Thermostable glycosyltransferase variants

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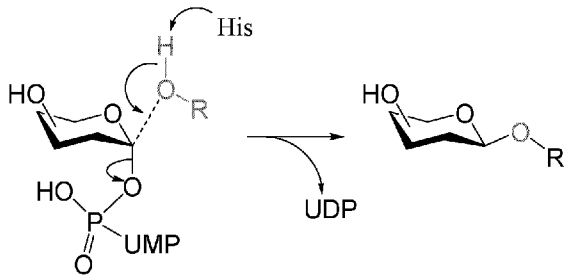
FIGURE 1



(57) Abstract: The present invention concerns glycosyltransferase enzyme mutants having improved half-lives and thermal stability compared to the parent enzyme UDP-glycosyltransferase (PtUGT) from the indigo producing plant Polygonum tinctorium/Persicaria tinctoria; and further provides a composition, kit, and methods employing these mutants for glycosylation of desired compounds, such as indoxyl compounds.

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TITLE: Thermostable glycosyltransferase variants

FIELD OF THE INVENTION

The present invention concerns enzyme mutants having improved temporal, thermal, and chemical stability, compared to the parent enzyme glycosyltransferase (*Pt*UGT1) from the indigo producing plant *Polygonum tinctorium*/*Persicaria tinctoria*; and their use in methods for glycosylation of desired compounds, such as indoxyl compounds and thereby providing a greener alternative to current industrial processes for colored fabrics and other products.

BACKGROUND OF THE INVENTION

Glycosyltransferases, such as UDP-glycosyltransferases (UGTs), can be used in biotech applications to attach a sugar molecule to a vast variety of industrial chemicals (*e.g.* fragrances, dyes, food additives), thereby enhancing their solubility and decreasing volatility and toxicity. This can also be used to enhance the bioavailability of pharmaceuticals. However, natural glycosyltransferases are not particularly stable, making them economically infeasible to use on industrial scale.

SUMMARY OF THE INVENTION

In one aspect, the present invention provides a **polypeptide** having **glycosyltransferase** activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75% sequence identity with SEQ ID NO. 2, and wherein said amino acid sequence comprises (i) one or more amino acid residue substitutions selected from: E75P, Q86K, S110V, I188L, G222D, G296L, V297G, F381V, T388A, S413K and G430K with respect to SEQ ID NO 2, and/or (ii) amino acid residue substitutions T388C and A399C with respect to SEQ ID NO 2.

In one preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75% sequence identity to SEQ ID NO.2, and wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, G430K, and T388A with respect to SEQ ID NO 2.

In another preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 2, and

wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, G430K, F381V and T388A with respect to SEQ ID NO 2.

In another preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, G430K, F381V, T388C, and A399C with respect to SEQ ID NO 2.

In a second aspect, the invention provides a **composition** comprising (i) a polypeptide of the present invention having glycosyltransferase enzyme, (ii) a compound comprising a reactive group, and (iii) a nucleotide sugar.

In a third aspect, the invention provides a **kit of parts** comprising (i) a polypeptide of the present invention having glycosyltransferase enzyme, and (ii) a polypeptide having beta-glucosidase enzyme activity (enzyme classification EC 3.2.1.21).

In a fourth aspect, the invention provides a **method for glycosylating a compound**, comprising the steps of

a. providing (i) a compound comprising a reactive group, (ii) a polypeptide of the present invention having glycosyltransferase enzyme, and (iii) a nucleotide sugar,
b. mixing the components provided in step (a)
c. letting the mixture react to obtain a glycosylated compound.

In a fifth aspect, the invention provides a **method for dying a product**, comprising the steps of

d. providing (i) an indoxyl compound, (ii) a polypeptide of the present invention having glycosyltransferase enzyme, (iii) a nucleotide sugar, and (iv) a polypeptide having beta-glucosidase enzyme activity (enzyme classification EC 3.2.1.21),
e. mixing components (i), (ii), and (iii) provided in step (a), preferably at reaction conditions wherein less than 2% free oxygen is present,
f. letting the mixture react to obtain a soluble glycosylated indoxyl dye-precursor,
g. mixing said dye precursor with said product and said beta-glucosidase under reaction conditions wherein free oxygen is present, to obtain a dyed textile.

wherein said product is elected from yarn, textiles, and fabrics,

In a sixth aspect, the invention provides **use** of a polypeptide of the present invention having glycosyltransferase enzyme, for glycosylating a compound, wherein said compound comprises a reactive group.

DESCRIPTION OF THE INVENTION

Brief description of the figures:

Figure 1: Scheme of glycosylation reactions catalyzed by UDP-glycosyltransferase.

Figure 2: (A) Current industrial process involving chemically synthesized indigo and addition of reducing agents (e.g. sodium dithionite) to the indigo vat for reduction to dye-competent, soluble leucoindigo. In the proposed chemo-enzymatic process, indoxyl is glucosylated at the C3 hydroxyl group giving indican as product. The glucoside, indican, is stable and can be stored. The glucosyl group is removed only on fabric, during the dyeing step, allowing the regenerated indoxyl to oxidize to indigo on the fabric. No reducing agent is required when dyeing with indican. BGL=β-glucosidase; UGT=UDP-dependent glycosyltransferase. **(B)** Glucose acts as a protecting group for indoxyl. Removal of the glucose by a β-glucosidase releases indoxyl which can be further oxidized by air exposure.

Figure 3: (A) and **(B)** Changes in the melting temperatures of mutants designed based on hypothetical disulfide bridge formation, in respect to *Pt*UGT1 WT.

Figure 4: Changes in the melting temperatures of double mutants and a triple mutant combining the different beneficial mutations based on hypothetical disulfide bridge formation, in respect to *Pt*UGT1 WT.

Figure 5: (A) and **(B)** Changes in the melting temperatures of mutants designed based on consensus mutagenesis, in respect to *Pt*UGT1 WT.

Figure 6: (A) and **(B)** Changes in the melting temperatures of mutants combining different beneficial mutations based on consensus mutagenesis, in respect to *Pt*UGT1 WT.

Figure 7: Relative activity of mutant 87, mutant 88 and mutant 90 at **(A)** 40°C, **(B)** 55°C, and **(C)** 60°C, in respect to *Pt*UGT1 WT activity at 40°C.

Figure 8: Relative activity of *Pt*UGT1 WT, mutant 87, mutant 88 and mutant 90 after pre-incubation for different period of time at 45°C, in respect to the activity without pre-incubation at 45°C.

Figure 9: Relative activity of *Pt*UGT1 WT, mutant 87, mutant 88 and mutant 90 after pre-incubation for different period of time at room temperature (22°C), in respect to the activity without pre-incubation at room temperature.

Figure 10: Relative activity at of *Pt*UGT1 WT, mutant 87, mutant 88 and mutant 90 in the presence of 15% organic solvent (either acetone, acetonitrile or isopropanol), in respect to the activity without organic solvents.

Figure 11: Reaction chromatograms showing the absence or presence of product DCP-glucoside in a reaction using 4 mM DCP and either *Pt*UGT1 WT or mutant 87 enzyme. The reaction with WT enzyme does not show any product, presumably due to low chemostability towards DCP. The reaction with mutant 87 shows a clear product peak, confirming the enhanced chemo-stability of this variant.

Figure 12: Kinetics of indican synthesis using 100 mM indoxyl-acetate as substrate, 2U of Esterase from *Bacillus subtilis* (Sigma Aldrich), and different concentrations of *Pt*UGT1/SuSy, at a constant molar ratio of 1:5 (50 µg, 20 µg, 10 µg, 5 µg for *Pt*UGT1 WT or Mut 87; and 432.5 µg, 173 µg, 86.7 µg, 43.3 µg for SuSy). The results clearly shows that higher concentrations of Mut 87 reached higher concentrations of indican, while the reactions using *Pt*UGT1 WT did not produce any indican, presumably due to chemical inactivation of the enzyme at high substrate concentration.

Figure 13: Discs of 20 square centimeters of ready-to-dye denims (radius 1.784 cm, diameter 3.57cm, weight 802+/- 2 mg) are dyed in 3 mL of water at pH 9 with various amounts of indican (indicated on the picture) and 1 mg of Rye β-glucosidase 1. Discs are turned over every 5 min at room temperature for 15 min, and left for 1 h at room temperature before being washed with water and soap and dried overnight at room temperature. Pictures are taken four days after dyeing and after multiple washing / drying cycles. (A) Front side of samples; (B) Back side of samples.

Figure 14: Indoxyl derivatives acceptors of *Pt*UGT1 WT and mutant variants.

Figure 15: Photo of color development in tubes comprising 6-Bromo-Indoxyl after 90 minutes incubation at 30°C. Tubes from left to right: Tube 1: (-) Control without UGT, Tube 2: (-) Control without UDP-Glc, Tube 3: (+) Control with *Pt*UGT1 WT, Tube 4: With *Pt*UGT1 Mut 87, Tube 5: With *Pt*UGT1 Mut 88, Tube 6: With *Pt*UGT1 Mut 90.

Figure 16: Photo of color development in tubes comprising 5-Bromo-4-chloro-indoxyl after 60 minutes incubation at 30°C. From left to right **(A)** Tube 1: (-) Control without UGT, Tube 2: With PtUGT1 WT, Tube 3: With Mut 87, Tube 4: With Mut 88. **(B)** Tube 1: (-) Control without UGT, Tube 2: With Mut 90.

Figure 17: Difference in the melting temperatures of prior art UGT enzymes compared to *Pt*UGT1 WT.

Figure 18: Chemostability. Reaction chromatograms showing the absence or presence of product DCP-glucoside in a reaction using 4 mM DCP and (a) *Pt*UGT1 WT, (b) *Pt*UGT2, (c) PtIGS, or (d) mutant 87 enzyme.

Abbreviations, terms, and definitions:

Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of similarity between two amino acid sequences of essentially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as ((Nref-Ndif)100)/(Nref), wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Sequence identity calculations are preferably automated using the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Sequence alignment may be performed using program MAFFT24 (Multiple Alignment using Fast Fourier Transform; Katoh et al 2019) using default parameters (SCORING MATRIX: blosum62, gap opening penalty: 1.53, gap extension penalty 0.123).

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: Proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine; Group 6: Histidine. Lysine, Arginine.

Melting temperature (*T*m (°C)) of a protein, as used herein, defines the temperature (*T*m) at which both the folded and unfolded states are equally populated at equilibrium (assuming two-state protein folding), which is the denaturation midpoint of the protein, and is measured by using a thermal shift assay, such as the Protein Thermal Shift Dye Kit (ThermoFisher Scientific) and a qPCR QuantStudio5 machine - see examples section.

Half/shelf-life times are defined as the amount of time that an enzyme can be pre-incubated at a defined temperature having as a result a 50% residual activity compared with the activity without the pre-incubation.

Indoxyl compound is herein defined as indoxyl, thioindoxyl, and any indoxyl or thioindoxyl derivative having an unprotected (reactive) thio or hydroxyl group in position 3. Indoxyl derivatives may comprise halogen substitution(s) on the ring structure. Examples of indoxyl derivatives include, but are not limited to: 6-Bromo-indoxyl, 5-Bromo-4-chloro-indoxyl, 6-Chloro-indoxyl, 5-bromo-indoxyl, 5-bromo-6-chloro-indoxyl, Thioindoxyl, 5-bromo-7-bromo-indoxyl.

Reactive group is herein defined as a chemical group that can be glycosylated by a glycosyltransferase enzyme.

Free oxygen is herein defined as molecular oxygen or dioxygen.

Dye precursor is herein defined as a compound that can give rise to dyed material upon one or more chemical transformations.

Nucleotide sugar is herein defined as a molecule in which a sugar is bound to a nucleotide via a glycosidic bond; wherein the sugar is a monosaccharide, such as glucose, rhamnose, xylose, arabinose. Nucleotide sugars act as glycosyl donors in glycosylation reactions; those reactions are catalyzed by glycosyltransferases.

Mutant enzyme (or enzyme variant) is an enzyme which compared to the wild type enzyme comprises one of more amino acid substitutions.

Detailed description of the invention:

The present invention provides improved glycosyltransferases.

As mentioned above, glycosyltransferases can be used in a variety of applications to attach a sugar molecule to different compounds, thereby enhancing their solubility, and decreasing volatility and potentially toxicity.

Specifically, UDP-dependent glycosyltransferase (UGT) is a superfamily of enzymes that catalyze glucosidation and help to transfer glycosyl from UDP-glycosyl donor to a variety of compounds. The enzymatic reaction is proposed to occur by deprotonation of the acceptor hydroxyl group by a highly conserved histidine residue in the UGT active site. The activated acceptor RO^- subsequently performs a nucleophilic attack at the C1 of the sugar donor to form a glycosidic bond (Figure 1).

UGTs glycosylate many different chemicals, including indoxyl (indigo dye precursor). In particular, UGT enzyme variants can be applied as a green biotech alternative to current industrial processes for blue denim production (Figure 2).

Blue denim is traditionally dyed with chemically synthesized indigo under harsh environmentally challenging conditions. As a final step in the synthesis, indigo forms spontaneously from indoxyl by oxidation by air, but for use in dying, indigo further needs to be solubilized with a strong reducing agent (e.g. $Na_2S_2O_4$), which is likewise environmentally challenging.

The improved, hyperstable glycosyltransferase enzyme variants described herein can be added to the current industrial process, thereby eliminating 'dirty chemistry' steps in blue denim dyeing. Specifically, the hydroxyl group of chemically synthesized indoxyl may be glycosylated by glycosyltransferase, thereby protecting the reactive functional group and generating the stable soluble (colorless) indican molecule. Indican may then later be hydrolyzed by beta-glucosidase (BGL) back to indoxyl which can then spontaneously oxidize to form blue indigo directly on the fabric. The invention thereby provides a "greener" alternative to the present industrial process, by providing an alternative solution to the final steps of the indigo dying process, whereby the use of the harsh strong reducing agent is avoided.

The application is equally applicable to similar indoxyl dye-compounds.

I. An improved UGT enzyme

In one aspect, the present invention provides an improved glycosyltransferase mutant enzyme which has improved functional properties relative to the parent (wild type) enzyme form which the mutant was derived. Specifically, the glycosyltransferase mutant enzymes of the present invention are derived from PtUGT1 (SEQ ID NO 2), and have the following properties:

- increased melting temperature (Figure 6)
- comparable activity at 40 °C (Figure 7A)
- activity at 55 °C and even 60 °C, while the wildtype enzyme has no activity at those temperatures (Figure 7B and 7C)
- increased half-life, by at least 216x for Mut 87, 144x for Mut 88, and 72x for Mut 90 at 45 °C (Figure 8)
- increased tolerance to different organic solvents (Figure 10)
- can stabilize different indoxyl compounds by formation of glycosylated soluble dye-precursors (Figure 12, 15 and 16), and thus be used in dyeing applications such as denim dyeing (Figure 13).

As part of natural processing of proteins in microbial organisms, the leading methionine amino acid residue is naturally removed and hence is not part of the final mature protein. Therefore, reference to specific amino acid positions in the amino acid sequence of the

wild type PtUGT1 enzyme is preferably done using the amino acid sequence without the leading methionine. In the present application, SEQ ID NO. 2 and SEQ ID NO. 195 both represent the amino acid sequence of wild type PtUGT1, the only difference being that SEQ ID NO. 2 does not comprise the leading methionine residue, while SEQ ID NO. 195 comprises the leading methionine residue.

The mutant glycosyltransferase enzyme of the present invention possesses glycosyltransferase activity (enzyme classification EC:2.4.1.-) for glycosylating a selected compound, said compound having a reactive group. The mutant enzyme has at least 75% sequence identity to wild type UDP-dependent glycosyltransferase (*Pt*UGT1, SEQ ID NO. 2) from *Polygonum tinctorium*/*Persicaria tinctoria*, but comprises one or more specific mutations relative to the sequence of *Pt*UGT1. Specifically, the mutant comprises (i) one or more amino acid residue substitutions selected from: E75P, Q86K, S110V, I188L, G222D, G296L, V297G, F381V, T388A, S413K and G430K relative to the amino acid sequence of *Pt*UGT1, and/or (ii) amino acid residue substitutions T388C and A399C relative to the amino acid sequence of *Pt*UGT1.

In one embodiment, the mutant glycosyltransferase enzyme of the present invention has glycosyltransferase activity, and the amino acid sequence of said enzyme comprises one or more of the amino acid substitutions disclosed above, relative to *Pt*UGT1 parent (wild type) enzyme, and further has at least 75% sequence identity to *Pt*UGT1 (SEQ ID NO.:2), such as at least 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to *Pt*UGT1 (SEQ ID NO.:2).

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises (i) one or more amino acid residue substitutions selected from: E75P, Q86K, S110V, I188L, G222D, G296L, V297G, F381V, T388A, S413K and G430K, and/or (ii) amino acid residue substitutions T388C and A399C, with respect to SEQ ID NO. 2.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, and G430K with respect to SEQ ID NO. 2.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises (i) amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, and G430K with respect to SEQ ID NO. 2, and (iia) one or more amino acid residue substitutions selected from F381V and T388A with respect to SEQ ID NO. 2.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequences comprises (i) amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, and G430K with respect to SEQ ID NO. 2, and (iib) amino acid residue substitutions T388C and A399C with respect to SEQ ID NO. 2.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises (i) amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, and G430K with respect to SEQ ID NO. 2, and (iia) one or more amino acid residue substitutions selected from F381V and T388A, and (iib) amino acid residue substitutions T388C and A399C with respect to SEQ ID NO. 2.

In one preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, G430K, and T388A with respect to SEQ ID NO. 2.

In one most preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide is SEQ ID NO. 4.

In the present application, SEQ ID NO. 4 and SEQ ID NO. 196 both represent the amino acid sequence of Mut97, the only difference being that SEQ ID NO. 4 comprises the

leading methionine residue, while SEQ ID NO. 196 does not comprise the leading methionine residue.

In another preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, G430K, F381V and T388A with respect to SEQ ID NO. 2.

In another most preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide is SEQ ID NO. 6.

In the present application, SEQ ID NO. 6 and SEQ ID NO. 197 both represent the amino acid sequence of Mut88, the only difference being that SEQ ID NO. 6 comprises the leading methionine residue, while SEQ ID NO. 197 does not comprise the leading methionine residue.

In another preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 2, and wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, G430K, F381V, T388C, and A399C with respect to SEQ ID NO. 2.

In another most preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide is SEQ ID NO. 8.

In the present application, SEQ ID NO. 8 and SEQ ID NO. 198 both represent the amino acid sequence of Mut90, the only difference being that SEQ ID NO. 8 comprises the leading methionine residue, while SEQ ID NO. 198 does not comprise the leading methionine residue.

In one preferred embodiment, the polypeptide of the invention has UPD-dependent glycosyltransferase activity. In a further preferred embodiment, the polypeptide of the invention has indoxyl-UDPG glucosyltransferase activity (enzyme classification EC: 2.4.1.220)

The mutant glycosyltransferase enzyme of the present invention possesses glycosyltransferase activity (enzyme classification EC:2.4.1.-) for glycosylating a

selected compound, said compound having a reactive group. The mutant enzyme has at least 75% sequence identity to wild type UDP-dependent glycosyltransferase (*Pt*UGT1, SEQ ID NO. 195) from *Polygonum tinctorium*/*Persicaria tinctoria*, but comprises one or more specific mutations relative to SEQ ID NO. 195. Specifically, the mutant comprises (i) one or more amino acid residue substitutions selected from: E76P, Q87K, S111V, I189L, G223D, G297L, V298G, F381V, T389A, S414K and G431K relative to SEQ ID NO. 195, and/or (ii) amino acid residue substitutions T389C and A400C relative to SEQ ID NO. 195.

