enzyme which result in improved synthesis of inulin-type fructooligosaccharides (FOS) from sucrose are described herein.

[0040] Semi-rational directed evolution of the *A. japonicus* β-fructofuranosidase using a combination of strategies was performed and is described below. Only loop regions were selected for engineering using a crystal structure-guided approach, and amino acid substitutions were selected based on scoring positions in homologous protein sequence alignments using sequence entropies and solvent accessibilities. 36 variants of the β-fructofuranosidase, each with a single amino acid substitution, were engineered and this library was screened in *Saccharomyces cerevisiae* for variants that produced higher levels of FOS than the parent (wild type) enzyme. Enzymes were further screened for those producing more FOS than the parent under glucose inhibiting conditions. This strategic combination likely resulted in the enriched functionality of the library - 58% of the first round library was active (these results are in contrast to the results of a similar strategy which focused on engineering loops, where site saturation mutagenesis at 90 loop residues in a lipase only yielded 10% of active mutants that were improved relative to the parent [65]).

[0041] Hits from the first round of screening were exhaustively combined to create a second library of combination variants with 2, 3 or 4 mutations, and a second round of screening was conducted to find combination variants with improved activity.

[0042] The amino acid substitutions mentioned below will be referred to relative to their position in the crystal structure of the mature polypeptide. The corresponding amino acid position in any of SEQ ID NOS: 3 - 21 can be calculated by subtracting 19 from the crystal position, and this position number is shown in parenthesis in some of the substitutions described below. In the claims, the position numbers of the amino acid residues into which substitutions are introduced correspond to the amino acid residues of the amino acid sequences represented by SEQ ID NOs: 3 - 21.

[0043] In the present invention, "substitution" means that a specific amino acid residue at a specific position is removed and another amino acid residue is inserted into the same position.

[0044] Modification to the *A. japonicus* β-fructofuranosidase polypeptide at positions 140, 178, 321 and/or 490 corresponding to the crystal structure of the mature polypeptide (corresponding to amino acids 121, 159, 302 and/or 471 of SEQ ID NOs: 3-21)) showed improved enzyme activity. Preferred modifications are substitution of the phenylalanine reside at position 140 (121) with a tyrosine residue, substitution of the alanine residue at position 178 (159) with a proline or serine residue, substitution of the glycine residue at position 321 (302) with an asparagine, aspartic acid, tyrosine or glutamic acid residue, and/or substitution of the glutamine residue at position 490 (471) with a serine, lysine or asparagine residue. More particularly, the substitution at position 140 (121) can be a tyrosine residue, the substitution at position 178 (159) can be a proline residue, the substitution at position 321 (302) can be an asparagine residue, and/or the substitution at position 490 (471) can be a serine residue. Consequently, a variant polypeptide of SEQ ID NO: 3 which has all four of the following amino acid residues does not fall within the scope of the present invention: phenylalanine at position 121, alanine at position 159, glycine at position 303 and glutamine at position 471. A178P and G321N were identified as positive contributors to thermostability. Proline substitutions in loops have been linked to improved thermostability due to backbone modifications that increase loop rigidity [58,66] and the data herein supports these findings.

[0045] 71% of active mutants (15 variants) were improved over the parent. Data showed that the effect of combining the top 4 first round substitutions was cumulative and delivered the best variant instead of a 3 or 2 combination or even single substitution variant. One particular combination variant with 4 amino acid substitutions displayed a combination of improved thermostability and catalytic activity. This variant was designated "V1" and had the following modifications: F140Y-A178P-G321 N-Q490S (F121Y-A159P-G302N-Q471S).

[0046] V1 had a specific activity that was 2-fold higher than that of the parent, and tested under conditions approximating its industrial application, V1 displayed an improved catalytic effectiveness than that of the parent by reducing the time to completion of the reaction by 22%. Extrapolation from the DSF thermostability data would suggest that V1 can be applied under reaction conditions at least 5°C higher than currently employed, and it is probable that time to completion can be further reduced, as it is accepted that reactions kinetics are enhanced at elevated temperatures.

[0047] Previous improvements to fructosyltransferase activity of fungal β-fructofuranosidases have been achieved by altering amino acids in the active site pocket [23,24] and in the non-catalytic β-sandwich domain [21]. Although catalytic pocket residues seem the obvious choice for amino acid substitutions, the applicant has demonstrated that substitutions in solvent exposed loops mediate long range interactions which alter active site geometry and in turn modify enzyme activity.

[0048] In order to optimise expression of the modified polypeptide in a host cell, such as a microbial cell from a yeast (e.g. *Pichia pastoris*), fungus or bacterium, the polynucleotide encoding the modified polypeptide can be codon-optimised according to the host cell. Methods for codon-optimisation are well known to those skilled in the art. The polynucleotide can optionally include a secretion signal, such as that for *T. reesei* endoxylanase 2 (*xln2*) or any other suitable secretion signal. A vector including the polynucleotide can be used to transform the host cell. The polypeptide may be expressed under the control of various constitutive or inducible promoters, such as the S. *cerevisiae* phosphoglycerate kinase 1 (PGK1) promoter, glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter or the alcohol oxidase (AOX1) promoter in *Pichia pastoris.* The transformed host cell can be added to a solution containing sucrose and be caused to express the polypeptide into the solution, possibly as whole cell catalysts, or alternatively a purified, partially purified or crude extract of the modified polypeptide can be added directly to the solution containing sucrose.

[0049] As described in more detail below, the β-fructofuranosidase gene (*fopA*) from *Aspergillus niger* ATCC 20611 (A. japonicus) was codon optimised for expression in *Pichia pastoris* DSMZ 70382 (purchased from the The Leibniz Institute DSMZ - German Collection of Microorganisms and Cell Cultures GmbH (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH)). The protein was further engineered for higher specific activity, decreased glucose inhibition and thermostability by introducing the mutations of V1. The original enzyme (FopA) produced by the *P. pastoris* fopA strain was then compared to the protein-engineered enzyme (fopA_V1) produced by the P. *pastoris* G250.2 strain for its ability to produce a similar sugar composition from sucrose to Actilight®, a prebiotic ingredient which has been proven to have multiple health benefits and is extensively used in a wide variety of functional foods. Actilight®, available from Beghin Meiji and Teros Syral, contains scFOS in a ratio of 37%, 53% and 10% for GF2, GF3 and GF4, respectively.

[0050] The invention will now be described in more detail by way of the following non-limiting examples.

Example 1: Modification of β-fructofuranosidase variants

Materials and methods

Microbial strains and media

[0051] *S. cerevisiae* EUROSCARFY02321 [BY4741;*Mat a; his3Δ1; leu2Δ0; met15Δ0; ura360; YIL162w(SUC2)::kanMX4]* served as host for the variant libraries [25]. The S. *cerevisiae* NI-C-D4 [*Matα*; *trp1; ura3; pep4*] oversecretion phenotype strain was used when heterologous proteins were to be purified [26]. *Escherichia coli* DH5α [*thuA2Δ(argF-lacZ)U169 phoA glnV44 Φ80Δ (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*] (New England Biolabs, Midrand, South Africa) was used for cloning and amplification of plasmids. *E. coli* cells were grown at 37 °C in Luria Bertani broth supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin, as appropriate.

DNA manipulations

[0052] All DNA manipulations were performed according to standard methods [27]. Restriction enzymes and T4 DNA ligase were used according the specifications of the supplier (ThermoScientific, Waltham, Massachusetts, USA). CLC Main Workbench version 6.8.1 (Qaigen) was used for sequence analyses.

Gene synthesis and mutagenesis

[0053] A codon-optimised (parent) gene was provided by DNA 2.0 (Menlo Park, CA, USA) as a synthetic construct

combining the *Trichoderma reesei* endoxylanase 2 (*xln2*) secretion signal [28] and the open reading frame of the *fopA* β-fructofuranosidase (GenBank accession number AB046383) (SEQ ID NO: 1 and 3). The native *fopA* secretion signal was excluded. Thirty-six variants of the β-fructofuranosidase gene were synthesised. The parent gene sequence was altered to produce gene products harbouring single amino acid substitutions. The substitutions were distributed throughout the protein sequence but were limited to loop regions as determined from the published crystal structure (3LF7) of Chuankhayan et al. [7]. The published crystal structures were determined for the *A. japonicus* β-fructofuranosidase. There is 99% homology on the DNA (AB046383, GU356596.1) and protein (BAB67771.1, ADK46938.1) levels of the β-fructofuranosidases of *A. niger* and *A. japonicus,* respectively. *Aspergillus niger* ATCC 20611 was reclassified by the curators of the ATCC culture collection (http://www.lgcpromochem-atcc. com) as *A. japonicus* and it was therefore assumed that the sequences and structures deposited in the databases are for the same gene/enzyme.

[0054] Positions for amino acid substitutions were selected by the strategy provided by DNA2.0. An alignment of homologous sequences to the 3LF7 structure was used from the HSSP database [29]. Positions within the MSA corresponding to secondary structural elements of the 3LF7 chain A were excluded. The solvent accessibility computed for each sequence position was normalised according to values obtained for Ala-X-Ala tripeptides [30,31]. This provided a relative solvent accessibility at each position normalized by the side chain type. The sequence entropy for each position in the multiple sequence alignment was also obtained from the HSSP file for RCSB Protein Data Bank entry 3LF7. Sequence positions with relative solvent accessibility (RSA) greater than 50% and sequence entropy (SE) greater than 1.0 provided a list of positions in the structure-based multiple sequence alignment. From these alignment data, the most commonly observed substitutions were selected for inclusion in a 36 variant first round library. The mutations are listed in Table 1. A further 18 genes were synthesised containing exhaustive combinations of 5 single mutations that were determined to improve enzyme performance during first round screening (Table 2). The amino acid sequences of these variants are shown in SEQ D NOS: 4 - 21.

Table 1: Single amino acid substitutions made to the parent enzyme to generate the first round library of β-fructofuranosidase variants.

Amino acid substitution	Position on crystal structure (3LF7A)	Position in SEQ ID NOs: 3-21	Module	Relative solvent accessibility (%)	Sequence Entropy	Enzyme activity *
P28A	28	9	β-propeller	64	1.0	Active
G109S	109	90	β-propeller	79	1.5	Inactive
F140Y	140	121	β-propeller	50	2.0	Active
F140S	140	121	β-propeller	50	2.0	Inactive
F140T	140	121	β-propeller	50	2.0	Inactive
F140R	140	121	β-propeller	50	2.0	Inactive
A178P	178	159	β-propeller	55	1.7	Active
A178S	178	159	β-propeller	55	1.7	Active
A178Y	178	159	β-propeller	55	1.7	Inactive
D185N	185	166	β-propeller	65	1.2	Inactive
D185H	185	166	β-propeller	65	1.2	Inactive
D185Q	185	166	β-propeller	65	1.2	Inactive
Y261S	261	242	β-propeller	67	2.0	Inactive
Y261N	261	242	β-propeller	67	2.0	Inactive
Y261T	261	242	β-propeller	67	2.0	Inactive
G321N	321	302	β-propeller	56	2.1	Active
G321D	321	302	β-propeller	56	2.1	Active
G321E	321	302	β-propeller	56	2.1	Active
G321Y	321	302	β-propeller	56	2.1	Active

E389N	389	370	β-propeller	100	1.0	Active
E389A	389	370	β-propeller	100	1.0	Inactive
E389K	389	370	β-propeller	100	1.0	Inactive
D454Q	454	435	β-propeller	78	2.1	Active
D454G	454	435	β-propeller	78	2.1	Active
D454T	454	435	β-propeller	78	2.1	Inactive
E485P	485	466	β-sandwich	96	2.2	Active
E485Q	485	466	β-sandwich	96	2.2	Active
E485N	485	466	β-sandwich	96	2.2	Active
E485S	485	466	β-sandwich	96	2.2	Active
Q490S	490	471	β-sandwich	69	2.4	Active
Q490K	490	471	β-sandwich	69	2.4	Active
Q490N	490	471	β-sandwich	69	2.4	Active
T569P	569	550	β-sandwich	56	1.7	Active
T569N	569	550	β-sandwich	56	1.7	Active
T569A	569	550	β-sandwich	56	1.7	Active
N648D	648	629	β-sandwich	65	1.3	Inactive

Cloning and yeast library generation

[0055] Cloning vectors (pJ227) containing the synthesised gene variants were digested with *Eco*RI and X*ho*I restriction enzymes (Fermentas) and directly ligated with the pJC1 yeast expression vector [32] digested with the same restriction enzymes. Selection of *E. coli* transformants on LB agar plates supplemented with 100 ⌠g/ml ampicillin ensured isolation of clones with the gene variant-pJC1 combination and not re-circularised gene variant-pJ227, as the cloning vector conferred resistance to kanamycin. The primer pair 5'GTTTAGTAGAACCTCGTGAAACTTA 3' (SEQ ID NO: 22) and 5'ACTTAAAATACGCTGAACCCGAACAT3' (SEQ ID NO: 23) was used to screen clones by polymerase chain reaction to ensure the presence of the 2000 base pair insert. Positive clones were further confirmed by restriction digest analysis. Yeast was transformed by the lithium acetate method described by Hill et al. [33]. The method was adapted to 96-well format by proportionally scaling down reagents.

