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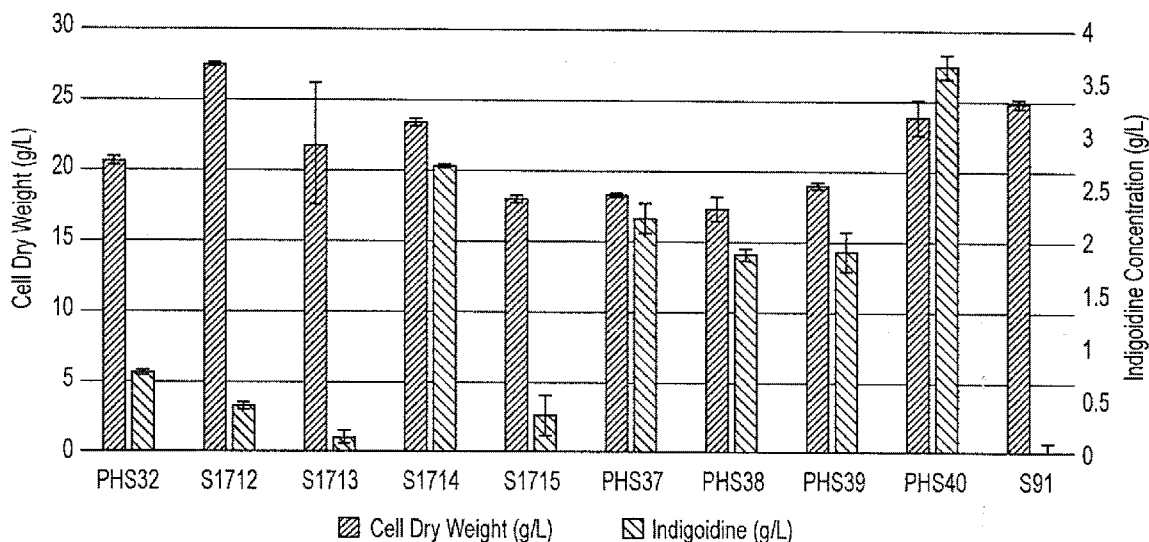


Figure 9

(57) Abstract: The present invention relates to bioproduction of dyes and dyed biomaterials, and in particular relates to cells and methods for producing indigoidine. The invention also provides a dyed cellulose material and methods for producing the same.



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Methods and Compositions

Field of the invention

- 5 The present invention is in the field of bioproduction of dyes and dyed biomaterials, and in particular cells and methods for producing the same.

Background

- 10 The textile industry is associated with severe water and other environmental pollution, due to the release of toxic chemicals as waste from fabric dye production and the dyeing process. Because of the climate crisis and ecological issues caused by non-sustainable industries – including dyeing and associated processes – public awareness of and support for more sustainable production processes is rapidly increasing. There
15 therefore exists an urgent need to develop new, sustainable processes of dye manufacturing that minimise environmental pollution and meet consumers' needs for ecologically friendly products.

- Indigoidine is a non-ribosomal peptide (NRP) with an intense blue colour that
20 resembles the industrial dye indigo. Indigoidine is produced naturally by several bacterial genera. Moreover, due to its molecular structure, indigoidine is an organic semiconductor with potential applications in bioelectronics. The pigment also exhibits antioxidant and antimicrobial activities. Indigoidine is therefore an attractive natural blue dye that may be used in the textile industry to replace polluting synthetic blue
25 dyes in the textile industry; and which may find uses in the electronics and medical fields.

- Indigoidine is produced by the condensation of two molecules of L-glutamine catalysed by a non-ribosomal peptide synthase (NRPS). L-glutamine itself is produced by the
30 condensation of glutamate and ammonia by a glutamine synthetase enzyme. α -ketoglutarate is converted into glutamate by glutamate dehydrogenase.

- Low production levels of non-ribosomal peptides (NRPs) in wild-type non-ribosomal peptide (NRP)-producing strains, and the complex structure of non-ribosomal peptides
35 (NRPs) and associated challenges in purifying non-ribosomal peptide (NRPs) from biological material, makes production of indigoidine production in native strains commercially unviable.

Attempts have been made in the art to develop synthetic biology approaches for improvement of indigoidine production. For example, engineered bacterial strains have been generated that are capable of expressing an NRPS such as the *Streptomyces lavendulae* NRPS BpsA and a phosphopantetheinyl transferase (PPTase) polypeptide, which is capable of activating the function of the NRPS polypeptide. Although condensation of L-glutamine to indigoidine is observed in these engineered bacterial strains, the level of production is limited, for example, by the availability of L-glutamine in the cell. Although supplementation with L-glutamine has separately been shown to increase indigoidine production, the cost of exogenous L-glutamine renders such an approach commercially unviable.

There therefore exists a need for engineered strains that provide an improved level of production of indigoidine.

Summary of the invention

The inventors have developed a robust indigoidine production platform in recombinant yeast. The inventors have surprisingly found that expressing a heterologous non-ribosomal peptide synthetase (NRPS) polypeptide and a heterologous phosphopantetheinyl transferase (PPTase) polypeptide in a yeast cell enables the yeast cell to produce indigoidine.

The inventors have further found that by overexpressing endogenous glutamate dehydrogenase and glutamine synthetase polypeptides in the recombinant yeast cell, it is possible to increase the production of indigoidine without the need to provide supplementary exogenous L-glutamine.

Production of indigoidine can also be increased by altering the carbon and nitrogen sources present in media used to culture the recombinant yeast cell; and by altering the carbon:nitrogen (C/N) ratio.

The inventors have also surprisingly found that it is possible to produce indigoidine-dyed bacterial cellulose by dyeing bacterial pellicles with indigoidine extracted from the recombinant yeast cells; and/or by co-culturing the recombinant yeast cell with a bacterial cellulose producing bacteria.

Detailed description of the invention

The invention is as set out in the claims.

- 5 A first aspect of the invention provides a recombinant yeast cell capable of producing indigoidine, wherein the cell is capable of expressing:
- a) a heterologous non-ribosomal peptide synthetase (NRPS) polypeptide capable of converting glutamine into indigoidine; and
 - b) a heterologous phosphopantetheinyl transferase (PPTase) polypeptide.

10

As will be appreciated by the skilled person, the recombinant yeast cell may be capable of expressing NRPS and PPTase by containing the corresponding RNA and/or DNA nucleic acids.

- 15 Accordingly, it will be clear to the skilled person that the cell comprises:
- a) a nucleic acid comprising a nucleotide sequence encoding the heterologous NRPS polypeptide; and
 - b) a nucleic acid comprising a nucleotide sequence encoding the heterologous PPTase polypeptide;
- 20 and
- wherein the NRPS polypeptide and the PPTase polypeptide are capable of being expressed from the nucleic acids.

25

The nucleic acid may be a DNA nucleic acid, or may be an RNA nucleic acid.

It is important that the nucleic acid is arranged so that the NRPS and PPTase polypeptides may be expressed from said nucleic acids, for example where the nucleic acid is a DNA, the nucleic acid is operably associated with an appropriate promoter, discussed elsewhere herein.

30

- The nucleic acid molecule that comprises a sequence that encodes the NRPS polypeptide may be the same nucleic acid molecule that comprises a sequence that encodes the PPTase – *i.e.*, both polypeptides are expressed from the sample nucleic acid molecule. For example, each sequence may be located on the same chromosome
- 35 in the genome, or may be located on the same vector, such as a plasmid. In other embodiments, the sequence that encodes the NRPS is located on a different nucleic acid molecule to the sequence that encodes the PPTase. For example, each sequence

may be located in different regions of the genome, may be located in different vectors such as plasmids, or a combination of the two.

Accordingly in some embodiments of the recombinant yeast cell,

5

The NRPS polypeptide may be any NRPS polypeptide, provided it is heterologous to the host yeast cell. In some embodiments the NRPS polypeptide is a naturally occurring, or wild-type polypeptide. In other embodiments the NRPS polypeptide is a non-naturally occurring polypeptide or engineered polypeptide that comprises one or more mutations, substitutions or deletions relative to a naturally occurring or wild-type polypeptide.

10

In some embodiments the heterologous NRPS polypeptide is:

15

- a) BpsA; optionally:
 - i) *Streptomyces lavendulae* BpsA; or
 - ii) a synthetic BpsA;
- b) IndC; optionally *Streptomyces chromofuscus* IndC; or
- c) IndB; optionally *Streptomyces chromofuscus* IndB.

20

In some embodiments the heterologous NRPS polypeptide:

25

- a) comprises or consists of an amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 6, or SEQ ID NO: 8; or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 4, SEQ ID NO: 6, or SEQ ID NO: 8; and/or
- b) is encoded by a nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 7; or of at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 5 or SEQ ID NO: 7.

30

The PPTase polypeptide may be any PPTase polypeptide – provided it is heterologous to the host yeast cell. In some embodiments the PPTase polypeptide is a naturally occurring, or wild-type polypeptide. In other embodiments the PPTase polypeptide is a non-naturally occurring polypeptide or engineered polypeptide that comprises one or more mutations, substitutions or deletions relative to a naturally occurring or wild-type polypeptide.

35

In some embodiments the heterologous PPTase polypeptide is *E. coli* PPTase or *Bacillus subtilis* PPTase.

In some embodiments the heterologous PPTase polypeptide:

- 5 a) comprises an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 17, or SEQ ID NO: 19; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 1 or SEQ ID NO: 3, SEQ ID NO: 17, or SEQ ID NO: 18; and/or
- 10 b) is encoded by a nucleotide sequence of SEQ ID NO: 2, SEQ ID NO: 16, or SEQ ID NO: 18; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 2, SEQ ID NO: 16, or SEQ ID NO: 18.

15

In some embodiments the recombinant yeast cell is further capable of expressing or overexpressing a glutamine dehydrogenase (GDH) polypeptide and/or a glutamine synthetase (GS) polypeptide. Each of the glutamine dehydrogenase (GDH) polypeptide and/or a glutamine synthetase (GS) polypeptide may be endogenous, or may be

20 heterologous.

In some preferred embodiments the recombinant yeast cell overexpresses an endogenous GDH. In the same or different embodiment the recombinant yeast cell overexpresses an endogenous GS.

- 25 In some preferred embodiments the recombinant yeast cell overexpresses an heterologous GDH. In the same or different embodiment the recombinant yeast cell overexpresses an heterologous GS.

- In some preferred embodiments the recombinant yeast cell overexpresses an endogenous GDH. In the same or different embodiment the recombinant yeast cell overexpresses an heterologous GS.
- 30

In some preferred embodiments the recombinant yeast cell overexpresses an heterologous GDH. In the same or different embodiment the recombinant yeast cell overexpresses an endogenous GS.

35

In some embodiments the recombinant yeast cell comprises:

a) an engineered nucleic acid comprising a nucleotide sequence encoding the GDH polypeptide; and/or

b) an engineered nucleic acid comprising a nucleotide sequence encoding the GS polypeptide;

5 and

wherein the GDH polypeptide and the GS polypeptide are capable of being expressed from the nucleic acids.

10 As will be appreciated, a nucleic acid may be an "engineered" nucleic acid if the nucleotide sequence comprised by the nucleic acid has been altered or edited, for example by the introduction of an exogenous nucleotide sequence into the nucleic acid, or by deletion of a nucleotide sequence from the nucleic acid. In some embodiments, the engineered nucleic acid is a synthetic nucleic acid. In some embodiments, the engineered nucleic acid is a heterologous nucleic acid that is not naturally comprised
15 by the yeast cell. Heterologous nucleic acids are known to the skilled person and include nucleic acids selected from the group comprising or consisting of: a plasmid, a phagemid, a yeast artificial chromosome (YAC), and a bacterial artificial chromosome (BAC).

20 As described above, any one or more proteins or polypeptides may be expressed from a single nucleic acid molecule, e.g., a single vector, or may be expressed from separate vectors. For example the nucleic acid molecule that comprises a sequence that encodes the GDH polypeptide may be the same nucleic acid molecule that comprises a sequence that encodes the GS – i.e. both polypeptides are expressed from the same
25 nucleic acid molecule. For example, each sequence may be located on the same chromosome in the genome, or may be located on the same vector, such as a plasmid. In other embodiments, the sequence that encodes the GDH is located on a different nucleic acid molecule to the sequence that encodes the GS. For example, each sequence may be located in different regions of the genome, may be located in different
30 vectors such as plasmids, or a combination of the two.

In some embodiments a single nucleic acid molecule may comprise the sequences that encode any one or more of all of: GDH, GS, PPTase and/or NRPS.

35 Also as described above, the nucleic acids that comprise the sequences that encode any one or more of these polypeptides must be capable of expressing the polypeptide from the nucleic acid, i.e. be capable of transcription and/or translation from the nucleic acid.

In some preferred embodiments, the NRPS polypeptide, PPTase polypeptide, GDH polypeptide, and/or GS polypeptide are overexpressed in the cell. The skilled person will appreciate what is meant by "overexpressed" and will understand the means by which that overexpression can be achieved. For example the polypeptides may be expressed from a strong promoter, as described herein; or may be expressed from a high copy plasmid.

In some embodiments, the GDH polypeptide and/or GS polypeptide are endogenous GDH and/or GS polypeptides – i.e. are GS and GDH polypeptides that are naturally found within the recombinant yeast cell.

In some embodiments the GDH polypeptide is encoded by the open reading frame (ORF) defined by YALIOE09603g or YALIOF17820g. The nucleic acid sequences of these ORFs can be found in many public databases, such as GRYC (infra.fr), NCBI (nih.gov), Uniprot (uniprot.org) and KEGG (genome.jp/kegg/).

In some embodiments the GDH polypeptide:

a) comprises an amino acid sequence of SEQ ID NO: 20 or SEQ ID NO: 21; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 20 or SEQ ID NO: 21; and/or

b) is encoded by a nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 12; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 11 or SEQ ID NO: 12.

In some embodiments the GS polypeptide is encoded by the open reading frame defined by YALIOD13024g or YALIOF00506g.

The GS polypeptide may comprise an amino acid sequence of SEQ ID NO: 22 or SEQ ID NO: 23; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 22 or SEQ ID NO: 23; and/or

may be encoded by a nucleotide sequence of SEQ ID NO: 9 or SEQ ID NO: 10; or or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 9 or SEQ ID NO: 10.

As described above, in each case, the nucleic acid that encodes the desired polypeptide must be capable of expressing that polypeptide. For example the skilled person will know that the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide must each be operably linked to a promoter. In some instances, the same promoter may drive expression of more than one polypeptide, though preferably and more usually, each polypeptide is expressed from a nucleic acid that is operably linked to a separate promoter.

The skilled person will be able to choose the most suitable promoter for the particular situation. For example in some embodiments the promoter is:

- a) a constitutive promoter; or
- b) an inducible promoter.

The skilled person also knows about the different strength of expression that can be obtained from different promoters. For example the skilled person will understand the concept of a "strong" promoter and in some embodiments the promoter is a strong promoter.

In some embodiments the promoter driving expression of any of the polypeptides described herein is selected from the group comprising or consisting of:

- a) a constitutive promoter selected from the group comprising or consisting of: an ATP1 promoter [SEQ ID NO: 24], an ATP2 promoter [SEQ ID NO: 25], an FBAln promoter [SEQ ID NO: 26], a PGK1 promoter [SEQ ID NO: 27], a GPM1 promoter [SEQ ID NO: 28], a HHF1 promoter [SEQ ID NO: 29], a CYC1 promoter [SEQ ID NO: 30], an HHT1 promoter [SEQ ID NO: 31], an HTB1 promoter [SEQ ID NO: 32], an EXP1 promoter [SEQ ID NO: 33], a TDH1 promoter [SEQ ID NO: 34], an RPL25 promoter [SEQ ID NO: 35], a TEF1 promoter [SEQ ID NO: 36], a TEFIn promoter [SEQ ID NO: 37], a TEF2UAS promoter [SEQ ID NO: 38], a TEF4UAS promoter [SEQ ID NO: 39], and a TEF8UAS promoter [SEQ ID NO: 40], a pTEF promoter [SEQ ID NO: 13 and 14], a GAP promoter [SEQ ID NO: 57], or

b) an inducible promoter selected from the group comprising or consisting of: a pPOX2 promoter [SEQ ID NO: 41], a pXPR2 promoter [SEQ ID NO: 42], a pFBP1 promoter [SEQ ID NO: 43], a pMDH1a promoter [SEQ ID NO: 44], an ACL2 promoter [SEQ ID NO: 45], an XPR2 promoter [SEQ ID NO: 58], , a POT1 promoter [SEQ ID NO: 46], a LIP2 promoter [SEQ ID NO: 47], an ICL promoter [SEQ ID NO: 48], a YAT1 promoter [SEQ ID NO: 49], a CTR1 promoter [SEQ ID NO: 50], a CTR2 promoter [SEQ ID NO: 51], a pYALI0B18194 promoter [SEQ ID NO: 52], a pYALI0C11165 promoter [SEQ ID NO: 53], a pYALI0C15004 promoter [SEQ ID NO: 54], a pYALI0E14256 promoter [SEQ ID NO: 55], and a pYALI0F13937 promoter [SEQ ID NO: 56].

These promoters are described in "A DNA assembly toolkit to unlock the CRISPR/Cas9 potential for metabolic engineering" Tigran Yuzbashev, Evgeniya Yuzbasheva, Olga Melkina, Davina Patel, Dmitrii Bubnov, Heiko Dietz, and Rodrigo Ledesma-Amaro Version 1 posted 04 Apr, 2023 <https://www.researchsquare.com/article/rs-2738543/v1>.

In some preferred embodiments, the promoter is a pTEF promoter; preferably the pTEF promoter has a nucleotide sequence of SEQ ID NO: 13 or SEQ ID NO: 14.

The skilled person will also appreciate that appropriate terminator sequences are required for proper protein expression. Accordingly the nucleotide sequences encoding the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide are operably linked to a terminator sequence. In some embodiments the terminator sequence is a tLIP2 terminator; for example the tLIP2 terminator with a nucleotide sequence of SEQ ID NO: 15.

The skilled person will understand that nucleic acids that express desired proteins, such as the NRPS and PPTase, GS and GDH described here, can be expressed from a chromosomal location, or can be expressed extra-chromosomally, for example from a plasmid.