In one embodiment, the mutant glycosyltransferase enzyme of the present invention has glycosyltransferase activity, and the amino acid sequence of said enzyme comprises one or more of the amino acid substitutions disclosed above, relative to SEQ ID NO. 195, and further has at least 75% sequence identity to SEQ ID NO. 195, such as at least 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 195.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises (i) one or more amino acid residue substitutions selected from: E76P, Q87K, S111V, I189L, G223D, G297L, V298G, F3812V, T389A, S414K and G431K, and/or (ii) amino acid residue substitutions T389C and A400C, with respect to SEQ ID NO. 195.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises amino acid residue substitutions E76P, Q87K, S111V, I189L, G223D, G297L, V298G, S414K, and G431K with respect to SEQ ID NO. 195.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises (i) amino acid residue substitutions E76P, Q87K, S111V, I189L, G223D, G297L, V298G, S414K, and G431K with respect to SEQ ID NO 195, and (iia) one or more amino acid residue substitutions selected from F382V and T389A with respect to SEQ ID NO. 195.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequences comprises (i) amino acid residue substitutions E76P, Q87K, S111V, I189L, G223D, G297L, V298G, S414K, and G431K with respect to SEQ ID NO 195, and (iib) amino acid residue substitutions T389C and A400C with respect to SEQ ID NO. 195.

In one embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises (i) amino acid residue substitutions E76P, Q87K, S111V, I189L, G223D, G297L, V298G, S414K, and G431K with respect to SEQ ID NO 195, and (iia) one or more amino acid residue substitutions selected from F382V and T389A, and (iib) amino acid residue substitutions T389C and A400C with respect to SEQ ID NO. 195.

In one preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises amino acid residue substitutions E76P, Q87K, S111V, I189L, G223D, G297L, V298G, S414K, G431K, and T389A with respect to SEQ ID NO. 195.

In one most preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide is SEQ ID NO. 196.

In the present application, SEQ ID NO. 4 and SEQ ID NO. 196 both represent the amino acid sequence of Mut97, the only difference being that SEQ ID NO. 4 comprises the leading methionine residue, while SEQ ID NO. 196 does not comprise the leading methionine residue.

In another preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises amino acid residue substitutions E76P,

Q87K, S111V, I189L, G223D, G297L, V298G, S414K, G431K, F382V and T389A with respect to SEQ ID NO. 195.

In another most preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide is SEQ ID NO. 197.

In the present application, SEQ ID NO. 6 and SEQ ID NO. 197 both represent the amino acid sequence of Mut88, the only difference being that SEQ ID NO. 6 comprises the leading methionine residue, while SEQ ID NO. 197 does not comprise the leading methionine residue.

In another preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, or 97% sequence identity to SEQ ID NO. 195, and wherein said amino acid sequence comprises amino acid residue substitutions E76P, Q87K, S111V, I189L, G223D, G297L, V298G, S414K, G431K, F382V, T389C, and A400C with respect to SEQ ID NO. 195.

In another most preferred embodiment, the present invention provides a polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide is SEQ ID NO. 198.

In the present application, SEQ ID NO. 8 and SEQ ID NO. 198 both represent the amino acid sequence of Mut90, the only difference being that SEQ ID NO. 8 comprises the leading methionine residue, while SEQ ID NO. 198 does not comprise the leading methionine residue.

II. A composition comprising the mutant UGT enzyme

In a second aspect, the present invention provides a composition comprising (i) a polypeptide as disclosed in section I having glycosyltransferase enzyme activity, (ii) a compound comprising a reactive group, and (iii) nucleotide sugar.

In some cases, the reactive group of the compound which is to be glycosylated may in the presence of oxygen react with the oxygen – such as in competition with the enzymatic glycosylation reaction. In such case, it may therefore be an advantage to provide an oxygen reduced, oxygen free, or substantially oxygen free environment for the glycosylation reaction to take place.

In one embodiment, the composition of the present invention is substantially oxygen free. In one embodiment, the composition of the present invention comprises less than

2% free oxygen, such as less than 1.5, 1, 0.5, or even less than 0.1% free oxygen and/or is maintained as a pressure less than 10, 9, 8, 7, 6, 5, 4, 3, 2 kPa or even less than 1 kPa, to reduce likelihood of oxidizing the reactive group of the compound.

In one embodiment, the reactive group of the compound in the composition is a hydroxyl group. In one embodiment, the compound comprising a reactive group is an indoxyl compound, and the composition is suitable for obtaining a stabilized dye precursor, as the indoxyl compound is glycosylated by the glycosyltransferase enzyme. In a preferred embodiment, the compound comprising a reactive group is selected from indoxyl, 6-bromo-indoxyl, 5-bromo-4-chloro-indoxyl, 6-Chloro-indoxyl, 5-bromo-indoxyl, 5-bromo-6-chloro-indoxyl, thioindoxyl, and 5-bromo-7-bromo-indoxyl.

In one embodiment, the polypeptide as disclosed in section I having glycosyltransferase enzyme activity is a UPD-dependent glycosyltransferase, and the nucleotide sugar is a UPD-sugar, such as UDP-glucose, UPD-rhamnose, UPD-xylose, and UPD-arabinose. In a preferred embodiment, the nucleotide sugar of the composition is UDP-glucose.

In a most preferred embodiment, the compound comprising a reactive group is indoxyl, which is converted to indican (a stable precursor of indigo) by the glycosyltransferase enzyme.

III. A kit of parts

In a third aspect, the present invention provides a kit of parts comprising (i) a polypeptide as disclosed in section I having glycosyltransferase enzyme activity, and (ii) a polypeptide encoding a beta-glucosidase (BGL) (enzyme classification EC 3.2.1.21).

The kits of parts of the present invention comprises a glycosyltransferase enzyme as defined herein and a BGL enzyme as defined herein. Exemplified by their action on indoxyl (Figure 2B), these enzymes catalyze separate reactions: Firstly the glycosyltransferase enzyme glycosylates and thereby stabilizes the indoxyl compound (i.e. forming indican), and then later at desired reaction conditions the BLG deglycosylates indican to regain the reactive indoxyl compound.

A person skilled in the art will be familiar with methods of providing the different enzymes for the kit of the present invention. Such enzymes may for example be microbially produced – such as recombinantly or by natural producers, or be synthesized. The enzymes may be provided in solution or dried form. The enzymes may be premixed or provided in separate containers.

III.i *Glycosyltransferase*

For details pertaining to the polypeptide having glycosyltransferase enzyme activity of the kit of the invention, see section I of the present application.

III.ii *Beta-glucosidase*

Beta-glucosidase (BGL) catalyzes the cleavage of glycoside bonds and is in regard to the present invention applied to remove glucose from the glycosylated compound. Such removal of the (protecting) sugar molecule will convert the compound back to its reactive form. Where the compound is an indoxyl compound, this may in its reactive form spontaneously dimerize under aerobic conditions.

In one embodiment, the BGL is selected from the group of enzymes classified as EC 3.2.1.21.

In one embodiment, the amino acid sequence of the BGL is one having at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO. 9, 10, or 11.

In one preferred embodiment, the amino acid sequence of the BGL is one having at least 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100 % sequence identity to SEQ ID NO. 9.

III.iii *Nucleotide sugar*

The kit may further comprise a nucleotide sugar, which is required for the glycosylation reaction catalyzed by glycosyltransferase. In one embodiment the nucleotide sugar is a UPD-sugar, such as UDP-glucose, UPD-rhamnose, UPD-xylose, and UPD-arabinose. In a preferred embodiment, the nucleotide sugar is UDP-glucose. UDP-glucose may be provided directly as UDP-glucose or indirectly in the form of other sugars or sugar-containing molecules which are then converted into UDP-glucose. One example of such indirect providing of UPD-glucose is by providing sucrose along with UDP, which then by enzymatic catalysis (such as using Sucrose synthase (SuSy) EC 2.4.1.13) is converted to UDP-glucose – as illustrated in example 4.1. Another example of indirect providing of UPD-glucose is by using sucrose phosphorylase (converts sucrose and phosphate into glucose-1-P and fructose), glucose-1-phosphate uridylyltransferase (converts UTP and glucose-1-P into UDP-glucose and PPi); and further to regenerate the UTP from UDP, using acetate kinase which requires acetyl-P as substrate in equimolar amounts (converts Acetyl-P + UDP to UTP + acetate) (Lee et al 2004, Bruyn et al 2015).

The kit may further comprise buffers and other relevant reagents for either maintaining activity of the enzymes and/or for enhancing the effect of the enzymes.

Finally, the kit may further comprise an instruction manual providing specifics for each kit component and/or a description of a method of using the kit components.

IV. Methods involving the improved UGT enzyme

In a fourth aspect, different methods involving the mutant glycosyltransferase enzymes of the present invention are provided, wherein the glycosyltransferase enzyme catalyzes glycosylation of a compound of interest.

IV.i *A method for glycosylating a compound*

In a fourth aspect, the present invention provides a method for glycosylating a compound, comprising the steps of:

a. providing (i) a compound comprising a reactive group, (ii) a polypeptide of the present invention (as disclosed in section I) having glycosyltransferase enzyme activity, and (iii) a nucleotide sugar,
b. mixing the components provided in step (a),
c. letting the mixture react to obtain the glycosylated compound.

As disclosed herein, the mutant glycosyltransferase enzyme of the present invention possesses glycosyltransferase activity for glycosylating a selected compound comprising a reactive group.

In one embodiment, the compound comprising a reactive group is an indoxyl compound. In a preferred embodiment, the indoxyl compound is selected from indoxyl, 6-bromo-indoxyl, 5-bromo-4-chloro-indoxyl, 6-Chloro-indoxyl, 5-bromo-indoxyl, 5-bromo-6-chloro-indoxyl, Thioindoxyl, and 5-bromo-7-bromo-indoxyl. In a most preferred embodiment, the compound comprising a reactive group is indoxyl.

Further, as disclosed previously, a nucleotide sugar must be present for the reaction to take place, but can be provided directly or indirectly as described in section III.iii. In one embodiment, the method of glycosylating a selected compound comprises providing UPD-glucose. In another embodiment in step (a) of the method, a sugar molecule is provided, which can be converted into a nucleotide sugar, preferably UDP-glucose, such as by enzymatic catalysis.

Reaction conditions of the method may depend on what the target compound for glycosylation is.

In some cases, the reactive group of the compound which is to be glycosylated may in the presence of oxygen react with the oxygen – such as in competition with the enzymatic glycosylation reaction. In such case, it may therefore be an advantage to

provide an oxygen reduced, oxygen free, or substantially oxygen free environment for the glycosylation reaction to take place.

In one embodiment, step (b) in the method of glycosylating a compound is performed under conditions, where less than 2% free oxygen, such as less than 1.5, 1, 0.5, or even less than 0.1% free oxygen and/or is maintained as a pressure less than 10, 9, 8, 7, 6, 5, 4, 3, 2 kPa or even less than 1 kPa, to reduce likelihood of oxidizing the reactive group of the compound. In one embodiment, the reactive group of the target compound for glycosylation is a hydroxyl group.

In one embodiment, where the target compound for glycosylation is an indoxyl compound, the reaction preferably takes place at oxygen reduced, substantially oxygen free, or even anaerobic conditions to ensure the reactive indoxyl compound does not spontaneously dimerize. In one embodiment, where the target compound for glycosylation is an indoxyl compound, the reaction preferably takes place at conditions comprising less than 2% free oxygen, such as less than 2, 1.5, 1, 0.5, or even less than 0.1%, and/or decreased pressure such as less than 10, 9, 8, 7, 6, 5, 4, 3, 2 or even less than 1 kPa - to reduce likelihood of the reactive indoxyl compound spontaneously dimerizing, such as indoxyl dimerizing to form indigo.

The compound comprising a reactive group is preferably incubated with the glycosyltransferase enzyme at temperature and pH conditions optimal for the enzyme. In one embodiment, the incubation temperature applied should be in the range 20–65°C, such as 20–60°C, such as 30–60°C, such as 40-55°C, preferably in the range 45–55°C, such as preferably around 50°C. In one embodiment, the incubation pH applied should be in the range pH 5–9, such as pH 5.5–8.5, such as preferably pH 6-8.

The enzymatic reaction may take place in buffered solution for stabilizing the enzymes, as a person skilled in the art would recognize and routinely optimize.

The glycosylated compounds produced by the method of the present invention may be detected by HLPC-UV, LC-MS, NMR, or similar equipment as recognized by a person skilled in the art.

IV.ii. *A method for producing a soluble dye precursor*

As disclosed previously, indoxyl compounds may under aerobic conditions dimerize and form colored compounds, which may be used as dyes, such as for dyeing fabrics or other products. These dimerized colored compounds are insoluble in an aqueous solution. Glycosylation of the indoxyl compound will stabilize the compound, prevent dimerization, and thereby provide a soluble dye precursor.

In one embodiment, a method for producing a soluble dye precursor is provided, comprising the steps:

a. providing (i) an indoxyl compound, (ii) a polypeptide of the present invention (as disclosed in section I) having glycosyltransferase enzyme activity, and (iii) a nucleotide sugar,
b. mixing the components provided in step (a), preferably at reaction conditions wherein less than 2% free oxygen is present,
c. letting the mixture react to obtain the glycosylated soluble indoxyl dye precursor.

Reaction conditions specified in section IV.i equally apply to this method for producing a soluble dye precursor.

IV.iii. *A method for dyeing a product, such as yarn or textile*

The mutant glycosyltransferase enzyme of the present invention is particularly useful in an enzyme-catalyzed method for dyeing products, such as yarn or textiles, thus proving an alternative to the current chemical process.

In one embodiment, the method for producing a soluble dye precursor disclosed in section IV.ii additionally comprises dying a product of instead, by further comprising the steps:

d. mixing the glycosylated soluble indoxyl dye precursor with a product of interest and a polypeptide having a beta-glucosidase enzyme activity (enzyme classification EC 3.2.1.21), at reaction conditions wherein free oxygen is present, to obtain a dyed product.

In one embodiment, the present invention provides a method for dying a product is provided, comprising the steps of

a. providing (i) an indoxyl compound, (ii) a polypeptide as disclosed in section I having glycosyltransferase enzyme activity, (iii) a nucleotide sugar, and (iv) a polypeptide having a beta-glucosidase enzyme activity,
b. mixing components (i), (ii), and (iii) provided in step (a), preferably at reaction conditions wherein less than 2% free oxygen is present,
c. letting the mixture react to obtain a soluble glycosylated indoxyl dye-precursor,
d. mixing said dye precursor with said product and said beta-glucosidase at reaction conditions wherein free oxygen is present, to obtain a dyed product.

In one embodiment, the product intended for dying by the methods disclosed herein, may be selected from yarn, textile, fabrics, and similar products. In a preferred embodiment, the product is a yarn or a textile.

In regard to steps (a), (b), and (c), reaction conditions specified in section IV.i equally apply to this method for dyeing a textile. In regard to step (d), the product intended for dyeing is mixed with the dye precursor, and the method further comprises the step of

mixing / adding a beta-glucosidase enzyme to then de-glycosylate the indoxyl compound. Such beta-glucosidase enzymes are described in section III.ii. Preferably, this part of the method takes place in aerobic conditions, whereby the indoxyl compound spontaneously dimerize and form a colored dye.

In a preferred embodiment, the indoxyl compound in the method for dyeing a product is indoxyl, the dye precursor is indican, and the final dyed product is dyed by indigo. In a much preferred embodiment, the final dyed product is a textile.

V. Use of the improved UGT enzyme

In a fifth aspect, the present invention discloses the use of a polypeptide having glycosyltransferase enzyme activity as disclosed in section I, in glycosylating a compound, wherein said compound comprises a reactive hydroxyl group.

In a preferred embodiment, the compound is an indoxyl compound, and the glycosylated compound is used as a dye-precursor in the process of dying textiles.

VI. Advantages and commercial application

As discussed previously, and further evidenced in the below examples, the glycosyltransferase enzymes of the present invention are improved compared to the wild type enzyme in several aspects, including melting temperature, half-life, solvent tolerance and chemo-stability. Such improvements are highly relevant commercially. The enzymes are particularly suited as a greener alternative to current fabric and textile dyeing processes, but may be used in many other

EXAMPLES

In the following, several different mutant UGT polypeptides are studied and characterized. Table 2, 3 and 4 provide an overview of these mutant enzymes and their amino acid mutations relative to the PtUGT1 wild type. The mutant enzymes are in this application generally referred to by their "mutant number".

General methodology

Mutant design

Variants of wild type PtUGT1 (SEQ ID NO. 2) were constructed using the original expression vector pTMH307 (SEQ ID NO. 12) as template (Hsu et. al 2018; GenBank accession No. MF688772). The mutations were introduced by PCR using USER cloning (NEB). The primers used for mutagenesis are shown in table 1. All constructs were

verified by DNA sequencing service (Eurofins) before transformation into chemically competent *E. coli* BL21 Star (DE3) (ThermoFisher Scientific) following manufacturer recommendations.