Yeast cultivation and media

[0056] *S. cerevisiae* was cultivated at 30 °C in YPD (1 % yeast extract, 2 % peptone and 2 % glucose) or in synthetic medium, SC without uracil [2% carbon source, 0.67% yeast nitrogen base without amino acids (with ammonium sulphate; Difco Laboratories, Detroit, Michigan, USA) and 0.13 % amino acid dropout pool [34]]. Glucose and galactose served as carbon source in solid and liquid SC^{-ura} media, respectively. Solid media contained 2% agar (Difco Laboratories).

[0057] Three yeast transformants per variant were manually transferred to individual wells of 2 ml round bottomed 96-deep-well plates (Merck, Modderfontein, South Africa) containing 1.25 ml SC^{-ura} media. Mixing was facilitated by a single 2-mm glass bead (Merck, Modderfontein, SouthAfrica) added to each well. Plates were sealed with sterile, breathable AeraSealTM film (Excel Scientific Inc., Victorville, California, USA) and shaken at 200 rpm for 4 days. Fifty microliters of each culture were transferred to a fresh plate and cultivated for a further 4 days. Master plates were generated using a 96-well replicator (Applikon Biotechnology, Delft, Netherlands). Culture supernatants were used in assays as source of enzyme after cell removal by centrifugation at 3000 rpm.

Table 2: Activity data for combination variants. Data are arranged in order of decreasing nystose per number of substitutions. Values in brackets are standard error (n = 3). The five most improved variants are coded.

Number of substitutions	F140Y *	A178P *	A178S *	G321N *	Q490S *	Glucose (g/l)	Fructose (g/l)	Sucrose (g/l)	1-Kestose (g/l)	Nystose (g/l)
4 (V1)	x	x		x	x	74.6 (± 3.4)	14.5 (± 0.9)	177.7 (± 1.3)	67.2 (± 0.5)	53.8 (± 2.0)
4	x		x	x	x	50.3 (± 19.6)	14.6 (± 6.1)	186.1 (± 3.0)	71.9 (± 6.4)	33.3 (± 1.5)
3 (V4)		x		x	x	68.1 (± 1.6)	11.2 (± 0.6)	177.8 (± 0.6)	63.1 (± 0.9)	50.8 (± 1.5)
3 (V5)	x	x			x	69.0 (± 3.9)	12.0 (± 1.2)	180.2 (± 3.0)	61.0 (± 3.7)	47.4 (± 2.5)
3 (V3)	x	x		x		67.0 (± 0.5)	14.1 (± 2.0)	176.5 (± 2.2)	65.3 (± 1.4)	47.0 (± 0.7)
3	x			x	x	64.8 (± 0.9)	10.2 (± 1.6)	173.4 (± 2.9)	75.0 (± 1.0)	42.4 (± 2.3)
3	x		x		x	60.6 (± 0.6)	12.0 (± 0.4)	168.4 (± 0.8)	80.7 (± 0.2)	34.2 (± 0.6)
3			x	x	x	58.7 (± 0.6)	22.3 (± 0.5)	167.1 (± 1.5)	80.6 (± 0.6)	34.0 (± 0.4)
3	x		x	x		47.3 (± 1.0)	18.4 (± 1.5)	119.9 (± 1.1)	86.5 (± 1.1)	15.6 (± 0.7)
2 (V8)		x		x		67.1 (± 1.4)	15.3 (± 0.8)	174.1 (± 0.9)	69.2 (± 2.5)	47.4 (± 0.6)
2				x	x	57.7 (± 7.5)	11.5 (± 0.6)	176.5 (± 3.2)	72.2 (± 1.1)	44.7 (± 1.6)
2	x			x		66.3 (± 1.1)	12.9 (±	173.1 (±	79.3 (± 1.1)	44.3 (± 1.2)

							1.9)	0.5)		
2	x	x				67.3 (± 5.8)	14.0 (± 0.8)	172.2 (± 0.2)	81.4 (± 1.3)	42.0 (± 0.9)
2		x			x	21.0 (± 9.4)	12.2 (± 1.8)	178.7 (± 3.5)	59.0 (± 8.0)	37.6 (± 5.2)
2	x				x	54.2 (± 9.2)	16.0 (± 5.9)	173.2 (± 3.3)	76.8 (± 2.4)	34.7 (± 4.1)
2			x	x		59.3 (± 1.3)	17.0 (± 1.2)	165.0 (± 0.7)	85.2 (± 1.8)	34.4 (± 0.6)
2	x		x			57.8 (± 1.7)	15.0 (± 1.4)	170.1 (± 4.4)	76.1 (± 7.1)	34.2 (± 4.4)
2			x		x	61.1 (± 1.8)	13.1 (± 0.3)	167.5 (± 2.7)	87.5 (± 2.4)	33.6 (± 0.9)
1		x				43.5 (± 21.3)	10.8 (± 0.4)	180.8 (± 5.7)	77.2 (± 2.6)	40.1 (± 2.1)
1				x		49.0 (± 11.5)	13.2 (± 0.6)	175.3 (± 6.2)	79.1 (± 1.8)	37.6 (± 1.4)
1					x	48.5 (± 13.0)	16.3 (± 3.1)	174.9 (± 3.0)	83.7 (± 3.0)	33.8 (± 3.0)
1	x					59.2 (± 1.0)	15.3 (± 1.2)	165.1 (± 2.9)	82.9 (± 1.3)	32.9 (± 1.9)
1			x			46.7 (± 6.4)	18.7 (± 4.0)	164.2 (± 4.7)	68.0 (± 19.7)	22.8 (± 2.3)
Parent	**-**	**-**	**-**		**-**	**44.1 (± 14.9)**	**17.7 (± 3.7)**	**166.1 (± 2.2)**	**69.1 (± 12.8)**	**32.6 (± 1.2)**

* Substract 19 to calculate amino acid position in SEQ ID NOs: 3-21

Library screening

[0058] Enzyme activity assays were performed in 96-well format by reacting 50 μl of culture supernatant with 50 μl of substrate at 55°C for 2 hours. The working concentration of substrate was 200 g/l sucrose (Fluka, Sigma-Aldrich, St. Louis, Missouri, USA) in 50 mM citrate phosphate buffer, pH 5.5. As determined previously [22], 54 g/l glucose was added to the substrate solution to test for variants insensitive to product inhibition. Saccharides in assay samples were quantified using high performance liquid chromatography and Fourier transform mid-infrared (FT-MIR) spectroscopy. The details of the methods were described previously [22]. The rationale for supplying galactose as carbon source in liquid cultures was the separation of glucose and galactose by HPLC and hence any glucose present in assay samples was attributed to enzyme activity. Inhibition was calculated as the difference between uninhibited variant activity and inhibited activity divided by uninhibited activity. Data were normalised to the parental activity. The parent and all variants were was cultivated and assayed with triplicate repeats. The cultivation and assay procedure was validated previously [22].

Protein purification

[0059] The top five performing variants were purified using immobilised metal affinity chromatography (IMAC). The proteins were N-terminal His-tagged by sub-cloning *Bgl*II-*Xho*I gene fragments into the same sites in a modified pJC1 yeast expression vector. The vector was modified by cloning a synthetic fragment (Geneart, Regensburg, Germany) encoding the *xyn2* secretion signal, six histidine residues and a factor Xa protease cleavage site into the *Eco*RI and *Bgl*II sites of the multiple cloning site (ATGGTTTCTTTCACATCCTTGTTGGCTGGTGTTGCTGCTATTTCCGGT-GTTTTG GCTGCTCCAGCTGCTGAAGTTGAATCCGTTGCTGTTGAGAAGagaCATCACCATCACCATCAC GGATC-

cGGCTCTGGATCTGGTATCGAGGGAAGA (SEQ ID NO: 24). Tagged gene variants were sequenced to verify integrity of the clones. Plasmids were transformed to S. *cerevisiae* NI-C-D4. Transformants were cultivated for 72 hours in 50 ml double strength SC^{-ura} buffered with succinic acid at pH 6.0 [(2% glucose, 1.34% yeast nitrogen base without amino acids (with ammonium sulphate; Difco Laboratories, Detroit, Michigan, USA) and 0.26 % amino acid dropout pool [34]]. Antifoam 204 (Sigma-Aldrich, St. Louis, Missouri, USA) was added after 48 hours of cultivation to a concentration of 0.025% (v/v). Following cell removal by centrifugation, supernatants were concentrated 50 times by ultrafiltration using Amicon ultra-15 centrifugal filters with 10 kDA MWCO (Millipore, Molsheim, France). IMAC protein purification was performed under native conditions using Ni-NTA spin columns supplied by Qaigen (Venlo, Netherlands). For the removal of imidazole, buffer exchange with 10 mM Bis-Tris, pH 6 was performed using the aforementioned ultrafiltration devices. Protein concentration was determined using the bicinchoninic acid assay (Pierce Chemical Company, Rockford, Illinois, USA) with bovine serum albumin as standard.

Enzyme assays

[0060] A unit of enzyme was defined as the amount of protein that produced 1 mmol 1-kestose per minute from 10% (w/v) sucrose at 40°C in 50 mM citrate phosphate buffer (pH 5.5). The definition approximates that of Hirayama et al. [35].

Protein electrophoresis

[0061] Samples were analysed by SDS-PAGE on an 8% resolving gel. Loading dye consisted of 60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14 mM β-mercaptoethanol and bromophenol blue and gels were run in Tris-glycine buffer (25 mM Tris-HCl, 250 mM glycine, 0.1% SDS). Protein bands were visualized with a silver-stain [36].

Isothermal denaturation (ITD) and differential scanning fluorimetry (DSF)

[0062] Protein thermal denaturation assays were performed by the method described by Niesen et al. [37]. SYPRO orange was supplied by Sigma-Aldrich (St. Louis, Missouri, USA) and used at a 5x working concentration. Each reaction utilised 65 ng of protein. Samples were incubated in a StepOnePlus Real-Time PCR machine (Applied Biosystems). IDT samples were incubated at 55°C for 10 hours while DSF samples were incubated with temperature increasing by 1 °C per minute from 25 - 95°C. Multicomponent data were exported from the StepOne software to Excel 2010 and ROX filter data were used. Data points beyond the maximum fluorescence +4 were discarded. First derivatives were calculated in Statistica version 12 (StatSoft Inc.). In cases where 2 peaks were obtained after application of the derivative, the temperature of the second peak was used. One-way analysis of variance (ANOVA) was conducted to test for differences between treatments applied to enzymes.

Computational analyses

[0063] Homology models for the single amino acid variants at the 4 positions yielding the most improved variants and the 5 best combination variants were generated by the SWISS-MODEL web server [38]. The crystal structure 3LF7 [7] served as template for the automated modelling mode. Template and target sequences shared 99% identity. Quality assessment of the models was performed by the QMEAN server (47).

[0064] Solvent accessible surface area (SASA) data for the most improved combination variant were generated from the homology model. SASAs were computed for the folded (from the homology models) and unfolded states (sequence specific theoretical calculations) by the ProtSA web server (51). Differences between the folded and mean unfolded ensembles were determined. To examine the influence of the amino acid substitutions on SASAs, differences between the parent and the variant were further calculated.

[0065] The Ligand-Protein Contacts (LPC) server was used to identify amino acid residues in contact with the ligand (first shell residues). Contacts with substituted residues in the variant were identified by the Contacts of Structural Units (CSU) server. Servers were accessed via http://ligin.weizmann.ac.il/cgi-bin/lpccsu/LpcCsu.cgi (67). Functional site predictions where made using 3LF7 structure as input for the Partial Order Optimal Likelihood (POOL) server (67). The top 8% of ranked residues were taken as active site residues.

FOS synthesis

[0066] FOS synthesis was performed with the parent enzyme and the variant displaying the highest specific activity. It was accomplished by reacting 10 U of parent enzyme per gram of sucrose and dosing the same amount of protein for the variant. The reaction was performed at 62°C with shaking at 120 rpm. Working concentrations were 600 g/l sucrose dissolved in 50 mM citrate phosphate buffer, pH 5.5. Samples were taken hourly for 12 hours and analysed by

HPLC after appropriate dilution.

Results

Round 1: single-amino-acid substitution library screening

[0067] Table 1 and Figure 1 list the amino acid substitutions selected to generate the first round of variants. Although the substitutions were limited to the loop regions, they were otherwise distributed across the entire protein sequence length. Substitutions were made at 13 positions with multiple substitutions at 10 of the positions. Of the 36 variants, two were deemed inactive as evidenced by a lack of growth on solid media supplemented with sucrose as sole carbon source. Active gene products complemented the suc2 knockout in the S. cerevisiae strain enabling growth on sucrose containing solid media. The activities of a further 10 variants were deemed severely compromised as HPLC and FT-MIR spectroscopy indicated negligible amounts of sucrose conversion. SDS-PAGE analyses showed consistent protein expression levels for the variants, barring three where protein production was abolished - expected bands were absent. The activities of these three variants remained unknown (data not shown). The sensitivity of the catalytic β propeller domain of FopA to structural alterations was highlighted by 48% of substitutions resulting in abolished or severely compromised activities as opposed to the β sandwich domain where all variants remained active bar one (9%), the activity of which remains unknown. Figure 2 shows the nystose and relative inhibition data for the 21 variants retaining good activity levels. The smaller the relative inhibition value the less sensitive the enzyme to glucose inhibition. Variant A178S appeared to tolerate glucose better than other variants but at the expense of its fructosyltransferase activity. First round screening data were used to design combination variants of the top five performing round 1 variants. The top four nystose producing variants F140Y, A178P, Q490S and G321N and the single variant showing the greatest relief from glucose inhibition (A178S) were combined exhaustively to produce another 18 variants which included combinations of two, three and four amino acid substitutions.