In some embodiments the nucleic acids comprising the nucleotide sequences encoding the NRPS polypeptide, the PPTase, GDH polypeptide, and/or GS polypeptide are integrated into the genome of the cell. Methods of integrating a nucleic acid into the genome of a cell are known to the skilled person. For example, a nucleic acid may be suitably integrated into a genome of a cell by targeted integration by homologous recombination or random genomic integration..

In some embodiments the nucleic acids comprising the nucleotide sequences encoding the NRPS polypeptide, the PPTase, GDH polypeptide, and/or GS polypeptide are maintained episomally, for example on one or more plasmids.

5

In some embodiments, the endogenous version of any one or more of these genes has been deleted from the host recombinant yeast cell. By deletion we include the meaning of a full deletion of the gene, but also a functional deletion, i.e. the gene is still present but harbours one or more mutations/insertions/deletions that means that a functional GDH and/or GS protein is not made.

10

For example in some embodiments, recombinant yeast cell comprises a deletion of the endogenous chromosomal GDH and/or GS genes.

15

In the same or different embodiments the recombinant yeast cell comprises a deletion of at least one gene selected from the group comprising or consisting of an open reading frame selected from: YALIOE09603g, YALIOF17820g, YALIOD13024g, YALIOF00506g, YALIOE18634g, YALIOB00572g, and/or YALIOF01606g, or any combination thereof.

20

Codon optimisation is the process of modifying the protein coding sequence to make best use of the codon bias within a given organism. In some embodiments the nucleotide sequences encoding the NRPS polypeptide and/or PPTase polypeptide, and/or when present the nucleotide sequences encoding the GDH and/or GS, are codon optimised for expression in yeast, for example have specifically been optimised for expression in *Yarrowia*.

25

The recombinant yeast cell of the invention may be of any genus or species. Preferably the recombinant yeast cell is an oleaginous yeast cell.

30

In some embodiments the oleaginous yeast cell is not a *Rhodospiridium* cell, for example is not a *Rhodospiridium toruloides* cell.

Preferably the yeast cell is a *Yarrowia* cell. In some embodiments the *Yarrowia* cell may be selected from the group comprising or consisting of: a *Yarrowia bubula* cell, a *Yarrowia deformans* cell, a *Yarrowia lipolytica* cell, a *Yarrowia porcina* cell, a *Yarrowia yakushimensis* cell, a *Yarrowia parophonii* cell, a *Yarrowia galli* cell, a *Yarrowia*

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oslonensis cell, a *Yarrowia alimentaria* cell, a *Yarrowia hollandica* cell, and a *Yarrowia phangngaensis*;

Preferably the recombinant *Yarrowia* cell is a *Yarrowia lipolytica* cell.

- 5 As is known in the art, *Yarrowia lipolytica* previously had the taxonomic name *Candida lipolytica*. Accordingly, the taxonomic names *Yarrowia lipolytica* and *Candida lipolytica* as used herein are interchangeable.

10 The recombinant yeast cell described above is able to produce indigoidine. It will be clear then that the invention also provides methods of producing indigoidine that make use of the recombinant yeast cell of the invention.

Accordingly the invention also provides a method of producing indigoidine comprising culturing a recombinant yeast cell of the invention.

15

For example the method of producing indigoidine comprises

expressing a heterologous non-ribosomal peptide synthetase (NRPS)
polypeptide capable of converting glutamine into indigoidine and a heterologous
phosphopantetheinyl transferase (PPTase) polypeptide in a recombinant yeast
20 cell of the invention;

wherein said expression results in the conversion of glutamine into indigoidine
thereby producing indigoidine.

25 It will be clear that the method requires culturing the cell under conditions suitable for expression of said NRPS polypeptide and PPTase polypeptide.

The recombinant yeast cell may be cultured in any culture media. In some embodiments the cell is cultured in a culture media, is selected from:

- 30 a) a rich media, optionally YPD or YP4D;
b) a minimal media, optionally YNB with glucose or YNB with glycerol; or
c) a super-minimal media; optionally wherein the super-minimal media is SM1, SM2, SM3, SM4, or SM5.

35 For example in some embodiments the culture media is selected from the group comprising or consisting of: YP4G media, YNB glucose media, and YNB glycerol media, optionally YNB glucose or YNB glycerol.

The skilled person will readily understand what these yeast culture media are, but briefly in some embodiments:

A) YP4G media comprises: [or YP4D replacing the glucose below with dextrose]

- 5 Yeast extract: 10 g/L
- Peptone: 20 g/L
- Glucose: 40 g/L

B) YNB glucose media comprises:

- YNB without amino acids: 6.8 g/L
- 10 - Phosphate buffer 500mM pH 6.8: 100 mL/L
- Glucose: 20 g/L

C) YNB Glycerol media comprises:

- YNB without amino acids: 6.8 g/L
- Phosphate buffer 500mM pH 6.8: 100 mL/L
- 15 - Glycerol: 20 g/L

D) SM1 super-minimal media comprises:

- 18 g/L Urea
- 20 g/L Glycerol

E) SM2 super-minimal media comprises:

- 20 - 18 g/L urea
- 25 g/L raw glycerol (which is a waste (crude) glycerol and produced as a by-product of biodiesel and soap production processes; Contents: 38-96% glycerol (80% in this work), Up to 50% methanol, 13% soaps, 2% water, 2-3% salts_

25 F) SM3 super-minimal media comprises:

- 18 g/L urea
- 25 g/L raw glycerol
- 0.17 g/L YNB

G) SM4 super-minimal media comprises:

- 30 - 18 g/L urea
- 25 g/L raw glycerol
- 1.2 g/L K₂PO₄

H) SM5 super-minimal media comprises:

- 18 g/L urea
- 35 - 25 g/L raw glycerol
- 1 g/L MnSO₄

I) YNB media comprises:

- YNB without amino acids: 6.8 g/L
- Phosphate buffer 500mM pH 6.8: 100 mL/L

40 J) YPD media comprises:

10 g/L yeast extract
20 g/L peptone
20 g/L glucose

5 Typically, culture media comprises a) a carbon source; b) a nitrogen source; and/or c) water. Carbon sources, nitrogen sources, and water that are suitable for use in culture media are known to the person skilled in the art.

10 The inventors have surprisingly found that the carbon to nitrogen ration (C/N) impacts indigoidine synthesis. In some embodiments therefore the recombinant yeast cell of the invention is cultured in a culture media that has a carbon to nitrogen (C/N) ratio of:

a) between about 2 and about 160, about 2 and about 150, about 2 and about 140, about 2 and about 130, about 2 and about 120, about 2 and about 110, about 2 and about 100, about 2 and about 90, about 2 and about 80, about 2 and about 70, about 2 and about 60, about 2 and about 50, about 2 and about 40, about 2 and about 30, about 2 and about 20, about 2 and about 10, about 2 and about 9, about 2 and about 8, about 2 and about 7, about 2 and about 6, about 2 and about 5, about 2 and about 4, about 2 and about 3, about 4 and about 160, about 4 and about 150, about 4 and about 140, about 4 and about 130, about 4 and about 120, about 4 and about 110, about 4 and about 100, about 4 and about 90, about 4 and about 80, about 4 and about 70, about 4 and about 60, about 4 and about 50, about 4 and about 40, about 4 and about 30, about 4 and about 20, about 4 and about 10, about 4 and about 9, about 4 and about 8, about 4 and about 7, about 4 and about 6, about 4 and about 5, about 6 and about 160, about 6 and about 150, about 6 and about 140, about 6 and about 130, about 6 and about 120, about 6 and about 110, about 6 and about 100, about 6 and about 90, about 6 and about 80, about 6 and about 70, about 6 and about 60, about 6 and about 50, about 6 and about 40, about 6 and about 30, about 6 and about 20, about 6 and about 10, about 6 and about 9, about 6 and about 8, about 6 and about 7, about 8 and about 160, about 8 and about 150, about 8 and about 140, about 8 and about 130, about 8 and about 120, about 8 and about 110, about 8 and about 100, about 8 and about 90, about 8 and about 80, about 8 and about 70, about 8 and about 60, about 8 and about 50, about 8 and about 40, about 8 and about 30, about 8 and about 20, about 8 and about 10, about 8 and about 9, about 8 and about 8, about 8 and about 7, about 8 and about 6, about 8 and about 5, about 8 and about 4, about 8 and about 3, about 10 and about 160, about 10 and about 150, about 10 and about 140, about 10 and about 130, about 10 and about

1100, about 10 and about 110, about 10 and about 100, about 10 and about 90, about 10 and about 80, about 10 and about 70, about 10 and about 60, about 10 and about 50, about 10 and about 40, about 10 and about 30, about 10 and about 20, about 20 and about 160, about 20 and about 150, about 20 and about 140, about 20 and about 130, about 20 and about 1200, about 20 and about 110, about 20 and about 100, about 20 and about 90, about 20 and about 80, about 20 and about 70, about 20 and about 60, about 20 and about 50, about 20 and about 40, about 20 and about 30, about 30 and about 160, about 30 and about 150, about 30 and about 140, about 30 and about 130, about 30 and about 1300, about 30 and about 110, about 30 and about 100, about 30 and about 90, about 30 and about 80, about 30 and about 70, about 30 and about 60, about 30 and about 50, about 30 and about 40, about 40 and about 160, about 40 and about 150, about 40 and about 140, about 40 and about 130, about 40 and about 1400, about 40 and about 110, about 40 and about 100, about 40 and about 90, about 40 and about 80, about 40 and about 70, about 40 and about 60, about 40 and about 50, about 50 and about 160, about 50 and about 150, about 50 and about 140, about 50 and about 130, about 50 and about 150, about 50 and about 110, about 50 and about 100, about 50 and about 90, about 50 and about 80, about 50 and about 70, or about 50 and about 60;

b) between 2 and 160, 2 and 150, 2 and 140, 2 and 130, 2 and 120, 2 and 110, 2 and 100, 2 and 90, 2 and 80, 2 and 70, 2 and 60, 2 and 50, 2 and 40, 2 and 30, 2 and 20, 2 and 10, 2 and 9, 2 and 8, 2 and 7, 2 and 6, 2 and 5, 2 and 4, 2 and 3, 4 and 160, 4 and 150, 4 and 140, 4 and 130, 4 and 140, 4 and 110, 4 and 100, 4 and 90, 4 and 80, 4 and 70, 4 and 60, 4 and 50, 4 and 40, 4 and 30, 4 and 20, 4 and 10, 4 and 9, 4 and 8, 4 and 7, 4 and 6, 4 and 5, 6 and 160, 6 and 150, 6 and 140, 6 and 130, 6 and 160, 6 and 110, 6 and 100, 6 and 90, 6 and 80, 6 and 70, 6 and 60, 6 and 50, 6 and 40, 6 and 30, 6 and 20, 6 and 10, 6 and 9, 6 and 8, 6 and 7, 8 and 160, 8 and 150, 8 and 140, 8 and 130, 8 and 180, 8 and 110, 8 and 100, 8 and 90, 8 and 80, 8 and 70, 8 and 60, 8 and 50, 8 and 40, 8 and 30, 8 and 20, 8 and 10, 8 and 9, 8 and 8, 8 and 7, 8 and 6, 8 and 5, 8 and 4, 8 and 3, 10 and 160, 10 and 150, 10 and 140, 10 and 130, 10 and 1100, 10 and 110, 10 and 100, 10 and 90, 10 and 80, 10 and 70, 10 and 60, 10 and 50, 10 and 40, 10 and 30, 10 and 20, 20 and 160, 20 and 150, 20 and 140, 20 and 130, 20 and 1200, 20 and 110, 20 and 100, 20 and 90, 20 and 80, 20 and 70, 20 and 60, 20 and 50, 20 and 40, 20 and 30, 30 and 160, 30 and 150, 30 and 140, 30 and 130, 30 and 1300, 30 and 110, 30 and 100, 30 and 90, 30 and 80, 30 and 70, 30 and 60, 30 and 50,

30 and 40, 40 and 160, 40 and 150, 40 and 140, 40 and 130, 40 and 1400, 40 and 110, 40 and 100, 40 and 90, 40 and 80, 40 and 70, 40 and 60, 40 and 50, 50 and 160, 50 and 150, 50 and 140, 50 and 130, 50 and 150, 50 and 110, 50 and 100, 50 and 90, 50 and 80, 50 and 70, or 50 and 60;

5

c) at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70, at least about 80, at least about 90, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150 or more;

10

d) less than about 160, less than about 150, less than about 140, less than about 130, less than about 120, less than about 110, less than about 100, less than about 90, less than about 80, less than about 70, less than about 60, less than about 50, less than about 40, less than about 30, less than about 20, less than about 10, less than about 9, less than about 8, less than about 7, less than about 6, less than about 5, less than about 4, less than about 3; and/or

15

e) at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150 or more;

20

f) less than 160, less than 150, less than 140, less than 130, less than 120, less than 110, less than about 100, less than 90, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, less than 3;

25

g) about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150; and/or

30

h) 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160.

35 Preferably the C/N ratio is between:

a) about 4 and about 20; and/or

b) 4 and 20.

Or is:

- c) 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about or 20; and/or
- 5 d) 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.

The carbon source may be any carbon source. Preferably the carbon-source is not derived from a food feedstock and is instead a waste product, or a feedstock grown specifically for the purposes of making indigoidine.

10

In some embodiments the carbon source is selected from the group comprising or consisting of: glucose, fructose, mannose, citrate, acetate, rapeseed oil, sunflower oil, palm oil, waste cooking oil, rapeseed oil, and sunflower oil, lignocellulosic hydrolysate, glycerol, and/or waste glycerol. In some embodiments the lignocellulosic hydrolysate is selected from the group comprising or consisting of: hydrolysed linen chaff, pepper, vegetable mix, and urban pruning, or any combination thereof.

15

In preferred embodiments the carbon source is lignocellulosic hydrolysate.

20

The water may be any water. As with the feedstock, there are certain environmental, and cost, advantages if the water used to culture the yeast does not need to have been purified or cleaned.

25

In some embodiments the water is selected from the group comprising or consisting of: sea water, tap water and distilled water; or any combination thereof.

30

The nitrogen source may be any nitrogen source. In some embodiments the nitrogen source is selected from the group comprising or consisting of: urine, synthetic urine, urea, ammonium chloride, peptone, and ammonium sulphate, or any combination thereof. Synthetic urine, in some embodiments, comprises 18.018g/L urea, 1.96g/L creatine, 1.44g/L citric acid, 0.28g/L glycine, 18.76g/L sodium nitrate, 1.36g/L hippuric acid, and 0.48g/L cysteine.

35

In some embodiments the nitrogen source is ammonium sulphate.

Yeast may be cultured in or on solid (agar) media, or in liquid media. Preferably the method of producing indigoidine comprises culturing the recombinant yeast cell of the invention in a liquid media. Preferably the liquid culture is incubated with some

agitation, such as shaking in a shaking incubator. The inventors found that increasing the agitation speed during culture impacted indigoidine production.

In some embodiments the said culturing comprises agitating the liquid media during cell growth; for example wherein the liquid media is agitated:

- 5
- a) at between about 150 and about 2000 RPM, about 150 and about 250 RPM, about 150 and about 200 RPM, about 200 and about 250 RPM, about 150 and about 800 RPM, about 200 and about 800 RPM, and about 250 and about 800 RPM, about 800 and about 2000 RPM, about 1000 and about 1800 RPM,
- 10
- about 1200 and about 1400 RPM;
- b) at between 150 and 2000 RPM, 150 and 250 RPM, 150 and 200 RPM, 200 and 250 RPM, 150 and 800 RPM, 200 and 800 RPM, and 250 and 800 RPM, 800 and 2000 RPM, 1000 and 1800 RPM, 1200 and 1400 RPM;
- c) at at least 150 RPM, at least 200 RPM, at least 250 RPM, at least 800 RPM, at least 1000 RPM, at least 1200 RPM, at least 1400 RPM, at least 1600 RPM, at least 1800 RPM;
- 15
- d) at less than 2000 RPM, less than 1800 RPM, less than 1600 RPM, less than 1400 RPM, less than 1200 RPM, less than 1000 RPM, less than 800RPM, less than 250 RPM, or less than 200 RPM;
- c) at about 150 RPM, about 200 RPM, about 250 RPM, about 800 RPM, about 1000 RPM, about 1200 RPM, about 1400 RPM, about 1600 RPM, about 1800 RPM, or about 2000 RPM; or
- 20
- d) at 150 RPM, 200 RPM, 250 RPM, 800 RPM, 1000 RPM, 1200 RPM, 1400 RPM, 1600 RPM, 1800 RPM, or 2000 RPM.