Table 1. Primers for making amino acid mutations in PtUGT1

Mutation	Primer forward	Primer reverse
P14C	ATG CCA CGU CAT AAT CGT GCC CTC CGC CGG C (SEQ ID NO. 15)	ACG TGG CAU GGT GGA GCG GTG GTT GGT (SEQ ID NO. 16)
A117C	ACC TTT UCG CCA CTG ATG CAA TCG ACG T (SEQ ID NO. 17)	AAA AGG UCG ACG ACG AGG GCG CAG ACG CGG CGG CCG GAG (SEQ ID NO. 18)
S21C	ATC GTG CCC UGC GCC GGC ATG GGC CAC CTC AT (SEQ ID NO. 19)	AGG GCA CGA UTA TGA CGT GCG GTG GTG G (SEQ ID NO. 20)
D122C	ACT GAT GCA AUC GAC GTC GCC CTT GAG CTC (SEQ ID NO. 21)	ATT GCA TCA GUG GCG AAA AGG CAG ACG ACG AGG GCG GCG (SEQ ID NO. 22)
V76C / R97C	ACG CCC AAA UCG AGA CTC TCA TGT CCC TCA TGG TTG TCT GCT CCC TCC CCT CGC TCC GC (SEQ ID NO. 23)	ATT TGG GCG UCG GAG GGG GCG TCG GAG AGG TCG CAC TCG GGG AGG AAG GAG GT (SEQ ID NO. 24)
L119C / A132C	CC TTT UCG CCA CTG ATG CAA TCG ACG TCT GCC TTG AGC TCG GCA TCC GCC CTT T (SEQ ID NO. 25)	AAA AGG UCG ACG ACG CAG GCG GCG ACG CGG CGG C (SEQ ID NO. 26)
V121C / A125C	ACC TTT UCT GCA CTG ATG CAA TCG ACG TCG CCC (SEQ ID NO. 27)	AAA AGG UCG CAG ACG AGG GCG GCG ACG CG (SEQ ID NO. 28)
T126C	ATG CAA UCG ACG TCG CCC TTG AGC T (SEQ ID NO. 29)	ATT GCA UCG CAG GCG AAA AGG TCG ACG AC (SEQ ID NO. 30)
R208C	AAG TGC TAU AAA TTG GCC GAG GGT GTT ATC GTA (SEQ ID NO. 31)	ATA GCA CTU GGA GTG GTG GAG GAG CCA CTT (SEQ ID NO. 32)
A132C / I137C	AGC TCG GCU GCC GCC CTT TCA TCT TCT TCC CCT CC (SEQ ID NO. 33)	AGC CGA GCU CAA GGC AGA CGT CGA TTG CAT CAG T (SEQ ID NO. 34)
A132C / P139C	AGC TCG GCA UCC GCT GCT TCA TCT TCT TCC CCT CCA CCG CC (SEQ ID NO. 35)	ATG CCG AGC UCA AGG CAG ACG TCG ATT GCA TCA GT (SEQ ID NO. 36)
A147C	ACC TGC AUG ACC CTC TCC TTC TTC CT (SEQ ID NO. 37)	ATG CAG GUG GAG GGG AAG AAG ATG AAA G (SEQ ID NO. 38)

P227C	AGG GGG GAU GCA TCA GGG AGC TTT TGC ACC CC (SEQ ID NO. 39)	ATC CCC CCU CCA AAC CCT CGA AGC TAT (SEQ ID NO. 40)
P173C / P181C	ATC CCC GGG UGT ATT TGC GTC CAC GGC AAG GAT TTG ATC GAC (SEQ ID NO. 41)	ACC CGG GGA UCT GAA CGC AGT CGG ACA GCT CGG CAA A (SEQ ID NO. 42)
I176C	AGT GCC CCG GGU GTA TTC CGG TCC ACG GCA AGG ATT (SEQ ID NO. 43)	ACC CGG GGC ACU GAA CCG GGT CGG ACA GCT (SEQ ID NO. 44)
D186C	AGT GCT UGA TCG ACC CGG TTC AGG ATA GGA (SEQ ID NO. 45)	AAG CAC UTG CCG TGG ACC GGA ATA C (SEQ ID NO. 46)
K396C	AAT GCA UGA ACG CTG TTA TGC TAA CCG AGG G (SEQ ID NO. 47)	ATG CAT UGC TCT GCA TAG AGG GGC CAT GT (SEQ ID NO. 48)
P190C / A198C	AGA ACG ACU GCT ACA AGT GGC TCC TCC ACC ACT CC (SEQ ID NO. 49)	AGT CGT TCU TCC TAT CCT GAA CGC AGT CGA TCA AAT CCT TGC C (SEQ ID NO. 50)
D193C / N196C	AGG AAG UGC GAC GCC TAC AAG TGG CTC CTC C (SEQ ID NO. 51)	ACT TCC UGC ACT GAA CCG GGT CGA TCA A (SEQ ID NO. 52)
A259C / L264C	AGT GCT GCA AGU GGT TGG ACC AGC AGC CAC GTG GAT (SEQ ID NO. 53)	ACT TGC AGC ACU CAG GCC GGC AAG CTG CCC CCT TCT CGC A (SEQ ID NO. 54)
P271C / S274C	AGT GCC GUG GAT GCG TCC TAT TCG TGA ATT TCG GGA GT (SEQ ID NO. 55)	ACG GCA CUG CTG GTC CAA CCA CTT CA (SEQ ID NO. 56)
G273C	ACG TTG CUC CGT CCT ATT CGT GAA TT (SEQ ID NO. 57)	AGC AAC GUG GCT GCT GGT CCA ACC ACT T (SEQ ID NO. 58)
G365C	ACG TGC GGG UTC TTG ACG CAT TGT GGG TGG AAT T (SEQ ID NO. 59)	ACC CGC ACG UCG ACT CAT GGC TTA AGA C (SEQ ID NO. 60)
V278C	ATT CTG CAA UTT CGG GAG TGG TGG GGT C (SEQ ID NO. 61)	ATT GCA GAA UAG GAC GGA TCC ACG TGG C (SEQ ID NO. 62)
W307C	ATG CGT GGU TAG GCC TCC AAA CGA CGG CAT TG (SEQ ID NO. 63)	ACC ACG CAU AGG AAC CTC TGC TGG CTG (SEQ ID NO. 64)
L286C / Q290C	AGT ACG GAG UGC CAG AAC GAG CTT GCA GGT GTG C (SEQ ID NO. 65)	ACT CCG TAC UGC AGA CCC CAC CAC TCC CGA AA (SEQ ID NO. 66)
G337C / E340C	AGT GCT TCU TGT GCC AGA CCG CGG GCA GGG GTT T (SEQ ID NO. 67)	AGA AGC ACU CGG GCA GGA GTT TCA ACG (SEQ ID NO. 68)
T342C / G346C	AGG TGC UTG GTC TTG CCA ATG TGG GCC CC (SEQ ID NO. 69)	AGC ACC UGC CCG CGC ACT GCT CCA AGA ACC CCT C (SEQ ID NO. 70)

L368C / I387C	AGA GCG UGT TCC ATG GGG TAC CAC TAT GCA CAT GGC CCC TCT ATG CAG AGC AA (SEQ ID NO. 71)	ACG CTC UCC AGT GTT GAA TTC CAC CCA CAA TGC GTG CAG AAC CCG CCC GTC GAC TCA (SEQ ID NO. 72)
T388C / A399C	AGC AAA AGA UGA ACT GCG TTA TGC TAA CCG AGG GCC TGA GG (SEQ ID NO. 73)	ATC TTT TGC UCT GCA TAG AGG GGC CAG CAA ATT AGT GGT ACC CCA TGG AAC A (SEQ ID NO. 74)
P412C / E424C	ATG GAA UCA TCC GAG GTG CTT GCA TCG CAC GAG TTA TAG GGG AGT TG (SEQ ID NO. 75)	ATT CCA UCC TTA CCC ACT GAG CAT CTG AGT CCC ACC CTC AG (SEQ ID NO. 76)
V455C / S462C	AGC AAA GAU GGA TCA TGC ACT CGA GCT CTT GAA GAG GTT GCA (SEQ ID NO. 77)	ATC TTT GCU CAA GCA AGC AGA AGC CGC ACG CTT (SEQ ID NO. 78)
S457C / G460C	AAA GAT UGC TCA TCT ACT CGA GCT CTT GAA GAG (SEQ ID NO. 79)	AAT CTT UGC ACA ATA CAG CAG AAG CCG C (SEQ ID NO. 80)
L64C / I68C	ACA CCT CCU TCC TCC CCG AGG TCG ACC TCT (SEQ ID NO. 81)	AGG AGG TGU CGC AGG AGG CAG GGC AGG AGG AGA GGA AGT CGC G (SEQ ID NO. 82)
T146C / M148C	ACC CTC UCC TTC TTC CTC CAC CTC GAG AAG C (SEQ ID NO. 83)	AGA GGG UGC AGG CGC AGG AGG GGA AGA AGA TGA AAG (SEQ ID NO. 84)
E224C	AGG GTT TGU GCG GGG GAC CGA TCA GGG AGC TTT (SEQ ID NO. 85)	ACA AAC CCU CGA AGC TAT TTA CGA TAA CA (SEQ ID NO. 86)
E235C / K238C	ATG CCC GCG GGU TTA CCC GGT CGG ACC GCT GAT T (SEQ ID NO. 87)	ACC CGC GGG CAU CCC GGG CAG GGG TGC AAA AGC TCC CTG AT (SEQ ID NO. 88)
L267C	AG TGG UGC GAC CAG CAG CCA CGT GGA T (SEQ ID NO. 89)	ACC ACT UCA AGC ACT CAG GCC GGG C (SEQ ID NO. 90)
S363C	AGC CAT GAG UGC ACG GGC GGG TTC TTG ACG CAT T (SEQ ID NO. 91)	ACT CAT GGC UTA AGA CAT CGA TCT GCG GGG (SEQ ID NO. 92)
N279C	TT CGT GUG CTT CGG GAG TGG TGG GGT C (SEQ ID NO. 93)	ACA CGA AUA GGA CGG ATC CAC GTG GC (SEQ ID NO. 94)
V308C	ATG GTG CGU TAG GCC TCC AAA CGA CGG CAT TG (SEQ ID NO. 95)	ACG CAC CAU AGG AAC CTC TGC TGG CTG (SEQ ID NO. 96)
P390C / Q395C	ATG CAG AGU GCA AGA TGA ACG CTG TTA TGC TAA CC (SEQ ID NO. 97)	ACT CTG CAU AGA GGC ACC ATG TAA TTA GTG GTA CCC (SEQ ID NO. 98)
G460C / T463C	ATT GCT CAU CTT GCC GAG CTC TTG AAG AGG TTG CAA A (SEQ ID NO. 99)	ATG AGC AAU CTT TGC TCA ATA CAG CAG AA (SEQ ID NO. 100)

P48C	ACC TTC GCC GUA TGC ACC AGC GGC CCG CCC TCA (SEQ ID NO. 101)	ACG GCG AAG GUG AAG GTG AAG CGC GGA AG (SEQ ID NO. 102)
S98C	ATG GTT GUC CGC TGC CTC CCC TCG CTC CGC GAC CTC AT (SEQ ID NO. 103)	CA ACC AUG AGG GAC ATG AGA GTC TCG ATT TG (SEQ ID NO. 104)
P13C	ACC GCT CCA UGC CCG CAC GTC ATA ATC GTG (SEQ ID NO. 105)	ATG GAG CGG UGG TTG GTG GAG CGG CGG G (SEQ ID NO. 106)
A66C	ATC GAC ACC UCC TTC CTC CCC GAG GTC GAC C (SEQ ID NO. 107)	AGG TGT CGA UGG AGC AAG GGA GGG AGG AGA GGA A (SEQ ID NO. 108)
T10C	ACC TGC GCU CCA CCA CCG CAC GTC ATA (SEQ ID NO. 109)	AGC GCA GGU TGG TGG AGC GGC GGG GGA (SEQ ID NO. 110)
S112C	ACT CCG CCU GCG GCC GCC GCG TCG CCG CC (SEQ ID NO. 111)	AGG CGG AGU AGG AGG CAA TGA GGT CGC (SEQ ID NO. 112)
A22P	ATC GTG CCC UCC CCG GGC ATG GGC CAC CTC ATC (SEQ ID NO. 113)	AGG GCA CGA UTA TGA CGT GCG GTG GTG G (SEQ ID NO. 114)
M91I	ATC TCC CUC ATG GTT GTC CGC TCC CTC CCC (SEQ ID NO. 115)	AGG GAG AUG AGA GTC TCG ATT TGG GCG (SEQ ID NO. 116)
E75P	ACC TCC TUC CTC CCC CCG GTC GAC CTC TCC GAC GCC CC (SEQ ID NO. 117)	AAG GAG GUG TCG ATG GAG GCA GGG AG (SEQ ID NO. 118)
E157P	ACC TCC CGA AGC UTG ATG AAA CGG TGT CAT GTG AGT T (SEQ ID NO. 119)	AGC TTC GGG AGG UGG AGG AAG AAG GAG AGG GTC AT (SEQ ID NO. 120)
G222D	AGG ATT UGG AGG GGG GAC CGA TCA GGG (SEQ ID NO. 121)	AAA TCC UCG AAG CTA TTT ACG ATA ACA (SEQ ID NO. 1222)
G405D	AGG ACC UGA GGG TGG GAC TCA GAC CCT CAG T (SEQ ID NO. 123)	AGG TCC UCG GTT AGC ATA ACA GCG TTC A (SEQ ID NO. 224)
G409A	AGG GTG GCA CUC AGA CCC TCA GTG GGT AAG GAT GG (SEQ ID NO. 125)	AGT GCC ACC CUC AGG CCC TCG GTT AGC AT (SEQ ID NO. 126)
G430K	ATA AAA GAG UTG ATG GAA GGT GAG GAA GGG AAA C (SEQ ID NO. 127)	ACT CTT TTA UAA CTC GTG CGA TCT CAG C (SEQ ID NO. 128)
G222D (45)	AGG ATT UGG AGG GGG GAC CGA TCA G (SEQ ID NO. 129)	AAA TCC UCG AAG CTA TTT ACG ATA ACA (SEQ ID NO. 130)

G405D / G409A / G430K	AGG ATG GAA UCA TCC GAG GTG CTG AGA TCG CAC GAG TTA TAA AAG AGT TGA TGG AAG GTG AGG AAG GG (SEQ ID NO. 131)	ATT CCA TCC UTA CCC ACT GAG GGT CTG AGT GCC ACC CTC AGG TCC TCG GTT AGC ATA ACA GCG (SEQ ID NO. 132)
E75P (46)	ACC TCC TUC CTC CCC CCG GTC GAC CTC TCC GAC GCC (SEQ ID NO. 133)	AAG GAG GUG TCG ATG GAG GCA GGG AG (SEQ ID NO. 134)
E157P (46)	ACC TCC CGA AGC UTG ATG AAA CGG TGT CAT G (SEQ ID NO. 135)	AGC TTC GGG AGG UGG AGG AAG AAG GAG AGG GTC A (SEQ ID NO. 136)
G222D (46)	AGG ATT UGG AGG GGG GAC CGA TCA G (SEQ ID NO. 137)	AAA TCC UCG AAG CTA TTT ACG ATA AC (SEQ ID NO. 138)
S50D	ACG GCC CGC CCU CAT CCT CCC AGC GCG ACT T (SEQ ID NO. 139)	AGG GCG GGC CGU CGG TGG GTA CGG CGA AGG T (SEQ ID NO. 140)
M94T	ACG GTT GUC CGC TCC CTC CCC TCG CT (SEQ ID NO. 141)	ACA ACC GUG AGG GAC ATG AGA GTC TCG ATT T (SEQ ID NO. 142)
Q86K	ACG CCA AAA UCG AGA CTC TCA TGT CCC TCA TGG T (SEQ ID NO. 143)	ATT TTG GCG UCG GAG GGG GCG TCG GAG A (SEQ ID NO. 144)
Q86R	ACG CCC GUA TCG AGA CTC TCA TGT CCC TCA TG (SEQ ID NO. 145)	ACG GGC GUC GGA GGG GGC GTC GGA GA (SEQ ID NO. 146)
A107K	ATT AAA UCC TAC TCC GCC TCC GGC CG (SEQ ID NO. 147)	ATT TAA UGA GGT CGC GGA GCG AGG G (SEQ ID NO. 148)
K210R	ATC GTT UGG CCG AGG GTG TTA TCG TAA ATA G (SEQ ID NO. 149)	AAA CGA UAC CTC TTG GAG TGG TGG A (SEQ ID NO. 150)
Q269E	AGC CAC GUG GAT CCG TCC TAT TCG TGA ATT TC (SEQ ID NO. 151)	ACG TGG CUG CTC GTC CAA CCA CTT CAA GCA (SEQ ID NO. 152)
V285T	ACC CTC AGU ACG GAG CAG CAG AAC GAG CTT (SEQ ID NO. 153)	ACT GAG GGU CCC ACC ACT CCC GAA ATT (SEQ ID NO. 154)
A299E	AGG TGT GCU GGA ACA CAG CCA GCA GAG GTT C (SEQ ID NO. 155)	AGC ACA CCU GCA AGC TCG TTC TGC TGC (SEQ ID NO. 156)
Q341R	AGG GGT TCU TGG AGC GTA CCG CGG GCA GGG GTT TGG (SEQ ID NO. 157)	AGA ACC CCU CGG GCA GGA GTT TCA ACG (SEQ ID NO. 158)
K332D / E336K / Q341R / A343K	AGG GTT CUT GGA GCG TAC CAA AGG CAG GGG TTT GGT CTT GCC AAT G (SEQ ID NO. 159)	AGA ACC CUT TGG GCA GGA GAT CCA ACG GGT CGA TCT CCC C (SEQ ID NO. 160)

S413K	ACC CAA AGU GGG TAA GGA TGG AAT CAT CCG AGG (SEQ ID NO. 161)	ACT TTG GGU CTG AGT CCC ACC CTC AGG (SEQ ID NO. 162)
I472K	AAA AAA UGG GAA AGC AAG GTT TAA GGA TCC T (SEQ ID NO. 163)	ATT TTT UTG CAA CCT CTT CAA GAG C (SEQ ID NO. 164)
A81L	ACC TGC CCU CCG ACG CCC AAA TCG AGA CTC T (SEQ ID NO. 165)	AGG GCA GGU CGG AGA GGT CGA CCT CGG (SEQ ID NO. 166)
S110V	CG TGG CCU CCG GCC GCC GCG TCG CCG (SEQ ID NO. 167)	AGG CCA CGU AGG AGG CAA TGA GGT CGC (SEQ ID NO. 168)
I129F	ATT CGA CGU CGC CCT TGA GCT CGG CAT C (SEQ ID NO. 169)	ACG TCG AAU GCA TCA GTG GCG AAA AGG (SEQ ID NO. 170)
I188L	ATT TGC UGG ACC CGG TTC AGG ATA GGA AGA AC (SEQ ID NO. 171)	AGC AAA UCC TTG CCG TGG ACC GGA AT (SEQ ID NO. 172)
L333F	AAT TCC UGC CCG AGG GGT TCT TGG AGC (SEQ ID NO. 173)	AGG AAT UTC AAC GGG TCG ATC TCC C (SEQ ID NO. 174)
M351S	AGG GGT TUG GTC TTG CCA AGC TGG GCC CCG CAG ATC GAT GT (SEQ ID NO. 175)	AAA CCC CUG CCC GCG GTC TGC TCC AA (SEQ ID NO. 176)
F381V	AGC GTG GUC CAT GGG GTA CCA CTA ATT ACA TGG (SEQ ID NO. 177)	ACC ACG CUC TCC AGT GTT GAA TTC CAC (SEQ ID NO. 178)
T388A	ATT GCA UGG CCC CTC TAT GCA GAG CAA AAG (SEQ ID NO. 179)	ATG CAA UTA GTG GTA CCC CAT GGA A (SEQ ID NO. 180)
S55A	AGC GCG ACU TCC TCT CCT CCC TCC CTG CCT (SEQ ID NO. 181)	AGT CGC GCU GGG AGG CTG AGG GCG GGC CGC TGG T (SEQ ID NO. 182)
A125G	ACC TTT UCG GCA CTG ATG CAA TCG ACG TCG CC (SEQ ID NO. 183)	AAA AGG UCG ACG ACG AGG GCG GCG A (SEQ ID NO. 184)
F140Y	ACA TCT UCT TCC CCT CCA CCG CCA T (SEQ ID NO. 185)	AAG ATG UAA GGG CGG ATG CCG AGC TC (SEQ ID NO. 186)
GV296 / 297LG	ACT GGG UCT GGC CCA CAG CCA GCA GAG GTT (SEQ ID NO. 187)	ACC CAG UGC AAG CTC GTT CTG CTG C (SEQ ID NO. 188)
H300M	AGG TGT GCU GGC CAT GAG CCA GCA GAG GTT CCT ATG GG (SEQ ID NO. 189)	AGC ACA CCU GCA AGC TCG TTC TGC TGC (SEQ ID NO. 190)
E340G	AGG GGT TCU TGG GCC AGA CCG CGG GCA GGG GTT T (SEQ ID NO. 191)	AGA ACC CCU CGG GCA GGA GTT TCA ACG (SEQ ID NO. 192)

F381V-Mut88	AGC GTG GUC CAT GGG GTA CCA CTA ATT GCA TGG (SEQ ID NO. 193)	ACC ACG CUC TCC AGT GTT GAA TTC CAC (=F381V-Reverse; SEQ ID NO. 178)
A388C / A399C-Mut90	AGC AAA AGA UGA ACT GCG TTA TGC TAA CCG AGG GCC TGA GG (=T388C / A399C Forward; SEQ ID NO. 73)	ATC TTT TGC UCT GCA TAG AGG GGC CAG CAA ATT AGT GGT ACC CCA TGG (SEQ ID NO. 194)

Construction of plasmid comprising Mut87

The plasmid of mutant 87 was prepared as a further development of plasmids of earlier mutants:

- The plasmid of mutant 87 was prepared using the primers "GV296/297LG" (Table 1) on the plasmid of mutant 86.
- The plasmid of mutant 86 was prepared using the primers "G222D" (Table 1) on the plasmid of mutant 85.
- The plasmid of mutant 85 was prepared using the primers "E75P" (Table 1) on the plasmid of mutant 81.
- The plasmid of mutant 81 was prepared using the primers "G430K" (Table 1) on the plasmid of mutant 80.
- The plasmid of mutant 80 was prepared using the primers "T388A" (Table 1) on the plasmid of mutant 77.
- The plasmid of mutant 77 was prepared using the primers "S413K" (Table 1) on the plasmid of mutant 74.
- The plasmid of mutant 74 was prepared using the primers "I188L" (Table 1) on the plasmid of mutant 71.
- The plasmid of mutant 71 was prepared using the primers "S110V" (Table 1) on the plasmid of mutant 48.
- The plasmid of mutant 48 was prepared using the primers "Q86K" (Table 1) on the original expression vector pTMH307.