Round 2: combination variant screening

[0068] Table 2 lists the combination variants grouped by number of substitutions and their activity data arranged per group in descending order for nystose. The parent (wild type) data is given in bold font. Sixteen of the 18 variants showed improved activity relative to the parent. Besides the single substitution variant A178S, only F140Y-A178S-G321N displayed poorer fructosyltransferase activity than the parent. Furthermore, all the variants with the A178S substitution performed worse than the variants with the A178P substitution. Although combining A178S with other substitutions did recover activity relative to the parent, it was generally assumed to be a deleterious substitution and did not prove permissive in a combinatorial context. No trend emerged for relative inhibition of combination variants and the A178S substitution (data not shown). Single substitutions ranked in decreasing order of nystose production A178P, G321N, Q490S, F140Y and A178S. A178P and G321N always proved to be good substitutions be it in isolation, in combination with each other and with either Q490S and/or F140Y. The contributions of Q490S and F140Y to enzyme activity were combination dependent. Together in a combination of 2 they were the poorest performers of the combination mutants. When combined with A178P, F140Y improved enzyme activity to a greater degree than Q490S - 37.6 g/l nystose produced as opposed to 34.7 g/l. In a combination of 2 with G321N, both F140Y and Q490S resulted in the same amount of nystose produced at approximately 44 g/l. However, the 1-kestose values differed with F140Y-G321N levels at 79.3 g/l while G321N-Q490S levels were 72.2 g/l. It is probable that G321N-Q490S was a more efficient enzyme as decreasing 1-kestose levels with similar nystose levels indicate GF4 production. No significant differences in the levels of sucrose, glucose and fructose supported this deduction. For a 3 combination variant the opposite for Q490S was true - A178P-G321N-Q490S produced more nystose than F140Y- A178P-G321N. The 4 combination variant F140Y-A178P-G321N-Q490S proved to be the most improved variant with nystose levels 65% higher than the parent - 53.8 g/l versus 32.6 g/l.

Improved variant characterisation

[0069] This section applies to the parent and the five most improved combination variants. For simplicity the combination variants were named V1: F140Y-A178P-G321N-Q490S (F121Y-A159P-G321N-Q471S), V3: F140Y-A178P-G321N (F121Y-A159P-G302N), V4: A178P-G321N-Q490S (A159P-G302N-Q471S), V5: F140Y-A178P-Q490S (F121Y-A159P-Q471S) and V8: A178P-G321N (A159P-G302N).

Protein purification and electrophoresis

[0070] Enzymes were purified using IMAC. Figure 3 shows the purified enzymes and crude yeast supernatants on a silver stained SDS-PAGE gel. The high degree of enzyme purity following IMAC is evident in gel A. The intense bands

between 100 and 130 kDa correspond to the parent and variant β-fructofuranosidases - the band was absent in the reference (gel B, lane 2). The proteins were larger than the expected 69 kDa as deduced from the protein sequence but the shift was attributed to N-glycosylation of the proteins by yeast. Following the removal of the glycosylation with PNGase F, bands of the expected size were obtained (gel C, lane 4). All variants had 8 putative N-glycosylation sites which was unchanged from the glycosylation pattern of the parent [40]. Chuankhayan et al. [7] proposed that altered N-glycosylation patterns from heterologous hosts may influence enzyme stability. As the parent and all variants were expressed in the same host this was irrelevant but altered stability was ruled out due to altered N-glycosylation patterns resulting from different primary protein structures of the variants.

Specific activity

[0071] Specific activities for the purified enzymes were determined. Results are shown in Figure 4. The specific activity of the parent enzyme was 1652 U/mg protein which was lower than the 2650 U/mg protein reported by Nishizawa et al. [19]. Heterologous enzyme production and experimental disparities could account for this. Differences in activity between the native enzyme and the heterologous *fopA* gene product were also reported by Yanai et al. [41]. All the variants had significantly ($p = 0.0023$ for V8) higher specific activities than the parent, with V1 as much as double.

Isothermal denaturation (ITD)

[0072] To investigate the stability of the improved variants, ITD (a method that quantifies protein stability by measuring protein unfolding caused by heat denaturation in the presence of SYPRO orange dye [42]) was employed. Typically this method is used to quantify stability and ligand affinity for a given protein. The method was employed in a modified sense in that the proteins were the variable factor. Purified proteins were incubated for 10 hours at 55°C to examine the influence of protracted exposure to temperatures routinely used for industrial FOS synthesis reactions. Figure 5 shows that after 2 hours the fluorescence measured for the parent enzyme increased dramatically whereas the variants showed more measured increases. The increased fluorescence can be interpreted as thermal denaturation of the enzymes. Upon protein unfolding, binding of the SYPRO orange dye to newly exposed hydrophobic amino acid residues results in increased fluorescence [43]. It thus appeared that the variant enzymes were more thermostable than the parent. Slight differences in stability were observed between the variants, which could be attributed to the differing combinations of amino acid substitutions.

Differential scanning fluorimetry (DSF)

Melting temperatures (T_m) and pH optima

[0073] To further investigate the thermostability of the variants, DSF [37] was employed. The principle is similar to ITD, but instead of maintaining a set temperature, it is increased by 1°C per minute. The T_m of a protein is the temperature at which half of the protein molecules are unfolded and reflects the transition midpoint of the fluorescence vs temperature curve [37,44,45]. Factors influencing protein stability include buffers, salts and detergents and also specific interactions with ligands. Factors that promote stability delay the thermally induced unfolding and result in increased T_m. DSF experiments were conducted for the parent and 5 variants at pHs ranging from 4 to 7. Fluorescence intensity curves for the parent displayed the typical two-state unfolding transition (folded to unfolded with no stable intermediates) [37,46] at all pH levels tested. However, all the variant profiles were pH dependent. At low pH the unfolding proceeded via the typical two-state transition while at higher pH unfolding occurred in via multiple-state transitions. This was attributed to a mutation(s) that stabilised a portion of the protein and thus more energy was required for complete unfolding. The maximum fluorescence at pH 4 was in excess of 60000 AU, whereas at pH 7 it was 22000 AU. Hirayama *et al.* [35] reported the optimum pH for the wild type enzyme in terms of activity and stability to be between 5 and 6 and 6.5, respectively. At pHs below 4.5 the stability and activity of the enzyme were severely compromised. The high fluorescence and two-state transition of the variant proteins at low pH were therefore attributed to the severely unfavourable conditions imposed on the enzyme at pH 4. The ionization states of amino acids were likely altered and hence contributed to extensive protein denaturation. T_ms were calculated from the first derivatives applied to the fluorescence data [37]. For samples presenting multiple transitions the maximum of the second peak was used to determine the T_m. The results at each pH for the enzymes are shown in Figure 6. Similar to the ITD results, it was clear that the variants were more thermostable than the parent. The pH optima profiles did not change due to amino acid substitution - the optima for all enzymes were at pH 5.0. At optimum pH V8 displayed a 5.7 °C increased T_m relative to the parent (73.7°C), making it the most thermostable variant. V1, V3 and V4 all displayed improvements of 5.3 °C, while V5 was improved by 4.3 °C. The differences between the three T_m groups were all statistically significant ($p<0.05$). Considering the combinations of substitutions in the variants, A178P and G321N appeared to be responsible for the improved thermostability. All variants

harboured the A178P substitution and they were all more stable than the parent. As V1 (F140Y-A178P-G321N-Q490S), V3 (F140Y-A178P-G321N) and V4 (A178P-G321N-Q490S) all displayed the same improvement, it appeared that F140Y and Q490S were marginally destabilising substitutions in terms of thermostability - inclusion of either one or both of them resulted in a decreased T_m relative to V8. As V8 (A178P-G321N) was the most improved and V5 (F140Y-A178P-Q490S) the least which was concluded was that G321N contributed positively to stability.

Ligand affinities

[0074] Although V8 was the most thermostable enzyme, it did not display the highest specific activity. DSF was used to perform comparative investigations into substrate interactions between the parent and variant proteins. More effective ligand binding to enzymes results in delayed thermal denaturation and higher T_ms. Results for ΔT_m (T_m at given substrate concentration minus T_m at 0 substrate concentration) at different substrate and product concentrations are shown in Figure 7. At 0.2 M sucrose increased T_ms were observed for all enzymes (Figure 7A). Further increased sucrose concentrations resulted in diminished substrate binding for all enzymes as evidenced by lower T_ms. At 0.5 M sucrose the parent, V5 and V8 displayed the same T_m as in the absence of substrate, whereas variants V1, V3 and V4 displayed increased T_ms - differences were small, $\otimes T_m$ of 1°C or less, but significant ($p<0.05$ for V1, V3 and V4). It was apparent that the substrate binding at 0.5 M sucrose was altered for enzymes with higher specific activities, namely V1, V3 and V4 (Figure 4). In the case of 1-kestose, all variants bound the substrate with higher affinity than the parent (Figure 7B). Increased 1-kestose concentrations increased the stability of all variants except V4 which did not display an altered T_m at all concentrations tested. Enzyme stabilisation as a result of nystose binding was not evident at concentrations up to 0.2 M - no significant changes in T_m were observed (Figure 7C). However, at 0.4 M the parent and V4 were stabilised significantly by nystose binding. The other variants did not show significant improvements in T_m as a result of substrate binding. Finally, binding of glucose (product) to enzymes was also examined and results indicated that the two most improved variants, V1 and V3, appeared to have diminished affinities for glucose - T_ms were not affected at glucose concentrations up to 0.3 M while the parent, V3, V4 and V5 were stabilised by 1°C. In summary, the most improved variant, V1, displayed increased affinities for sucrose and 1-kestose and decreased affinities for nystose and glucose at the highest substrate concentrations tested. The T_m of the parent was unaffected by sucrose or 1-kestose while it was increased by nystose and glucose, again at the highest concentrations tested. Taken in combination, these data indicated that subtle changes to the range of substrate and product affinities as well as improved thermostability were responsible for improved enzyme activities. It is plausible that the amino acid substitutions altered the active site such that the enzymes were relieved to an extent from substrate and product inhibition, conditions that are well documented in the literature for β-fructofuranosidases, albeit not this specific enzyme. Further kinetic characterisation is required to fully understand the impact of substitutions on the enzymes' activities.

FOS synthesis

[0075] As an ultimate test of variant performance, equal amounts of purified parent enzyme and V1 were used to produce FOS under conditions similar to those used in industry. In commercial FOS products the ratio of GF2:GF3:GF4 is approximately 42:47:10 and depending on the product, compromise up to 95% of the dry mass after chromatographic separation of glucose, fructose and sucrose [19,60-62]. Figure 8 shows HPLC data for all the relevant sugars. It was evident that V1 has a higher catalytic activity relative to the parent, as the levels of nystose and GF4 were higher. Higher 1-kestose levels for the parent highlighted the difference in activities as this is the initial FOS species produced from sucrose. It in turn serves as substrate for the formation of nystose and GF4. As equal amounts of protein were dosed, it can be deduced that V1 has a higher turnover number (k_{cat}) than the parent. The fructose data for both enzymes were virtually identical, which indicated that the hydrolytic activity was unchanged in the variant. V1 consumed marginally more sucrose than the parent, but it was not reflected in the amount of total FOS produced. As the differences in molecular weights between sucrose and FOS are large, experimental error could account for the discrepancy (a given amount of sucrose translates into a small amount of FOS which could be missed given a maximum of 10% error of quantification). Similarly for glucose, which is an indicator of global enzyme activity, clear differences at all the time points were not evident. Although the reaction was followed for 12 hours, the endpoint for a typical industrial reaction was regarded as the time when total FOS composition comprised 10% GF4. V1 reached 10% GF4 in 4.6 h while it took the parent 6.2 h to produce FOS of similar composition. This difference represented a 26% reduction in time required to complete the reaction. Otherwise stated, enzyme dosage could be reduced to achieve the same result in 6.2 h.

[0076] The hydrolytic activity of the enzyme dominated after 11 h as reflected by the sharp increase in fructose and corresponding decreases in GF4, nystose, sucrose and to a lesser extent, 1-kestose levels.

Example 2: Expression of V1 in *Pichia oastoris* and conversion of sucrose to FOS

Materials and methods

Bioreactor cultivations

[0077] *P. pastoris* cultivations were performed in 1.3L New Brunswick Bioflo 110 Fermenters/Bioreactors with a working volume of 1L. Biocommand version 3.30 plus software was used for monitoring and feed rate control. Pre-cultures were prepared by inoculating a streak of colonies from YPD agar plates into 4 ml of buffered minimal glycerol medium (BMGH) as described in the *Pichia* Expression Kit (Invitrogen) with the exception of a final YNB concentration of 0.17 %. Following overnight incubation at 30°C cultures were diluted to an optical density $(OD)_{600}$ = 0.1 in 40 ml of fresh BMGH medium. Subsequently, these seed-cultures were grown overnight at 200 rpm to an OD_{600} = 10-15, before inoculating the entire volume to the medium to a final volume of 400 ml in the bioreactor, obtaining a starting OD_{600} = 1.0-1.5.