25

The inventors also found that supplementation of the culture media with glutamine and/or glutamic acid improves indigoidine production. Accordingly in some embodiments the culture media is supplemented with glutamine and/or glutamic acid, for example supplemented with:

30

glutamine at concentrations of:

- i) between about 1mM and about 100mM, about 2mM and about 90mM, about 3mM and about 80mM, about 4mM and about 70mM, about 5mM and about 60mM, about 6mM and about 50mM, about 7mM and about 40mM, about 8mM and about 30mM, about 9mM and about 20mM, about 10 mM and about 15mM,
- 35
- about 1mM and about 10mM, about 2mM and about 9mM, about 3mM and about 8mM, about 4mM and about 7mM, about 5mM and about 6 mM, about 10 mM and about 100mM, about 20mM and about 90mM, about 30mM and about 80mM,

about 40mM and about 70mM, about 50mM and about 60mM, about 50mM and about 150mM, about 60mM and about 140mM, about 70mM and about 130mM, about 80mM and about 120mM, or about 90mM and about 110mM glutamine;

ii) between 1mM and 100mM, 2mM and 90mM, 3mM and 80mM, 4mM and 70mM, 5mM and 60mM, 6mM and 50mM, 7mM and 40mM, 8mM and 30mM, 9mM and 20mM, 10 mM and 15mM, 1mM and 10mM, 2mM and 9mM, 3mM and 8mM, 4mM and 7mM, 5mM and 6 mM, 10 mM and 100mM, 20mM and 90mM, 30mM and 80mM, 40mM and 70mM, 50mM and 60mM, 50mM and 150mM, 60mM and 140mM, 70mM and 130mM, 80mM and 120mM, or 90mM and 110mM glutamine;

iii) at least 1mM, at least 2 mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 20mM, at least 30mM, at least 40mM, at least 50mM, at least 60mM, at least 70mM, at least 80mM, at least 90mM, at least 100mM, at least 110mM, at least 120mM, at least 130mM, at least 140mM, at least 150mM, or more glutamine;

iv) less than 150mM, less than 140mM, less than 130mM, less than 120mM, less than 120mM, less than 110mM, less than 100mM, less than 90mM, less than 80mM, less than 70mM, less than 60mM, less than 50mM, less than 40mM, less than 30mM, less than 20mM, less than 10mM, less than 9mM, less than 8mM, less than 7mM, less than 6mM, less than 5mM, less than 4mM, less than 3mM, less than 2mM, or less glutamine;

v) about 1mM, about 2 mM, about 3mM, about 4mM, about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about 10mM, about 20mM, about 30mM, about 40mM, about 50mM, about 60mM, about 70mM, about 80mM, about 90mM, about 100mM, about 110mM, about 120mM, about 130mM, about 140mM, or about 150mM glutamine; and/or

vi) 1mM, 2 mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM, 140mM, or 150mM glutamine;

and/or

supplemented with glutamic acid at concentrations of:

i) between about 1mM and about 100mM, about 2mM and about 90mM, about 3mM and about 80mM, about 4mM and about 70mM, about 5mM and about 60mM, about 6mM and about 50mM, about 7mM and about 40mM, about 8mM and about 30mM, about 9mM and about 20mM, about 10 mM and about 15mM, about 1mM and about 10mM, about 2mM and about 9mM, about 3mM and about 8mM, about 4mM and about 7mM, about 5mM and about 6 mM, about 10 mM and about 100mM, about 20mM and about 90mM, about 30mM and about 80mM,

about 40mM and about 70mM, about 50mM and about 60mM, about 50mM and about 150mM, about 60mM and about 140mM, about 70mM and about 130mM, about 80mM and about 120mM, or about 90mM and about 110mM glutamic acid;

ii) between 1mM and 100mM, 2mM and 90mM, 3mM and 80mM, 4mM and 70mM, 5mM and 60mM, 6mM and 50mM, 7mM and 40mM, 8mM and 30mM, 9mM and 20mM, 10 mM and 15mM, 1mM and 10mM, 2mM and 9mM, 3mM and 8mM, 4mM and 7mM, 5mM and 6 mM, 10 mM and 100mM, 20mM and 90mM, 30mM and 80mM, 40mM and 70mM, 50mM and 60mM, 50mM and 150mM, 60mM and 140mM, 70mM and 130mM, 80mM and 120mM, or 90mM and 110mM glutamic acid;

iii) at least 1mM, at least 2 mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 20mM, at least 30mM, at least 40mM, at least 50mM, at least 60mM, at least 70mM, at least 80mM, at least 90mM, at least 100mM, at least 110mM, at least 120mM, at least 130mM, at least 140mM, at least 150mM, or more glutamic acid;

iv) less than 150mM, less than 140mM, less than 130mM, less than 120mM, less than 120mM, less than 110mM, less than 100mM, less than 90mM, less than 80mM, less than 70mM, less than 60mM, less than 50mM, less than 40mM, less than 30mM, less than 20mM, less than 10mM, less than 9mM, less than 8mM, less than 7mM, less than 6mM, less than 5mM, less than 4mM, less than 3mM, less than 2mM, or less glutamic acid;

v) about 1mM, about 2 mM, about 3mM, about 4mM, about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about 10mM, about 20mM, about 30mM, about 40mM, about 50mM, about 60mM, about 70mM, about 80mM, about 90mM, about 100mM, about 110mM, about 120mM, about 130mM, about 140mM, or about 150mM glutamic acid; and/or

vi) 1mM, 2 mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM, 140mM, or 150mM glutamic acid.

In some embodiments of the recombinant yeast cell of the invention, the yeast cell expresses an endogenous glutamate dehydrogenase (GDH) polypeptide and/or an endogenous glutamine synthase (GS) polypeptide. In some embodiments the GDH and GS may be heterologous to the yeast cell. In some preferred embodiments the expression of GDH and/or GS is an overexpression, for example via a strong promoter, or via expression from a multicopy plasmid.

The method of producing indigoidine may comprise a step relating to the extraction or purification of the indigoidine from the cell or the cell culture. For example in some embodiments the extracting and/or purifying the indigoidine from the cell or cell culture comprises lysing the cell. Lysing the cell may be performed by any means. In some
5 embodiments the cell is lysed by homogenisation in DMSO. Such methods are known, and in some embodiments involve the use of glass beads, cell disruption at 8,000 rpm and centrifugation.

Yeast cells may be collected from the culture by centrifugation or filtration and are in
10 some embodiments dried before lysis by grinding.

Other methods and uses of the recombinant yeast cell of the invention will also be apparent to the skilled person and form part of the invention.

For example, the invention provides the use of the recombinant yeast cell of the
15 invention in a method of producing indigoidine. The method may be the method of producing indigoidine of the invention as described herein.

The invention also provides indigoidine that was obtained or is obtainable from a
20 recombinant yeast cell of the invention.

The invention also provides indigoidine that was obtained or is obtainable via the method of producing indigoidine of the invention.

As mentioned elsewhere here, the inventors have found that the indigoidine produced
25 by the recombinant yeast cell of the invention can be used to produce indigoidine-stained bacterial cellulose, through either co-culture of the cellulose producing bacteria and the recombinant yeast cell of the invention, or via a stepwise separate culture process.

For example, the invention provides a method of producing indigoidine-stained
30 bacterial cellulose, or an indigoidine-stained bacterial cellulose pellicle, where the method comprises co-culturing the recombinant yeast cell of the invention with a cellulose-producing second cell.

35 The second cell is a bacterial cell, and is:

- a) is capable of producing bacterial cellulose;
- b) expresses all of bcsA, bcsD, bscC and bscD;

- c) belongs to a genus selected from the group comprising or consisting of: *Komagataeibacter*, *Escherichia*, *Gluconacetobacter*, *Acetobacter*, *Sarcina*, *Agrobacterium*, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and *Alcaligenes*;
- 5 d) are selected from the group comprising or consisting of: *Komagaeibacter rhaeticus*; *Komagaeibacter xylinus*, *Komagaeibacter hansenii*, *Komagaeibacter medellinensis*, *Komagaeibacter europaeus*, *Komagaeibacter maltaceti*, *Komagaeibacter pomaceti*, *Komagaeibacter oboediens*, or *Komagaeibacter saccharivoans*;
- 10 e) are selected from the group comprising or consisting of:
- i) a strain of *Komagaeibacter rhaeticus* selected from the group comprising or consisting of: *Komagaeibacter rhaeticus* iGEM. *Komagaeibacter rhaeticus* AF1; *Komagaeibacter rhaeticus* LMG22126; or
- 15 ii) *Gluconacetobacter xylinus* CGMCC 2995;
- and/or
- f) are *Komagaeibacter rhaeticus* iGEM cells.

The method of producing indigoidine-stained bacterial cellulose or a cellulose pellicle comprises, in some embodiments, growing the recombinant yeast cell and the cellulose-producing second cell under conditions that allows the cellulose-producing second cell to produce cellulose or a cellulose pellicle. In some embodiments the cellulose produced can be considered to be a pellicle, and so in some embodiments the conditions are such that allows the cellulose-producing second cell to produce a pellicle.

25 Exemplary conditions that allow the production of bacterial cellulose, and a bacterial cellulose pellicle, are described in WO 2023/285800, the entirety of which is hereby incorporated by reference. Specifically, page 13 lines 21-28; Example 1 page 88; Example 2 page 91 lines 37 – page 92 line 15 are hereby incorporated by reference.

30 For example, in some embodiments the conditions that allow the cellulose-producing second cell to produce cellulose or a cellulose pellicle comprise culturing the cell:

- a) at a pH of:
- i) between 3-7, optionally a pH of between 3.25 and 6.75, 3.5 and 6.5, 3.5 and 6.25, 3.75 and 6, 4 and 5.75, 4.25 and 5.5, 4.5 and 5.25; pH 5.8; and/or
- 35 ii) at least 3 but less than or equal to pH 7, for example at least 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 5.8, 6, 6.25, 6.5, 6.75, but less than or equal to pH 7;

and/or

b) in culture media that is:

i) HS media;

ii) YPD media; or

5 iii) Coconut water media.

The invention also provides a method of producing indigoidine-stained bacterial cellulose or producing an indigoidine-stains pellicle, the method comprising:

10 a) culturing a cellulose-producing second cell to form bacterial cellulose and/or a cellulose pellicle; and subsequently

b) contacting the cellulose or cellulose pellicle produced by the cellulose-producing second cell in (a) with the recombinant yeast cell of the invention and as described elsewhere herein.

15 Preference for the cellulose-producing second cell are as described elsewhere herein.

By contacting we include the meaning of incubating the recombinant yeast cell of the invention with the cellulose or the pellicle. The cellulose or the pellicle may be incubated with the recombinant yeast cell of the invention under suitable conditions
20 for cultivating the recombinant yeast cell as described herein.

Preferences for the culture conditions to produce bacterial cellulose or a cellulose pellicle are as described elsewhere herein.

25 In some embodiments of the methods for producing indigoidine-stained cellulose or a pellicle, the cellulose or pellicle may be sterilised, for example where the sterilisation is selected from the group comprising or consisting of: autoclaving, heating, and desiccation, or any combination thereof.

30 The skilled person will appreciate that the indigoidine produced by the recombinant yeast cell of the invention can be used to dye textiles and other materials (including the bacterial cellulose/pellicle which can itself be used as a textile). Accordingly in some embodiments the invention provides a method of producing indigoidine-dyed textile or thread, comprising:

35 a) producing indigoidine according to the method the invention;

and

b) contacting the textile or thread with the indigoidine obtained in (a).

In some embodiments, the indigoidine produced by the recombinant yeast cell is extracted and purified as described elsewhere herein, for example by lysing the cells. Lysing the cell may be performed by any means. In some embodiments the cell is lysed by homogenisation in DMSO. Such methods are known, and in some
5 embodiments involve the use of glass beads, cell disruption at 8,000 rpm and centrifugation.

Yeast cells may be collected from the culture by centrifugation or filtration and are in some embodiments dried before lysis by grinding.

10 In some embodiments in contacting the textile or thread with the indigoidine obtained in (a) comprises soaking the textile or thread in the indigoidine. In some embodiments the textile or thread is soaked in the dyeing mixture for:

- 15 a) between about 4 h and about 12 h, about 5 h and about 11 h, about 6 h and about 10 h, about 7 h and about 9 h;
- b) between 4 h and 12 h, 5 h and 11 h, 6 h and 10 h, 7 h and 9 h;
- c) at least 4 h, at least 5 h, at least 6 h, at least 7 h, at least 8 h, at least 9 h, at least 10 h, at least 12 h;
- 20 d) less than 12 h, less than 11 h, less than 10 h, less than 9 h, less than 8 h, less than 7 h, less than 6 h, less than 5 h;
- e) about 4 h, about 5 h, about 6 h, about 7 h, about 8 h, about 9 h, about 10 h, about 11 h, about 12 h; and/or
- f) 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h.

25 In some embodiments the textile or thread is then

- c) autoclaved; and/or
- d) washed.

30 The invention also provides indigoidine-stained bacterial cellulose obtained or obtainable from any of the methods of the invention.

The invention also provides a garment comprising the indigoidine-stained bacterial cellulose, the indigoidine-stained pellicle, or the indigoidine-stained textile or thread of the invention.

35 The invention also provides an expression construct comprising a nucleic acid comprising:

- a) a nucleotide sequence encoding an NRPS polypeptide, for example as described herein;
- b) a nucleotide sequence encoding a PPTase polypeptide, for example as described herein;
- 5 c) a nucleotide sequence encoding a GDH polypeptide, for example as described herein; and/or
- d) a nucleotide sequence encoding a GS polypeptide, for example as described herein;

10 wherein the nucleotide sequences encoding the NRPS polypeptide, the PPTase polypeptide, the GDH polypeptide, and/or the GS polypeptide are operably linked to a promoter.

15 The expression construct, including any associated sequences such as promoter, or homology regions required to direct homologous recombination into the genome, may be provided as a PCR product, *i.e.*, amplified out from a temple vector.

Preferences for the nucleic acid, the NRPS, PPTase, GDH and GS and any associated promoters and terminators are as described elsewhere herein.

20 The invention also provides a vector that comprises the expression construct of the invention. The vector may be any vector, for example may be selected from the group comprising or consisting of: a plasmid, a phagemid, a YAC, and a BAC.

25 The invention also provides an engineered non-ribosomal peptide synthetase (NRPS) polypeptide that converts glutamine into indigoidine, wherein the engineered NRPS:

- a) comprises:
 - 30 i) an amino acid sequence of SEQ ID NO: 8 or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 8;
 - ii) an amino acid subsequence of SEQ ID NO: 8 capable of converting glutamine into indigoidine or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 8; or
 - 35 iii) an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical thereto;

and/or

b) is encoded by:

i) a nucleotide sequence of SEQ ID NO: 7 or a nucleotide sequence that is at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 7;

ii) a nucleotide subsequence of SEQ ID NO: 7 or of a nucleotide subsequence that is at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 7; or

iii) a nucleic acid sequence that is at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical thereto.

Also provided is a nucleic acid comprising a nucleotide sequence encoding the engineered non-ribosomal peptide synthetase (NRPS) polypeptide of the invention. The nucleic acid may be operably linked to a promoter and/or terminator as defined herein.

The invention provides a cell comprising the expression construct, the nucleic acid, and/or the vector of the invention. The cell may be any cell, for example may be any cell described herein. In some embodiments the cell is a cell such as *E. coli* and is used for cloning purposes. In some embodiments the cell is a recombinant yeast cell as described herein and is used for making indigoidine.

25

The invention also provides a plurality of cells of the invention.

In some embodiments the plurality of cells comprises a first plurality of cells that are recombinant yeast cells, and a second plurality of cells that are cellulose-producing cells.

30

As will be apparent to the skilled person, many of the methods and compositions of the invention lend themselves to being provided in the form of a kit, or kit of parts. Accordingly the invention also provides a kit comprising:

35

- a) the recombinant yeast cell of the invention;
- b) culture media as described herein;
- c) a second cellulose-producing cell of the invention;
- d) the indigoidine of the invention;

- e) a bacterial cellulose of the invention;
- f) a pellicle of the invention;
- g) a textile or thread of the invention;
- h) the garment of the invention;
- 5 i) the expression construct of the invention;
- j) the vector of the invention;
- k) the engineered non-ribosomal peptide synthetase (NRPS) of the invention;
- l) the nucleic acid of the invention;
- 10 and/or
- m) the plurality of cells of the invention.

The invention also provides the following numbered embodiments:

- 15 1. A recombinant yeast cell capable of producing indigoidine, wherein the cell expresses or is capable of expressing:
 - a) a heterologous non-ribosomal peptide synthetase (NRPS) polypeptide capable of converting glutamine into indigoidine; and
 - b) a heterologous phosphopantetheinyl transferase (PPTase) polypeptide.
- 20 2. The recombinant yeast cell of embodiment 1, wherein the cell comprises:
 - a) a nucleic acid comprising a nucleotide sequence encoding the heterologous NRPS polypeptide; and
 - b) a nucleic acid comprising a nucleotide sequence encoding the heterologous PPTase polypeptide;
 - 25 and
 - wherein the NRPS polypeptide and the PPTase polypeptide are capable of being expressed from the nucleic acids.
- 30 3. The recombinant yeast cell of embodiment 1, wherein the cell comprises a nucleic acid comprising:
 - a) a nucleotide sequence encoding the heterologous NRPS polypeptide; and
 - b) a nucleotide sequence encoding the heterologous PPTase polypeptide;
 - and
 - 35 wherein the NRPS polypeptide and the PPTase polypeptide are capable of being expressed from the nucleic acid.

4. The recombinant yeast cell of any of embodiments 1-3, wherein the heterologous NRPS polypeptide is:

- a) BpsA; optionally:
 - i) *Streptomyces lavendulae* BpsA; or
 - ii) a synthetic BpsA;
- b) IndC; optionally *Streptomyces chromofuscus* IndC; or
- c) IndB; optionally *Streptomyces chromofuscus* IndB.

5. The recombinant yeast cell of any of embodiments 1-4, wherein the heterologous NRPS polypeptide:

- a) comprises or consists of an amino acid sequence of SEQ ID NO: 4, SEQ ID NO: 6, or SEQ ID NO: 8; or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 4, SEQ ID NO: 6, or SEQ ID NO: 8; and/or
- b) is encoded by a nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 7; or of at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 5 or SEQ ID NO: 7.

6. The recombinant yeast cell of any of embodiments 1-5, wherein the heterologous PPTase polypeptide is *E. coli* PPTase or *Bacillus subtilis* PPTase.

7. The recombinant yeast cell of any of embodiments 1-6, wherein the heterologous PPTase polypeptide:

- a) comprises an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 17, or SEQ ID NO: 19; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 1 or SEQ ID NO: 3, SEQ ID NO: 17, or SEQ ID NO: 18; and/or
- b) is encoded by a nucleotide sequence of SEQ ID NO: 2, SEQ ID NO: 16, or SEQ ID NO: 18; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 2, SEQ ID NO: 16, or SEQ ID NO: 18.

8. The recombinant yeast cell of any of embodiments 1-7, wherein the cell further expresses or overexpresses, or is capable of expressing or overexpressing, a glutamine dehydrogenase (GDH) polypeptide and/or a glutamine synthetase (GS) polypeptide.

5 9. The recombinant yeast cell of embodiment 8, wherein the cell comprises:
a) an engineered nucleic acid comprising a nucleotide sequence encoding the GDH polypeptide; and/or
b) an engineered nucleic acid comprising a nucleotide sequence encoding the GS polypeptide;
10 and
wherein the GDH polypeptide and the GS polypeptide are capable of being expressed from the nucleic acids.

10. The recombinant yeast cell of embodiment 8, wherein the cell comprises an
15 engineered nucleic acid comprising:
a) a nucleotide sequence encoding the GDH polypeptide; and/or
b) a nucleotide sequence encoding the GS polypeptide;
and
wherein the GDH polypeptide and the GS polypeptide are capable of being
20 expressed from the nucleic acid.

11. The recombinant yeast cell of any of embodiments 1-8, wherein the GDH polypeptide and/or GS polypeptide are endogenous GDH and/or GS polypeptides.