Construction of plasmid comprising Mut88

The plasmid of mutant 88 was prepared using the primers "F381V-Mut88" (Table 1) on the plasmid of mutant 87. See above for details of how the plasmid of mutant 87 was prepared.

Construction of plasmid comprising Mut90

The plasmid of mutant 90 was generated using the primers "A388C/A399C-Mut90" (Table 1) on the plasmid of mutant 88. See above for details of how the plasmid of mutant 88 was prepared.

Expression and purification of PtUGT1 WT and variants

For the expression of PtUGT1 variants 10 ml pre-cultures cells carrying the corresponding expression vector were grown overnight in 2xYT media containing ampicillin (100 µg/ml) and used to inoculate 1 L cultures of 2xYT media with ampicillin selection. Cultures were grown at 37 °C in an MaxQ8000 incubator (Thermo Fisher

Scientific, Germany) at 200 rpm and induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD600 ~ 1. Cultures were then grown at 18 °C for 21 h for protein expression, and the cells were harvested by centrifugation. The cell pellets were resuspended in 50 mM HEPES pH 7.0, 300 mM NaCl, and 40 mM imidazole pH 8.0. The cell suspension was lysed with 2 cycles through an Avestin Emulsiflex C5 (ATA Scientific Pty Ltd., Australia) homogenizer and treated with DNAse I (Merck). Cells debris was removed by centrifugation at 15000 *g* for 20 min at 4 °C. The cleared extracts were loaded onto Ni Sepharose Fast Flow columns (HisTrap affinity columns, GE Healthcare, U.S.) and the protein was purified using an Äkta FPLC system (GE Healthcare, U.S.). After washing the columns with 20 volumes of buffer (50 mM HEPES pH 7.0, 300 mM NaCl, and 40 mM imidazole pH 8.0), elution was carried out with a 40–500 mM imidazole gradient on the same buffer. The peak fractions were analyzed by SDS-PAGE using NuPAGETM 4–12% Bis-Tris Protein Gels (Thermo Fisher Scientific, U.S.) stained with Instant Blue (Expedeon Ltd. U.K.), pooled, concentrated using a 50,000 MWCO Amicon Ultra-15 Centrifugal Filter Unit (Merck Millipore, Germany) and stored in 25 mM HEPES pH 7, 50 mM NaCl, and 1 mM DTT.

Final protein concentrations were determined by absorbance measurements at 280 nm using a ND-1000 spectrophotometer (Fischer scientific) and the corresponding theoretical extinction coefficient.

Tm experiments – Differential scanning fluorometry (DSF)

Melting temperatures (Tm) of PtUGT1 and the variants were measured by DSF using the Protein Thermal Shift Dye Kit (ThermoFisher Scientific) and a qPCR QuantStudio5 machine. Dye solution (1000x) was diluted to final (2x) in Buffer 2x (100 mM HEPES pH7, 100 mM NaCl). 10 µL of dye solution 2x was mixed with 10 µL of protein samples at 0,8 mg/mL in H2O and pipetted in the qPCR multiwell plate. Multiwell plate was centrifuged 30 seconds at 1000 rpm and transferred to the qPCR machine. The protocol initiate with 2 minutes incubation at 25 °C, followed by a temperature increase of 0,05 °C/second up to 99 °C, and a final incubation of 2 minutes at 99 °C. The thermal shift assay is a technique that quantifies change in protein denaturation temperature, and is thus used herein to identify mutations beneficial for protein thermal stability. Measurements were carried out in triplicate/quadruplet. Raw data was analyzed with Protein Thermal Shift™ Software v1.x.

Example 1: Disulfide bridge mutants

1.1 Mutant design

First we focus on **introduction of disulfide bridges**. In order to do this we use the program SSBOND (Hazes & Dijkstra, 1988) that analyzes protein structures and identifies pairs of residues that could form disulfide bridges if they were mutated to cysteines, based on the distance of Cβ atoms, Cβ/Sγ angles, and Sγ1/Sγ2 angle, and the web-based tool Disulfide by Design 2.0 (Douglas B Craig & Alan A Dombkowski). The programs identified 35 pair of residues having the potential to form intramolecular disulfide bridges and 2 pair of residues having the potential to form intermolecular disulfide bridges. See Table 2 for details of these mutants.

Table 2. Disulfide bridge mutants

Mutant N°	AA mutated
1	P14C, A117C
2	S21C, D122C
3	V76C, R97C
4	L119C, A132C
5	V121C, A125C
6	T126C, R208C
7	A132C, I137C
8	A132C, P139C
9	A147C, P227C
10	P173C, P181C
11	I176C
12	D186C, K396C
13	P190C, A198C
14	D193C, N196C
15	A259C, L264C
16	P271C, S274C
17	G273C, G365C
18	V278C, W307C
19	L286C, Q290C
20	G337C, E340C
21	T342C, G346C

22	**L368C, I387C**
23	**T388C, A399C**
24	**P412C, E424C**
25	**V455C, S462C**
26	**S457C, G460C**
27	**L64C, I68C**
28	**T146C, M148C**
29	**A147C, E224C**
30	**E235C, K238C**
31	**L267C, S363C**
32	**N279C, V308C**
33	**P390C, Q395C**
34	**G460C, T463C**
35	**P48C, S98C**
36	**A66C, P13C**
37	**S112C, T10C**
13+23	**P190C, A198C, T388C, A399C**
13+28	**P190C, A198C, T146C, M148C**
23+28	**T388C, A399C, T146C, M148C**
13+23+28	**P190C, A198C, T388C, A399C, T146C, M148C**

1.2 Melting temperature

Melting temperatures (*T*m) of PtUGT1 wild type and the disulfide bridge mutants were measured as described above. The results are illustrated in Figure 3, where it can be seen that though most mutants performed about the same (or worse) than the wildtype, there were also a few mutants which had increased Tm compared to the wild type - such as mut13, mut23, and mut28.

These best performing mutations were combined as different double mutants or a triple mutant. These mutants had higher *T*m than any of single mutants (see Figure 4), but unfortunately performed poorly in terms of activity (data not shown).

Example 2: Consensus mutants

2.1 Mutant design

Secondarily a consensus approach was used. A multiple sequence homology alignment was performed by first collecting sequences of PtUGT1 homologues of 60% or higher sequence identity, using NCBI sequence blast search. Subsequently, a multiple sequence alignment of all the sequences was created using Multiblast ClustalW2 (ref?), the alignment columns were manually/visually scanned and the positions where the original PtUGT1 amino acid was under-represented identified. Leveraging the structure of PtUGT1 (5NLM) and of two of the homologs (2ACV and 2VG8) used for the consensus approach, a rational analysis of the potential mutations was performed and the final number of variants was set on 34. Mutants of PtUGT1 were constructed to make a specific position more alike the majority of known homologues. See Table 3 for details of these mutants.

Table 3. Consensus mutagenesis mutants

Mutant No	**AA mutated**	**Comments**
PtUGT1 WT	Wild type enzyme (no mutations)	
38	A22P, M91I	Increase Pro/Gly ratio
39	E75P	
40	E157P	
41	G222D	
42	G405D	
43	G409A	
44	G430K	
45*	G222D, G405D, G409A, G430K	
46**	E75P, E157P, G222D, G405D, G409A, G430K	
47	S50D, M94T	Extra polar interactions
48	Q86K	
48B	Q86R	
49	A107K	
50	K210R	
51	Q269E	
52	V285T	
53	A299E, Q341R	
54	K332D, E336K, Q341R, A343K	
55	S413K	
56	S472K	
57	A81L	

58	S110V	Improve hydrophobic packing
59	I129F	
60	I188L	
61	L333F	
62	M351S	
63	F381V	
64	T388A	
65	S55A	Unclasified
66	A125G	
67	F140Y, I472K	
68	GV296/297LG	
69	H300M	
70	E340G	

*Mut45 = Mut(41+42+43+44)

**Mut46= Mut(39+40+41+42+43+44)

2.2 Melting temperature

Melting temperatures (*T*m) of PtUGT1 wild type and the consensus mutants were measured as described above. The results are illustrated in Figure 5. For the consensus mutagenesis enzymes, more mutants were found which had increased *T*m compared to the wild type - such as mut39, mut41, mut44, mut45, mut46, mut48, mut48B, mut55, mut58, mut60, mut63, mut64, and mut68.

Example 3: Combination of mutations

3.1 Mutant design

Different combinations of mutations were tested, as specified in Table 4.

Table 4. Combination of mutants

Mutant No	**AA mutated**
PtUGT1 WT	Wild type enzyme (no mutations)
71	Q86K, S110V
72	S413K, T388A
73	F381V, GV296/297LG
74	Q86K, S110V, I188L, T388A
75	S413K, T388A, I188L
76	F381V, GV296/297LG, I188L
77	Q86K, S110V, I188L, S413K

78	Q86K, S110V, I188L
79	F381V, GV296/297LG, I188L, G430K
80	Q86K, S110V, I188L, S413K, T388A
81	Q86K, S110V, I188L, S413K, T388A, G430K
82	E75P, G430K
83	E75P, G222D
84	E75P, G222D, G430K
85	Q86K, S110V, I188L, S413K, T388A, G430K, E75P
86	Q86K, S110V, I188L, S413K, T388A, G430K, E75P, G222D
87	Q86K, S110V, I188L, S413K, T388A, G430K, E75P, G222D, GV296/297LG
88	Q86K, S110V, I188L, S413K, T388A, G430K, E75P, G222D, GV296/297LG, F381V
89	Q86K, S110V, I188L, S413K, T388A, G430K, E75P, G222D, GV296/297LG, F381V, T146C, M148C
90	Q86K, S110V, I188L, S413K, G430K, E75P, G222D, GV296/297LG, F381V, T388C, A399C
91	E75P, G430K, Q86K
92	E75P, G430K, Q86K, GV296/297LG
93	E75P, G430K, Q86K, T388C, A399C
94	E75P, G430K, Q86K, T146C, M148C
96	Q86K, S110V, I188L, S413K, G430K, E75P, G222D, T388C, A399C
97	Q86K, S110V, I188L, S413K, T388A, G430K, E75P, G222D, T146C, M148C
98	Q86K, S110V, I188L, S413K, G430K, E75P, G222D, GV296/297LG, T388C, A399C
99	Q86K, S110V, I188L, S413K, T388A, G430K, E75P, G222D, GV296/297LG, T146C, M148C

3.2 Melting temperature

Melting temperatures (*T*m) of PtUGT1 wild type and the combination mutants were measured as described above. The results are illustrated in Figure 6 and further summarized in table 4. For the combination mutant enzymes, mutants were found which had further increased *T*m compared to the wild type - as high as 15 °C increase in melting temperature was obtained for mut90, compared to wild type.

The best performing mutants were selected for further studies: mut87, mut88, mut90. The selection was primarily based on increase in melting temperature as well as relative activity measured compared to wild type.

Table 4

Enzyme mutants tested	Mutations in tested mutant enzyme																Melting temperature		Relative activity
	Mut39 E75P	Mut41 G222D	Mut44 G430K	Mut48 Q86K	Mut55 S413K	Mut58 S110V	Mut60 I188L	Mut63 F381V	Mut64 T388A	Mut68 G296L	Mut23 T388C	Mut28 146C					TmB	ΔTmB	
Wild type																	**49,7**	**0**	**100%**
Mut 71																	52,52	2,82	102%
Mut 72																	51,5	1,8	127%
Mut 73																	56,69	6,99	68%
Mut 74																	54,31	4,61	159%
Mut 75																	53,19	3,49	163%
MUT 76																	57,1	7,4	132%
MUT 77																	55,58	5,88	168%
Mut 78																	54,58	4,88	190%
MUT 79																	57,52	7,82	118%
MUT 80																	55,73	6,03	180%
MUT 81																	56,46	6,76	185%
Mut 82																	51,77	2,07	116%
Mut 83																	50,03	0,33	54%
Mut 84																	52,38	2,68	74%
MUT 85																	57,53	7,83	218%
MUT 86																	58,61	8,91	181%
MUT 91																	54,43	4,73	126%
MUT 92																	58,18	8,48	63%
MUT 93																	57,13	7,43	129%
MUT 94																	56,12	6,42	67%
MUT 96																	60,08	10,38	171%
MUT 97																	59,32	9,62	67%

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Table 4 (continued)

Enzyme mutants tested	Mutations in tested mutant enzyme															Melting temperature		Relative activity
	Mut39 E75P	Mut41 G222D	Mut44 G430K	Mut48 Q86K	Mut55 S413K	Mut58 S110V	Mut60 I188L	Mut63 F381V	Mut64 T388A	Mut68 G296L V297G	Mut23 T388C A399C	Mut28 146C 148C				TmB	ΔTmB	
Wild type																**49,7**	**0**	**100%**
MUT 87																62,84	**13,14**	104%
MUT 88																63,04	**13,34**	97%
MUT 89																63,84	14,14	86%
MUT 90																65,34	**15,64**	86%
MUT 98																64,02	14,32	98%
MUT 99																62,15	12,45	83%

All specified mutations are with reference to wild type PtUGT (SEQ ID NO 2). Black shade in the table means that the mutation is present in the mutant.

ΔTm is change in Tm compared to wild type enzyme (in 50 mM HEPES, 50 mM NaCl at pH 7). Relative activity is mutant activity compared to wildtype activity at 40°C (in Citrate-Phosphate buffer at pH 7)

3.3 Relative activity at different temperatures

Relative activity experiments were also carried out at 40, 55 and 60 °C.

Calculation of relative activity of PtUGT1 variants (mut87, mut88, and mut90) compared with WT activity were performed in reactions (triplicate) with end point measurements of product formation using the model substrate 3,4-Dichlorophenol (DCP). Product peak was monitored via reverse phase HPLC, using an Ultimate 3000 Series apparatus (Thermo Scientific) and a kinetex 2.6 µm C18 100 Å 100x4.6 mm analytical column (Phenomenex). MilliQ water and acetonitrile containing 0.1% formic acid were used as mobile phases A and B, respectively. PCR strip tubes containing 200 µl of reaction mixture (1 mM UDP-glucose, 50 mM citrate-phosphate buffer pH 7, 500 µM DCP and 1 ug enzyme) were incubated at 40 °C for 10 minutes. Reactions were stopped and analyzed at 290 nm using a multi-step program (starting at 5% B, ramp up to 25% B at 1.5 min, ramp up to 30% B at 3.5 min, ramp to up 100% B at 6.25 min, stay at 100% B until 7 min, gradient decrease to 0% B at 8 min, stay at 0% B until 9 min). Peak integration and data handling was performed using the Chromeleon software (Thermo Scientific).

The three mutants have comparable or slightly reduced activity to the wildtype enzyme at 40 °C (Figure 7A). However, at 55 °C and 60 °C the mutants significantly outperform the wild type enzyme, which has no activity at these temperatures (Figure 7B and 7C). At 55°C the mutants perform better or at least comparable to the wildtype activity measured at 40 °C. At 60°C the mutants still maintain half the activity as recorded for the wildtype at 40 °C.

3.4 Half-life

Half/shelf-life times are defined as the amount of time that an enzyme can be pre-incubated at a defined temperature having as a results a 50% residual activity compared with the activity without the pre-incubation.

For determination of the half/shelf-life of PtUGT1 WT and variants, stocks of free enzyme in buffer (100 mM HEPES pH7, 100mM NaCl) were incubated either at 45 °C or room temperature for different period of times, and their residual activities were analyzed using the same procedure described for the "relative activity experiment" and compared against the activity without the pre-incubation step.

At 45 °C, the residual activity of the wild-type enzyme is reduced by >90% after 5 h 20 min. Meanwhile, at this same incubation period, the residual activity of the mutant enzymes remain rather constant. A drop to approx. 60% is seem after 24 hours, and after 96h the residual activity is down to approx. between 35-45%. See Figures 8 and 9

3.5 Solvent tolerance

For determination of the solvent tolerance of PtUGT1 WT and variants, relative activities were analyzed using the same procedure described for the "relative activity experiment" with the addition of either 15% v/v acetone, acetonitrile or isopropanol, and compared against the activity without addition of any organic solvent. As seen in Figure 10, the mutants pertain some activity in all three solvents, while the wildtype shows no activity in acetonitrile and isopropanol.

3.6 Chemo-stability

Chemo-stability is defined as the property of a polypeptide to retain structural integrity and activity in presence of chemicals such as indoxyl, indoxyl derivatives or DCP. Chemo-stability is here tested against DCP, which is usually considered "harsh" for the enzyme, and may reduce or destroy the activity of the enzyme.

Reactions were performed in presence of 15 mg/L enzyme (*Pt*UGT1 WT or Mutant 87), 4 mM 3,4-dichlorophenol (DCP), 6 mM UDP-Glc and 0.5 M citrate pH 6.2. 50 µL reactions were set up in HPLC vials with insert and incubated at 20 degrees for 48h prior to analysis via reverse phase HPLC, using an Ultimate 3000 Series apparatus (Thermo Scientific) and a kinetex 2.6 µm C18 100 Å 100x4.6 mm analytical column (Phenomenex). MilliQ water and acetonitrile containing 0.1% formic acid were used as mobile phases A and B, respectively. Monitoring and data handling was operated using the Chromeleon software (Thermo Scientific). The method used for the separation of analytes had a flow rate of 1 mL/min and started at 2% B for 30 seconds, followed for 1 minute of 35% B and then a gradient from 35% to 80% B for 1.5 min. After, B was increased to 98% for 1.2 min and finally reduced to 2% B for the last 0.8 minute. DCP and its glucoside were detected at 280 nm.

In figure 11, the HPLC chromatogram shows that in the reaction done with the WT enzyme, there is only substrate present, no product at all. So the WT enzyme is not able to withstand the higher concentration of DCP and therefore it is inactive. Similar lack of chemostability is reported by Petermeier et al 2021. In the reaction done with Mut87, there is a clear peak of the product DCP-glucoside, which means the Mut87 its chemo-stable against this compounds, whereas the WT is not.