[0078] Fermentation basalt salt medium (BSM), supplemented with 4% glycerol and PTM_1 trace salts, was used as culture media as described by the *Pichia* Fermentation protocol (Invitrogen). Culture conditions were maintained as follows: temperature of 30°C and a pH of 5.0 was maintained with 28% ammonium hydroxide, aeration rate of 1.0 volume of oxygen per volume of fermentation culture per minute (vvm), the dissolved oxygen (DO) was maintained at 30% controlled by a cascade effect between agitation (200-1000 rpm) and sparging O_2 when agitation was not sufficient. Fermentations were performed as per the *Pichia* Fermentation protocol (Invitrogen) with the following exceptions: During the glycerol fed-batch phase the glycerol was fed via a DO-stat feeding strategy (feeding started when DO >= 30 % and stopped when it was < 30 %). This was continued for 72 hrs after the batch phase at which point the bioreactor volume was harvested, centrifuged for 3 min at 3000 rpm and then filtered through 22 μm filter and stored at 4°C for determining the enzyme activity.

Enzyme activity assay

[0079] To determine the fructofuranosidase activity, sucrose was used as the substrate and prepared in a 50 mM citrate phosphate buffer (pH 5.5) and used at a working concentration of 100 $g.l^{-1}$. The substrate solution was equilibrated at 40°C for 10 min where after culture supernatant was added to a final of 25% [v/v] and incubated for 60 min. To stop the reaction, perchloric acid (PCA) was added to a final concentration of 2.14% followed by the addition of 7 N KOH to precipitate the proteins prior to chemical analysis. Negative control reactions containing all the assay constituents except for either sucrose or enzyme were included. The samples were diluted appropriately and subjected to HPLC analysis using an external glucose standard calibration. The method has been decribed previously [69]. The concentration of glucose liberated during the assays was indicative of global fructofuranosidase activity. A unit of enzyme was defined as the amount of enzyme required to produce 1 μmol glucose per minute under the described conditions [14].

Fructooligosaccharide (FOS) production

[0080] To produce scFOS, a 60% sucrose solution (w/v) was prepared in a 50 mM citrate phosphate buffer (pH 5.0). The substrate solution was equilibrated at the required temperature in a Gyrotory Water Bath Shaker (New Brunswick Scientific Co. Inc., Edison N.J., USA) for 2 min while shaking at 120 rpm where after culture supernatant was added at a predetermined dosage according to central composite design. Samples were taken every 2 hrs and the reaction stopped by adding perchloric acid (PCA) to a final concentration of 2.14% followed by the addition of 7 N KOH to precipitate the proteins prior to chemical analysis in a Dionex UltiMate 3000 (Thermo Fisher Scientific, Waltham Mass., USA).

Central composite design

[0081] The production of scFOS was optimised as a function of temperature and enzyme dosage using response surface methodology (RSM) with a two-factor central composite design using Design Expert® software (Stat-Ease Inc., Minneapolis, USA). The input factors in the design were selected in the ranges of $57°C \leq A \leq 67°C$ and 8 U/g sucrose $\leq B \leq$ 12 U/g sucrose, where A represents the temperature and B the enzyme dosage. This design gave a total of 11 experiments for each enzyme (Tables 3 and 4).

Table 3: Central composite design of temperature (A) and enzyme dosage (B) for scFOS production by the enzyme fopA_V1 using 60% (w/v) sucrose solution for 8 hrs. Only time points where ~10% GF4 was produced are shown or where conditions did not achieve this the values for the final two time points are shown.

Run no.	Factors		Time (hrs)	% of total scFOS		
	A (°C)	B (U/g sucrose)		GF2	GF3	GF4
1	62.00	12.83	4	44.05	47.50	8.44
			6	35.34	51.16	13.50
2	62.00	10.00	6	41.88	48.93	9.19
			8	36.63	50.97	12.40
3	67.00	8.00	6	47.45	45.40	7.15
			8	42.00	48.60	9.40
4	57.00	8.00	6	50.20	45.01	4.79
			8	43.07	51.52	5.42
5	54.93	10.00	6	58.33	35.23	6.44
			8	52.17	38.10	9.73
6	62.00	10.00	6	40.74	49.51	9.74
			8	34.30	53.70	12.00
7	69.07	10.00	6	41.83	50.56	7.60
			8	38.34	51.60	10.06
8	62.00	7.17	6	49.26	44.64	6.10
			8	42.72	48.79	8.50
9	57.00	12.00	6	40.29	51.69	8.03
			8	34.05	54.49	11.46
10	67.00	12.00	4	44.90	47.46	7.64
			6	36.94	51.02	12.03
11	62.00	10.00	6	41.51	48.00	10.50
			8	35.18	50.44	14.38

Table 4: Central composite design of temperature (A) and enzyme dosage (B) for scFOS production by the enzyme fopA using 60% (w/v) sucrose solution for 8 hrs. Only time points where ~10% GF4 was produced are shown or where conditions did not achieve this the values for the final two time points are shown.

Run no.	Factors		Time (hrs)	% of total scFOS		
	A (°C)	B (U/g sucrose)		GF2	GF3	GF4
1	62.00	12.83	6	40.43	49.60	9.97
			8	35.05	51.54	13.41
2	62.00	10.00	6	45.60	45.44	8.96
			8	39.81	49.41	10.78
3	67.00	8.00	6	54.89	40.25	4.86
			8	50.81	43.18	6.02
4	57.00	8.00	6	56.22	38.94	4.83
			8	49.40	45.05	5.55
5	54.93	10.00	6	60.02	34.31	5.67
			8	51.25	42.41	6.33

(continued)

Run no.	Factors		Time (hrs)	% of total scFOS		
	A (°C)	B (U/g sucrose)		GF2	GF3	GF4
6	62.00	10.00	6	45.43	47.77	6.80
			8	39.39	51.07	9.54
7	69.07	10.00	6	53.55	42.39	4.06
			8	50.69	44.47	4.84
8	62.00	7.17	6	54.71	41.10	4.19
			8	48.24	45.70	6.06
9	57.00	12.00	6	43.51	49.91	6.58
			8	36.72	53.63	9.65
10	67.00	12.00	6	43.79	48.42	7.79
			8	40.80	50.06	9.14
11	62.00	10.00	6	44.25	47.46	8.29
			8	38.54	50.99	10.47

Results

[0082] The culture volume in the bioreactors was harvested after 94 hrs and the biomass separated from the volume. The enzyme assays of the two strains yielded enzyme activities of 1202 U/ml for P. *pastoris* fopA and 1124 U/ml for G250.2.

[0083] The optimum temperature for both enzymes is at ~62°C (Figures 9 to 11), but fopA_V1 was still able to produce 10.06% GF4 after 8 hrs at 69.07°C. This is more than double that for the fopA enzyme, which only produced 4.84% GF4 after the same amount of time. This would indicate that the fopA_V1 enzyme is more thermotolerant compared to the fopA enzyme. Both these enzymes struggled to produce 10% GF4 at the lower temperatures and required an increased enzyme dosage to achieve this (Tables 3 and 4). At enzyme dosages less than 10.00 U/g sucrose, both these enzymes were unable to produce a sugar composition similar to that of Actilight®. At the higher enzyme dosages of 12.83 U/g sucrose (62°C) and 12.00 U/g sucrose (67°C), fopA_V1 was able to produce sugar compositions similar to Actilight® at 6 hrs decreasing the incubation time by 2 hrs compared to fopA (Figure 10 and Table 3).

[0084] Generally, the fopA_V1 enzyme produced higher percentage GF4 or similar amounts in shorter time periods than the fopA enzyme at all the conditions tested. It was also able to tolerate higher temperatures and decrease incubation times at the higher enzyme dosages.

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[0085]

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Sequences

[0086]

SEQ ID No. 1: DNA codon optimised bv DNA 2.0 for parent enzyme fopA. including *xln2* secretion signal