25 12. The recombinant yeast cell of embodiment 11, wherein the GDH polypeptide is encoded by the open reading frame defined by YALIOE09603g or YALIOF17820g.

13. The recombinant yeast cell of any of embodiments 11-12, wherein the GDH polypeptide:
30 a) comprises an amino acid sequence of SEQ ID NO: 20 or SEQ ID NO: 21; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 20 or SEQ ID NO: 21; and/or
35 b) is encoded by a nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 12; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least

95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 11 or SEQ ID NO: 12.

14. The recombinant yeast cell of any of embodiments 11-13, wherein the GS polypeptide is encoded by the open reading frame defined by YALI0D13024g or YALI0F00506g.

15. The recombinant yeast cell of any of embodiments 11-14, wherein the GS polypeptide:

- 10 a) comprises an amino acid sequence of SEQ ID NO: 22 or SEQ ID NO: 23; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 22 or SEQ ID NO: 23; and/or
- 15 b) is encoded by a nucleotide sequence of SEQ ID NO: 9 or SEQ ID NO: 10; or or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 9 or SEQ ID NO: 10.

20

16. The recombinant yeast cell of any of embodiments 2-15, wherein the nucleotide sequences encoding the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide are each operably linked to a promoter, optionally each operably linked to a separate promoter.

25

17. The recombinant yeast cell of embodiment 16, wherein the promoter is:

- a) a constitutive promoter; or
- b) an inducible promoter;
- optionally

30

wherein the promoter is a strong promoter..

18. The recombinant yeast cell of either of embodiments 16 or 17, wherein the promoter is selected from the group comprising or consisting of:

- 35 a) a constitutive promoter selected from the group comprising or consisting of: an ATP1 promoter [SEQ ID NO: 24], an ATP2 promoter [SEQ ID NO: 25], an FBAln promoter [SEQ ID NO: 26], a PGK1 promoter [SEQ ID NO: 27], a GPM1 promoter [SEQ ID NO: 28], a HHF1 promoter [SEQ ID NO: 29], a CYC1 promoter [SEQ ID NO: 30], an HHT1 promoter [SEQ ID NO: 31], an HTB1

- promoter [SEQ ID NO: 32], an EXP1 promoter [SEQ ID NO: 33], a TDH1 promoter [SEQ ID NO: 34], an RPL25 promoter [SEQ ID NO: 35], a TEF1 promoter [SEQ ID NO: 36], a TEF_{in} promoter [SEQ ID NO: 37], a TEF2UAS promoter [SEQ ID NO: 38], a TEF4UAS promoter [SEQ ID NO: 39], and a TEF8UAS promoter [SEQ ID NO: 40], a pTEF promoter [SEQ ID NO: 13 and 14], a GAP promoter [SEQ ID NO: 57], or
- 5 b) an inducible promoter selected from the group comprising or consisting of: a pPOX2 promoter [SEQ ID NO: 41], a pXPR2 promoter [SEQ ID NO: 42], a pFBP1 promoter [SEQ ID NO: 43], a pMDH1a promoter [SEQ ID NO: 44], an
- 10 ACL2 promoter [SEQ ID NO: 45], an XPR2 promoter [SEQ ID NO: 58], , a POT1 promoter [SEQ ID NO: 46], a LIP2 promoter [SEQ ID NO: 47], an ICL promoter [SEQ ID NO: 48], a YAT1 promoter [SEQ ID NO: 49], a CTR1 promoter [SEQ ID NO: 50], a CTR2 promoter [SEQ ID NO: 51], a pYALI0B18194 promoter [SEQ ID NO: 52], a pYALI0C11165 promoter [SEQ ID NO: 53], a pYALI0C15004
- 15 promoter [SEQ ID NO: 54], a pYALI0E14256 promoter [SEQ ID NO: 55], and a pYALI0F13937 promoter [SEQ ID NO: 56].
19. The recombinant yeast cell of any of embodiments 16-18, wherein the promoter
- 20 is a pTEF promoter; optionally
- wherein the pTEF promoter has a nucleotide sequence encoded by SEQ ID NO: 13 or SEQ ID NO: 14.
20. The recombinant yeast cell of any of embodiments 2-15, wherein the nucleotide
- 25 sequences encoding the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide are operably linked to a terminator sequence.
21. The recombinant yeast cell of embodiment 20, wherein the terminator sequence
- 30 is a tLIP2 terminator; optionally
- wherein the tLIP2 terminator has a nucleotide sequence of SEQ ID NO: 15.
22. The recombinant yeast cell of any of embodiments 2-21, wherein the nucleic
- 35 acids comprising the nucleotide sequences encoding the NRPS polypeptide and/or the PPTase polypeptide are integrated into the genome of the cell.

23. The recombinant yeast cell of any of embodiments 8-22, wherein the nucleic acids comprising the nucleotide sequences encoding the GDH polypeptide, and/or GS polypeptide are integrated into the genome of the cell....

5 24. The recombinant yeast cell of any of embodiments 2-23 comprising a deletion of the endogenous chromosomal GDH and/or GS genes.

25. The recombinant yeast cell of any of embodiments 2-24 comprising a deletion of at least one gene selected from the group comprising or consisting of an open
10 reading frame selected from: YALIOE09603g, YALIOF17820g, YALIOD13024g, YALIOF00506g, YALIOE18634g, YALIOB00572g, and/or YALIOF01606g, or any combination thereof.

26. The recombinant yeast cell of any of embodiments 2-25, wherein the nucleotide
15 sequences encoding the NRPS polypeptide and/or PPTase polypeptide, and/or when present the nucleotide sequences encoding the GDH and/or GS, are codon optimised for expression in yeast, optionally optimised for expression in *Yarrowia*.

27. The recombinant yeast cell of any of the preceding embodiments wherein the
20 yeast cell is an oleaginous yeast cell.

28. The recombinant yeast cell of embodiment 27 wherein the oleaginous yeast cell is not a *Rhodospiridium* cell,
optionally
25 wherein the cell is not a *Rhodospiridium toruloides* cell.

29. The recombinant yeast cell of any of the preceding embodiments, wherein the yeast cell is a *Yarrowia* cell, optionally is selected from the group comprising or consisting of: a *Yarrowia bubula* cell, a *Yarrowia deformans* cell, a *Yarrowia lipolytica*
30 cell, a *Yarrowia porcina* cell, a *Yarrowia yakushimensis* cell, a *Yarrowia parophonii* cell, a *Yarrowia galli* cell, a *Yarrowia oslonensis* cell, a *Yarrowia alimentaria* cell, a *Yarrowia hollandica* cell, and a *Yarrowia phangngaensis*;
preferably
wherein the recombinant *Yarrowia* cell is a *Yarrowia lipolytica* cell.

35

30. A method of producing indigoidine comprising:

expressing a heterologous non-ribosomal peptide synthetase (NRPS) polypeptide capable of converting glutamine into indigoidine and a heterologous phosphopantetheinyl transferase (PPTase) polypeptide in a recombinant yeast cell according to any of embodiments 1-29;

5

wherein said expression results in the conversion of glutamine into indigoidine thereby producing indigoidine.

31. The method of embodiment 30, wherein the method comprises culturing the cell under conditions suitable for expression of said NRPS polypeptide and PPTase polypeptide.

32. The method of embodiment 31, wherein the cell is cultured in a culture media, optionally wherein the media is selected from:

- 15
- a) a rich media, optionally YPD or YP4D;
 - b) a minimal media, optionally YNB with glucose or YNB with glycerol; or
 - c) a super-minimal media; optionally wherein the super-minimal media is SM1, SM2, SM3, SM4, or SM5.

20 33. The method of any of embodiments 30-32, wherein the media comprises:

- a) a carbon source;
- b) a nitrogen source; and/or
- c) water.

25 34. The method of any of embodiments 32 or 33, wherein the culture media has a carbon to nitrogen (C/N) ratio of:

- 30
- a) between about 2 and about 160, about 2 and about 150, about 2 and about 140, about 2 and about 130, about 2 and about 120, about 2 and about 110, about 2 and about 100, about 2 and about 90, about 2 and about 80, about 2 and about 70, about 2 and about 60, about 2 and about 50, about 2 and about 40, about 2 and about 30, about 2 and about 20, about 2 and about 10, about 2 and about 9, about 2 and about 8, about 2 and about 7, about 2 and about 6, about 2 and about 5, about 2 and about 4, about 2 and about 3, about 4 and about 160, about 4 and about 150, about 4 and about 140, about 4 and about 130, about 4 and about 120, about 4 and about 110, about 4 and about 100, about 4 and about 90, about 4 and about 80, about 4 and about 70, about 4 and about 60, about 4 and about 50, about 4 and about 40, about 4 and about 30, about 4 and about 20, about 4 and about 10, about 4 and about
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9, about 4 and about 8, about 4 and about 7, about 4 and about 6, about 4 and about 5, about 6 and about 160, about 6 and about 150, about 6 and about 140, about 6 and about 130, about 6 and about 160, about 6 and about 110, about 6 and about 100, about 6 and about 90, about 6 and about 80, about 6 and about 70, about 6 and about 60, about 6 and about 50, about 6 and about 40, about 6 and about 30, about 6 and about 20, about 6 and about 10, about 6 and about 9, about 6 and about 8, about 6 and about 7, about 8 and about 160, about 8 and about 150, about 8 and about 140, about 8 and about 130, about 8 and about 180, about 8 and about 110, about 8 and about 100, about 8 and about 90, about 8 and about 80, about 8 and about 70, about 8 and about 60, about 8 and about 50, about 8 and about 40, about 8 and about 30, about 8 and about 20, about 8 and about 10, about 8 and about 9, about 8 and about 8, about 8 and about 7, about 8 and about 6, about 8 and about 5, about 8 and about 4, about 8 and about 3, about 10 and about 160, about 10 and about 150, about 10 and about 140, about 10 and about 130, about 10 and about 1100, about 10 and about 110, about 10 and about 100, about 10 and about 90, about 10 and about 80, about 10 and about 70, about 10 and about 60, about 10 and about 50, about 10 and about 40, about 10 and about 30, about 10 and about 20, about 20 and about 160, about 20 and about 150, about 20 and about 140, about 20 and about 130, about 20 and about 1200, about 20 and about 110, about 20 and about 100, about 20 and about 90, about 20 and about 80, about 20 and about 70, about 20 and about 60, about 20 and about 50, about 20 and about 40, about 20 and about 30, about 30 and about 160, about 30 and about 150, about 30 and about 140, about 30 and about 130, about 30 and about 1300, about 30 and about 110, about 30 and about 100, about 30 and about 90, about 30 and about 80, about 30 and about 70, about 30 and about 60, about 30 and about 50, about 30 and about 40, about 40 and about 160, about 40 and about 150, about 40 and about 140, about 40 and about 130, about 40 and about 1400, about 40 and about 110, about 40 and about 100, about 40 and about 90, about 40 and about 80, about 40 and about 70, about 40 and about 60, about 40 and about 50, about 50 and about 160, about 50 and about 150, about 50 and about 140, about 50 and about 130, about 50 and about 150, about 50 and about 110, about 50 and about 100, about 50 and about 90, about 50 and about 80, about 50 and about 70, or about 50 and about 60;

b) between 2 and 160, 2 and 150, 2 and 140, 2 and 130, 2 and 120, 2 and 110, 2 and 100, 2 and 90, 2 and 80, 2 and 70, 2 and 60, 2 and 50, 2 and 40, 2 and 30, 2 and 20, 2 and 10, 2 and 9, 2 and 8, 2 and 7, 2 and 6, 2 and 5, 2

- and 4, 2 and 3, 4 and 160, 4 and 150, 4 and 140, 4 and 130, 4 and 140, 4 and 110, 4 and 100, 4 and 90, 4 and 80, 4 and 70, 4 and 60, 4 and 50, 4 and 40, 4 and 30, 4 and 20, 4 and 10, 4 and 9, 4 and 8, 4 and 7, 4 and 6, 4 and 5, 6 and 160, 6 and 150, 6 and 140, 6 and 130, 6 and 160, 6 and 110, 6 and 100, 6 and 90, 6 and 80, 6 and 70, 6 and 60, 6 and 50, 6 and 40, 6 and 30, 6 and 20, 6 and 10, 6 and 9, 6 and 8, 6 and 7, 8 and 160, 8 and 150, 8 and 140, 8 and 130, 8 and 180, 8 and 110, 8 and 100, 8 and 90, 8 and 80, 8 and 70, 8 and 60, 8 and 50, 8 and 40, 8 and 30, 8 and 20, 8 and 10, 8 and 9, 8 and 8, 8 and 7, 8 and 6, 8 and 5, 8 and 4, 8 and 3, 10 and 160, 10 and 150, 10 and 140, 10 and 130, 10 and 1100, 10 and 110, 10 and 100, 10 and 90, 10 and 80, 10 and 70, 10 and 60, 10 and 50, 10 and 40, 10 and 30, 10 and 20, 20 and 160, 20 and 150, 20 and 140, 20 and 130, 20 and 1200, 20 and 110, 20 and 100, 20 and 90, 20 and 80, 20 and 70, 20 and 60, 20 and 50, 20 and 40, 20 and 30, 30 and 160, 30 and 150, 30 and 140, 30 and 130, 30 and 1300, 30 and 110, 30 and 100, 30 and 90, 30 and 80, 30 and 70, 30 and 60, 30 and 50, 30 and 40, 40 and 160, 40 and 150, 40 and 140, 40 and 130, 40 and 1400, 40 and 110, 40 and 100, 40 and 90, 40 and 80, 40 and 70, 40 and 60, 40 and 50, 50 and 160, 50 and 150, 50 and 140, 50 and 130, 50 and 150, 50 and 110, 50 and 100, 50 and 90, 50 and 80, 50 and 70, or 50 and 60;
- 20 ;
- 25 c) at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70, at least about 80, at least about 90, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150 or more;
- 30 d) less than about 160, less than about 150, less than about 140, less than about 130, less than about 120, less than about 110, less than about, less than about 100, less than about 90, less than about 80, less than about 70, less than about 60, less than about 50, less than about 40, less than about 30, less than about 20 , less than about 10, less than about 9, less than about 8, less than about 7, less than about 6, less than about 5, less than about 4, less than about 3; and/or
- 35 e) at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150 or more;

- 5 f) less than 160, less than 150, less than 140, less than 130, less than 120, less than 110, less than about, less than 100, less than 90, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, less than 3;
- g) about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150; and/or
- 10 h) 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160.
35. The method of any of embodiments 32-34, wherein the C/N ratio is between:
- 15 a) about 4 and about 20; and/or
- b) 4 and 20.
36. The method of embodiment 35, wherein the C/N ratio is:
- 20 a) 4, about 5, about 6, about 7, about 8, about 9, about 10, about 11, about 12, about 13, about 14, about 15, about 16, about 17, about 18, about 19, about or 20; and/or
- b) 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20.
37. The method of any of embodiments 32-36, wherein the culture media is selected from the group comprising or consisting of: YP4G media, YNB glucose media, and YNB glycerol media, optionally YNB glucose or YNB glycerol.
- 25 38. The method of any of embodiments 32-36, wherein the carbon source is selected from the group comprising or consisting of: glucose, fructose, mannose, citrate, acetate, rapeseed oil, sunflower oil, palm oil, waste cooking oil, rapeseed oil, and sunflower oil, lignocellulosic hydrolysate, glycerol, and/or waste glycerol;
- 30 optionally
- wherein the lignocellulosic hydrolysate is selected from the group comprising or consisting of: hydrolysed linen chaff, pepper, vegetable mix, and urban pruning, or any combination thereof.
- 35 39. The method of any of embodiments 33-37, wherein the carbon source is lignocellulosic hydrolysate.

40. The method of any of embodiments 33-37, wherein the water is selected from the group comprising or consisting of: sea water, tap water and distilled water; or any combination thereof.

5 41. The method of any of embodiments 33-40, wherein the nitrogen source is selected from the group comprising or consisting of: urine, synthetic urine, urea, ammonium chloride, peptone, and ammonium sulphate, or any combination thereof.

42. The method of any of embodiments 33-41, wherein the nitrogen source is
10 ammonium sulphate.

43. The method of any of embodiments 32-42, wherein the culture media is a liquid media.

15 44. The method of embodiment 43, wherein said culturing comprises agitating the liquid media during cell growth; optionally wherein the liquid media is agitated:

a) at between about 150 and about 2000 RPM, about 150 and about 250 RPM, about 150 and about 200 RPM, about 200 and about 250 RPM, about 150 and about 800 RPM, about 200 and about 800 RPM, and about 250 and about
20 800 RPM, about 800 and about 2000 RPM, about 1000 and about 1800 RPM, about 1200 and about 1400 RPM;

b) at between 150 and 2000 RPM, 150 and 250 RPM, 150 and 200 RPM, 200 and 250 RPM, 150 and 800 RPM, 200 and 800 RPM, and 250 and 800 RPM, 800 and 2000 RPM, 1000 and 1800 RPM, 1200 and 1400 RPM;

25 c) at at least 150 RPM, at least 200 RPM, at least 250 RPM, at least 800 RPM, at least 1000 RPM, at least 1200 RPM, at least 1400 RPM, at least 1600 RPM, at least 1800 RPM;

d) at less than 2000 RPM, less than 1800 RPM, less than 1600 RPM, less than 1400 RPM, less than 1200 RPM, less than 1000 RPM, less than 800RPM, less than 250 RPM, or less than 200 RPM;
30

c) at about 150 RPM, about 200 RPM, about 250 RPM, about 800 RPM, about 1000 RPM, about 1200 RPM, about 1400 RPM, about 1600 RPM, about 1800 RPM, or about 2000 RPM; or

d) at 150 RPM, 200 RPM, 250 RPM, 800 RPM, 1000 RPM, 1200 RPM, 1400
35 RPM, 1600 RPM, 1800 RPM, or 2000 RPM.

45. The method of any of embodiments 32-44, wherein the culture media is supplemented with glutamine and/or glutamic acid.