Example 4: Demin dying

4.1 Synthesis of indican from Indoxyl acetate

The proof of concept for the synthesis of indican from high concentrations of indoxyl-acetate (100 mM) was performed in triplicate inside an anaerobic chamber, using glass HPLC vials stirred with small magnets and at 30 °C. Reaction consisted on 3.5 mg

indoxyl-acetate, 90 mM buffer phosphate-citrate pH8, 1 mM UDP, 200 mM sucrose, 2U of Esterase from *Bacillus subtilis* (Sigma Aldrich), and different concentrations of PtUGT1/ SuSy always at a molar ratio of 1:5 (50 µg, 20 µg, 10 µg, 5 µg for PtUGT1 WT or Mut 87; and 432,5 µg, 173 µg, 86,7 µg, 43,3 µg for SuSy). Sucrose synthase (SuSy) converts sucrose and uridine 5'-diphosphate (UDP) into UDP-glucose. The reaction was started by the addition of all three enzymes (Esterase, PtUGT1 and SuSy) and the progression was followed by HPLC using the same method used in "Relative activity experiment". Samples were collected at 1, 2, 3, 6, 12, 24, and 32 hours.

Figure 12 shows that WT enzymes does not produce any indican from indoxyl acetate under the conditions tested herein, with Mut87 enzyme does indeed produce indican from indoxyl acetate. It is speculated that the high concentration of substrate inactivates the WT enzyme; while this does not seem to pose a problem for the mutant enzyme.

4.2 Demin dyeing

Discs of 20 square centimeters of ready-to-dye denims (radius 1.784 cm, diameter 3.57cm, weight 802+/- 2 mg) are dyed in 3 mL of water at pH 9 with 30 µmol indican (prepared above) and 1 mg of Rye β-glucosidase 1 (SEQ ID NO. 9). Discs are turned over every 5 min at room temperature for 15 min, and left for 1 h at room temperature before being washed with water and soap and dried overnight at room temperature.

As seen in figure Figure 13, the produced indican is capable of dyeing denim. The coloration is further specified in CIEL table 6.

Table 6. CIEL values for dyed textiles

Sample	**L***	**a***	**b***
10 µmol Indican Back	69.74	-5.77	-11.18
10 µmol Indican Front	74.84	-5.27	-8.26
20 µmol Indican Back	64.25	-5.84	-11.37
20 µmol Indican Front	66.20	-5.70	-12.12
30 µmol Indican Back	56.57	-5.22	-14.09
30 µmol Indican Front	55.31	-7.03	-15.14
40 µmol Indican Back	52.37	-5.41	-15.05
40 µmol Indican Front	51.98	-6.30	-14.86
60 µmol Indican Back	53.04	-6.50	-14.79
60 µmol Indican Front	52.16	-5.56	-16.32
100 µmol Indican Back	46.48	-6.54	-14.35
100 µmol Indican Front	48.42	-4.56	-18.03

Example 5: Glycosylation of other indoxyl derivatives

In the example above, it was demonstrated that the UGT mutants of the present invention are capable of glycosylating indoxyl. We herein further demonstrate that the UGT mutants are also able to glycosylating other indoxyl derivatives. See Figure 14 for graphical illustration of a selection of such indoxyl derivatives of interest.

5.1 6-Bromo-Indoxyl

Reactions performed in strip tubes of PCR at 30°C. Reaction components are specified in table 7. Initiated with addition of esterase from *Bacillus subtilis* in buffer with multichannel pipette.

Table 7 Reaction components for glycosylation of 6-bromo-indoxyl

Component	Stock concentration	End concentration	Added volume
6-Bromo-Indoxyl acetate**	3,5mM in H20	1,75mM	75 ul
UDP-Glucose	100 mM in H20	5 mM	7,5ul
UGT*			1,5ul
Esterase from *Bacillus subtilis*	0,1U/ul in buffer 2x	0,2U reaction	2ul
Buffer 2x	HEPES 100mM pH7; 100mM NaCl	HEPES 44mM pH7, NaCl 44mM	64ul
		TOTAL VOLUME	150 ul

*UGT stocks: WT=9,04mg/ml; Mut 87=1,57mg/ml; Mut 88=12,32mg/ml; Mut 90=4,4mg/ml. Storage buffer: 25mM HEPES pH7, 50mM NaCl.

**6-Bromo-Indoxyl-Acetate 3,5mM in water dissolved in bath sonicator at room temperature.

When 6-Bromo-Indoxyl acetate is treated with esterase enzyme, acetate and 6-Bromo-Indoxyl form. If further exposed to air, a dimer spontaneously forms from 6-bromo-indoxyl, which has a distinct purple color (known as Tyrian purple or Royal purple). As evidenced in Figure 15, it is clear that this purple color develops for the two negative controls, where either no UGT enzyme or no UDP-glucose is added. Meanwhile, for the samples where UGT enzyme is added, the 6-Bromo-Indoxyl is glycosylated (after acetate removal) and thereby "prevented" from forming the dimer, hence no color formation.

It is thereby shown that PtUGT1 (wild type) and all tested mutants are active on 6-Bromo-Indoxyl.

5.2 5-Bromo-4-chloro-3-Indoxyl

Reactions performed in strip tubes of PCR at 30°C. Reaction components are specified in table 8. Initiated with addition of esterase from *Bacillus subtilis* in buffer with multichannel pipette.

Table 8 Reaction components for glycosylation of 5-bromo-4-chloro-3-indoxyl			
Component	Stock concentration	End concentration	Added volume
5-bromo-4-chloro-3-indoxyl-Acetate	2,5mg/ml (8,6mM) in DMSO	0,057 mM	1 ul
UDP-Glucose	100 mM in H20	5 mM	7,5ul
UGT	see above*		5ul
Esterase from *Bacillus subtilis*	0,1U/ul in buffer 2x	0,05U reaction	0,5ul
H2O			61 ul
Buffer 2x	HEPES 100mM pH7; 100mM NaCl	HEPES 50mM pH7, NaCl 50mM	75ul
		TOTAL VOLUME	150 ul

*UGT stocks: WT=9.04 mg/ml; Mut 87=1.57 mg/ml; Mut 88=12.32 mg/ml; Mut 90=4.4 mg/ml. Storage buffer: 25mM HEPES pH7, 50 mM NaCl.

**5-bromo-4-chloro-indoxyl-acetate 3,5 mM in water dissolved in bath sonicator at room temperature.

When 5-Bromo-4chloro-Indoxyl acetate is treated with esterase enzyme, acetate and 5-Bromo-4chloro-Indoxyl form. If further exposed to air, a dimer spontaneously forms from 5-Bromo-4chloro-Indoxyl, which has a bright blue color. As evidenced in Figure 16, it is clear that this blue color develops for the negative control, where no UGT enzyme is added. Meanwhile, for the samples where UGT enzyme is added, the 5-Bromo-4chloro-Indoxyl is glycosylated (after acetate removal) and thereby "prevented" from forming the dimer, hence no color formation.

It is thereby shown that PtUGT1 (wild type) and all tested mutants are active on 5-Bromo-4chloro-Indoxyl.

5.3 Further indoxyl derivatives

6-chloro-indoxyl, 5-bromo-indoxyl, 5-bromo-6-chloro-indoxyl, thioindoxyl, and 5,7-dibromo-indoxyl are also glycosylated by an enzyme of the present invention. This may be demonstrated in a similar manner as shown above in section 5.1 and 5.2, where the acetate-form of the molecules are used as starting material, and an esterase enzyme is used in combination with the UTG enzyme. The spontaneous dimerization of the indoxyl-derivatives is prevented by the action of the UGT enzyme, resulting in glycosylation of the compounds.

Example 6: Melting temperatures and chemostability of prior art UGT enzymes

The following prior art UGT enzymes were tested:

- PtUGT2 (SEQ ID NO. 200): P. tinctorium UGT isoform 2 (disclosed as sequence #4 in WO2016/141207). PtUGT2 has five mutations compared to SEQ ID NO. 2: delS1, V19M, G225A, E230Q, and A423D.

- PtIGS (SEQ ID NO. 202): Persicaria tinctoria glycosyltransferase; Uniprot ref. A0A2L2R220. PtIGS has three mutations compared to SEQ ID NO. 2: delS1, V19M, and A423D.

The enzymes were expressed and purified as disclosed herein.

Melting temperatures (*T*m) of prior art UGT enzymes were measured as described herein (section 'general methodology'). The results are summarized in table 9 and illustrated in Figure 17, where the Δ(Tm) is with respect to the PtUGT1 WT (SEQ ID NO. 2).

Table 9 Melting temparatures

	Tm (°C)	**ΔTm (°C) compared to WT PtUGT1**
WT PtUGT1	52,45 ± 0,05	
PtUGT2	52,49 ± 0,34	0.04
PtIGS	51,55 ± 0,56	-0.90

It was found that the melting temperature of PtUGT2 did not differ significantly from the WT PtUGT1. while PtIGS had lower melting temperatures than PtUGT1 WT.

Chemostability was measured as described herein (example 3.6). The results are presented in figure 18. The HPLC chromatograms show that in the reaction done with Mut87, there is a clear peak of the product DCP-glucoside, which means Mut87 its chemo-stable against this compounds; whereas in the reactions done with the wild type enzyme and other prior art enzymes, only a very small DCP-glucoside peak is found, hence the prior art enzymes are not able to withstand the high concentration of DCP and are therefore inactive.

REFERENCES

Lee et al 2004. One-pot enzymatic synthesis of UDP-D-glucose from UMP and Glucose-1-Phosphase using ATP regeneration system. Journal of Biochemistry and Molecular Biology, Vol. 37, No. 4, July 2004, pp. 503-506

Frederik De Bruyn et al. Development of an in vivo glucosylation platform by coupling production to growth: Production of phenolic glucosides by a glycosyltransferase of Vitis

vinifera. *Biotechnol. Bioeng* (2015) 112, 1594– 1603, https://doi.org/10.1002/bit.25570

Craig, D.B., Dombkowski, A.A. Disulfide by Design 2.0: a web-based tool for disulfide engineering in proteins. *BMC Bioinformatics* **14,** 346 (2013). doi.org/10.1186/1471-2105-14-346

B W Dijkstra. Model building of disulfide bonds in proteins with known three-dimensional structure B Hazes 1, PMID: 3244694 DOI: 10.1093/protein/2.2.119

Hsu, T. M. et al. Employing a biochemical protecting group for a sustainable indigo dyeing strategy. Nat. Chem. Biol. 14, 256–261 (2018).

Inoue et al 2017. Characterization of UDP-glucosyltransferase from Indigofera tinctoria. Plant Physiol Biochem. 2017 Dec;121:226-233. doi: 10.1016/j.plaphy.2017.11.002. Epub 2017 Nov 6.

Philipp Petermeier, Cristina Fortuna, Kathrine M. Hübschmann, Gonzalo N. Bidart, Thomas Tørring, David Teze, Ditte H. Welner, and Selin Kara. ACS Sustainable Chemistry & Engineering 2021 9 (25), 8497-8506. DOI: 10.1021/acssuschemeng.1c01536

CLAIMS

1. A polypeptide having glycosyltransferase activity (enzyme classification EC:2.4.1.-), wherein the amino acid sequence of said polypeptide has at least 75% sequence identity with SEQ ID NO. 2, and wherein said amino acid sequence comprises (i) one or more amino acid residue substitutions selected from: E75P, Q86K, S110V, I188L, G222D, G296L, V297G, F381V, T388A, S413K and G430K with respect to SEQ ID NO. 2, and/or (ii) amino acid residue substitutions T388C and A399C with respect to SEQ ID NO. 2.

2. The polypeptide according to claim 1, wherein the half-life at 45 °C of said glycosyltransferase activity of said polypeptide is increased, compared to SEQ ID NO. 2.

3. The polypeptide according to claim 1 or 2, wherein said amino acid sequence comprises amino acid residue substitutions E75P, Q86K, S110V, I188L, G222D, G296L, V297G, S413K, and G430K with respect to SEQ ID NO. 2.

4. The polypeptide according to claim 3, wherein said amino acid sequence further comprises (i) one or more amino acid residue substitutions selected from F381V and T388A with respect to SEQ ID NO 2, and/or (ii) amino acid residue substitutions T388C and A399C with respect to SEQ ID NO. 2.

5. The polypeptide according to claim 3, wherein said amino acid sequence further comprises

   (i) amino acid residue substitution T388A with respect to SEQ ID NO. 2, or

   (ii) amino acid residue substitutions F381V and T388A with respect to SEQ ID NO. 2, or

   (iii) amino acid residue substitutions F381V, T388C, and A399C with respect to SEQ ID NO. 2.

6. A composition comprising (i) a polypeptide having glycosyltransferase enzyme activity according to any one of claims 1-5, (ii) a compound comprising a reactive group, and (iii) a nucleotide sugar.

7. A composition according to claim 6, wherein the compound is an indoxyl compound; and preferably wherein said composition comprises less than 2% free oxygen.

8. A composition according to claim 6 or 7, wherein the nucleotide sugar is an UPD-glucose.

9. A kit of parts comprising (i) a polypeptide having glycosyltransferase enzyme activity according to any one of claims 1-5, and (ii) a polypeptide having beta-glucosidase enzyme activity (enzyme classification EC 3.2.1.21).

10. A method for glycosylating a compound, comprising the steps of
    a. providing (i) a compound comprising a reactive group, (ii) a polypeptide having glycosyltransferase activity according to any one of claims 1–5, and (iii) a nucleotide sugar,
    b. mixing the components provided in step (a)
    c. letting the mixture react to obtain a glycosylated compound.

11. The method according to claim 10, wherein the compound provided in step (a) is an indoxyl compound, wherein the glycosylated compound obtained in step (c) is a soluble glycosylated indoxyl dye-precursor, and wherein steps (b) and (c) are preferably carried out under reaction conditions wherein less than 2% free oxygen is present.

12. The method according to claim 11, wherein the indoxyl compound is selected from indoxyl, 6-bromo-indoxyl, 5-bromo-4-chloro-indoxyl, 6-chloro-indoxyl, 5-bromo-indoxyl, 5-bromo-6-chloro-indoxyl, thioindoxyl, and 5-bromo-7-bromo-indoxyl.

13. The method according to any one of claims 10-12, wherein the nucleotide sugar is UDP-glucose.

14. A method for dying a product, comprising the steps of
    a. providing (i) an indoxyl compound, (ii) a polypeptide having glycosyltransferase enzyme activity according to anyone of claims 1-5, (iii) a nucleotide sugar, and (iv) a polypeptide having beta-glucosidase enzyme activity (enzyme classification EC 3.2.1.21),

b. mixing components (i), (ii), and (iii) provided in step (a), preferably at reaction conditions wherein less than 2% free oxygen is present,
c. letting the mixture react to obtain a soluble glycosylated indoxyl dye-precursor,
d. mixing said dye precursor with said product and said beta-glucosidase under reaction conditions wherein free oxygen is present, to obtain a dyed textile.

wherein said product is elected from yarn, textiles, and fabrics,

15. Use of a polypeptide having glycosyltransferase enzyme activity according to anyone of claims 1-5 for glycosylating a compound, wherein said compound comprises a reactive group.

16. The use according to claim 15, wherein the compound is an indoxyl compound, and wherein the glycosylated compound is for use in a textile dying process.

FIGURE 1

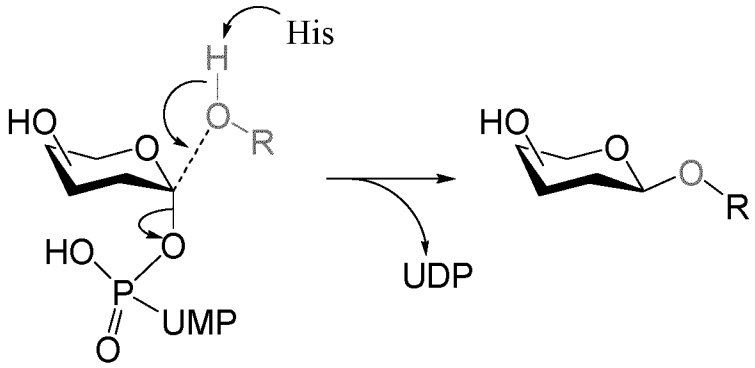

FIGURE 2

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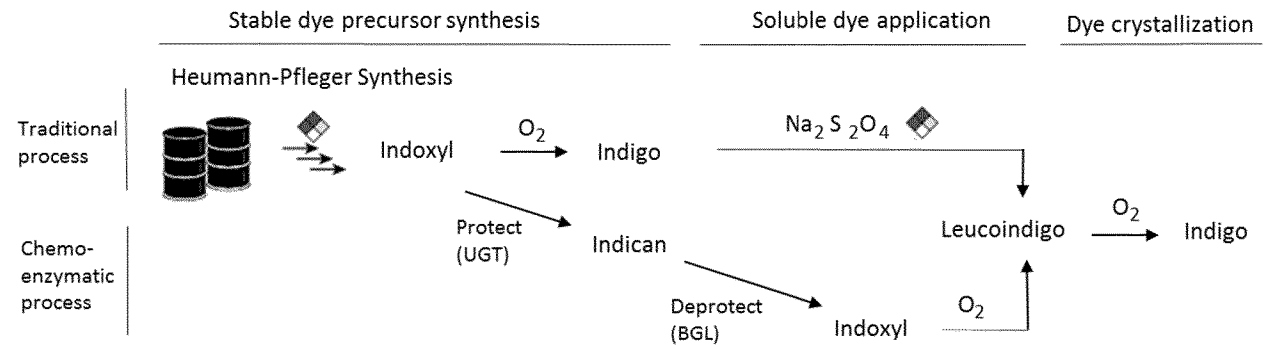

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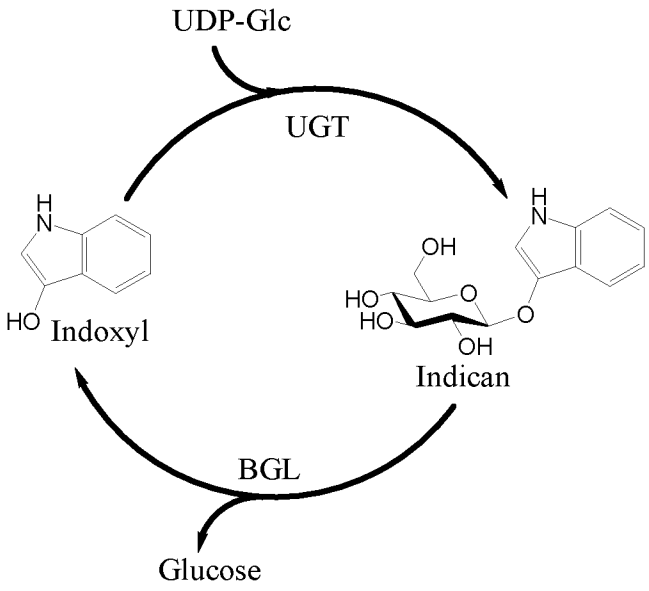