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ATGGTCAGCTTCACTAGTCTGTTGGCTGGAGTTGCAGCTATCTCCGGTGTGCTGGCAGCTCCTGCTGCCGAAG
TAGAGTCTGTAGCAGTGGAAAAGAGATCTTACCACCTAGATACAACAGCCCCTCCTCCAACAAATTTGAGTAC
ACTTCCAAACAATACACTGTTCCATGTTTGGAGGCCAAGAGCACATATTTTGCCAGCCGAAGGTCAAATCGGT
GATCCCTGTGCACACTACACTGACCCATCAACTGGTCTGTTTCATGTTGGATTTTTGCACGACGGTGATGGAA
TTGCAGGAGCCACCACCGCTAATTTGGCTACTTATACGGACACGTCCGACAACGGTTCATTTTTGATTCAACC
CGGTGGCAAAAACGATCCTGTCGCTGTGTTTGACGGTGCAGTTATACCTGTCGGTGTGAATAATACTCCAACG
TTGCTGTATACTTCTGTTAGCTTTTTGCCAATACATTGGTCCATCCCGTACACTCGTGGAAGCGAGACTCAAT
CATTGGCTGTTGCCAGAGATGGTGGTAGAAGATTCGATAAACTGGATCAAGGTCCCGTAATCGCAGATCACCC
ATTTGCAGTGGATGTGACCGCCTTCAGAGATCCTTTTGTTTTCCGTTCGGCTAAGCTAGATGTCCTGTTATCT
CTTGACGAGGAAGTAGCTAGGAATGAAACCGCAGTGCAACAGGCTGTTGACGGATGGACTGAGAAAAATGCTC
CTTGGTACGTGGCCGTATCCGGAGGAGTTCATGGAGTCGGACCCGCACAATTCCTATACAGACAAAATGGTGG
AAATGCAAGCGAGTTCCAATACTGGGAGTATTTGGGAGAATGGTGGCAGGAAGCTACTAATAGTTCATGGGGT
GACGAGGGAACATGGGCTGGCAGATGGGGTTTCAATTTCGAAACAGGAAATGTGTTATTTCTAACTGAGGAAG
GCCACGATCCTCAAACAGGCGAGGTGTTCGTTACTTTGGGTACAGAGGGATCAGGTTTGCCAATTGTTCCCCA
AGTGAGTAGCATTCATGACATGTTGTGGGCAGCTGGTGAAGTTGGTGTTGGTTCCGAGCAGGAAGGCGCCAAA
GTGGAGTTCTCCCCTTCCATGGCTGGTTTCCTTGACTGGGGCTTTTCTGCATACGCAGCCGCTGGTAAAGTTT
TGCCTGCCTCATCCGCAGTTTCTAAGACATCTGGTGTTGAAGTTGACAGATATGTCTCCTTCGTTTGGTTGAC
TGGTGACCAGTATGAGCAAGCTGACGGTTTTCCAACCGCTCAGCAAGGATGGACAGGTTCCTTGTTATTGCCA
CGTGAGCTTAAGGTCCAAACTGTTGAAAACGTGGTTGACAACGAACTGGTGCGTGAAGAGGGTGTTTCCTGGG
TCGTTGGTGAGAGTGATAACCAGACTGCCAGGTTACGAACTTTAGGTATCACTATCGCTAGAGAAACAAAAGC
TGCATTGTTGGCAAACGGTTCCGTGACAGCTGAGGAGGATAGAACCCTACAAACCGCTGCTGTTGTTCCATTC
GCTCAGTCACCATCGTCCAAGTTTTTCGTTTTGACGGCTCAGCTTGAATTTCCTGCTTCTGCTAGATCATCAC
CTCTGCAATCCGGTTTTGAAATTCTCGCTTCGGAATTGGAGAGAACCGCTATCTACTACCAATTTTCTAACGA
AAGCCTAGTGGTTGACCGTTCTCAGACTAGCGCAGCAGCTCCAACTAATCCTGGTCTTGATTCGTTCACAGAA
TCTGGTAAGCTGAGACTTTTCGATGTCATCGAAAATGGTCAAGAGCAAGTCGAGACATTAGACTTGACGGTAG
TGGTAGACAACGCTGTGGTCGAAGTCTATGCTAATGGAAGATTTGCTTTGTCTACTTGGGCCAGGTCATGGTA
TGATAATTCCACACAAATTCGTTTCTTTCATAACGGAGAGGGAGAGGTACAGTTTAGAAATGTTAGCGTTAGT
GAAGGTCTGTACAATGCATGGCCAGAACGTAACTAA
```

SEQ ID No. 2: DNA for parent enzyme fopA (codon-optimised by DNA 2.0), with Geneart® codon-optimised *xln2* secretion signal and his-tagging sequence

ATGGTTTCTTTCACATCCTTGTTGGCTGGTGTTGCTGCTATTTCCGGTGTTTTGGCTGCTCCAGCTGCTGAAG
TTGAATCCGTTGCTGTTGAGAAGAGATCCTACCACTTGGATACTACTGCTCCACCACCAACTAACTTGTCCAC
TTTGCCAAACAACACTTTGTTCCATGTTTGGAGACCAAGAGCACACATTTTGCCAGCTGAGGGTCAAATTGGT
GATCCATGTGCTCACTACACTGACCCATCCACTGGTTTGTTTCATGTTGGTTTCTTGCACGACGGTGATGGTA
TTGCTGGTGCTACTACTGCTAACTTGGCTACTTACACTGACACTTCCGACAACGGTTCCTTCTTGATTCAACC
TGGTGGAAAGAACGATCCAGTTGCTGTTTTCGACGGTGCTGTTATCCCAGTTGGTGTTAACAACACTCCAACT
TTGTTGTACACTTCCGTTTCTTTCTTGCCAATCCACTGGTCCATTCCATACACTAGAGGTTCCGAGACTCAAT
CTTTGGCTGTTGCTAGAGATGGTGGTAGAAGATTCGACAAGTTGGACCAGGGTCCAGTTATTGCTGATCACCC
ATTCGCTGTTGACGTTACTGCTTTCAGAGATCCATTCGTTTTCAGATCCGCTAAGTTGGACGTTTTGTTGTCC
TTGGACGAGGAAGTTGCTAGAAACGAGACTGCTGTTCAACAAGCTGTTGACGGATGGACTGAAAAGAACGCTC
CTTGGTACGTTGCTGTTTCTGGTGGTGTTCATGGTGTTGGTCCAGCTCAGTTCTTGTACAGACAGAACGGTGG
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GATGAGGGAACTTGGGCTGGTAGATGGGGTTTCAACTTCGAGACTGGTAACGTTTTGTTCTTGACTGAAGAGG
GTCACGATCCACAAACTGGTGAAGTTTTCGTTACTTTGGGTACTGAAGGTTCCGGATTGCCAATTGTTCCTCA
GGTTTCCTCCATTCACGATATGTTGTGGGCTGCTGGTGAAGTTGGTGTTGGTTCTGAACAAGAGGGTGCTAAG
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TGCCAGCTTCTTCTGCTGTTTCCAAGACTTCCGGTGTTGAGGTTGACAGATACGTTTCCTTTGTTTGGTTGAC
TGGTGACCAATACGAACAAGCTGACGGTTTTCCAACTGCTCAACAGGGATGGACTGGTTCTTTGTTGTTGCCA
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TTGTCGGTGAATCCGACAATCAGACTGCTAGATTGAGAACTTTGGGTATCACTATCGCTAGAGAGACTAAGGC
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GCTCAATCTCCATCCTCCAAGTTCTTCGTTTTGACTGCTCAGTTGGAGTTTCCAGCTTCTGCTAGATCCTCTC
CATTGCAATCCGGTTTCGAGATTTTGGCTTCCGAGTTGGAGAGAACTGCTATCTACTACCAGTTCTCCAACGA
GTCTTTGGTTGTTGACAGATCCCAAACTTCTGCTGCTGCTCCAACTAACCCAGGATTGGACTCTTTCACTGAG
TCCGGTAAGTTGAGATTGTTCGACGTTATCGAGAACGGTCAAGAGCAAGTTGAGACTTTGGACTTGACTGTTG
TTGTTGATAACGCTGTTGTTGAGGTTTACGCTAACGGTAGATTCGCTTTGTCTACTTGGGCTAGATCCTGGTA
CGACAACTCCACTCAGATCAGATTCTTCCACAACGGTGAAGGTGAAGTTCAGTTCAGAAACGTTTCCGTTTCC
GAGGGTTTGTACAACGCTTGGCCAGAGAGAAACTAA

SEQ ID No. 3: Protein sequence for mature parent enzyme FopA

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 4: Variant 1 - F140Y-A178P-G321N-Q490S, crystal structure numbering (F121Y-A159P-G302N-Q471S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 5: Variant 2 - F140Y-G321N-Q490S, crystal structure numbering (F121Y-G302N-Q471S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 6: Variant 3 - G321N-F140Y-A178P, crystal structure numbering (G302N-F121Y-A159P)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 7: Variant 4 - G321N-Q490S-A178P. crystal structure numbering (G302N-Q471S-A159P)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 8: Variant 5 - F140Y-Q490S-A178P, crystal structure numbering (F121Y-Q471 S-A159P)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 9: Variant 6 - G321N-F140Y, crystal structure numbering (G302N-F121 Y)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 1 0: Variant 7 - G321N-Q490S, crystal structure numbering (G302N-Q471S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 11: Variant 8 - G321N-A178P, crystal structure numbering (G302N-A159P)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 12: Variant 9 - F140Y-Q490S, crystal structure numbering (F121Y-Q471S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 13: Variant 10 - F140Y-A178P, crystal structure numbering (F121Y-A159P)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 14: Variant 11 - Q490S-A178P, crystal structure numbering (Q471S-A159P)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVIPDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 15: Variant 12 - G321N-F140Y-Q490S-A178S, crystal structure numbering (G302N-F121Y-Q471S-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 16: Variant 14 - G321N-F140Y-A178S, crystal structure numbering (G302N-F121Y-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 17: Variant 15 - G321N-Q490S-A178S, crystal structure numbering (G302N-Q471S-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL

ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 18: Variant 16 - F140Y-Q490S-A178S, crystal structure numbering (F121Y-Q471S-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 19: Variant19 - G321N-A178S, crystal structure numbering (G302N-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSNLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 20: Variant 21 - F140Y-A178S, crystal structure numbering (F121Y-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSYLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 21: Variant 22 - Q490S-A178S, crystal structure numbering (Q471S-A159S)

SYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANL
ATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIPYTRGSETQSLAVARDGG
RRFDKLDQGPVISDHPFAVDVTAFRDPFVFRSAKLDVLLSLDEEVARNETAVQQAVDGWTEKNAPWYVAVSGG
VHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEV
FVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSK
TSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELKVQTVENVVDNELVREEGVSWVVGESDNQT
ARLRTLGITIARETKAALLANGSVTAEEDRTLSTAAVVPFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEIL
ASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLTVVVDNAVVEV
YANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVSEGLYNAWPERN*

SEQ ID No. 25: *Xln2* secretion signal
MVSFTSLLAGVAAISGVLAAPAAEVESVAVEKR

SEQ ID No. 26: Protein sequence for parent enzyme FopA including synthetic *Xln2* secretion signal

MVSFTSLLAGVAAISGVLAAPAAEVESVAVEKRSYHLDTTAPPPTNLSTLPNNTLFHVWRPRAHILPAEGQIG
DPCAHYTDPSTGLFHVGFLHDGDGIAGATTANLATYTDTSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPT
LLYTSVSFLPIHWSIPYTRGSETQSLAVARDGGRRFDKLDQGPVIADHPFAVDVTAFRDPFVFRSAKLDVLLS
LDEEVARNETAVQQAVDGWTEKNAPWYVAVSGGVHGVGPAQFLYRQNGGNASEFQYWEYLGEWWQEATNSSWG
DEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEVFVTLGTEGSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAK
VEFSPSMAGFLDWGFSAYAAAGKVLPASSAVSKTSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWIGSLLLP
RELKVQTVENVVDNELVREEGVSWVVGESDNQTARLRTLGITIARETKAALLANGSVTAEEDRTLQTAAVVPF
AQSPSSKFFVLTAQLEFPASARSSPLQSGFEILASELERTAIYYQFSNESLVVDRSQTSAAAPTNPGLDSFTE
SGKLRLFDVIENGQEQVETLDLTVVVDNAVVEVYANGRFALSTWARSWYDNSTQIRFFHNGEGEVQFRNVSVS
EGLYNAWPERN

Claims

1. A modified polypeptide having fructofuranosidase activity, wherein the modified polypeptide comprises an amino acid sequence which is at least 90% identical to SEQ ID NO: 3 and has at least one of the following amino acid substitutions:

   the phenylalanine (F) at amino acid position 121 is substituted by tyrosine (Y);
   the alanine (A) at amino acid position 159 is substituted by proline (P) or serine (S);
   the glycine (G) at amino acid position 302 is substituted by asparagine (N), aspartic acid (D), tyrosine (Y) or glutamic acid (E); and/or
   the glutamine (Q) at amino acid position 471 is substituted by serine (S), lysine (K) or asparagine (N).

2. The modified polypeptide according to claim 1, which further includes a secretion signal at its 5' end, the secretion signal having an amino acid sequence which is at least 90% identical to SEQ ID NO: 25, and which optionally further comprises
   an amino acid sequence which is at least 90% identical to SEQ ID NO: 26 and which has at least one amino acid substitution at position 154, 192, 335 and/or 504 of SEQ ID NO: 26.

3. The modified polypeptide according to either one of claims 1 or 2, which has at least two of said amino acid substitutions at any of amino acid positions 121, 159, 302 and/or 471 of SEQ ID NO: 3, more preferably at least three of said amino acid substitutions at positions 121, 159, 302 and/or 471 of SEQ ID NO: 3, or more preferably amino acid substitutions at all of positions 121, 159, 302 and 471 of SEQ ID NO: 3.

4. The modified polypeptide according to any one of claims 1 to 3, which includes the following modifications:

   a. F121Y-A159P-G302N-Q471S;
   b. F121Y-A159P-G302N;
   c. A159P-G302N-Q471S;
   d. F121Y-A159P-Q471S; or

e. A159P-G302N.

5. The modified polypeptide according to any one of claims 1 to 4, which comprises an amino acid sequence of any one of SEQ ID NOS: 4 - 21.

6. The modified polypeptide according to any one of claims 1 to 5, which comprises amino acid sequence SEQ ID NO: 4.

7. A polynucleotide which encodes a modified polypeptide as claimed in any one of claims 1 to 6.

8. The polynucleotide according to claim 7, which comprises a nucleotide sequence which is at least 90% identical to the sequence of nucleotides 100 to 2007 of SEQ ID NO: 1 or SEQ ID NO: 2, or a complement thereof, wherein SEQ ID NO: 1 or SEQ ID NO: 2 has been modified so that the polynucleotide encodes a modified polypeptide as claimed in any one of claims 1 to 6.

9. The polynucleotide according to claim 7 or 8, which includes a *T. reesei* endoxylanase 2 *(xln2)* secretion signal, optionally wherein the secretion signal is encoded by nucleotides 1-99 of SEQ ID NO: 1 or 2.

10. A vector comprising a polynucleotide of any one of claims 7 to 9.

11. A transformed host cell comprising a vector of claim 10, wherein the transformed host cell is preferably a yeast, bacterium or fungus, such as *Pichia pastoris*.

12. A process for producing a modified polypeptide of any one of claims 1 to 6, the process comprising the steps of transforming a host cell with a polynucleotide of any one of claims 7 to 9 and causing the polypeptide to be expressed, optionally further comprising the step of recovering the polypeptide from the transformed host cell or a supernatant into which the polypeptide has been secreted.

13. The process according to claim 12, wherein the host cell is a *Pichia pastoris* cell.

14. The process for producing a fructooligosaccharide (FOS), the process comprising the steps of contacting sucrose with a modified polypeptide of any one of claims 1 to 6 under conditions which cause the sucrose to be converted into one or more fructooligosaccharides, optionally