46. The method of any of embodiments 32-45, wherein:

a) the culture media is supplemented with glutamine at concentrations of:

i) between about 1mM and about 100mM, about 2mM and about 90mM, about 3mM and about 80mM, about 4mM and about 70mM, about 5mM and about 60mM, about 6mM and about 50mM, about 7mM and about 40mM, about 8mM and about 30mM, about 9mM and about 20mM, about 10 mM and about 15mM, about 1mM and about 10mM, about 2mM and about 9mM, about 3mM and about 8mM, about 4mM and about 7mM, about 5mM and about 6 mM, about 10 mM and about 100mM, about 20mM and about 90mM, about 30mM and about 80mM, about 40mM and about 70mM, about 50mM and about 60mM, about 50mM and about 150mM, about 60mM and about 140mM, about 70mM and about 130mM, about 80mM and about 120mM, or about 90mM and about 110mM glutamine;

ii) between 1mM and 100mM, 2mM and 90mM, 3mM and 80mM, 4mM and 70mM, 5mM and 60mM, 6mM and 50mM, 7mM and 40mM, 8mM and 30mM, 9mM and 20mM, 10 mM and 15mM, 1mM and 10mM, 2mM and 9mM, 3mM and 8mM, 4mM and 7mM, 5mM and 6 mM, 10 mM and 100mM, 20mM and 90mM, 30mM and 80mM, 40mM and 70mM, 50mM and 60mM, 50mM and 150mM, 60mM and 140mM, 70mM and 130mM, 80mM and 120mM, or 90mM and 110mM glutamine;

iii) at least 1mM, at least 2 mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 20mM, at least 30mM, at least 40mM, at least 50mM, at least 60mM, at least 70mM, at least 80mM, at least 90mM, at least 100mM, at least 110mM, at least 120mM, at least 130mM, at least 140mM, at least 150mM, or more glutamine;

iv) less than 150mM, less than 140mM, less than 130mM, less than 120mM, less than 120mM, less than 110mM, less than 100mM, less than 90mM, less than 80mM, less than 70mM, less than 60mM, less than 50mM, less than 40mM, less than 30mM, less than 20mM, less than 10mM, less than 9mM, less than 8mM, less than 7mM, less than 6mM, less than 5mM, less than 4mM, less than 3mM, less than 2mM, or less glutamine;

v) about 1mM, about 2 mM, about 3mM, about 4mM, about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about 10mM, about 20mM, about 30mM, about 40mM, about 50mM, about 60mM, about

70mM, about 80mM, about 90mM, about 100mM, about 110mM, about 120mM, about 130mM, about 140mM, or about 150mM glutamine; and/or

vi) 1mM, 2 mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM, 140mM, or 150mM glutamine;

and/or

b) the culture media is supplemented with glutamic acid at concentrations

of:

i) between about 1mM and about 100mM, about 2mM and about 90mM, about 3mM and about 80mM, about 4mM and about 70mM, about 5mM and about 60mM, about 6mM and about 50mM, about 7mM and about 40mM, about 8mM and about 30mM, about 9mM and about 20mM, about 10 mM and about 15mM, about 1mM and about 10mM, about 2mM and about 9mM, about 3mM and about 8mM, about 4mM and about 7mM, about 5mM and about 6 mM, about 10 mM and about 100mM, about 20mM and about 90mM, about 30mM and about 80mM, about 40mM and about 70mM, about 50mM and about 60mM, about 50mM and about 150mM, about 60mM and about 140mM, about 70mM and about 130mM, about 80mM and about 120mM, or about 90mM and about 110mM glutamic acid;

ii) between 1mM and 100mM, 2mM and 90mM, 3mM and 80mM, 4mM and 70mM, 5mM and 60mM, 6mM and 50mM, 7mM and 40mM, 8mM and 30mM, 9mM and 20mM, 10 mM and 15mM, 1mM and 10mM, 2mM and 9mM, 3mM and 8mM, 4mM and 7mM, 5mM and 6 mM, 10 mM and 100mM, 20mM and 90mM, 30mM and 80mM, 40mM and 70mM, 50mM and 60mM, 50mM and 150mM, 60mM and 140mM, 70mM and 130mM, 80mM and 120mM, or 90mM and 110mM glutamic acid;

iii) at least 1mM, at least 2 mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 20mM, at least 30mM, at least 40mM, at least 50mM, at least 60mM, at least 70mM, at least 80mM, at least 90mM, at least 100mM, at least 110mM, at least 120mM, at least 130mM, at least 140mM, at least 150mM, or more glutamic acid;

iv) less than 150mM, less than 140mM, less than 130mM, less than 120mM, less than 120mM, less than 110mM, less than 100mM, less than 90mM, less than 80mM, less than 70mM, less than 60mM, less than 50mM, less than 40mM, less than 30mM, less than 20mM, less than

10mM, less than 9mM, less than 8mM, less than 7mM, less than 6mM, less than 5mM, less than 4mM, less than 3mM, less than 2mM, or less glutamic acid;

v) about 1mM, about 2 mM, about 3mM, about 4mM, about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about 10mM, about 20mM, about 30mM, about 40mM, about 50mM, about 60mM, about 70mM, about 80mM, about 90mM, about 100mM, about 110mM, about 120mM, about 130mM, about 140mM, or about 150mM glutamic acid; and/or

vi) 1mM, 2 mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM, 140mM, or 150mM glutamic acid.

47. The method of any of embodiments 30-46, wherein the method further comprises:

expressing an endogenous glutamate dehydrogenase (GDH) polypeptide and/or an endogenous glutamine synthetase (GS) polypeptide in a cell according to any of embodiments 1-29.

48. The method of any of embodiments 30-47, wherein the NRPS polypeptide, PPTase polypeptide, GDH polypeptide, and/or GS polypeptide are overexpressed in the cell.

49. The method of any of embodiments 30-48, wherein:

a) the NRPS polypeptide is an NRPS polypeptide defined in any of embodiments 4-5;

b) the PPTase polypeptide is a PPTase polypeptide defined in any of embodiments 6-7;

c) the GDH polypeptide is a GDH polypeptide defined in any of embodiments 11-13; and/or

d) the GS polypeptide is a GS polypeptide defined in any of embodiments 11, 14-15.

50. The method of any of embodiments 30-49, wherein the cell comprises a nucleic acid as defined in any of embodiments 2-7, 9-23; and

the NRPS polypeptide, PPTase polypeptide, GDH polypeptide, and/or GS polypeptide are overexpressed from the nucleic acid.

51. The method of any of embodiments 30-50, wherein the method further comprises extracting and/or purifying the indigoidine from the cell; optionally wherein extracting and/or purifying the indigoidine from the cell comprises lysing the cell; optionally

5 wherein the cell is lysed by homogenisation in DMSO.

52. Use of the recombinant yeast cell of any of embodiments 1-30 in a method of producing indigoidine.

10 53. The use according to embodiment 52, wherein the method is a method defined in any of embodiments 30-51.

54. Indigoidine obtained or obtainable from a cell according to any of embodiments 1-30 or a method according to any of embodiments 31-51.

15 55. A method of producing indigoidine-stained bacterial cellulose, the method comprising co-culturing the recombinant yeast cell according to any of embodiments 1-30 with a cellulose-producing second cell.

20 56. The method of embodiment 55, wherein the method comprises growing the recombinant yeast cell and the cellulose-producing second cell under conditions that allows the cellulose-producing second cell to produce cellulose; optionally wherein the conditions allow the cellulose-producing second cell to produce a pellicle.

25 57. The method of embodiment 56, wherein the conditions that allow the cellulose-producing second cell to produce cellulose comprise culturing the cell:

a) at a pH of:

30 i) between 3-7, optionally a pH of between 3.25 and 6.75, 3.5 and 6.5, 3.5 and 6.25, 3.75 and 6, 4 and 5.75, 4.25 and 5.5, 4.5 and 5.25; pH 5.8; and/or

ii) at least 3 but less than or equal to pH 7, for example at least 3, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 4.75, 5, 5.25, 5.5, 5.75, 5.8, 6, 6.25, 6.5, 6.75, but less than or equal to pH 7;

35 and/or

b) in culture media that is:

i) HS media;

ii) YPD media; or

- iii) Coconut water media.

58. The method of any of embodiments 55-57, wherein the cellulose-producing second cell is a bacterial cell.

5

59. The method of embodiment 58, wherein the bacterial cell:

- a) is capable of producing bacterial cellulose;
- b) expresses all of *bcsA*, *bcsD*, *bscC* and *bscD*;
- c) belongs to a genus selected from the group comprising or consisting of:
10 *Komagataeibacter*, *Escherichia*, *Gluconacetobacter*, *Acetobacter*, *Sarcina*,
Agrobacterium, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and
Alcaligenes;
- d) are selected from the group comprising or consisting of: *Komagaeibacter*
rhaeticus; *Komagaeibacter xylinus*, *Komagaeibacter hansenii*, *Komagaeibacter*
15 *medellinensis*, *Komagaeibacter europaeus*, *Komagaeibacter maltaceti*,
Komagaeibacter pomaceti, *Komagaeibacter oboediens*, or *Komagaeibacter*
saccharivoans;
- e) are selected from the group comprising or consisting of:
 - i) a strain of *Komagaeibacter rhaeticus* selected from the group
20 comprising or consisting of: *Komagaeibacter rhaeticus* iGEM.
Komagaeibacter rhaeticus AF1; *Komagaeibacter rhaeticus* LMG22126;
or
 - ii) *Gluconacetobacter xylinus* CGMCC 2995;
 and/or
- f) are *Komagaeibacter rhaeticus* iGEM cells.
25

60. A method of producing indigoidine-stained bacterial cellulose, the method comprising:

- a) culturing a cellulose-producing second cell to form bacterial cellulose;
30 and subsequently
 - b) contacting the cellulose produced by the cellulose-producing second cell
in (a) with the recombinant yeast cell according to any of embodiments 1-30;
- optionally wherein the cellulose-producing second cell is a bacterial cell,
35 optionally wherein the bacterial cell:
- a) is capable of producing bacterial cellulose;
 - b) expresses all of *bcsA*, *bcsD*, *bscC* and *bscD*;

- c) belongs to a genus selected from the group comprising or consisting of: *Komagataeibacter*, *Escherichia*, *Gluconacetobacter*, *Acetobacter*, *Sarcina*, *Agrobacterium*, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and *Alcaligenes*;
- 5 d) are selected from the group comprising or consisting of: *Komagaeibacter rhaeticus*; *Komagaeibacter xylinus*, *Komagaeibacter hansenii*, *Komagaeibacter medellinensis*, *Komagaeibacter europaeus*, *Komagaeibacter maltaceti*, *Komagaeibacter pomaceti*, *Komagaeibacter oboediens*, or *Komagaeibacter saccharivoans*;
- 10 e) are selected from the group comprising or consisting of:
- i) a strain of *Komagaeibacter rhaeticus* selected from the group comprising or consisting of: *Komagaeibacter rhaeticus* iGEM. *Komagaeibacter rhaeticus* AF1; *Komagaeibacter rhaeticus* LMG22126; or
- 15 ii) *Gluconacetobacter xylinus* CGMCC 2995;
- and/or
- f) are *Komagaeibacter rhaeticus* iGEM cells.
- 20 61. A method of producing an indigoidine-stained pellicle, the method comprising co-culturing the recombinant yeast cell according to any of embodiments 1-30 with a cellulose-producing second cell,
- optionally wherein the cellulose-producing second cell is a bacterial cell, optionally wherein the bacterial cell:
- 25 a) is capable of producing bacterial cellulose;
- b) expresses all of *bcsA*, *bcsD*, *bscC* and *bscD*;
- c) belongs to a genus selected from the group comprising or consisting of: *Komagataeibacter*, *Escherichia*, *Gluconacetobacter*, *Acetobacter*, *Sarcina*, *Agrobacterium*, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and
- 30 *Alcaligenes*;
- d) are selected from the group comprising or consisting of: *Komagaeibacter rhaeticus*; *Komagaeibacter xylinus*, *Komagaeibacter hansenii*, *Komagaeibacter medellinensis*, *Komagaeibacter europaeus*, *Komagaeibacter maltaceti*, *Komagaeibacter pomaceti*, *Komagaeibacter oboediens*, or *Komagaeibacter saccharivoans*;
- 35 e) are selected from the group comprising or consisting of:
- i) a strain of *Komagaeibacter rhaeticus* selected from the group comprising or consisting of: *Komagaeibacter rhaeticus* iGEM.

Komagaeibacter rhaeticus AF1; *Komagaeibacter rhaeticus* LMG22126;

or

ii) *Gluconacetobacter xylinus* CGMCC 2995;

and/or

5 f) are *Komagaeibacter rhaeticus* iGEM cells.

62. A method of producing an indigoidine-stained pellicle, the method comprising:

a) culturing a cellulose-producing second cell to form a cellulose pellicle;
and subsequently

10 b) contacting the resultant pellicle with the cell according to any of
embodiments 1-29,

optionally wherein the cellulose-producing second cell is a bacterial cell,
optionally wherein the bacterial cell:

a) is capable of producing bacterial cellulose;

15 b) expresses all of *bcsA*, *bcsD*, *bcsC* and *bcsD*;

c) belongs to a genus selected from the group comprising or consisting of:
Komagataeibacter, *Escherichia*, *Gluconacetobacter*, *Acetobacter*, *Sarcina*,
Agrobacterium, *Azotobacter*, *Rhizobium*, *Pseudomonas*, *Salmonella* and
Alcaligenes;

20 d) are selected from the group comprising or consisting of: *Komagaeibacter*
rhaeticus; *Komagaeibacter xylinus*, *Komagaeibacter hansenii*, *Komagaeibacter*
medellinensis, *Komagaeibacter europaeus*, *Komagaeibacter maltaceti*,
Komagaeibacter pomaceti, *Komagaeibacter oboediens*, or *Komagaeibacter*
saccharivoans;

25 e) are selected from the group comprising or consisting of:

i) a strain of *Komagaeibacter rhaeticus* selected from the group
comprising or consisting of: *Komagaeibacter rhaeticus* iGEM.
Komagaeibacter rhaeticus AF1; *Komagaeibacter rhaeticus* LMG22126;
or

30 ii) *Gluconacetobacter xylinus* CGMCC 2995;

and/or

f) are *Komagaeibacter rhaeticus* iGEM cells.

35 63. The method of any of embodiments 55-62, wherein the cellulose-producing
second cell is cultured in the conditions defined in embodiment 56.

64. The method of any of embodiments 55-63, wherein contacting the cellulose or contacting the pellicle comprises incubating the recombinant yeast cell of embodiments 1-30 with the cellulose or the pellicle.

5 65. The method of embodiment 64, wherein incubating the recombinant yeast cell of embodiments 1-30 with the cellulose or the pellicle comprises culturing the recombinant yeast cell as defined in any of embodiments 31-54.

10 66. The method of any of embodiments 55-65, wherein the method further comprises sterilising the cellulose or pellicle, optionally wherein the sterilisation is selected from the group comprising or consisting of: autoclaving, heating, and desiccation, or any combination thereof.

67. A method of producing indigoidine-dyed textile or thread, comprising:

- 15 a) producing indigoidine according to the method of any of embodiments 31-54;
; and
b) contacting the textile or thread with the indigoidine obtained in (a).

20 68. The method of embodiment 67, wherein producing indigoidine according to (s) further comprises:

- i) collecting the yeast cells;
optionally by centrifugation or filtration;
ii) drying the yeast cells; and/or
25 iii) lysing the dried yeast cells;

optionally wherein lysing the yeast cells comprises grinding the yeast cells. In some embodiments the cell is lysed by homogenisation in DMSO. Such methods are known, and in some embodiments involve the use of glass beads, cell disruption at 8,000 rpm and centrifugation.

30

69. The method of any of embodiments 67-68, wherein b) contacting the textile or thread with the indigoidine obtained in (a) comprises soaking the textile or thread in the indigoidine.

35 70. The method of embodiment 69, wherein the textile or thread is soaked in the dyeing mixture for:

- a) between about 4 h and about 12 h, about 5 h and about 11 h, about 6 h and about 10 h, about 7 h and about 9 h;

- b) between 4 h and 12 h, 5 h and 11 h, 6 h and 10 h, 7 h and 9 h;
 c) at least 4 h, at least 5 h, at least 6 h, at least 7 h, at least 8 h, at least 9 h, at least 10 h, at least 12 h;
 d) less than 12 h, less than 11 h, less than 10 h, less than 9 h, less than 8 h, less than 7 h, less than 6 h, less than 5 h;
 e) about 4 h, about 5 h, about 6 h, about 7 h, about 8 h, about 9 h, about 10 h, about 11 h, about 12 h; and/or
 f) 4 h, 5 h, 6 h, 7 h, 8 h, 9 h, 10 h, 11 h, 12 h.
- 10 71. The method of any of embodiments 67-70, wherein the method further comprises after step b):
 c) autoclaving textile or thread; and/or
 d) washing the textile or thread.
- 15 72. Indigoidine-stained bacterial cellulose obtained or obtainable from the method of any of embodiments 55-66.
73. An indigoidine-stained pellicle obtainable from the method of any of embodiments 55-66.
- 20 74. An indigoidine-stained textile or thread obtainable from the method of any of embodiments 67-71.
75. A garment comprising the indigoidine-stained bacterial cellulose of embodiment 25 72, the indigoidine-stained pellicle of embodiment 73, or the indigoidine-stained textile or thread of embodiment 74.
76. An expression construct comprising a nucleic acid comprising:
 a) a nucleotide sequence encoding an NRPS polypeptide;
 30 b) a nucleotide sequence encoding a PPTase polypeptide;
 c) a nucleotide sequence encoding a GDH polypeptide; and/or
 d) a nucleotide sequence encoding a GS polypeptide;
 wherein the nucleotide sequences encoding the NRPS polypeptide, the PPTase polypeptide, the GDH polypeptide, and/or the GS polypeptide are operably linked to a
 35 promoter.
77. The expression construct of embodiment 76, wherein:

a) the NRPS polypeptide is an NRPS polypeptide defined in any of embodiments 4-5;

b) the PPTase polypeptide is a PPTase polypeptide defined in any of embodiments 6-7;

5 c) the GDH polypeptide is a GDH polypeptide defined in any of embodiments 11-13; and/or

d) the GS polypeptide is a GS polypeptide defined in any of embodiments 11, 14-15.

10 78. The expression construct of any of embodiments 76-77, wherein the promoter is a promoter defined in any of embodiments 17-19.

79. The expression construct of any of embodiments 76-78, wherein the nucleotide sequences encoding the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide are operably linked to a terminator sequence.

80. The expression construct of embodiment 79, wherein the terminator sequence is a terminator sequence according to embodiment 21.