FIGURE 3

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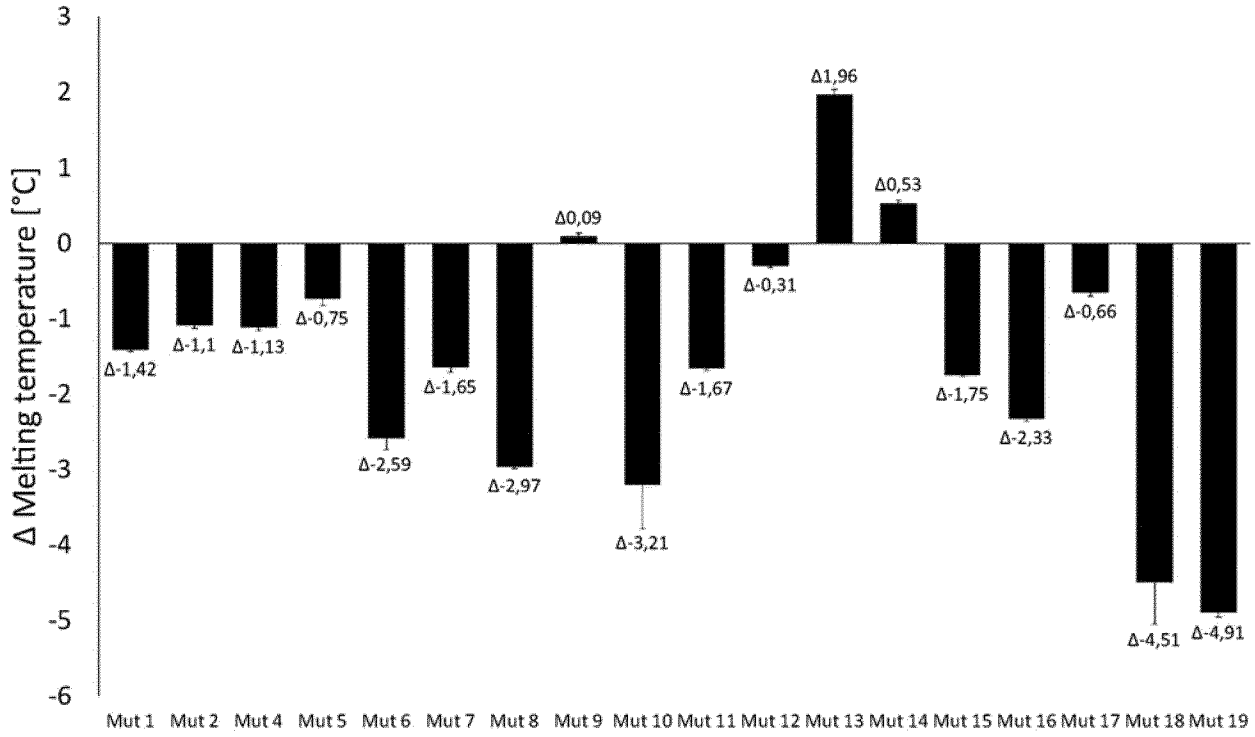
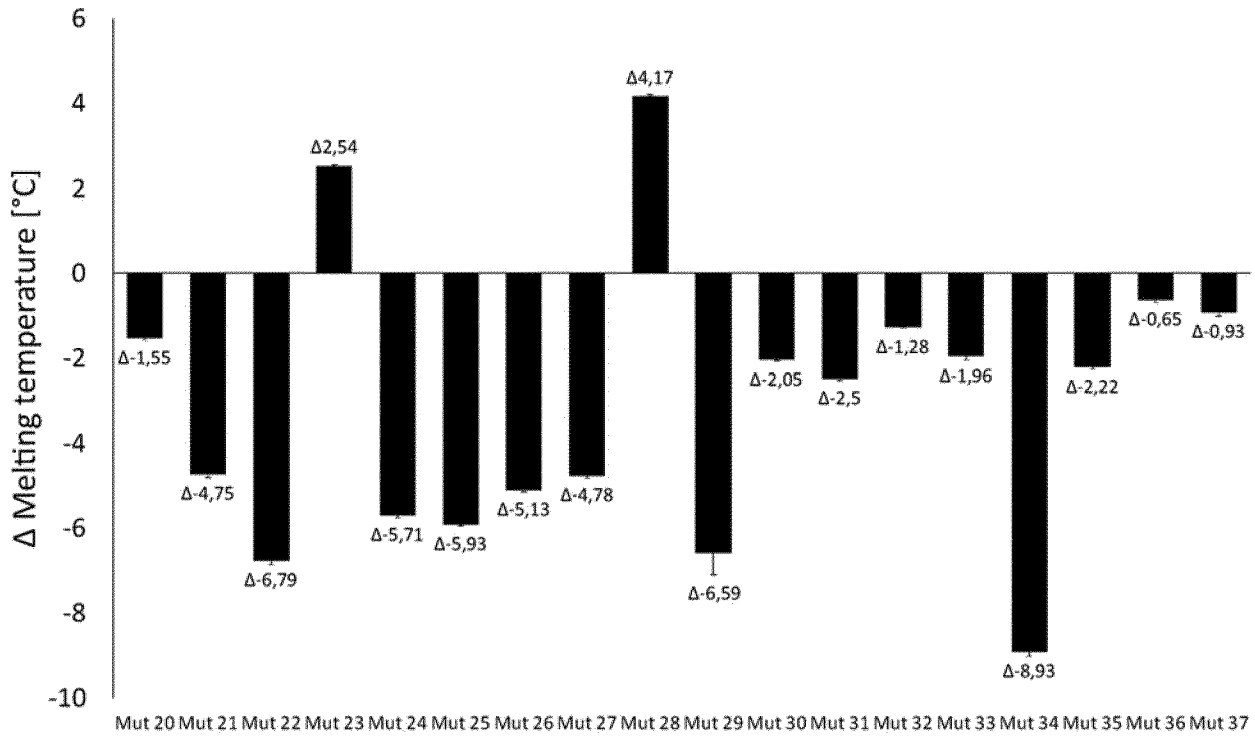

Δ Melting temperature [°C]
3
2
1
0
-1
-2
-3
-4
-5
-6
Δ-1,42
Δ-1,1
Δ-1,13
Δ-0,75
Δ-2,59
Δ-1,65
Δ-2,97
Δ0,09
Δ-3,21
Δ-1,67
Δ-0,31
Δ1,96
Δ0,53
Δ-1,75
Δ-2,33
Δ-0,66
Δ-4,51
Δ-4,91
Mut 1 Mut 2 Mut 4 Mut 5 Mut 6 Mut 7 Mut 8 Mut 9 Mut 10 Mut 11 Mut 12 Mut 13 Mut 14 Mut 15 Mut 16 Mut 17 Mut 18 Mut 19

B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6
4
2
0
-2
-4
-6
-8
-10
Δ-1,55
Δ-4,75
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Δ2,54
Δ-5,71
Δ-5,93
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Δ-4,78
Δ4,17
Δ-6,59
Δ-2,05
Δ-2,5
Δ-1,28
Δ-1,96
Δ-8,93
Δ-2,22
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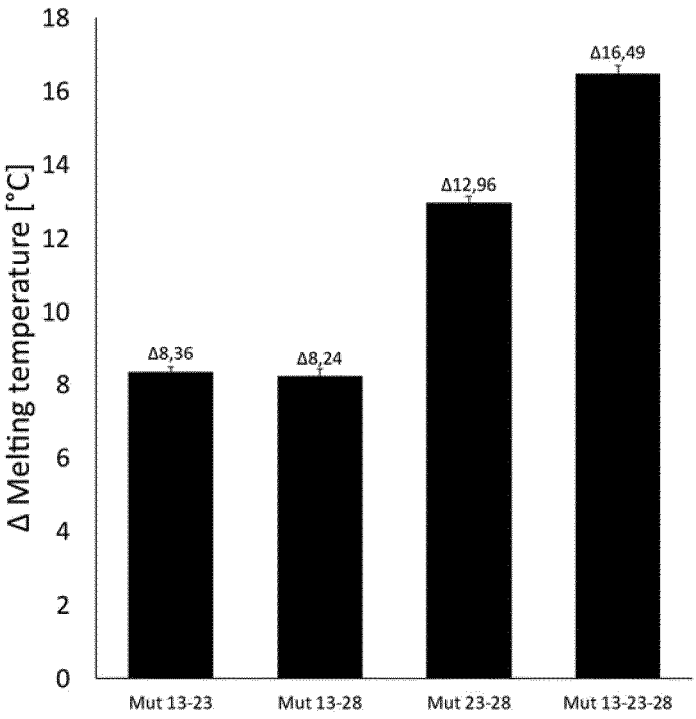
FIGURE 4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14
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10
8
6
4
2
0
Δ Melting temperature [°C]
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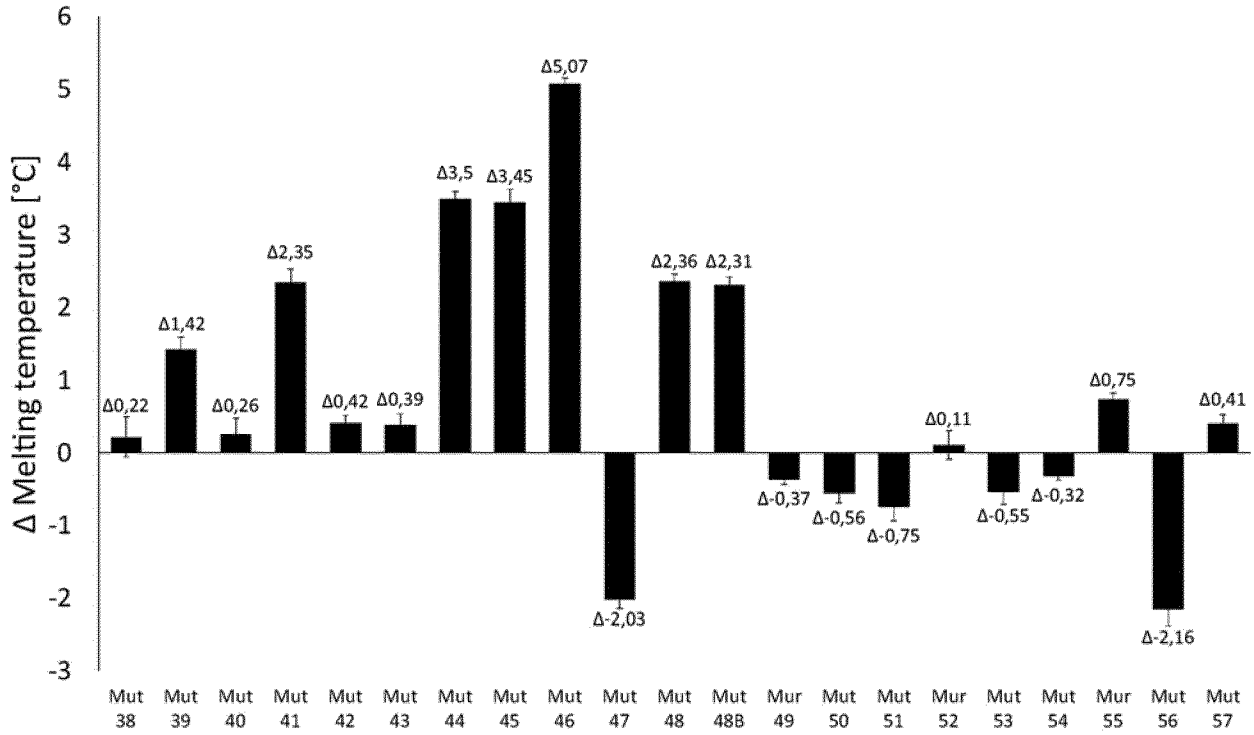
Δ12,96
Δ16,49
Mut 13-23
Mut 13-28
Mut 23-28
Mut 13-23-28

FIGURE 5

A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5
4
3
2
1
0
-1
-2
-3
Δ0,22
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Δ0,39
Δ3,5
Δ3,45
Δ5,07
Δ-2,03
Δ2,36
Δ2,31
Δ-0,37
Δ-0,56
Δ-0,75
Δ0,11
Δ-0,55
Δ-0,32
Δ0,75
Δ-2,16
Δ0,41
Mut 38
Mut 39
Mut 40
Mut 41
Mut 42
Mut 43
Mut 44
Mut 45
Mut 46
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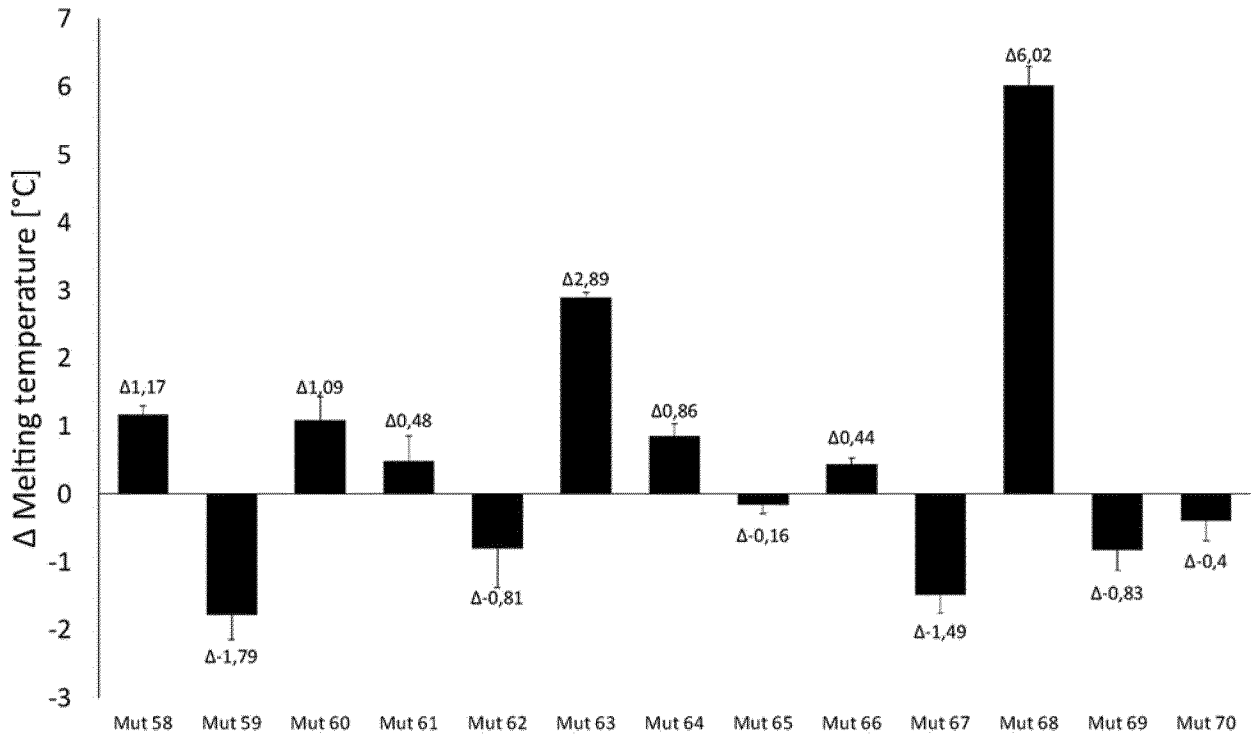
Mur 52
Mut 53
Mut 54
Mur 55
Mut 56
Mut 57

B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6
5
4
3
2
1
0
-1
-2
-3
Δ1,17
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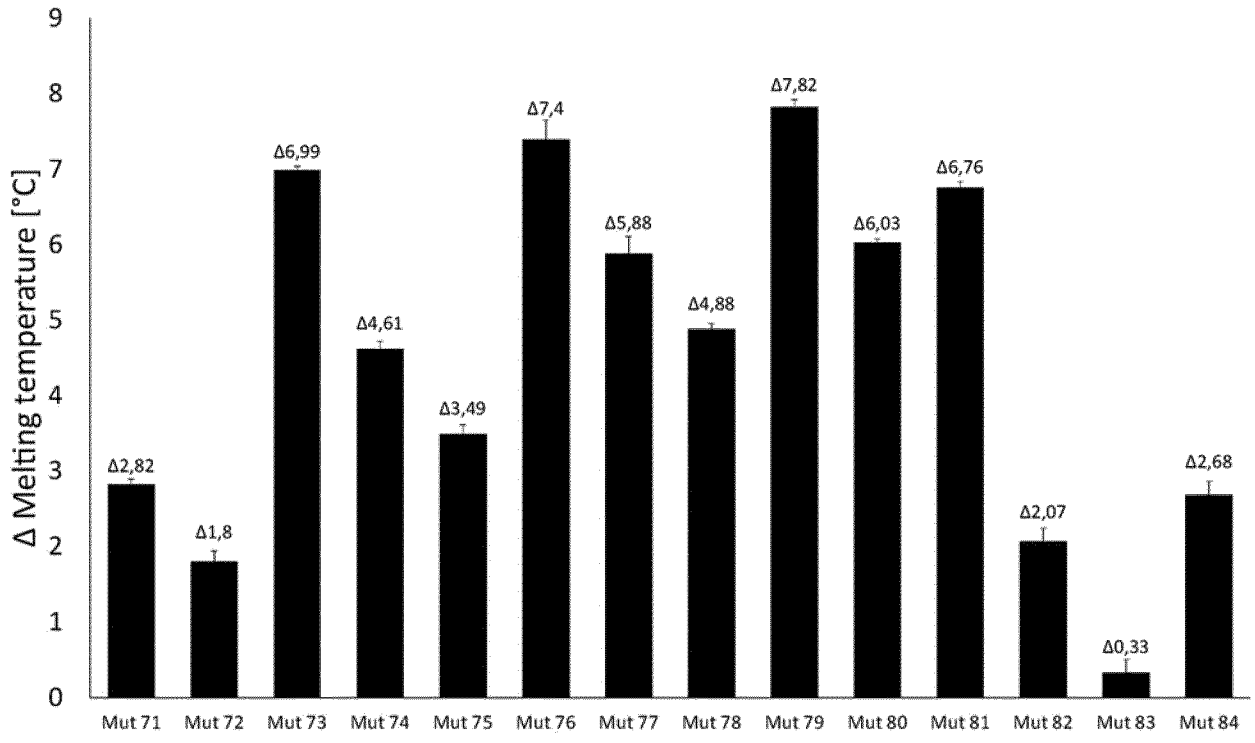
Δ0,48
Δ-0,81
Δ2,89
Δ0,86
Δ-0,16
Δ0,44
Δ-1,49
Δ6,02
Δ-0,83
Δ-0,4
Mut 58
Mut 59
Mut 60
Mut 61
Mut 62
Mut 63
Mut 64
Mut 65
Mut 66
Mut 67
Mut 68
Mut 69
Mut 70