wherein the transformed host cell of claim 11 is brought into contact with the sucrose and the polypeptide is caused to be expressed.

15. The process according to any one of claims 12 to 14, wherein the fructooligosaccharides include 1-kestose (GF2), nystose (GF3) and/or β-fructofuranosyl nystose (GF4).

Patentansprüche

1. Modifiziertes Polypeptid, welches eine Fructofuranosidase-Aktivität aufweist, wobei das modifizierte Polypeptid eine Aminosäuresequenz umfasst, die mindestens zu 90 % identisch mit SEQ ID NO: 3 ist und mindestens eine der folgenden Aminosäuresubstitutionen aufweist:

   das Phenylalanin (F) in der Aminosäureposition 121 ist durch Tyrosin (Y) substituiert;
   das Alanin (A) in der Aminosäureposition 159 ist durch Prolin (P) oder Serin (S) substituiert;
   das Glycin (G) in der Aminosäureposition 302 ist durch Asparagin (N), Asparaginsäure (D), Tyrosin (Y) oder Glutaminsäure (E) substituiert; und/oder
   das Glutamin (Q) in der Aminosäureposition 471 ist durch Serin (S), Lysin (K) oder Asparagin (N) substituiert.

2. Modifiziertes Polypeptid nach Anspruch 1, das ferner ein Sekretionssignal an seinem 5'-Ende enthält, wobei das Sekretionssignal eine Aminosäuresequenz aufweist, die mindestens zu 90 % identisch mit SEQ ID NO: 25 ist, und gegebenenfalls ferner eine Aminosäuresequenz umfasst, die mindestens zu 90 % identisch mit SEQ ID NO: 26 ist und die mindestens eine Aminosäuresubstitution in den Positionen 154, 192, 335 und/oder 504 von SEQ ID NO: 26 aufweist.

3. Modifiziertes Polypeptid nach einem der Ansprüche 1 oder 2, das mindestens zwei der Aminosäure-Substitutionen

an einer beliebigen der Aminosäurepositionen 121, 159, 302 und/oder 471 von SEQ ID NO: 3 aufweist, bevorzugter mindestens drei der Aminosäuresubstitutionen an den Positionen 121, 159, 302 und/oder 471 von SEQ ID NO: 3 aufweist oder stärker bevorzugt Aminosäuresubstitutionen an allen Positionen 121, 159, 302 und 471 von SEQ ID NO: 3 aufweist.

4. Modifiziertes Polypeptid nach einem der Ansprüche 1 bis 3, welches die folgenden Modifikationen enthält:

   a. F121Y-A159P-G302N-Q471S;
   b. F121Y-A159P-G302N;
   c. A159P-G302N-Q471S;
   d. F121Y-A159P-Q471S; oder
   e. A159P-G302N.

5. Modifiziertes Polypeptid nach einem der Ansprüche 1 bis 4, das eine Aminosäuresequenz nach einer der SEQ ID NOS: 4 bis 21 umfasst.

6. Modifiziertes Polypeptid nach einem der Ansprüche 1 bis 5, das die Aminosäuresequenz SEQ ID NO: 4 umfasst.

7. Polynukleotid, das ein modifiziertes Polypeptid nach einem der Ansprüche 1 bis 6 kodiert.

8. Polynukleotid nach Anspruch 7, das eine Nukleotidsequenz umfasst, die mindestens zu 90 % identisch mit der Sequenz der Nukleotide 100 bis 2007 von SEQ ID NO: 1 oder SEQ ID NO: 2 oder einem Komplement davon ist, wobei SEQ ID NO: 1 oder SEQ ID NO: 2 derart modifiziert wurde, dass das Polynukleotid ein modifiziertes Polypeptid nach einem der Ansprüche 1 bis 6 kodiert.

9. Polynukleotid nach Anspruch 7 oder 8, das ein *T.-reesei*-Endoxylanase-2-(*xln2*)-Sekretionssignal enthält, wobei wahlweise das Sekretionssignal der Nucleotide 1 bis 99 von SEQ ID NO: 1 oder 2 kodiert wird.

10. Vektor, der ein Polynukleotid nach einem der Ansprüche 7 bis 9 umfasst.

11. Transformierte Wirtszelle, die einen Vektor von Anspruch 10 umfasst, wobei die transformierte Wirtszelle vorzugsweise eine Hefe, ein Bakterium oder ein Pilz wie *Pichia pastoris* ist.

12. Verfahren zum Herstellen eines modifizierten Polypeptids nach einem der Ansprüche 1 bis 6, wobei das Verfahren die folgenden Schritte umfasst: Transformieren einer Wirtszelle mit einem Polynukleotid nach einem der Ansprüche 7 bis 9 und Bewirken, dass das Polypeptid exprimiert wird, wahlweise ferner umfassend den Schritt des Gewinnens des Polypeptids aus der transformierten Wirtszelle oder einem Überstand, in den das Polypeptid sekretiert wurde.

13. Verfahren nach Anspruch 12, wobei die Wirtszelle eine *Pichia-pastoris*-Zelle ist.

14. Verfahren zum Herstellen eines Fructooligosaccharides (FOS), wobei das Verfahren die Schritte des Kontaktierens der Saccharose mit einem modifizierten Polypeptid nach einem der Ansprüche 1 bis 6 unter Bedingungen umfasst, die veranlassen, dass die Saccharose in ein oder mehrere Fructooligosaccharid(e) umgewandelt wird, wobei wahlweise die transformierte Wirtszelle nach Anspruch 11 mit der Saccharose in Kontakt gebracht wird und das Polypeptid exprimiert wird.

15. Verfahren nach einem der Ansprüche 12 bis 14, wobei die Fructooligosaccharide 1-Kestose (GF2), Nystose (GF3) und/oder β-Fructofuranosylnystose (GF4) enthalten.

Revendications

1. Polypeptide modifié présentant une activité fructofuranosidase, le polypeptide modifié comprenant une séquence d'acides aminés identique à au moins 90 % à la SEQ ID NO: 3 et comportant au moins l'une des substitutions d'acides aminés suivantes :

   la phénylalanine (F) au niveau de la position 121 de l'acide aminé est substituée par la tyrosine (Y) ;
   l'alanine (A) au niveau de la position 159 de l'acide aminé est substituée par la proline (P) ou la sérine (S) ;

la glycine (G) au niveau de la position 302 de l'acide aminé est substituée par l'asparagine (N), l'acide aspartique (D), la tyrosine (Y) ou l'acide glutamique (E) ; et/ou
la glutamine (Q) au niveau de la position 471 de l'acide aminé est substituée par la sérine (S), la lysine (K) ou l'asparagine (N).

2. Polypeptide modifié selon la revendication 1, qui comprend en outre un signal de sécrétion au niveau de son extrémité 5', le signal de sécrétion comportant une séquence d'acides aminés qui est identique à au moins 90 % à la SEQ ID NO: 25, et qui comprend éventuellement en outre
une séquence d'acides aminés qui est identique à au moins 90 % à la SEQ ID NO: 26 et qui comporte au moins une substitution d'acides aminés au niveau de la position 154, 192, 335 et/ou 504 de la SEQ ID NO: 26.

3. Polypeptide modifié selon l'une quelconque des revendications 1 ou 2, qui comporte au moins deux desdites substitutions d'acides aminés au niveau de l'une quelconque des positions d'acides aminés 121, 159, 302 et/ou 471 de la SEQ ID NO: 3, plus préférablement au moins trois desdites substitutions d'acides aminés au niveau des positions 121, 159, 302 et/ou 471 de SEQ ID NO: 3, ou plus préférablement des substitutions d'acides aminés au niveau de toutes les positions 121, 159, 302 et 471 de la SEQ ID NO: 3.