20 81. A vector comprising the expression construct according to any of embodiments 76-80.

82. The vector according to embodiment 81, wherein the vector is selected from the group comprising or consisting of: a plasmid, a phagemid, a YAC, and a BAC.

83. An engineered non-ribosomal peptide synthetase (NRPS) polypeptide converting glutamine into indigoidine, wherein the engineered NRPS:

a) comprises:

30 i) an amino acid sequence of SEQ ID NO: 8 or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 8;

35 ii) an amino acid subsequence of SEQ ID NO: 8 capable of converting glutamine into indigoidine or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 8; or

iii) an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical thereto;

and/or

5 b) is encoded by:

i) a nucleotide sequence of SEQ ID NO: 7 or a nucleotide sequence that is at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 7;

10 ii) a nucleotide subsequence of SEQ ID NO: 7 or of a nucleotide subsequence that is at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 7; or

15 iii) a nucleic acid sequence that is at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical thereto.

84. A nucleic acid comprising a nucleotide sequence encoding the engineered non-ribosomal peptide synthetase (NRPS) polypeptide of embodiment 85.

20

85. The nucleic acid of embodiment 84, wherein the nucleotide sequence encoding the engineered NRPS polypeptide is operably linked to a promoter.

86. The nucleic acid of embodiment 85, wherein the promoter is a promoter defined in any of embodiments 17-19.

25

87. The nucleic acid of embodiments 85-86, wherein the nucleotide sequence encoding the engineered NRPS polypeptide is operably linked to a terminator sequence.

30 88. The nucleic acid of embodiment 87, wherein the terminator sequence is a terminator sequence according to embodiment 21.

89. A vector comprising the expression construct according to any of embodiments 84-88.

35

90. The vector according to embodiment 89, wherein the vector is selected from the group comprising or consisting of: a plasmid, a phagemid, a YAC, and a BAC.

91. A cell comprising the expression construct of any of embodiments 76-80, the nucleic acid of any of embodiments 84-88, or the vector of any of embodiments 81-82, 89-90.

5 92. The cell of embodiment 91, wherein the cell is a cell as defined in any of embodiments 1-29.

93. A plurality of cells, wherein each cell of the plurality is a cell according to embodiment 92.

10

94. A plurality of cells comprising a first plurality of cells as defined in embodiment 93, and a second plurality of second cells as defined in any of embodiments 55-63.

95. A kit comprising:

15

- a) the cell of any of embodiments 1-29;
- b) culture media of as defined in any of embodiments 32-43, 45-46;
- c) a second cell as defined in any of embodiments 55-63;
- d) the indigoidine of embodiment 54;
- e) a bacterial cellulose as defined in any of embodiments 55-66, 72;

20

- f) a pellicle as defined in any of embodiments 61-66, 73;
- g) a textile or thread as defined in any of embodiments 67-71, 74;
- h) the garment of embodiment 75;
- i) the expression construct of any of embodiments 76-80;
- j) the vector of any of embodiments 81-82, 89-90;

25

k) the engineered non-ribosomal peptide synthetase (NRPS) of embodiment 83;

- l) the nucleic acid of any of embodiments 84-88;
- m) the cell of any of embodiments 91-92; and/or
- n) the plurality of cells of any of embodiments 93-94.

30

The listing or discussion of an apparently prior-published document in this specification should not necessarily be taken as an acknowledgement that the document is part of the state of the art or is common general knowledge.

35

Preferences and options for a given aspect, feature or parameter of the invention should, unless the context indicates otherwise, be regarded as having been disclosed in combination with any and all preferences and options for all other aspects, features and parameters of the invention. For example, the invention provides a *Yarrowia*

lipolytica cell expressing a synthetic BpsA and a GDH polypeptide encoded by YALIOF17820g.

Figure legends

5

Figure 1 – Microscopic image of the indigoidine-producing *Y. lipolytica* strain. (A) Blue spots show the pigment distribution within the cells. (B) Indigoidine extract obtained from the biomass of the engineered *Y. lipolytica* cells. (C) Biosynthesis of indigoidine from α -ketoglutarate in an engineered *Y. lipolytica* strain. Endogenous glutarate dehydrogenase and glutamine synthetase enzymes produce L-glutamine, which is condensed by heterologously expressed BpsA (activated by a heterologously expressed PPTase) to generate indigoidine.

10

Figure 2 – (A) Indigoidine titers, (B) growth profiles, (C) and pH changes during cultivation of the indigoidine-producing *Y. lipolytica* strain in rich and minimal media in flasks. (D) Cell morphology and (E) flasks after 72 and 120 h of culture.

15

Figure 3 - The influence of carbon source on indigoidine production. (A) analytical grade substrates, (B) lignocellulosic hydrolysates, (C) waste substrates. The cultures were carried out for 120h in deep-well plates.

20

Figure 4 – The impact of (A) nitrogen quality and (B) availability on indigoidine production. The cultures were carried out for 120h in deep-well plates.

25

Figure 5 – The influence of agitation speed on the (A) indigoidine biosynthesis and (B) growth of the indigoidine-producing *Y. lipolytica* proof-of-concept strain.

Figure 6 - The impact of (A) temperature and (B) water type on indigoidine production. The indigoidine concentrations after 120h cultivation in (C) super-minimal media (SMm).

30

Figure 7 – (A) Indigoidine production, growth and substrate consumption of the indigoidine-producing *Y. lipolytica* strain during cultivation in bioreactors in media containing urea and ammonium sulphate. (B) The indigoidine-producing strain (left) and *Y. lipolytica* wild-type strain (right) during cultivation in bioreactors.

35

Figure 8 – (A) Indigoidine biosynthesis pathway and (B) the effect of glutamine and glutamate supplementation on indigoidine production after 120h of cultivation. The red

rectangle represents enzymatic reactions native to *Y. lipolytica*, while the blue rectangle indicates heterologous reaction. YNB – minimal medium with 20 g/L glucose.

Figure 9 - The effect of endogenous precursor supply on indigoidine production in *Y. lipolytica*.

Figure 10 - Blue bacterial cellulose (BC) fibres obtained through co-cultivation of indigoidine-producing *Y. lipolytica* strain and bacterial-cellulose producing bacteria strain.

Figure 11 - . Production of pigmented BC pellicles. (A) Bacterial pellicle prior to inoculation with indigoidine producing *Y. lipolytica*. Pellicle size can be tuned by selection of A cultivation vessel. (B) Development of pigmented bacterial cellulose biomaterials after shaking for 72-120h. Non-pigmented *Y. lipolytica* cells appear as grey areas on the biomaterial. Microscope images of pigmented bacterial cellulose pellicles. (C) Microscopic top view of the biomaterial surface and (D) Contour view. White arrows highlight filamentous *Y. lipolytica* inside/proximal to the bacterial cellulose network. Indigoidine is deposited as blue crystals throughout the biomaterial.

Figure 12 - Textile dyeing with *Y. lipolytica* biomass. (A) Effect of multiple applications of dyeing paste to cotton. (B) and (C) Indigoidine containing biomass can be used as a robust dyeing method.

Figure 13 - Comparison of native (BpsA_X5) and designed (BpsA_X5-UNC) proteins. (A) Amino acid sequence alignment; red regions indicate identical amino acids; blue regions show similar amino acids. (B) Three-dimensional structures of the BpsA proteins.

Sequences referred to herein

```
>SEQ_ID_NO: 1_PPTase(wt)
MKIYGIYMDRPLSQEENERFMFTFISPEKREKORRFYHKEDAHRTLLGDVLVRSVISRQYQLDKSDIRFSTQBYGKPCIPDLPAHFNISH
SGRWVIGAFDSQPIGIDIEKTKPISLEIAKRFFSKTEYSDLLAKDKDEQTDYFYHLWSMKESFIKQEGKGLSLPLDSFSVRLHQDGGQVSI
ELPDSHSPCYIKTYEVDPGYKMAVCAHPDFPEDITMVSYEELL
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CGATTCTTCAGCAAGACCGAGTACTCTGACCTGCTGGCCAAGGACAAGGACGAGCAGACCGACTACTTCTACCACCTGTGGTCTATGAAG
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GAGCTGCCCGACTCTCACTCGCCCTGCTACATCAAGACCTACGAGGTGGACCCCGGCTACAAGATGGCCGTGTGCGCTGCTCACCCGAC
TTCCCCGAGGACATTACCATGGTGTCTTACGAGGAAGTCTGTAA
```

```
>SEQ_ID_NO: 3_PPTase(codon_opt)
```

5 >SEQ_ID_NO: 4_BpsA(wt)
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GAAYLPLSPPEYFEDRLRYMIENSQAKIILAQQLRLVTRLRELAPQDVVRVVTLRESEAFVLPEQGVAPAEIGARPDSLAVIYTSGSTGKPKY
GMIEHSHSVSQLGLWLRETFSDSKTIILQKTPMSFDAQWEILSPANGATVVMGAPGVYADPEGLIETIVKYGVTLQCVPTLLQGLLD
TEKFPECTSLQQIFSGGEALSRLLAIQTTFQEMPGRALINVYGPTECTINSSSYAVDPAELGEAPQSSISIGAPVADTEYHILGKEDLKPVG
10 VGEIGELYVGGGQLARGYLHRPDLTAERFLEIEVTEGAGPVRILYKTDGLGQWNPDGTVQFAGRADNQVKLRGVRVLEDEISLAENHDWV
RNAFVYKNDGRTGFQNLIACVLESEKEAALMDQGNHSGHHAKSKKQLQVRAQLNSPGLRDDADLAARVAYDLPGAEPTEPQRSRVFARK
TYRVEYEGGVAETADLLALLGGQVPAAYSRAADLAPALGQILHRFGGQVLSERLLPKYGYASPGALYATQLYFELEGVGLQPGVYVYQ
PQRHQVLVLISEKAATGRPTAHIHFIGRGGIEFPVYKNNIQEVLEIETGHIVGLFEQVLPAAYGLDIRDLAYEPAVRDLDDVFEEDFYLGTF
ELVPHPTGRREDHAEEVYVQTHGSKVANLPPEGQYRYADGTLTRFSDDILVLRKQVIAINGSVYQAA5FGISVISAPEEMHWVTVLGKLLQHL
15 MNNGLGLFMSSGYSKTKNPLASRRIDLSVLQANGVESGSPSYFFVGGKVSDEQLGHEGMRDESVHMRGPAELIRDLDLVSFLPDMYIPNR
VVVFERLPLSANGKIDAKALASQDVNAELVERFVAPRTESEKETAIVWAKSLRRSSVSVQDDFFESGGNSLIAGLIRLNSRLGVSGL
PLQSVLESPTVEKLSRRLREVAQESSRLVRHLAETGKDRPVLWCWPGLGGYPMNLRTLAGEIGLGRSFGYIQAHGINEGEAPYATITEMA
RADIEAIKELQPKGPYTLWGYSFGARVAFETAQLEQAGEKVDNLFLLAPGSPTVRAENGVRVYGREASFANRAYTTTLFSVFTGTLISGPD
LEKCLSATDEESFAGFISELKGIIDVDLAKRIISVVGQTYEFEYSFRELAERTLAPVTFIKARGDDYSFIENSGYSAEPTVIDDLAD
20 HYSLLRTPDIGELVKHIRYLLGE

[illegible]

70
GATLEFSEFTPEFEDKRLFENSTALITADQXLSVRKRLAEKRVLTVEFSEAEVVFPEFZAFARAKFEDTALVITVTSSTGKRA
GVMIHRSINVQGLWLRETYAIVDRSKVILQKTFMSFADQAQWEILSPANGATVVMGAPGVYADPEGLIETIVHNNVTFQLQCVPTLQGLID
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75
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[illegible]

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CONSTITUTIVE PROMOTERS

> SEQ_ID_NO: 24_ATP1_promoter

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> SEQ_ID_NO: 26_FBAin_promoter

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> SEQ_ID_NO: 27_PGK1_promoter

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> SEQ_ID_NO: 28_GPM1_promoter

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15 > SEQ ID NO: 29 HHF1 promoter

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30 > SEQ ID NO: 30 CYC1 promoter

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40 > SEQ ID NO: 31 HHT1 promoter

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50 > SEQ ID NO: 32 HTB1 promoter

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60 > SEQ ID NO: 33 EXP1 promoter

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75 > SEQ ID NO: 34 TDH1 promoter

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INDUCIBLE PROMOTERS

> SEQ ID NO: 41 pPOX2

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> SEQ ID NO: 42 pXPR2

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> SEQ ID NO: 43 pFBF1

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> SEQ ID NO: 44 pMDH1a

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> SEQ ID NO: 45 ACL2

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> SEQ ID NO: 46 POT1

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>SEQ ID NO: 47 LIP2 promoter

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> SEQ ID NO: 50 CTRL promoter

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>SEQ ID NO: 60 - consensus sequence between BpsA-X5 and BaspA-X5-UNC as per Figure 13:

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 I#L#aDHYqlReadIqELkqhIr..hdLeE...

Where:

! - isoleucine or valine
 \$ - leucine or methionine
 % - phenylalanine or tyrosine
 # - asparagine, aspartic acid, glutamine, or glutamic acid
 . - gap

Examples

Example 1 – generating an indigoidine-producing *Yarrowia lipolytica* strain

A proof-of-concept strain overexpressing codon-optimized genes encoding BpsA and PPTase enzymes was generated by integrating expression cassettes into the genome of *Y. lipolytica*.

Figure 1 shows microscopic analysis of indigoidine-producing *Y. lipolytica* and indigoidine extract obtained from the biomass of the engineered *Y. lipolytica* cells.

Example 2 - Carbon source impact on the indigoidine biosynthesis in an engineered *Y. lipolytica* strain

The *Y. lipolytica* strain generated in Example 1, overexpressing *BpsA* and *PPTase*, was cultivated in two types of liquid media – rich (YPD and YP4D) and minimal (YNB glucose and YNB glycerol) in flasks. The tested media contained 20 g/L (40 g/L in YP4D) of carbon source. The highest product titers were achieved in minimal YNB media, reaching 2.6 g/L and 2.5 g/L from glucose and glycerol, respectively (**Fig. 2A**). The amounts of indigoidine produced in rich media, were significantly lower, reaching respectively 1.5 g/L and 1.1 g/L in YP4D and YPD. Contrary to the product formation, the growth of the cells was much better in nutrient-rich media, as shown in **Figure 2B**. The pH of the cultures was tracked over time (**Fig. 2C**). In nutrient-rich media, there was a slight increase in pH between 72 and 96 hours. However, in minimal media, there was a significant drop from pH 7 to 3 at 48 hours, which then levelled off at pH 5.

The morphology of the cells varied when comparing those grown in nutrient-rich media to those grown in minimal media. The transformant grown in YPD had an oval shape, while in YNB, there was a clear variation in shape with both oval and elongated hyphal cells present (as shown in **Figure 2D**). Additionally, when grown in YNB, blue rings of indigoidine were observed on the walls of the flasks (**Fig. 2E**), probably resulting from the breakage of the elongated cells and subsequent dye release.

Next, a range of carbon sources was tested to identify the best feedstock for indigoidine production. The proof-of-concept strain was cultivated in deep-well plates in minimal YNB medium with analytical grade (20 g/L carbon; **Fig. 3A**), as well as renewable and waste carbon sources (varying concentrations, **Fig. 3B, C**) for 120h. Thus, the best substrate was glucose (5.2 g/L indigoidine), followed by monosaccharides fructose and mannose, and the polyol glycerol. Acetate, alongside vegetable oils allowed for indigoidine production in the 0.34-0.56 g/L range. Negligible titre was achieved with citrate as the sole carbon source (0.07 g/L).

The cost of carbon source can constitute up to 30% of the total bioprocess' costs, hence it is relevant to search for cost-effective feedstocks. The lignocellulosic hydrolysates provide a cheap and sustainable feedstock source for bioprocesses. The proof-of-concept strain was able to convert carbon present in the hydrolysates to indigoidine with highest titre obtained from the urban pruning waste (2.9 g/L, **Fig. 3B**). Additionally, waste glycerol from biodiesel plant as well as waste cooking oil (WCO) from the local restaurant were used, yielding 0.73-0.79 g/L indigoidine (**Fig. 3C**).

Example 3 - Nitrogen quality and availability impact on indigoidine biosynthesis in an engineered *Y. lipolytica* strain

The indigoidine molecule contains four nitrogen atoms (23% of the total mass), thus this element is an important constituent of the pigment. In order to assess the impact of nitrogen quality (reduced or oxidized forms) on indigoidine biosynthesis in *Y. lipolytica*, several nitrogen sources were tested (**Fig. 4A**). The experiment identified ammonium sulphate and urea as the most suitable nitrogen sources for indigoidine biosynthesis.

The C/N ratio (nitrogen availability) is another important parameter in microbial bioprocesses as it influences both biosynthesis and growth. Thus, the impact of C/N ratio on indigoidine production and growth was analyzed (**Fig. 4B**). The most suitable C/N ratios for the process were in the range 2-20.

Example 4 – Effect of aeration on indigoidine biosynthesis in an engineered *Y. lipolytica* strain

The oxygen availability is a significant factor in biosynthesis and growth. In order to assess the impact of aeration on indigoidine production, the cultivations of *Y. lipolytica* proof-of-concept strain were carried out in flasks at three different shaking speeds (150, 200 and 250 rpm) and in 250 mL flat-bottom Erlenmeyer flasks. The experiments showed that higher aeration rates (induced by higher agitation speeds) are beneficial for indigoidine production (**Fig. 5**).

Example 5 - Sustainability of the bioprocess

The maintenance of the optimal bioprocess temperature (30°C in this case) is a significant contributor to the total production cost, as significant amounts of energy are required either for heating (colder climates) or cooling (warmer climates). The impact of three different temperatures (ambient, 25°C, 30°C) on the indigoidine production in the YNB medium with 20 g/L glucose was analyzed (**Fig. 6A**). The final titres decreased with the decreasing temperatures, yet even without the thermostat as much as 1.1 g/L was achieved.

Water could also be a serious cost contributor, as most often used distilled water (Milli-Q) requires energy to be produced. We analyzed three different types of water – Milli-Q, tap water (TW), and sea water (SW) in the YNB medium with 20 g/L glucose (**Fig.**

6B). Insignificant decrease in the final titres in favour to milli-Q water was observed, indicating that both tap water and sea water can be readily used for this bioprocess. This is of particular importance if the future production plant has its own water well or is located close to the coast.