FIGURE 6

A


Δ Melting temperature [°C]
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1
2
3
4
5
6
7
8
9
Δ2,82
Δ1,8
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Δ3,49
Δ7,4
Δ5,88
Δ4,88
Δ7,82
Δ6,03
Δ6,76
Δ2,07
Δ0,33
Δ2,68
Mut 71
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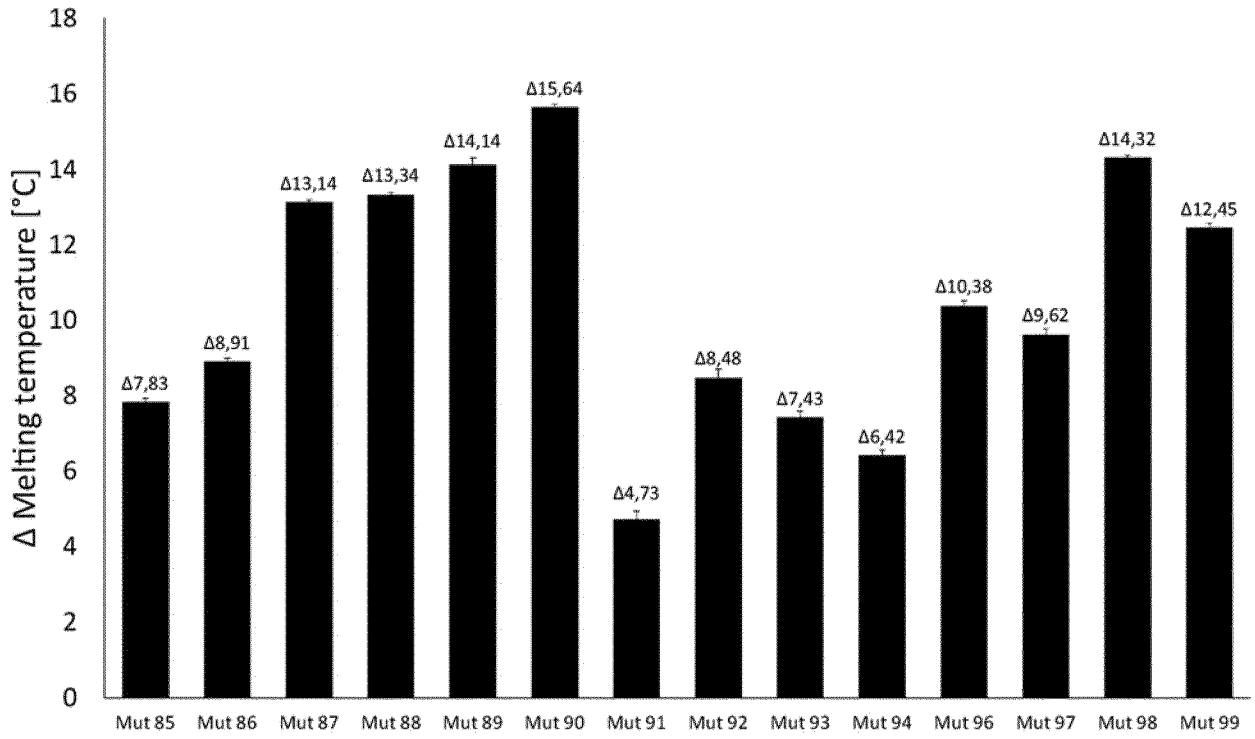
Mut 74
Mut 75
Mut 76
Mut 77
Mut 78
Mut 79
Mut 80
Mut 81
Mut 82
Mut 83
Mut 84

B


Δ Melting temperature [°C]
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4
6
8
10
12
14
16
18
Δ7,83
Δ8,91
Δ13,14
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Δ14,14
Δ15,64
Δ4,73
Δ8,48
Δ7,43
Δ6,42
Δ10,38
Δ9,62
Δ14,32
Δ12,45
Mut 85
Mut 86
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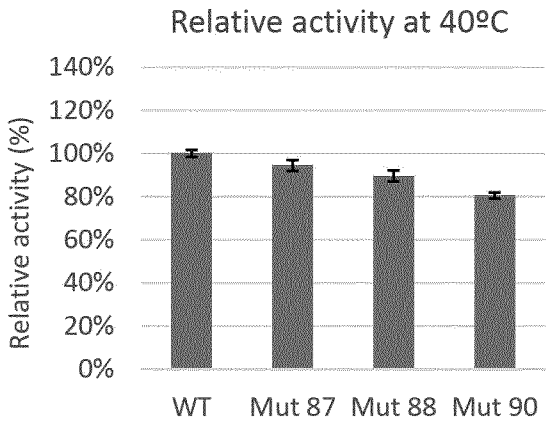
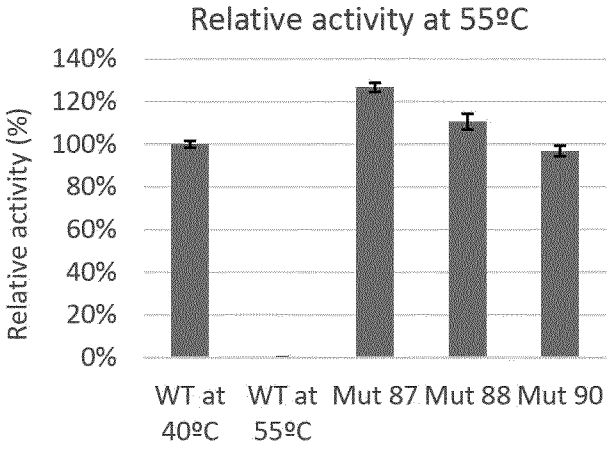
Mut 88
Mut 89
Mut 90
Mut 91
Mut 92
Mut 93
Mut 94
Mut 96
Mut 97
Mut 98
Mut 99

FIGURE 7

A


Relative activity at 40ºC
Relative activity (%)
140%
120%
100%
80%
60%
40%
20%
0%
WT
Mut 87
Mut 88
Mut 90

B


Relative activity at 55ºC
Relative activity (%)
140%
120%
100%
80%
60%
40%
20%
0%
WT at 40ºC
WT at 55ºC
Mut 87
Mut 88
Mut 90

C

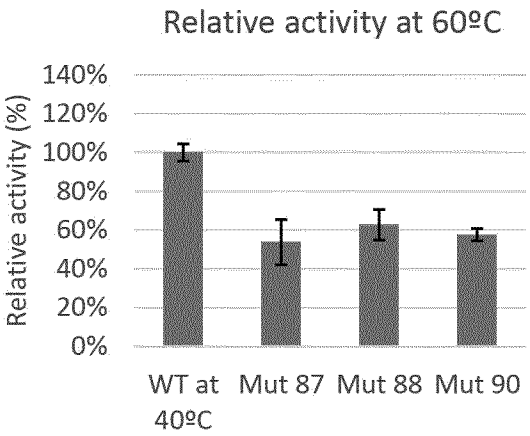

Relative activity at 60ºC
Relative activity (%)
140%
120%
100%
80%
60%
40%
20%
0%
WT at 40ºC
Mut 87
Mut 88
Mut 90

FIGURE 8

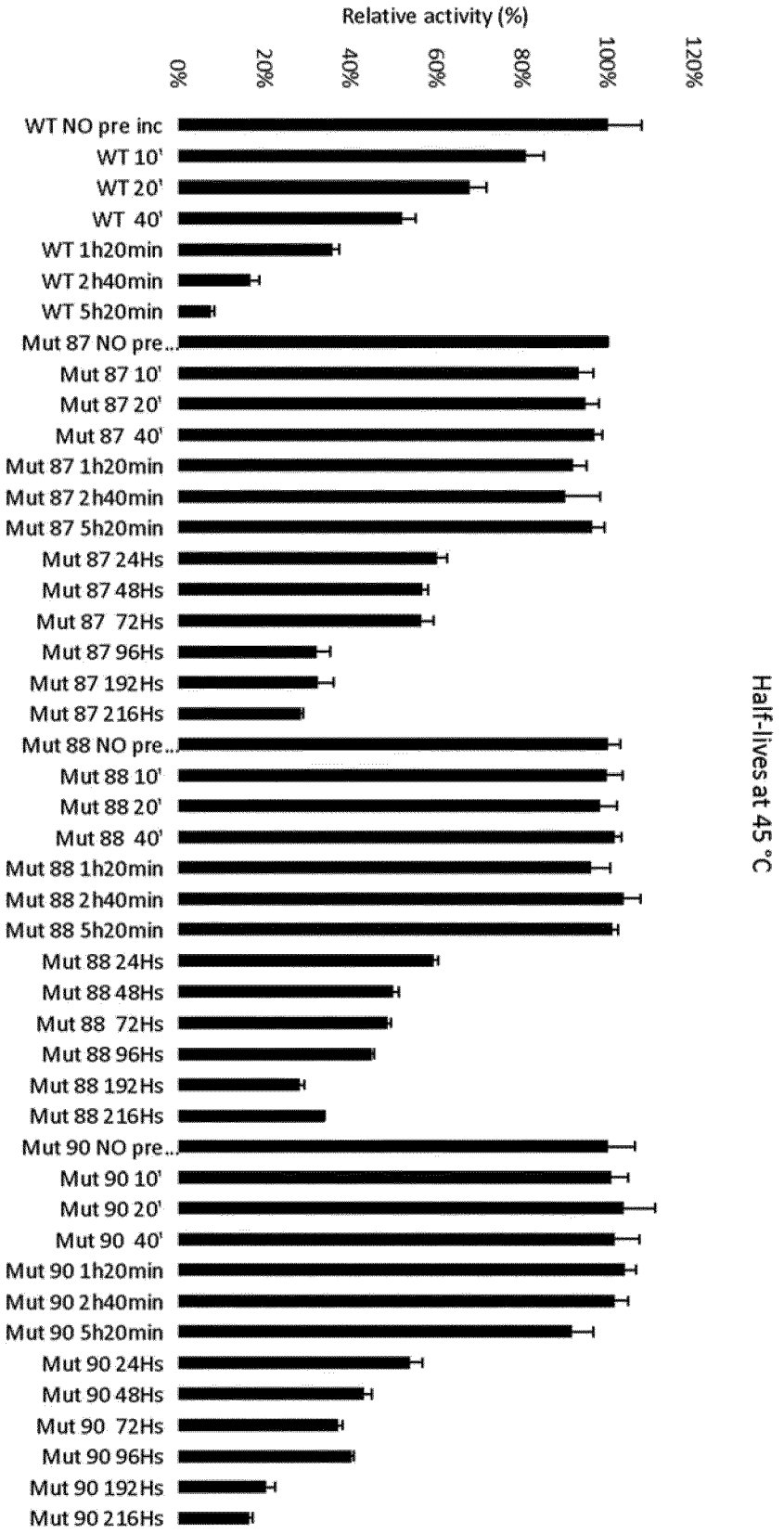

Relative activity (%)
0%
20%
40%
60%
80%
100%
120%
WT NO pre inc
WT 10'
WT 20'
WT 40'
WT 1h20min
WT 2h40min
WT 5h20min
Mut 87 NO pre..
Mut 87 10'
Mut 87 20'
Mut 87 40'
Mut 87 1h20min
Mut 87 2h40min
Mut 87 5h20min
Mut 87 24Hs
Mut 87 48Hs
Mut 87 72Hs
Mut 87 96Hs
Mut 87 192Hs
Mut 87 216Hs
Mut 88 NO pre..
Mut 88 10'
Mut 88 20'
Mut 88 40'
Mut 88 1h20min
Mut 88 2h40min
Mut 88 5h20min
Mut 88 24Hs
Mut 88 48Hs
Mut 88 72Hs
Mut 88 96Hs
Mut 88 192Hs
Mut 88 216Hs
Mut 90 NO pre..
Mut 90 10'
Mut 90 20'
Mut 90 40'
Mut 90 1h20min
Mut 90 2h40min
Mut 90 5h20min
Mut 90 24Hs
Mut 90 48Hs
Mut 90 72Hs
Mut 90 96Hs
Mut 90 192Hs
Mut 90 216Hs
Half-lives at 45 °C

FIGURE 9

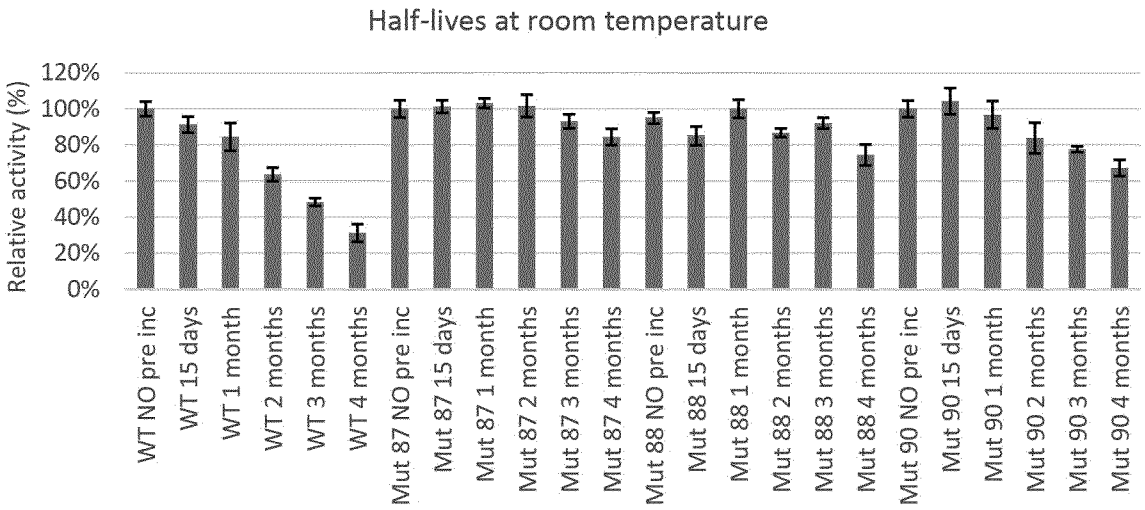


FIGURE 10

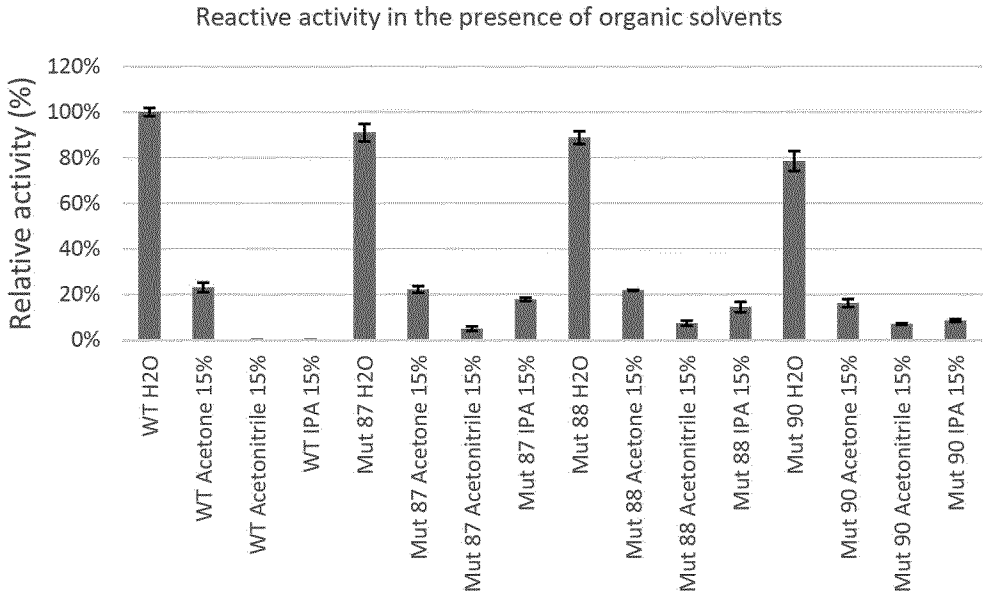


FIGURE 11

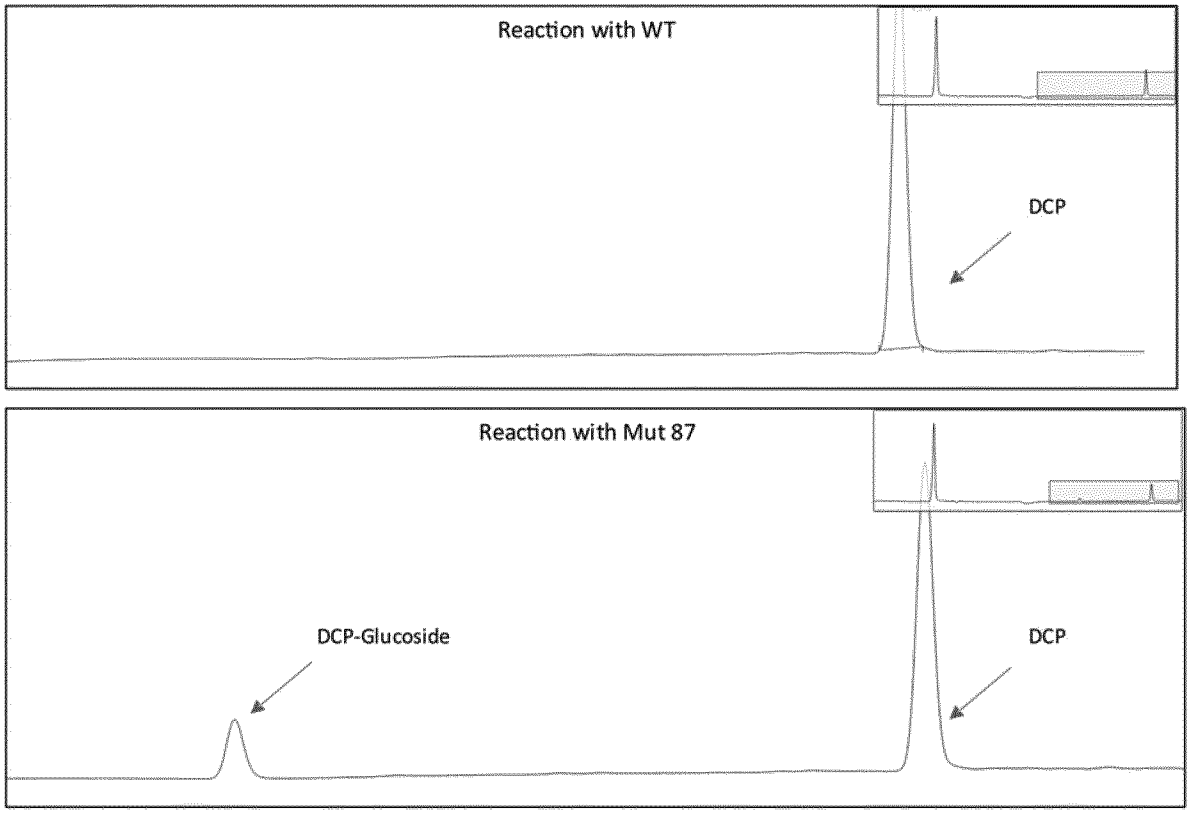


FIGURE 12

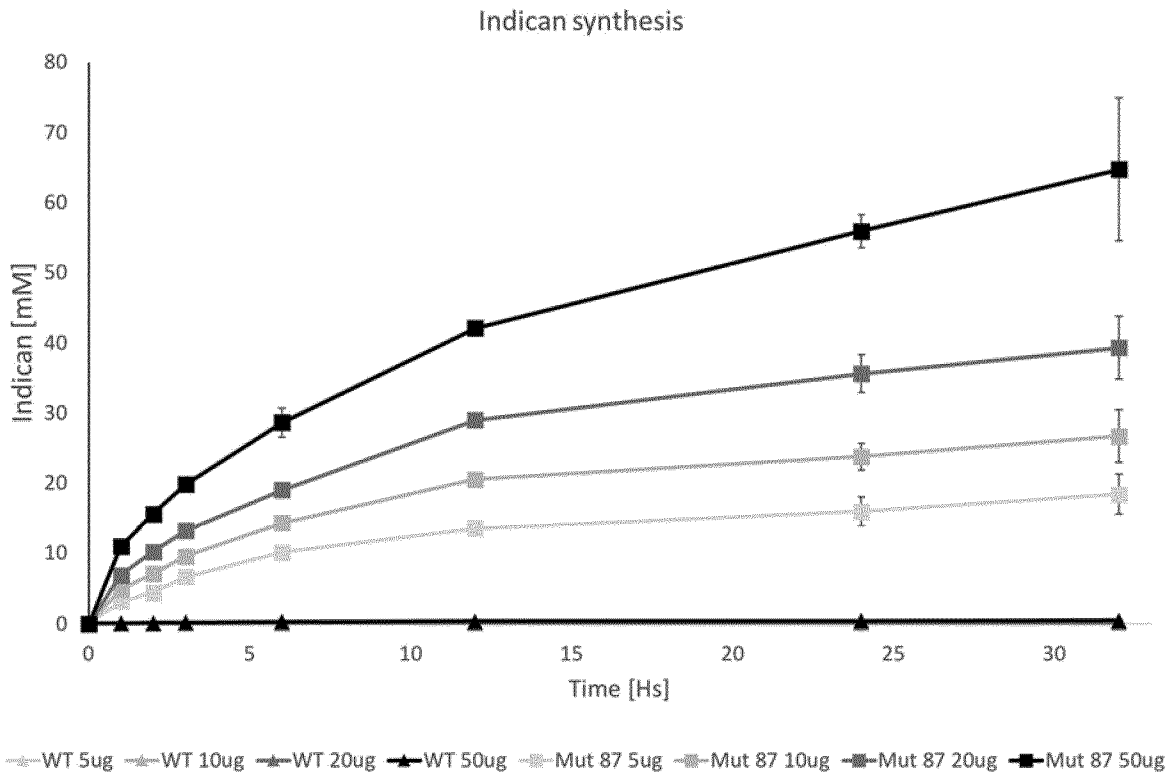
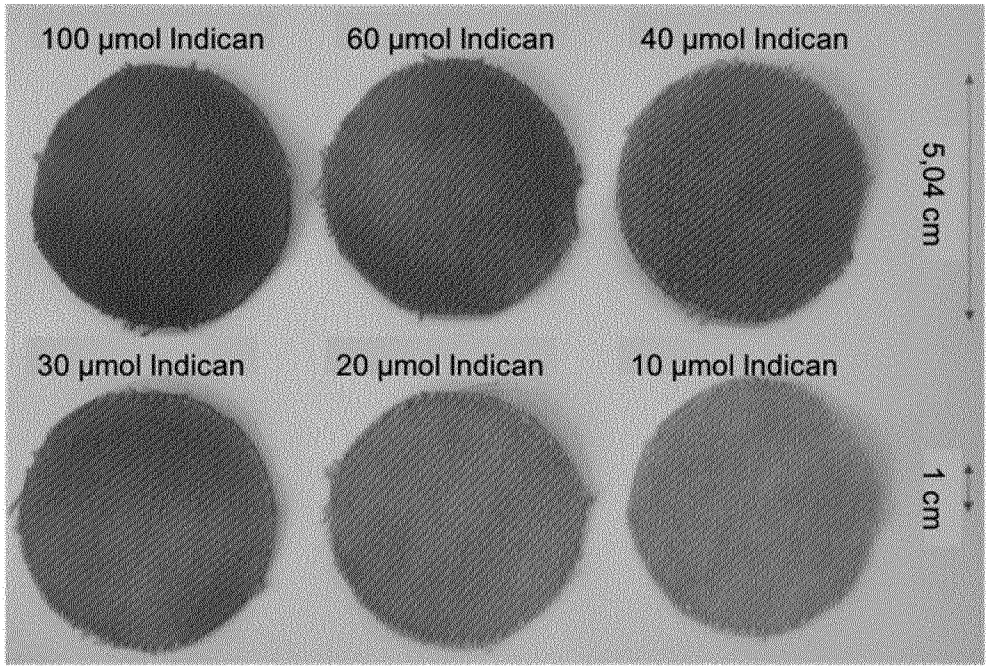