4. Polypeptide modifié selon l'une quelconque des revendications 1 à 3, qui comprend les modifications suivantes :

a. F121Y-A159P-G302N-Q471S ;
b. F121Y-A159P-G302N ;
c. A159P-G302N-Q471S ;
d. F121Y-A159P-Q471S ; ou
e. A159P-G302N.

5. Polypeptide modifié selon l'une quelconque des revendications 1 à 4, qui comprend une séquence d'acides aminés selon l'une quelconque des SEQ ID NOS: 4 - 21.

6. Polypeptide modifié selon l'une quelconque des revendications 1 à 5, qui comprend la séquence d'acides aminés SEQ ID NO: 4.

7. Polynucléotide qui code un polypeptide modifié selon l'une quelconque des revendications 1 à 6.

8. Polynucléotide selon la revendication 7, qui comprend une séquence nucléotidique qui est identique à au moins 90 % à la séquence des nucléotides 100 à 2007 de la SEQ ID NO: 1 ou la SEQ ID NO: 2, ou un complément de celle-ci, la SEQ ID NO: 1 ou la SEQ ID NO: 2 ayant été modifiées pour que le polynucléotide code un polypeptide modifié selon l'une quelconque des revendications 1 à 6.

9. Polynucléotide selon la revendication 7 ou 8, qui comprend un signal de sécrétion de T. *reesei* endoxylanase 2 (*xln2*), le signal de sécrétion étant éventuellement codé par les nucléotides 1 à 99 de la SEQ ID NO: 1 ou 2.

10. Vecteur comprenant un polynucléotide selon l'une quelconque des revendications 7 à 9.

11. Cellule hôte transformée comprenant un vecteur selon la revendication 10, la cellule hôte transformée étant de préférence une levure, une bactérie ou un champignon, tel que *Pichia pastoris.*

12. Procédé de production d'un polypeptide modifié selon l'une quelconque des revendications 1 à 6, le procédé comprenant les étapes de transformation d'une cellule hôte à l'aide d'un polynucleotide selon l'une quelconque des revendications 7 à 9 et amenant le polypeptide à s'exprimer, comprenant éventuellement en outre l'étape de récupération du polypeptide de la cellule hôte transformée ou d'un surnageant dans lequel le polypeptide a été sécrété.

13. Procédé selon la revendication 12, dans lequel la cellule hôte est une cellule de *Pichia pastoris.*

14. Procédé de production d'un fructooligosaccharide (FOS), le procédé comprenant les étapes de mise en contact du saccharose avec un polypeptide modifié selon l'une quelconque des revendications 1 à 6 dans des conditions qui amènent le saccharose à se convertir en un ou plusieurs fructooligosaccharides, la cellule hôte transformée selon la revendication 11 étant éventuellement mise en contact avec le saccharose et le polypeptide étant amené à s'exprimer.

15. Procédé selon l'une quelconque des revendications 12 à 14, dans lequel les fructooligosaccharides incluent le 1-kestose (GF2), le nystose (GF3) et/ou le β-fructofuranosyl nystose (GF4).


RE 1.0 RSA 64
----------SYHLDTTAPPPTNLSTLPNNTL 41
FHVWRPRAHILPAEGQIGDPCAHYTDPSTGLFHVGFLHDGDGIAGATTANLATYTD 97
RE 1.5 RSA 79
RE 2 RSA 50
TSDNGSFLIQPGGKNDPVAVFDGAVIPVGVNNTPTLLYTSVSFLPIHWSIFYTRG 152
RE 1.7 RSA 55
RE 1.2 RSA 65
SETQSLAVARDGGRRFDKLDQGPVLADHPFAVDVTAFRDPFVFRSAKLDVLLSLD 207
RE 2.0 RSA 67
EEVARNETAVQQAVDGWTEKNAPWYVAVSGGVHGVGPAQFLYRQNGGNASEFQYW 262
EYLGEWWQEATNSSWGDEGTWAGRWGFNFETGNVLFLTEEGHDPQTGEVFVTLGTE 318
RE 2.1 RSA 56
GSGLPIVPQVSSIHDMLWAAGEVGVGSEQEGAKVEFSPSMAGFLDWGFSAYAAAG 373
RE 1.0 RSA 100
KVLPASSAVSKTSGVEVDRYVSFVWLTGDQYEQADGFPTAQQGWTGSLLLPRELK 428
RE 2.1 RSA 78
VQTVENVVDNELVREEGVSWVVGESDNQTARLRTLGITIARETKAALLANGSVTA 483
RE 2.2 RSA 96
RE 2.4 RSA 69
EEDRTLQTAAVVFFAQSPSSKFFVLTAQLEFPASARSSPLQSGFEILASELERTA 538
RE 1.7 RSA 56
IYYQFSNESLVVDRSQTSAAAPTNPGLDSFTESGKLRLFDVIENGQEQVETLDLT 593
RE 1.3 RSA 65
VVVDNAVVEVYANGRFALSTWARSNYDNSTQIRFFHNGEGEVQFRNVSVSEGLYN 648
AWPERN 654

Fig. 1

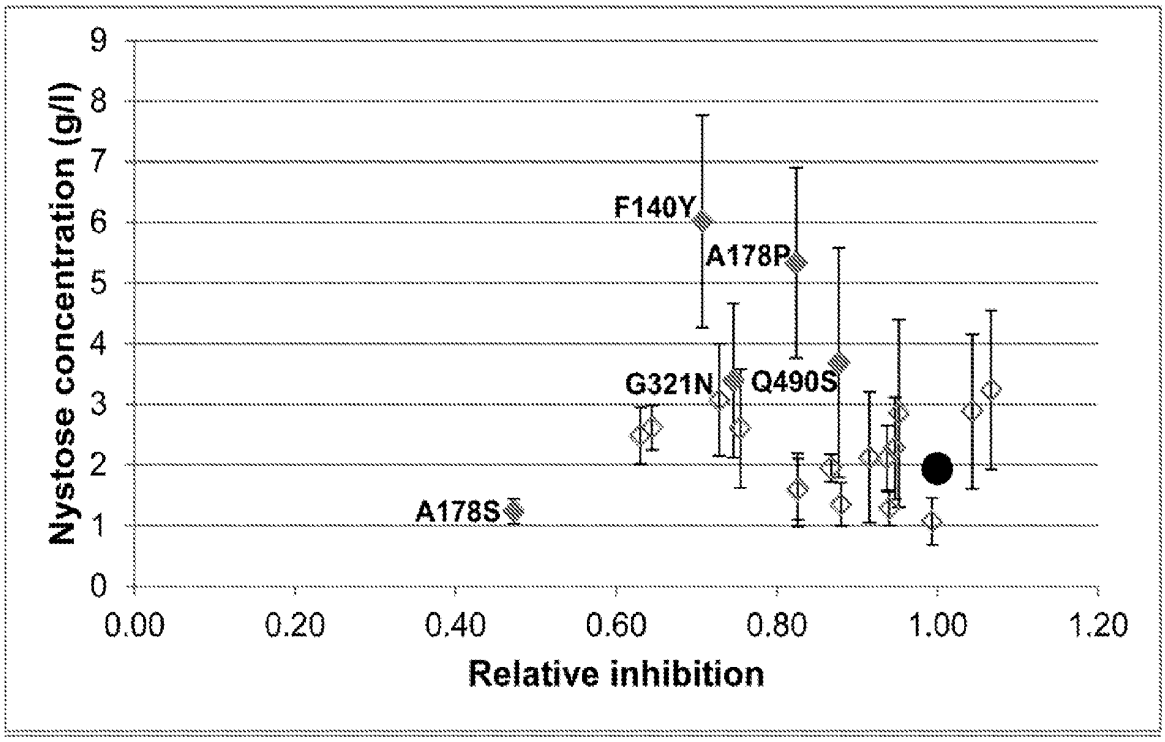
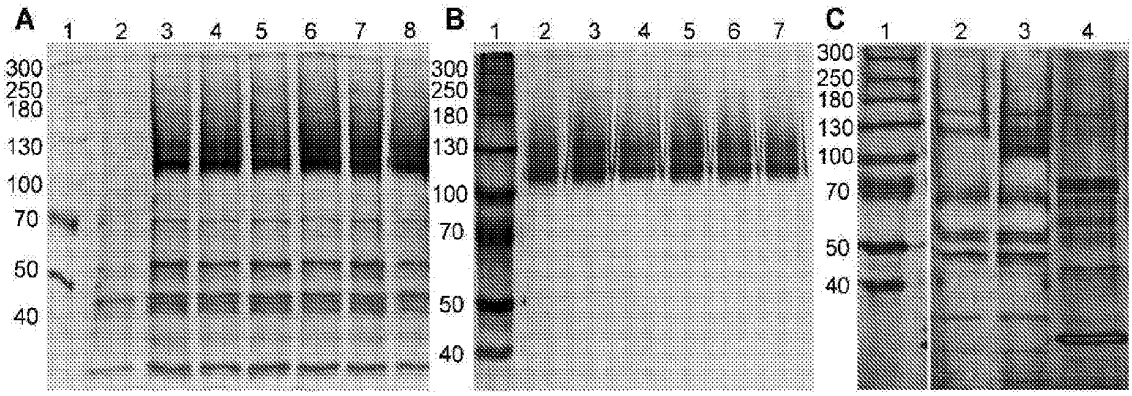

Nystose concentration (g/l)
9
8
7
6
5
4
3
2
1
0
F140Y
A178P
G321N
Q490S
A178S
0.00
0.20
0.40
0.60
0.80
1.00
1.20
Relative inhibition

Fig. 2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250
180
130
100
70
50
40
B
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70
50
40
C
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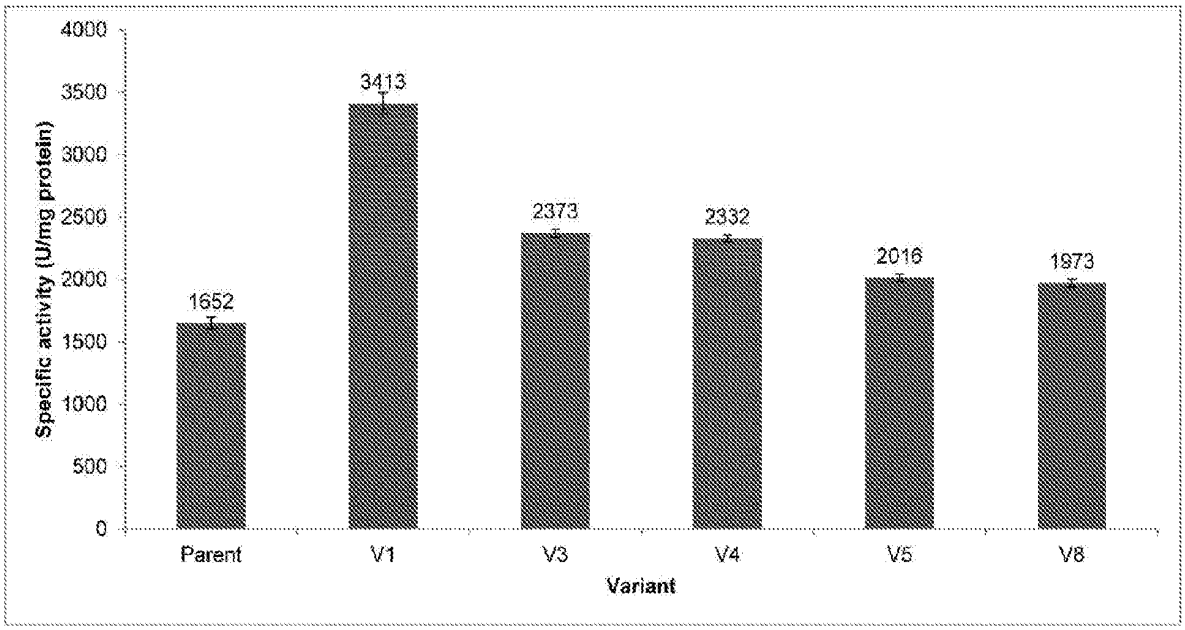
100
70
50
40

Fig. 3


Specific activity (U/mg protein)
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3000
2500
2000
1500
1000
500
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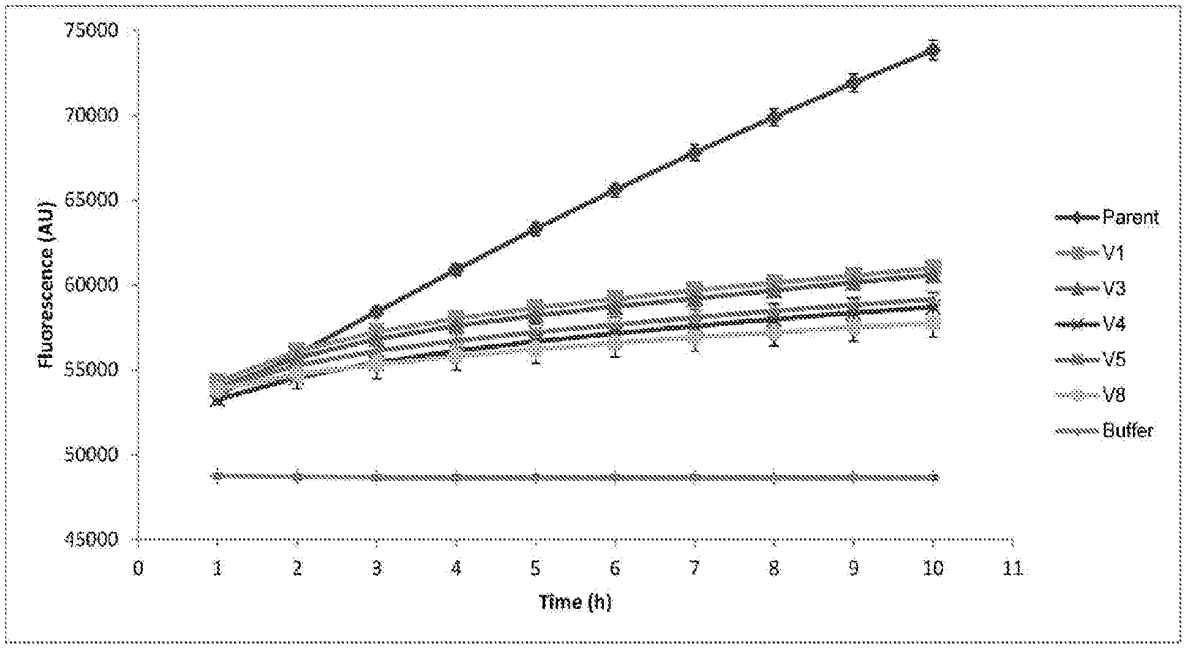
2373
2332
2016
1973
Parent
V1
V3
V4
V5
V8
Variant

Fig. 4


Fluorescence (AU)
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70000
65000
60000
55000
50000
45000
0
1
2
3
4
5
6
7
8
9
10
11
Time (h)
Parent
V1
V3
V4
V5
V8
Buffer

Fig. 5

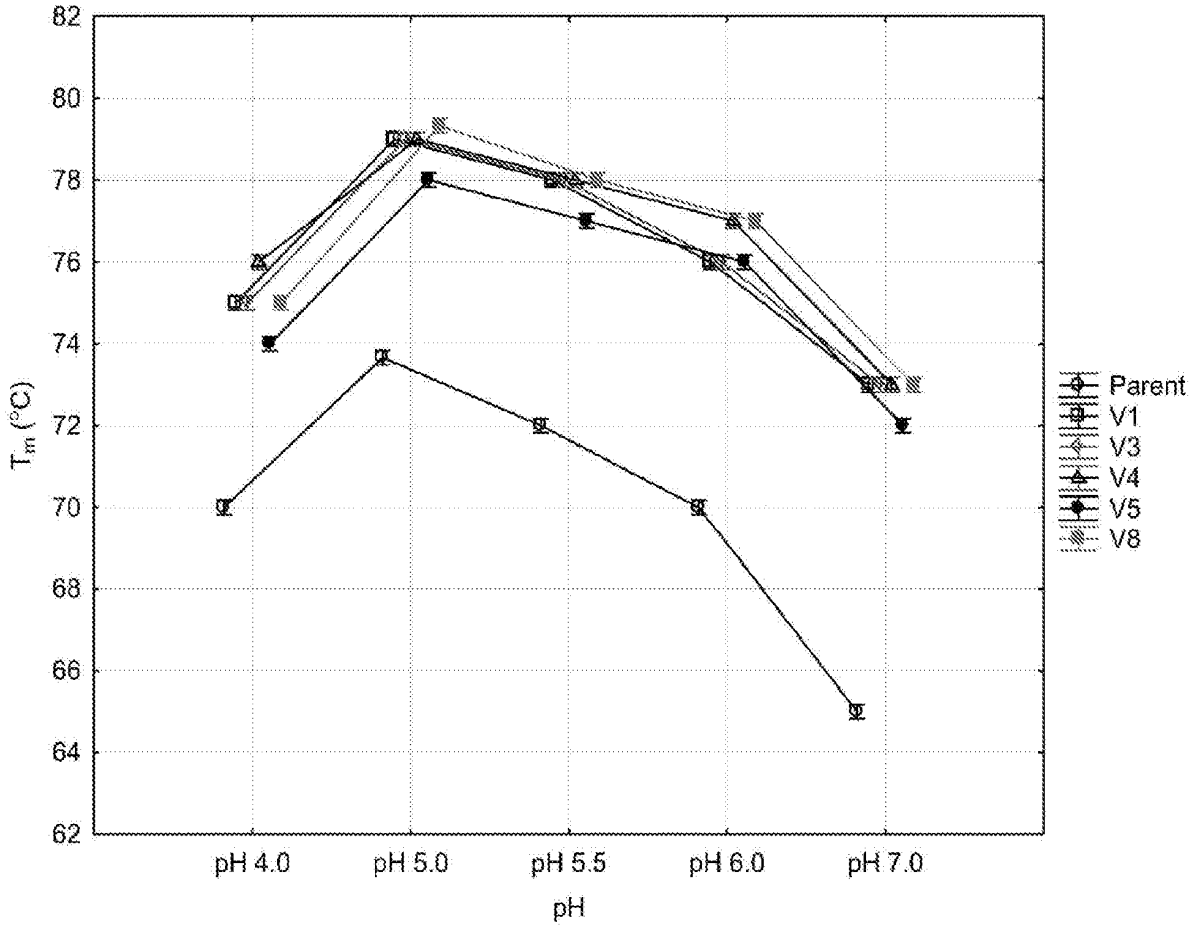
82
80
78
76
74
72
70
68
66
64
62
T_m (°C)
pH 4.0
pH 5.0
pH 5.5
pH 6.0
pH 7.0
pH
Parent
V1
V3
V4
V5
V8

Fig. 6

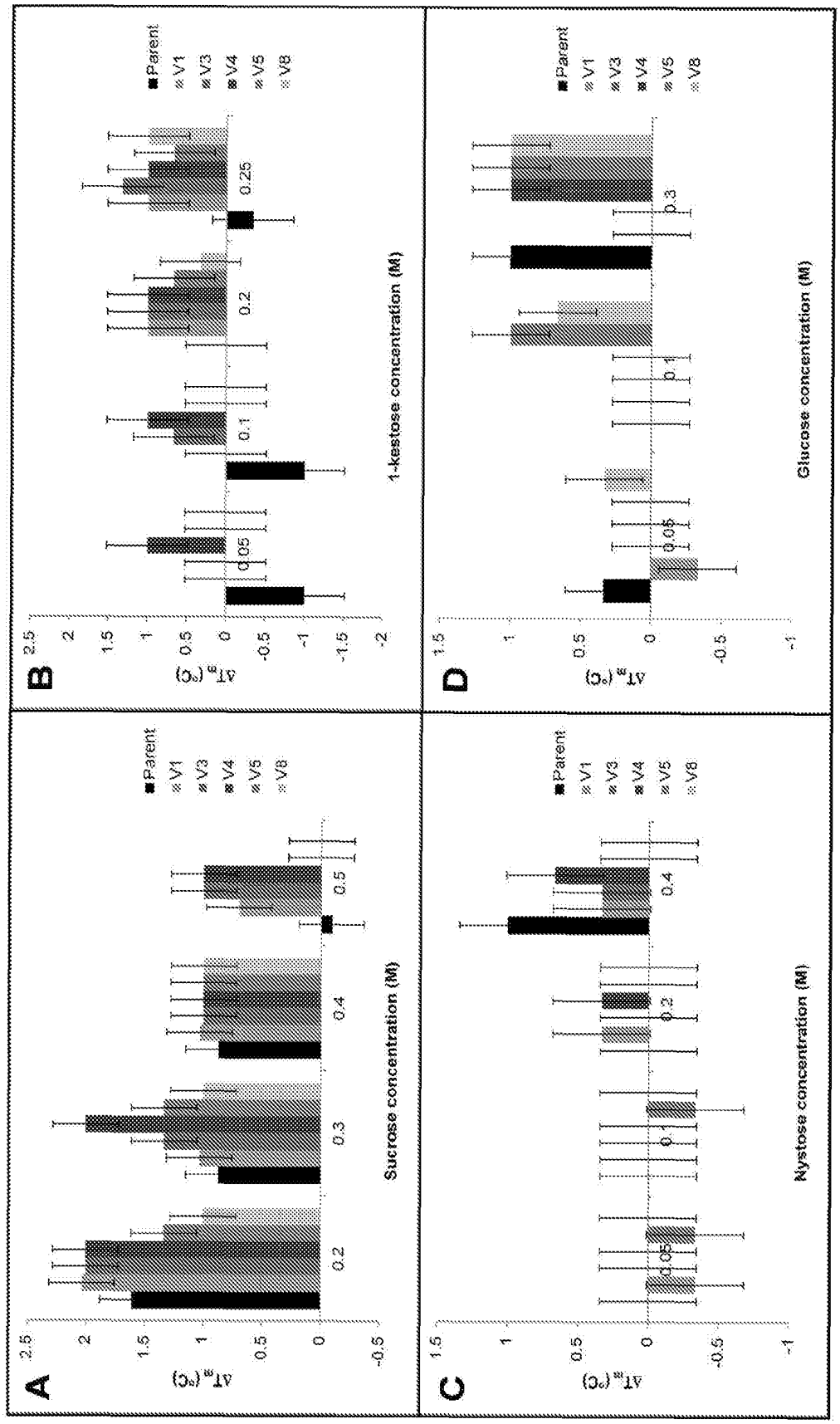
A
B
C
D
Parent
V1
V3
V4
V5
V8
ΔTm (°C)
Sucrose concentration (M)
1-kestose concentration (M)
Nystose concentration (M)
Glucose concentration (M)

Fig. 7

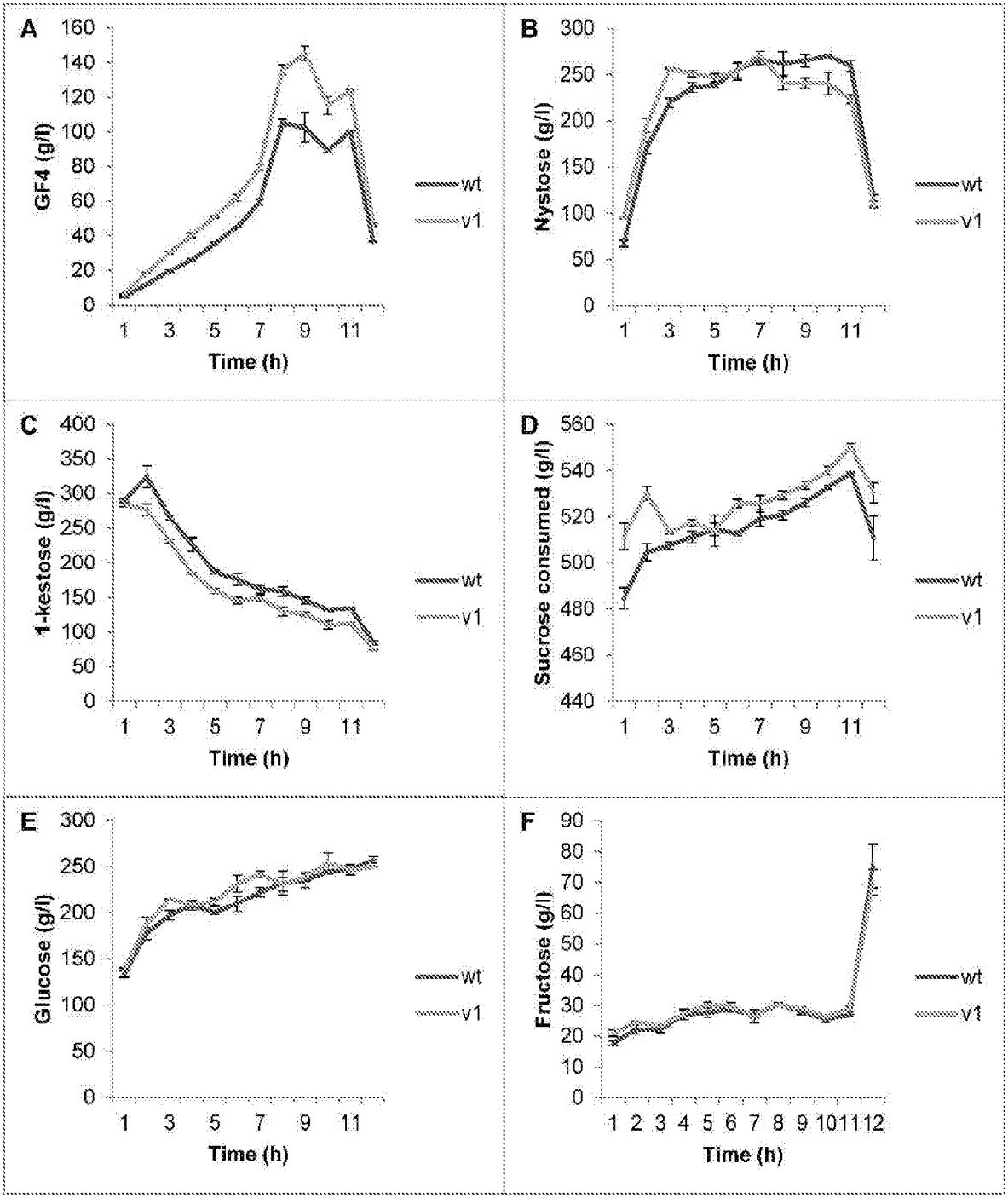
A
GF4 (g/l)
Time (h)
wt
v1
B
Nystose (g/l)
Time (h)
wt
v1
C
1-kestose (g/l)
Time (h)
wt
v1
D
Sucrose consumed (g/l)
Time (h)
wt
v1
E
Glucose (g/l)
Time (h)
wt
v1
F
Fructose (g/l)
Time (h)
wt
v1

Fig. 8

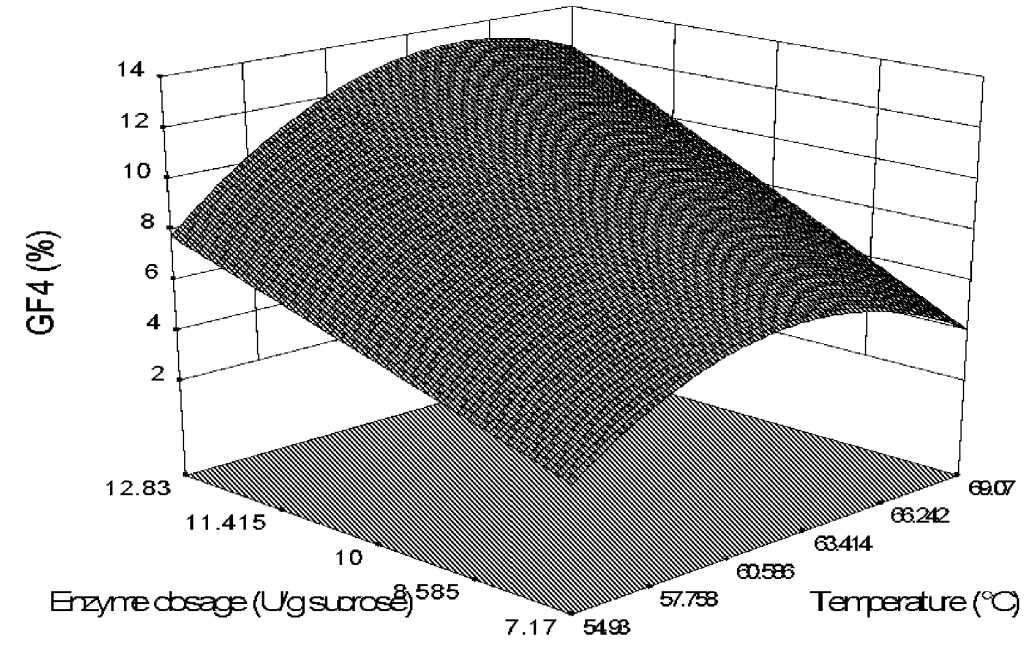
GF4 (%)
14
12
10
8
6
4
2
12.83
11.415
10
8.585
7.17
Enzyme dosage (U/g sucrose)
54.93
57.758
60.586
63.414
66.242
69.07
Temperature (°C)

Fig. 9

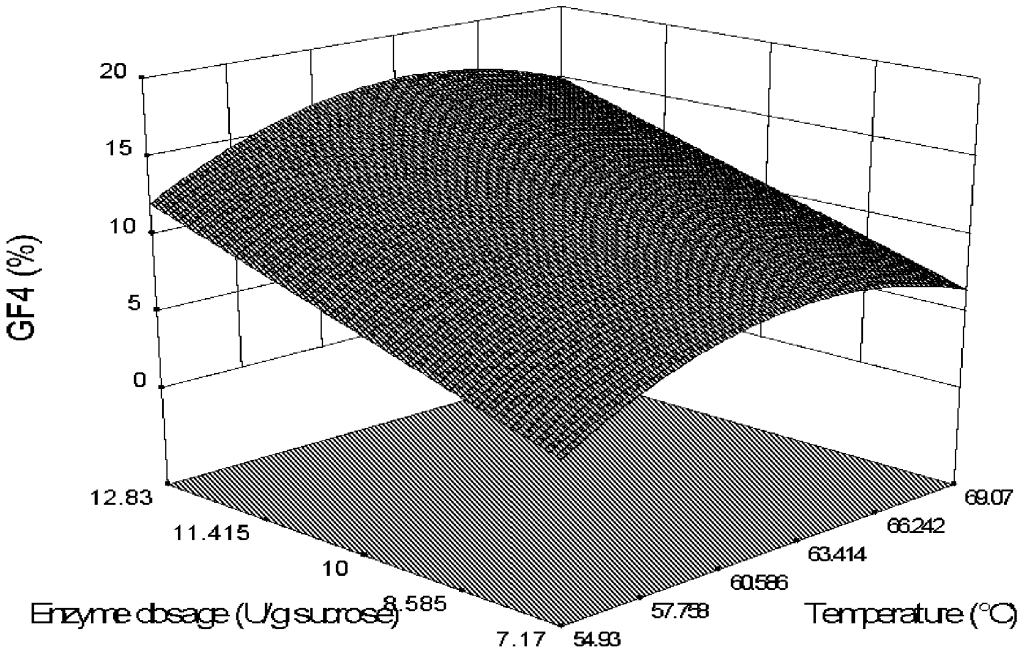
GF4 (%)
20
15
10
5
0
12.83
11.415
10
8.585
7.17
Enzyme dosage (U/g sucrose)
54.93
57.758
60.586
63.414
66.242
69.07
Temperature (°C)

Fig. 10

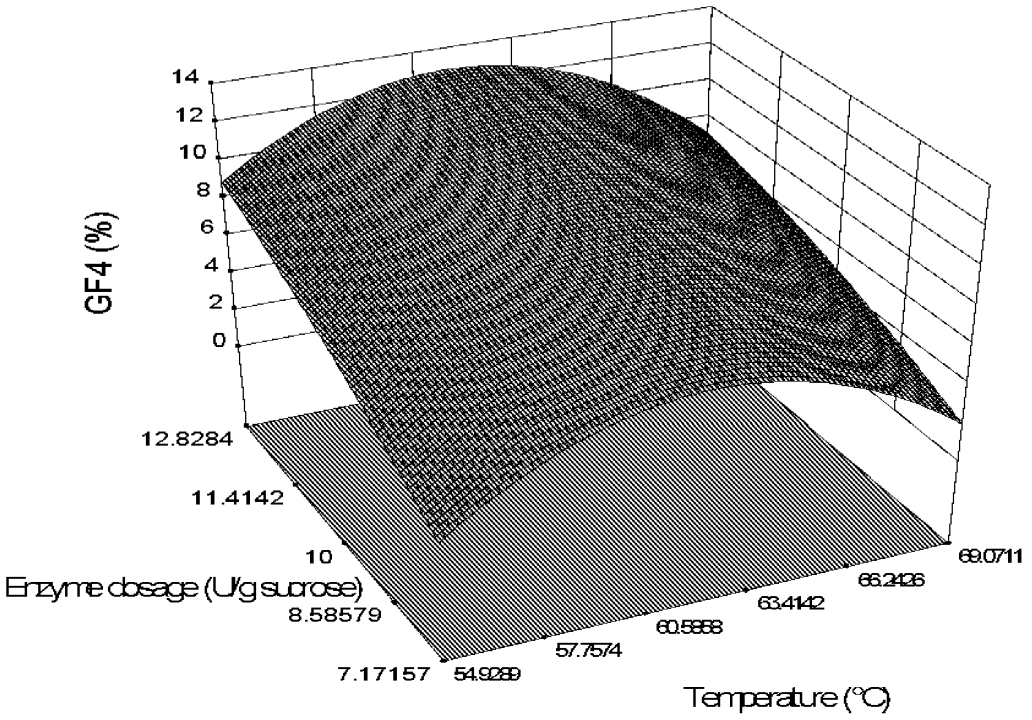
GF4 (%)
14
12
10
8
6
4
2
0
12.8284
11.4142
10
8.58579
7.17157
Enzyme dosage (U/g sucrose)
54.9289
57.7574
60.5858
63.4142
66.2426
69.0711
Temperature (°C)

Fig. 11

REFERENCES CITED IN THE DESCRIPTION

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