5

On top of that, we designed super-minimal media SMm, containing easily available and renewable feedstocks (**Table 1**). The indigoidine-producing *Y. lipolytica* strain was then cultivated in deep-well plates containing these 5 varieties of SMm. As shown in **Figure 6C**, the indigoidine was produced in all of them, while the highest titres were achieved in SM4 and SM5 after 120h of cultivation (1.8 and 2.3 g/L, respectively).

10

Table 1. The composition of the super-minimal media.

INGREDIENT	SUPERMINIM AL 1 (SM1)	SUPERMINIMAL 2 (SM2)	SUPERMINIMAL 3 (SM3)	SUPERMINIMAL 4 (SM4)	SUPERMINIMAL 5 (SM5)
UREA	18g/L	18g/L	18g/L	18g/L	18g/L
RAW GLYCEROL	-	25g/L	25g/L	25g/L	25g/L
GLYCEROL	20g/L	-	-	-	-
YNB	-	-	0.17g/L	-	-
K ₂ PO ₄	-	-	-	1.2g/L	1.2g/L
MNSO ₄	-	-	-	-	1g/L
TAP WATER	To amount	To amount	To amount	To amount	To amount

Example 6 - Bioreactor cultivations in the optimized conditions

15

The results from the experiments described above were used to carry out bioreactor cultivations. The proof-of-concept strain was cultivated in batch mode using 40 g/L of glucose, urea or ammonium sulphate as nitrogen sources, C/N 4 and high aeration rate maintained by shaking speed (800 rpm) and sterile air influx (1 lpm; **Fig. 7**). The highest indigoidine titre was obtained in media containing ammonium sulphate as nitrogen source and peaked at 1.9 g/L at 72 h.

20

Example 7 – The influence of precursor supply on indigoidine biosynthesis

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The direct precursors for indigoidine biosynthesis are amino acids L-glutamate and L-glutamine (**Fig. 8A**). In order to identify the bottleneck in the pathway, the indigoidine-producing *Y. lipolytica* strain was supplemented with glutamine and glutamate (**Fig. 8B**). The results suggest that the L-glutamate to L-glutamine conversion reaction is a rate-limiting step in the pathway. When compared to the

medium without supplementation (YNB with 20 g/L glucose), a 2.5- and 5.8-fold increase in production levels was observed for 100 mM glutamate, and 100 mM glutamine supplementation, respectively.

5 Example 8 – Indigoidine biosynthesis in the *Y. lipolytica* strain overexpressing GS and GDH

Supplementation with the direct precursors for biosynthesis is an effective strategy, however the cost of these precursors would render the process economically unviable.

10 Therefore, a genome-wide search for glutamate dehydrogenase (GDH)- and glutamine synthase (GS)-encoding genes was carried out in order to increase the endogenous precursor supply. Four target genes, two for each enzymatic reaction were identified *in silico*, cloned and expressed in the proof-of-concept strain (**Table 2**).

15 Table 2. *Y. lipolytica* strains overexpressing GDH- and GS-encoding genes.

Strain ID	Genotype
PHS32	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase</i>
S1712	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10D13024g (GS)</i>
S1713	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10F00506g (GS)</i>
S1714	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10E09603g (GDH)</i>
S1715	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10F17820g (GDH)</i>
PHS37	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10D13024g (GS)-YAL10E09603g (GDH)</i>
PHS38	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10D13024g (GS)-YAL10F17820g (GDH)</i>
PHS39	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10F00506g (GS)-YAL10E09603g (GDH)</i>
PHS40	<i>Y. lipolytica</i> S3 <i>BpsA_V1254M-PPTase-YAL10F00506g (GS)-YAL10F17820g (GDH)</i>
S91	Wild-type <i>Y. lipolytica</i> strain

Strains were made according to standard lab protocols, such as those described in the YaliCraft manual at <https://assets.researchsquare.com/files/rs-1570357/v1/9174b2d69b59349a43c2c41e.pdf> (researchsquare.com).

20 The constructed strains were cultivated in media containing glucose as a sole carbon source. The highest indigoidine titre was obtained with the strain PHS40 overexpressing putative GS- and GDH-encoding genes after 120 h cultivation (3.65 g/L; **Fig. 9**).

Example 9 - Co-cultivation of *Y. lipolytica* and *K. rhaeticus* to produce blue bacterial cellulose fibres

5 The pigment produced by the engineered *Y. lipolytica* strains has a direct application in the textile industry. However, we decided to go a step further and fabricate a sustainable, blue-coloured biomaterial using a co-cultivation strategy. The proof-of-concept strain was cultivated with bacterial-cellulose (BC) producing *K. rhaeticus* bacteria strain. Within 7 days, this preliminary experiment yielded evenly pigmented
10 cellulose fibres (**Fig. 10**), a biomaterial which could be readily used for example in shoe manufacturing. Alas, we were able to obtain only small and thin BC pieces, what could result from an antibacterial activity of indigoidine. We hypothesize that the indigoidine produced by the engineered *Y. lipolytica* strain inhibits the growth of *K. rhaeticus*, leading to low BC yields.

15 Because of the above, we were optimizing the protocol, to allow for more reliable BC production and pigmentation. On top of co-cultures, we explored decoupling BC formation and pigmentation in a two-step process. Firstly, *K. rhaeticus* is cultivated in static conditions for 5 days in a nutrient rich media (YPD). At the end of this process,
20 the bacteria produce a thick layer of cellulose at the liquid-air interface, referred to as pellicle (**Fig. 11A**). Secondly, the pellicle is isolated, sterilised in ethanol and washed with sterile water before being inoculated in minimal YNB media (20g/L glucose) together with a *Y. lipolytica* indigoidine-producing strain. We then apply shaking conditions (e.g., incubator or rotary shaking) to facilitate pellicle colonisation by the
25 indigoidine producing yeast. We hypothesize that filamentation of *Y. lipolytica* is increased when in minimal media, potentially leading to better colonisation patterns.

Example 10 - The use of pigmented *Y. lipolytica* biomass as a textile dyeing agent

30 At the end of the indigoidine production process (e.g., bioreactor cultivations), biomass is collected and left to dry overnight at room temperature. The dried cells are then ground via mechanical means, in our case through a pestle and mortar setup. The end product is a fine, dark blue powder. Addition of small (<1mL) volumes of DMSO is then carried out with the intention to create a dyeing paste.

35 Different textiles, either untreated or washed with soap, are then soaked for 4-12h in the dyeing paste. Shaking conditions can be applied to facilitate dye incorporation into

the fabric. The fabric is then sterilised by autoclaving and subsequently washed with soap and water to remove excess dye.

Successive treatment applications can be carried out to tune the colour and hue of the textile, with repeated applications leading to a higher dye incorporation in the textile (Fig. 12). We are also testing in situ dyeing approaches, adding textiles to a growing culture of *Y. lipolytica*. The yeast filamentation in minimal media may increase dye incorporation due to entanglement between the fibres.

10 Example 11 - Cloning and expression of the semi-synthetic BpsA protein in *Y. lipolytica*

A semi-synthetic BpsA protein (BpsA_X5-UNC) was designed *in silico* using an in-house methodology. The amino acid sequence is 50% identical to the native sequence from *S. lavendulae* (BpsA_X5; Fig. 13A). Both native and designed protein structures were predicted using AlphaFold. (see for example <https://alphafold.ebi.ac.uk/> Jumper et al 2021 Nature 596: 583-589; and Varadi et al 2022 NAR D439-D444). The designed protein shows nearly identical structure to the natural protein (Fig. 13B).

The BpsA_X5-UNC-encoding DNA sequence was synthesized by an external company and cloned into the expression vector. Currently, several vector variants are being constructed which will be used to transform *Y. lipolytica*. The constructed BpsA_X5-UNC-expressing strains will be tested for indigoidine production. This work will be completed within approx. 2 months from filing this form.

25 Example 12 - Cloning and expression of the FBA-identified target genes

We aim to further increase the indigoidine titers by overexpression and knock-out of the target genes identified using flux-balance analysis (FBA, Table 3). For gene over-expressions, the native *Y. lipolytica* genes will be cloned into expression vectors which will be subsequently used to transform indigoidine-producing *Y. lipolytica* strain. For gene knock-outs, the disruption cassettes will be constructed, cloned into carrier vector and transformed into *Y. lipolytica*. The impact of the introduced modifications on indigoidine production will be then analyzed.

Table 3 – Overexpression target genes

Target Name	Gene ID	Predicted reaction
<i>Glutamine Synthetase</i>	<i>YALJ0D13024g</i>	$L\text{-glutamate} + NH_3 + ATP \rightarrow L\text{-glutamine} + ADP + \text{Phosphate}$
<i>Glutamate Dehydrogenase (NAD⁺)</i>	<i>YALJ0E09603g</i>	$\alpha\text{-ketoglutarate} + NH_3 + NADH + H^+ \leftrightarrow L\text{-glutamate} + H_2O + NAD^+$
<i>Glutamate Dehydrogenase (NADP⁺)</i>	<i>YALJ0F17820g</i>	$\alpha\text{-ketoglutarate} + NH_3 + NAD(P)^+H + H^+ \leftrightarrow L\text{-glutamate} + H_2O + NAD(P)^+$
<i>isocitrate dehydrogenase (NAD⁺)</i>	<i>YALJ0E05137g</i>	$isocitrate + NAD^+ \leftrightarrow \alpha\text{-ketoglutarate} + CO_2 + NADH$
<i>isocitrate dehydrogenase (NAD⁺)</i>	<i>YALJ0D06303g</i>	Isocitrate to α -ketoglutarate
<i>isocitrate dehydrogenase (NADP⁺)</i>	<i>YALJ0F04095g</i>	Isocitrate to α -ketoglutarate
<i>aspartate transaminase (cyt)</i>	<i>YALJ0F29137g</i>	$\alpha\text{-ketoglutarate} + aspartate \rightarrow L\text{-glutamate} + oxaloacetate$
<i>malate dehydrogenase</i>	<i>YALJ0D16753g</i>	Malate to oxaloacetate
<i>3,4 dihydroxy-2-butanone-4-phosphate synthase</i>	<i>YALJ0B08965g</i>	$D\text{-ribulose 5-phosphate} \rightarrow formate + L\text{-3,4-dihydroxybutan-2-one 4-phosphate}$
<i>Malate Dehydrogenase</i>	<i>YALJ0E14190g</i>	Malate to oxaloacetate
<i>α-KG transporter</i>	<i>YALJ0D02629g</i>	Cytoplasmic transport of α -KG

Equivalents

- 5 The foregoing embodiments, instances, and examples are applicable to any of the aspects of the present disclosure and should be construed as such.

While the present disclosure has been described in terms of various aspects, embodiments, and examples, it is understood that variations, improvements, and equivalents will occur to the person skilled in the art. Such variations, improvements, and equivalents are contemplated by the present disclosure and fall within the scope of the matter disclosed and claimed herein.

Claims

1. A recombinant yeast cell capable of producing indigoidine, wherein the cell expresses:

- 5 a) a heterologous non-ribosomal peptide synthetase (NRPS) polypeptide capable of converting glutamine into indigoidine from a nucleic acid that encodes said NRPS; and
- b) a heterologous phosphopantetheinyl transferase (PPTase) polypeptide from a nucleic acid that encodes said PPTase.

10

2. The recombinant yeast cell of claim 1, wherein the cell overexpresses a glutamine dehydrogenase (GDH) polypeptide from a nucleic acid encoding said GDH polypeptide and a glutamine synthase (GS) polypeptide from a nucleic acid encoding said GS polypeptide.

15

3. The recombinant yeast cell of claim 1, wherein the cell overexpresses a glutamine dehydrogenase (GDH) polypeptide from a nucleic acid encoding said GDH polypeptide or a glutamine synthase (GS) polypeptide from a nucleic acid encoding said GS polypeptide.

20

4. The recombinant yeast cell of any one of claims 1-3, wherein the heterologous NRPS polypeptide:

- a) is BpsA; optionally:
- 25 i) *Streptomyces lavendulae* BpsA; or
- ii) a synthetic BpsA;
- b) is IndC; optionally *Streptomyces chromofuscus* IndC;
- c) is IndB; optionally *Streptomyces chromofuscus* IndB;
- d) comprises or consists of an amino acid sequence of SEQ ID NO: 4, SEQ
- 30 ID NO: 6, or SEQ ID NO: 8; or of an amino acid sequence at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 4, SEQ ID NO: 6, or SEQ ID NO: 8; and/or
- e) is encoded by a nucleotide sequence of SEQ ID NO: 5 or SEQ ID NO: 7;
- 35 or of at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 5 or SEQ ID NO: 7.

5. The recombinant yeast cell of any of claims 1-4, wherein the heterologous PPTase polypeptide:

a) is *E. coli* PPTase;

b) is *Bacillus subtilis* PPTase;

5 c) comprises an amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 17, or SEQ ID NO: 19; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 1 or SEQ ID NO: 3, SEQ ID NO: 17, or SEQ ID NO: 18; and/or

10 d) is encoded by a nucleotide sequence of SEQ ID NO: 2, SEQ ID NO: 16, or SEQ ID NO: 18; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 2, SEQ ID NO: 16, or SEQ ID NO: 18.

15

6. The recombinant yeast cell of any of claims 1-5, wherein the GDH polypeptide and/or GS polypeptide are endogenous GDH and/or GS polypeptides.

20 7. The recombinant yeast cell of any of claims 1-5, wherein the GDH polypeptide and/or GS polypeptide are heterologous GDH and/or GS polypeptides.

8. The recombinant yeast cell of any of claims 1-7, wherein:

25

the GDH polypeptide:

a) is encoded by the open reading frame defined by YALIOE09603g or YALIOF17820g;

30 b) comprises an amino acid sequence of SEQ ID NO: 20 or SEQ ID NO: 21; or an 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 20 or SEQ ID NO: 21; and/or

35 c) is encoded by a nucleotide sequence of SEQ ID NO: 11 or SEQ ID NO: 12; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 11 or SEQ ID NO: 12; and/or

the GS polypeptide:

- a) is encoded by the open reading frame defined by YALIOD13024g or YALIOF00506g;
- 5 b) comprises an amino acid sequence of SEQ ID NO: 22 or SEQ ID NO: 23; or an amino acid sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identity to SEQ ID NO: 22 or SEQ ID NO: 23; and/or
- 10 c) is encoded by a nucleotide sequence of SEQ ID NO: 9 or SEQ ID NO: 10; or is encoded by a nucleic acid that has a sequence that has at least 50%, at least 60%, at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% sequence identity to SEQ ID NO: 9 or SEQ ID NO: 10.

15

9. The recombinant yeast cell of any of claims 1-8, wherein the nucleotide sequences encoding the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide are each operably linked to a promoter that is selected from the group comprising or consisting of:

- 20 a) a constitutive promoter selected from the group comprising or consisting of: an ATP1 promoter [SEQ ID NO: 24], an ATP2 promoter [SEQ ID NO: 25], an FBAln promoter [SEQ ID NO: 26], a PGK1 promoter [SEQ ID NO: 27], a GPM1 promoter [SEQ ID NO: 28], a HHF1 promoter [SEQ ID NO: 29], a CYC1 promoter [SEQ ID NO: 30], an HHT1 promoter [SEQ ID NO: 31], an HTB1
- 25 promoter [SEQ ID NO: 32], an EXP1 promoter [SEQ ID NO: 33], a TDH1 promoter [SEQ ID NO: 34], an RPL25 promoter [SEQ ID NO: 35], a TEF1 promoter [SEQ ID NO: 36], a TEFIn promoter [SEQ ID NO: 37], a TEF2UAS promoter [SEQ ID NO: 38], a TEF4UAS promoter [SEQ ID NO: 39], and a TEF8UAS promoter [SEQ ID NO: 40], a pTEF promoter [SEQ ID NO: 13 and 14],
- 30 a GAP promoter [SEQ ID NO: 57], or
- b) an inducible promoter selected from the group comprising or consisting of: a pPOX2 promoter [SEQ ID NO: 41], a pXPR2 promoter [SEQ ID NO: 42], a pFBP1 promoter [SEQ ID NO: 43], a pMDH1a promoter [SEQ ID NO: 44], an ACL2 promoter [SEQ ID NO: 45], an XPR2 promoter [SEQ ID NO: 58], , a POT1
- 35 promoter [SEQ ID NO: 46], a LIP2 promoter [SEQ ID NO: 47], an ICL promoter [SEQ ID NO: 48], a YAT1 promoter [SEQ ID NO: 49], a CTR1 promoter [SEQ ID NO: 50], a CTR2 promoter [SEQ ID NO: 51], a pYALIOB18194 promoter [SEQ ID NO: 52], a pYALIOC11165 promoter [SEQ ID NO: 53], a pYALIOC15004

promoter [SEQ ID NO: 54], a pYALI0E14256 promoter [SEQ ID NO: 55], and a pYALI0F13937 promoter [SEQ ID NO: 56].

10. The recombinant yeast cell of any of claims 1-9, wherein the nucleotide sequences encoding the heterologous NRPS polypeptide, the heterologous PPTase polypeptide, the GDH polypeptide, and/or GS polypeptide are each operably linked to a promoter that is a pTEF promoter; optionally wherein the pTEF promoter has a nucleotide sequence encoded by SEQ ID NO: 13 or SEQ ID NO: 14.

11. The recombinant yeast cell of any of claims 1-10, wherein the nucleic acids that encode the NRPS polypeptide, the PPTase polypeptide, the GDH polypeptide and/or the GS polypeptide are integrated into the genome of the cell.

12. The recombinant yeast cell of any of claims 1-10 comprising a deletion of:

the endogenous chromosomal GDH and/or GS genes; and/or
at least one gene selected from the group comprising or consisting of an open reading frame selected from: YALI0E09603g, YALI0F17820g, YALI0D13024g, YALI0F00506g, YALI0E18634g, YALI0B00572g, and/or YALI0F01606g, or any combination thereof.

13. The recombinant yeast cell of any of the preceding claims wherein the yeast cell is an oleaginous yeast cell, but that is not a *Rhodospiridium* cell, optionally wherein the cell is not a *Rhodospiridium toruloides* cell.