FIGURE 13

A


100 µmol Indican
60 µmol Indican
40 µmol Indican
30 µmol Indican
20 µmol Indican
10 µmol Indican
5,04 cm
1 cm

B

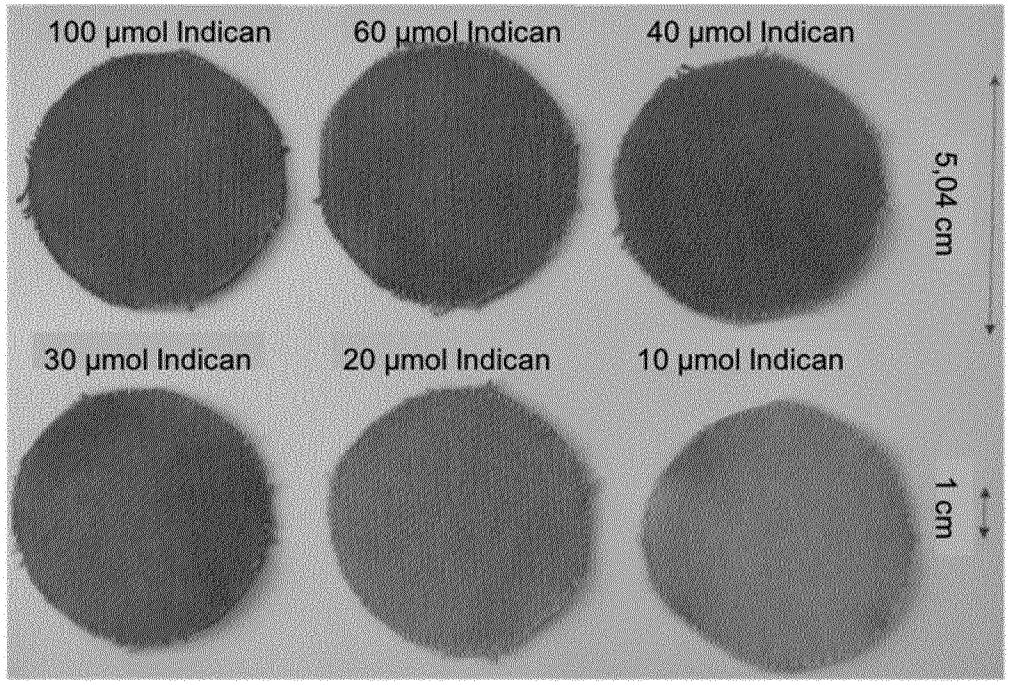

100 µmol Indican
60 µmol Indican
40 µmol Indican
30 µmol Indican
20 µmol Indican
10 µmol Indican
5,04 cm
1 cm

FIGURE 14

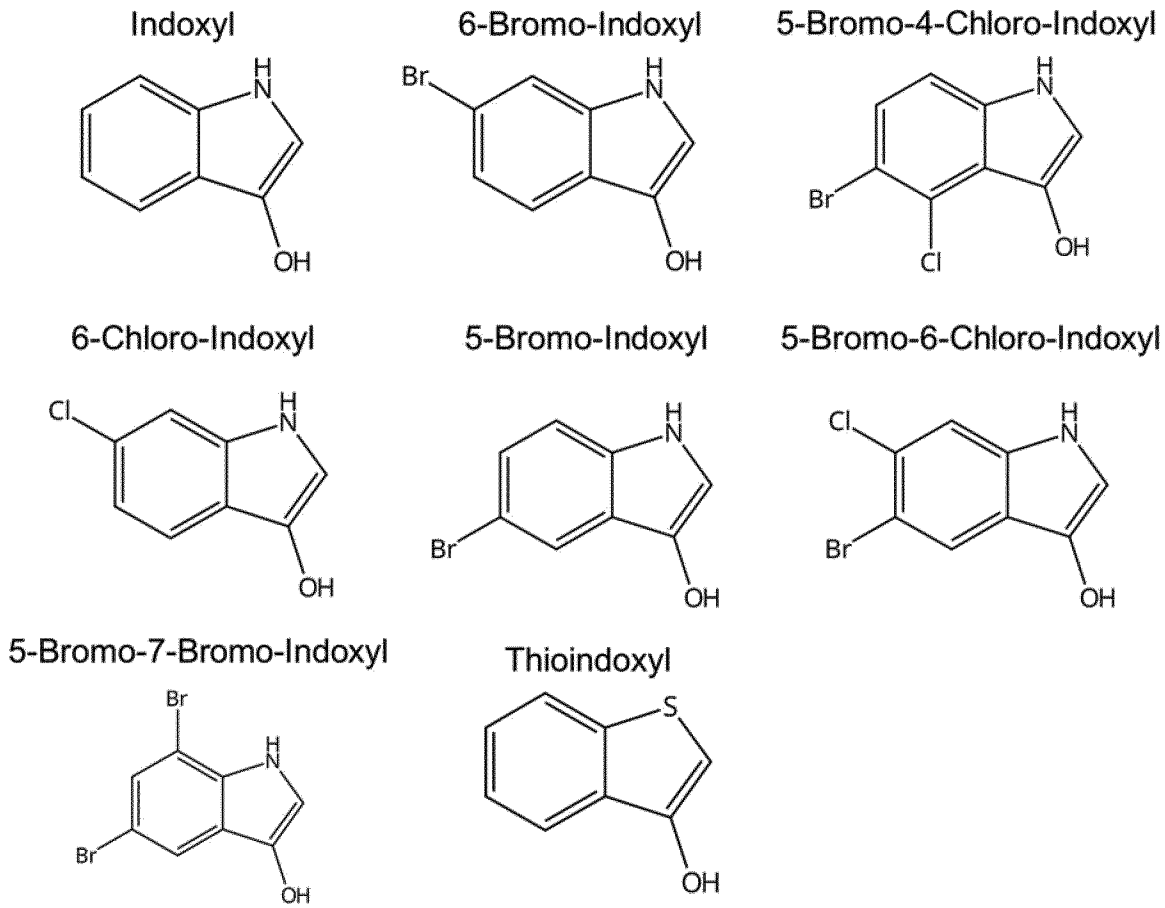


FIGURE 15

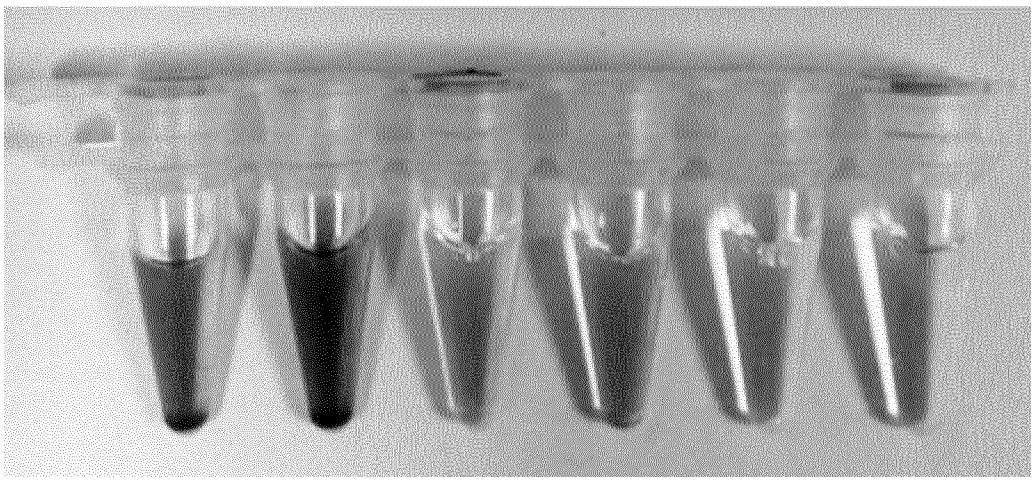


FIGURE 16

A **B**

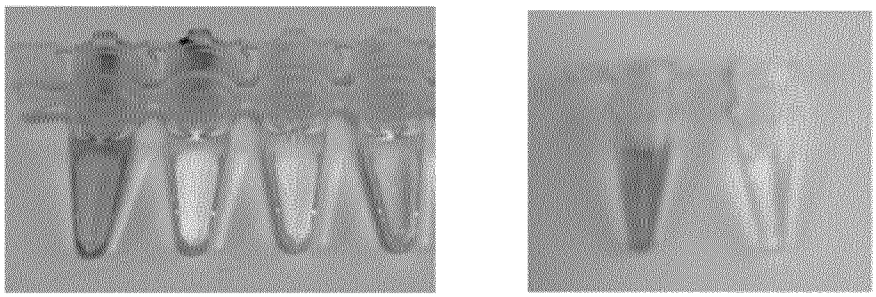


FIGURE 17

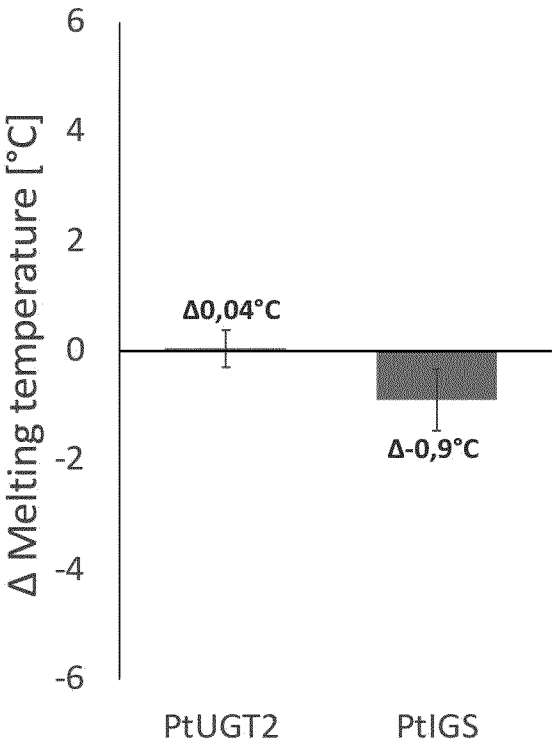
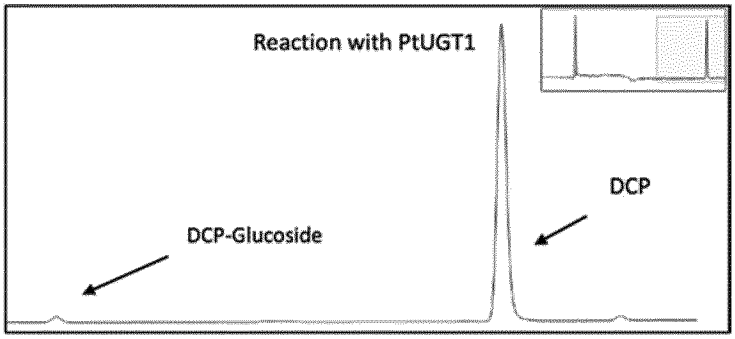
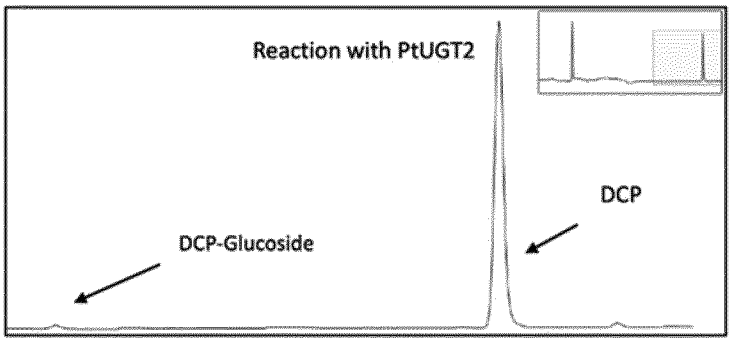


FIGURE 18

A



B



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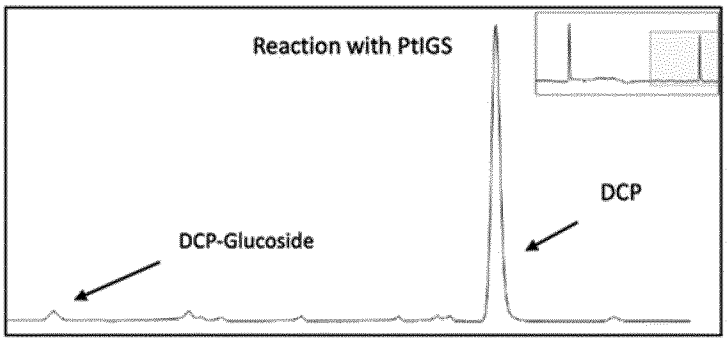
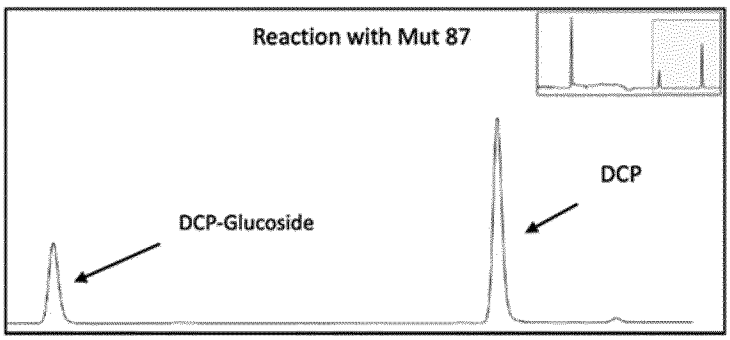


D



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/054318

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/10 C12P17/16
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, WPI Data, BIOSIS, CHEM ABS Data, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE UniProt [Online] 23 May 2018 (2018-05-23), "RecName: Full=Glycosyltransferase {ECO:0000256¦RuleBase:RU362057}; EC=2.4.1.- {ECO:0000256¦RuleBase:RU362057};", XP055947497, retrieved from EBI accession no. UNIPROT:A0A2R2JFJ4 Database accession no. A0A2R2JFJ4 sequence -----	1-16
X	WO 2016/141207 A1 (UNIV CALIFORNIA [US]) 9 September 2016 (2016-09-09) page 70, paragraph 1-2; examples 1-2; table 1; sequences 3, 4 -----	1-16
	-/--	

[x] Further documents are listed in the continuation of Box C.

[x] See patent family annex.

* Special categories of cited documents :

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 April 2023	11/05/2023

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Petri, Bernhard

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HSU TAMMY M ET AL: "Employing a biochemical protecting group for a sustainable indigo dyeing strategy", NATURE CHEMICAL BIOLOGY , vol. 14, no. 3 1 March 2018 (2018-03-01), pages 256-261, XP055947492, New York ISSN: 1552-4450, DOI: 10.1038/nchembio.2552 Retrieved from the Internet: URL:https://www.nature.com/articles/nchembio.2552.pdf page 257, left-hand column, paragraph 3 - page 258, right-hand column, paragraph 1; figure 2a	1-16
X	DATABASE UniProt [Online] 25 April 2018 (2018-04-25), "RecName: Full=Glycosyltransferase {ECO:0000256|RuleBase:RU362057}; EC=2.4.1.- {ECO:0000256|RuleBase:RU362057};", XP002807215, retrieved from EBI accession no. UNIPROT:A0A2L2R220 Database accession no. A0A2L2R220 sequence	1-16
X	INOUE SHINTARO ET AL: "Characterization of UDP-glucosyltransferase from Indigofera tinctoria", PLANT PHYSIOLOGY AND BIOCHEMISTRY, ELSEVIER, AMSTERDAM, NL, vol. 121, 6 November 2017 (2017-11-06), pages 226-233, XP085251882, ISSN: 0981-9428, DOI: 10.1016/J.PLAPHY.2017.11.002 page 229, right-hand column, paragraph 3.2; figure 2	1-16

-/--

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2023/054318

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PETERMEIER PHILIPP ET AL: "Exploring the in Vitro Operating Window of Glycosyltransferase Pt UGT1 from Polygonum tinctorium for a Biocatalytic Route to Indigo Dye", ACS SUSTAINABLE CHEMISTRY & ENGINEERING , vol. 9, no. 25 28 June 2021 (2021-06-28), pages 8497-8506, XP055947542, US ISSN: 2168-0485, DOI: 10.1021/acssuschemeng.1c01536 Retrieved from the Internet: URL:https://backend.orbit.dtu.dk/ws/portalfiles/portal/265868755/acssuschemeng.1c01536.pdf abstract -----	1-16

1

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2023/054318

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2023/054318

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2016141207 A1	09-09-2016	EP 3265577 A1	10-01-2018
		US 2018037917 A1	08-02-2018
		US 2020325516 A1	15-10-2020
		US 2022136024 A1	05-05-2022
		WO 2016141207 A1	09-09-2016