14. The recombinant yeast cell of any of the preceding claims, wherein the yeast cell is a *Yarrowia* cell, optionally is selected from the group comprising or consisting of: a *Yarrowia bubula* cell, a *Yarrowia deformans* cell, a *Yarrowia lipolytica* cell, a *Yarrowia porcina* cell, a *Yarrowia yakushimensis* cell, a *Yarrowia parophonii* cell, a *Yarrowia galli* cell, a *Yarrowia oslonensis* cell, a *Yarrowia alimentaria* cell, a *Yarrowia hollandica* cell, and a *Yarrowia phangngaensis*; optionally wherein the recombinant *Yarrowia* cell is a *Yarrowia lipolytica* cell.

15. A method of producing indigoidine comprising culturing a recombinant yeast cell according to any of claims 1-14.

16. The method of claim 15, wherein the cell is cultured in a culture media selected from:

- a) a rich media, optionally YPD or YP4D;
- b) a minimal media, optionally YNB with glucose or YNB with glycerol; or
- c) a super-minimal media; optionally wherein the super-minimal media is SM1, SM2, SM3, SM4, or SM5.

5

17. The method of any of claims 15 or 16, wherein the culture media has a carbon to nitrogen (C/N) ratio of:

- a) between about 2 and about 160, about 2 and about 150, about 2 and about 140, about 2 and about 130, about 2 and about 120, about 2 and about 110, about 2 and about 100, about 2 and about 90, about 2 and about 80, about 2 and about 70, about 2 and about 60, about 2 and about 50, about 2 and about 40, about 2 and about 30, about 2 and about 20, about 2 and about 10, about 2 and about 9, about 2 and about 8, about 2 and about 7, about 2 and about 6, about 2 and about 5, about 2 and about 4, about 2 and about 3, about 4 and about 160, about 4 and about 150, about 4 and about 140, about 4 and about 130, about 4 and about 120, about 4 and about 110, about 4 and about 100, about 4 and about 90, about 4 and about 80, about 4 and about 70, about 4 and about 60, about 4 and about 50, about 4 and about 40, about 4 and about 30, about 4 and about 20, about 4 and about 10, about 4 and about 9, about 4 and about 8, about 4 and about 7, about 4 and about 6, about 4 and about 5, about 6 and about 160, about 6 and about 150, about 6 and about 140, about 6 and about 130, about 6 and about 120, about 6 and about 110, about 6 and about 100, about 6 and about 90, about 6 and about 80, about 6 and about 70, about 6 and about 60, about 6 and about 50, about 6 and about 40, about 6 and about 30, about 6 and about 20, about 6 and about 10, about 6 and about 9, about 6 and about 8, about 6 and about 7, about 8 and about 160, about 8 and about 150, about 8 and about 140, about 8 and about 130, about 8 and about 120, about 8 and about 110, about 8 and about 100, about 8 and about 90, about 8 and about 80, about 8 and about 70, about 8 and about 60, about 8 and about 50, about 8 and about 40, about 8 and about 30, about 8 and about 20, about 8 and about 10, about 8 and about 9, about 8 and about 8, about 8 and about 7, about 8 and about 6, about 8 and about 5, about 8 and about 4, about 8 and about 3, about 10 and about 160, about 10 and about 150, about 10 and about 140, about 10 and about 130, about 10 and about 120, about 10 and about 110, about 10 and about 100, about 10 and about 90, about 10 and about 80, about 10 and about 70, about 10 and about 60, about 10 and about 50, about 10 and about 40, about 10 and about 30, about 10 and about 20, about 20 and about 160, about 20 and about 150, about 20

and about 140, about 20 and about 130, about 20 and about 1200, about 20 and about 110, about 20 and about 100, about 20 and about 90, about 20 and about 80, about 20 and about 70, about 20 and about 60, about 20 and about 50, about 20 and about 40, about 20 and about 30, about 30 and about 160, about 30 and about 150, about 30 and about 140, about 30 and about 130, about 30 and about 1300, about 30 and about 110, about 30 and about 100, about 30 and about 90, about 30 and about 80, about 30 and about 70, about 30 and about 60, about 30 and about 50, about 30 and about 40, about 40 and about 160, about 40 and about 150, about 40 and about 140, about 40 and about 130, about 40 and about 1400, about 40 and about 110, about 40 and about 100, about 40 and about 90, about 40 and about 80, about 40 and about 70, about 40 and about 60, about 40 and about 50, about 50 and about 160, about 50 and about 150, about 50 and about 140, about 50 and about 130, about 50 and about 150, about 50 and about 110, about 50 and about 100, about 50 and about 90, about 50 and about 80, about 50 and about 70, or about 50 and about 60;

b) between 2 and 160, 2 and 150, 2 and 140, 2 and 130, 2 and 120, 2 and 110, 2 and 100, 2 and 90, 2 and 80, 2 and 70, 2 and 60, 2 and 50, 2 and 40, 2 and 30, 2 and 20, 2 and 10, 2 and 9, 2 and 8, 2 and 7, 2 and 6, 2 and 5, 2 and 4, 2 and 3, 4 and 160, 4 and 150, 4 and 140, 4 and 130, 4 and 140, 4 and 110, 4 and 100, 4 and 90, 4 and 80, 4 and 70, 4 and 60, 4 and 50, 4 and 40, 4 and 30, 4 and 20, 4 and 10, 4 and 9, 4 and 8, 4 and 7, 4 and 6, 4 and 5, 6 and 160, 6 and 150, 6 and 140, 6 and 130, 6 and 160, 6 and 110, 6 and 100, 6 and 90, 6 and 80, 6 and 70, 6 and 60, 6 and 50, 6 and 40, 6 and 30, 6 and 20, 6 and 10, 6 and 9, 6 and 8, 6 and 7, 8 and 160, 8 and 150, 8 and 140, 8 and 130, 8 and 180, 8 and 110, 8 and 100, 8 and 90, 8 and 80, 8 and 70, 8 and 60, 8 and 50, 8 and 40, 8 and 30, 8 and 20, 8 and 10, 8 and 9, 8 and 8, 8 and 7, 8 and 6, 8 and 5, 8 and 4, 8 and 3, 10 and 160, 10 and 150, 10 and 140, 10 and 130, 10 and 1100, 10 and 110, 10 and 100, 10 and 90, 10 and 80, 10 and 70, 10 and 60, 10 and 50, 10 and 40, 10 and 30, 10 and 20, 20 and 160, 20 and 150, 20 and 140, 20 and 130, 20 and 1200, 20 and 110, 20 and 100, 20 and 90, 20 and 80, 20 and 70, 20 and 60, 20 and 50, 20 and 40, 20 and 30, 30 and 160, 30 and 150, 30 and 140, 30 and 130, 30 and 1300, 30 and 110, 30 and 100, 30 and 90, 30 and 80, 30 and 70, 30 and 60, 30 and 50, 30 and 40, 40 and 160, 40 and 150, 40 and 140, 40 and 130, 40 and 1400, 40 and 110, 40 and 100, 40 and 90, 40 and 80, 40 and 70, 40 and 60, 40 and 50, 50 and 160, 50 and 150, 50 and 140, 50 and 130, 50 and 150, 50 and 110, 50 and 100, 50 and 90, 50 and 80, 50 and 70, or 50 and 60;

- 5 c) at least about 2, at least about 3, at least about 4, at least about 5, at least about 6, at least about 7, at least about 8, at least about 9, at least about 10, at least about 20, at least about 30, at least about 40, at least about 50, at least about 60, at least about 70, at least about 80, at least about 90, at least about 100, at least about 110, at least about 120, at least about 130, at least about 140, at least about 150 or more;
- 10 d) less than about 160, less than about 150, less than about 140, less than about 130, less than about 120, less than about 110, less than about 100, less than about 90, less than about 80, less than about 70, less than about 60, less than about 50, less than about 40, less than about 30, less than about 20, less than about 10, less than about 9, less than about 8, less than about 7, less than about 6, less than about 5, less than about 4, less than about 3; and/or
- 15 e) at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 110, at least 120, at least 130, at least 140, at least 150 or more;
- 20 f) less than 160, less than 150, less than 140, less than 130, less than 120, less than 110, less than about 100, less than 90, less than 80, less than 70, less than 60, less than 50, less than 40, less than 30, less than 20, less than 10, less than 9, less than 8, less than 7, less than 6, less than 5, less than 4, less than 3;
- 25 g) about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 20, about 30, about 40, about 50, about 60, about 70, about 80, about 90, about 100, about 110, about 120, about 130, about 140, about 150; and/or
- h) 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160.

30 18. The method of any of claims 15-17, wherein:

35 The culture media has a carbon source that is selected from the group comprising or consisting of: lignocellulosic hydrolysate, glucose, fructose, mannose, citrate, acetate, rapeseed oil, sunflower oil, palm oil, waste cooking oil, rapeseed oil, and sunflower oil, glycerol, and/or waste glycerol; optionally wherein the lignocellulosic hydrolysate is selected from the group comprising or consisting of: hydrolysed linen chaff, pepper, vegetable mix, and urban pruning, or any combination thereof;

The culture media comprises water that is selected from the group comprising or consisting of: sea water, tap water and distilled water; or any combination thereof; and

5 The culture media comprises a nitrogen source that is selected from the group comprising or consisting of: ammonium sulphate, urine, synthetic urine, urea, ammonium chloride, peptone, and, or any combination thereof.

19. The method of claim 15-18, wherein said culturing comprises agitating the liquid media during cell growth; wherein the liquid media is agitated:

- 10 a) at between about 150 and about 2000 RPM, about 150 and about 250 RPM, about 150 and about 200 RPM, about 200 and about 250 RPM, about 150 and about 800 RPM, about 200 and about 800 RPM, and about 250 and about 800 RPM, about 800 and about 2000 RPM, about 1000 and about 1800 RPM, about 1200 and about 1400 RPM;
- 15 b) at between 150 and 2000 RPM, 150 and 250 RPM, 150 and 200 RPM, 200 and 250 RPM, 150 and 800 RPM, 200 and 800 RPM, and 250 and 800 RPM, 800 and 2000 RPM, 1000 and 1800 RPM, 1200 and 1400 RPM;
- c) at at least 150 RPM, at least 200 RPM, at least 250 RPM, at least 800 RPM, at least 1000 RPM, at least 1200 RPM, at least 1400 RPM, at least 1600 RPM, at least 1800 RPM;
- 20 d) at less than 2000 RPM, less than 1800 RPM, less than 1600 RPM, less than 1400 RPM, less than 1200 RPM, less than 1000 RPM, less than 800RPM, less than 250 RPM, or less than 200 RPM;
- c) at about 150 RPM, about 200 RPM, about 250 RPM, about 800 RPM, about 1000 RPM, about 1200 RPM, about 1400 RPM, about 1600 RPM, about 1800 RPM, or about 2000 RPM; or
- 25 d) at 150 RPM, 200 RPM, 250 RPM, 800 RPM, 1000 RPM, 1200 RPM, 1400 RPM, 1600 RPM, 1800 RPM, or 2000 RPM.

30 20. The method of any of claims 15-19, wherein:

- a) the culture media is supplemented with glutamine at concentrations of:
- 35 i) between about 1mM and about 100mM, about 2mM and about 90mM, about 3mM and about 80mM, about 4mM and about 70mM, about 5mM and about 60mM, about 6mM and about 50mM, about 7mM and about 40mM, about 8mM and about 30mM, about 9mM and about 20mM, about 10 mM and about 15mM, about 1mM and about 10mM, about 2mM and about 9mM, about 3mM and about 8mM, about 4mM and about 7mM, about 5mM and about 6 mM, about 10 mM and about 100mM,

about 20mM and about 90mM, about 30mM and about 80mM, about 40mM and about 70mM, about 50mM and about 60mM, about 50mM and about 150mM, about 60mM and about 140mM, about 70mM and about 130mM, about 80mM and about 120mM, or about 90mM and about 110mM glutamine;

ii) between 1mM and 100mM, 2mM and 90mM, 3mM and 80mM, 4mM and 70mM, 5mM and 60mM, 6mM and 50mM, 7mM and 40mM, 8mM and 30mM, 9mM and 20mM, 10 mM and 15mM, 1mM and 10mM, 2mM and 9mM, 3mM and 8mM, 4mM and 7mM, 5mM and 6 mM, 10 mM and 100mM, 20mM and 90mM, 30mM and 80mM, 40mM and 70mM, 50mM and 60mM, 50mM and 150mM, 60mM and 140mM, 70mM and 130mM, 80mM and 120mM, or 90mM and 110mM glutamine;

iii) at least 1mM, at least 2 mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 20mM, at least 30mM, at least 40mM, at least 50mM, at least 60mM, at least 70mM, at least 80mM, at least 90mM, at least 100mM, at least 110mM, at least 120mM, at least 130mM, at least 140mM, at least 150mM, or more glutamine;

iv) less than 150mM, less than 140mM, less than 130mM, less than 120mM, less than 120mM, less than 110mM, less than 100mM, less than 90mM, less than 80mM, less than 70mM, less than 60mM, less than 50mM, less than 40mM, less than 30mM, less than 20mM, less than 10mM, less than 9mM, less than 8mM, less than 7mM, less than 6mM, less than 5mM, less than 4mM, less than 3mM, less than 2mM, or less glutamine;

v) about 1mM, about 2 mM, about 3mM, about 4mM, about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about 10mM, about 20mM, about 30mM, about 40mM, about 50mM, about 60mM, about 70mM, about 80mM, about 90mM, about 100mM, about 110mM, about 120mM, about 130mM, about 140mM, or about 150mM glutamine; and/or

vi) 1mM, 2 mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM, 140mM, or 150mM glutamine;

and/or

b) the culture media is supplemented with glutamic acid at concentrations

of:

- 5 i) between about 1mM and about 100mM, about 2mM and about 90mM, about 3mM and about 80mM, about 4mM and about 70mM, about 5mM and about 60mM, about 6mM and about 50mM, about 7mM and about 40mM, about 8mM and about 30mM, about 9mM and about 20mM, about 10 mM and about 15mM, about 1mM and about 10mM, about 2mM and about 9mM, about 3mM and about 8mM, about 4mM and about 7mM, about 5mM and about 6 mM, about 10 mM and about 100mM, about 20mM and about 90mM, about 30mM and about 80mM, about 40mM and about 70mM, about 50mM and about 60mM, about 50mM and about 150mM, about 60mM and about 140mM, about 70mM and about 130mM, about 80mM and about 120mM, or about 90mM and about 110mM glutamic acid;
- 10 ii) between 1mM and 100mM, 2mM and 90mM, 3mM and 80mM, 4mM and 70mM, 5mM and 60mM, 6mM and 50mM, 7mM and 40mM, 8mM and 30mM, 9mM and 20mM, 10 mM and 15mM, 1mM and 10mM, 2mM and 9mM, 3mM and 8mM, 4mM and 7mM, 5mM and 6 mM, 10 mM and 100mM, 20mM and 90mM, 30mM and 80mM, 40mM and 70mM, 50mM and 60mM, 50mM and 150mM, 60mM and 140mM, 70mM and 130mM, 80mM and 120mM, or 90mM and 110mM glutamic acid;
- 15 iii) at least 1mM, at least 2 mM, at least 3mM, at least 4mM, at least 5mM, at least 6mM, at least 7mM, at least 8mM, at least 9mM, at least 10mM, at least 20mM, at least 30mM, at least 40mM, at least 50mM, at least 60mM, at least 70mM, at least 80mM, at least 90mM, at least 100mM, at least 110mM, at least 120mM, at least 130mM, at least 140mM, at least 150mM, or more glutamic acid;
- 20 iv) less than 150mM, less than 140mM, less than 130mM, less than 120mM, less than 120mM, less than 110mM, less than 100mM, less than 90mM, less than 80mM, less than 70mM, less than 60mM, less than 50mM, less than 40mM, less than 30mM, less than 20mM, less than 10mM, less than 9mM, less than 8mM, less than 7mM, less than 6mM, less than 5mM, less than 4mM, less than 3mM, less than 2mM, or less glutamic acid;
- 25 v) about 1mM, about 2 mM, about 3mM, about 4mM, about 5mM, about 6mM, about 7mM, about 8mM, about 9mM, about 10mM, about 20mM, about 30mM, about 40mM, about 50mM, about 60mM, about 70mM, about 80mM, about 90mM, about 100mM, about 110mM, about 120mM, about 130mM, about 140mM, or about 150mM glutamic acid; and/or
- 30
- 35

vi) 1mM, 2 mM, 3mM, 4mM, 5mM, 6mM, 7mM, 8mM, 9mM, 10mM, 20mM, 30mM, 40mM, 50mM, 60mM, 70mM, 80mM, 90mM, 100mM, 110mM, 120mM, 130mM, 140mM, or 150mM glutamic acid.

5 21. The method of any of claims 15-20, wherein the method further comprises extracting and/or purifying the indigoidine from the cell; optionally

wherein extracting and/or purifying the indigoidine from the cell comprises a) lysing the cell; optionally wherein the cell is lysed by homogenisation in DMSO;

10 b) collecting the yeast cells, drying the yeast cells, and lysing the yeast by grinding the yeast cells.

22. A method of producing indigoidine-stained bacterial cellulose or an indigoidine-stained cellulose pellicle, the method comprising co-culturing the recombinant yeast cell according to any of claims 1-14 with a cellulose-producing bacterial cell, wherein the cells are co-cultured under conditions that allows the cellulose-producing bacterial cell to produce cellulose or a cellulose pellicle.

20 23. A method of producing indigoidine-stained bacterial cellulose or an indigoidine-stained cellulose pellicle, the method comprising:

a) culturing a cellulose-producing bacterial cell to form bacterial cellulose or a cellulose pellicle; and subsequently

b) contacting the cellulose or cellulose pellicle produced by the cellulose-producing second cell in (a) with the recombinant yeast cell according to any of claims 1-14.

24. Indigoidine-stained bacterial cellulose or indogoidine-stained cellulose pellicle obtained or obtainable from the method of any of claims 22 or 23.

30 25. Indigoidine obtained or obtainable from the recombinant yeast cell of any of claims 1-14 or the method according to any of claim 15-23.

26. A method of producing a dyed textile or garment comprising contacting said textile or garment with the indigoidine of claim 25.

35 27. A kit comprising any one or more of:

a) the recombinant yeast cell of any of claims 1-14;

b) bacterial cellulose;

- c) a cellulose pellicle;
- d) indigoidine of claim 25;
- e) a textile or garment or thread; and/or
- f) cellulose-producing bacterial cell.

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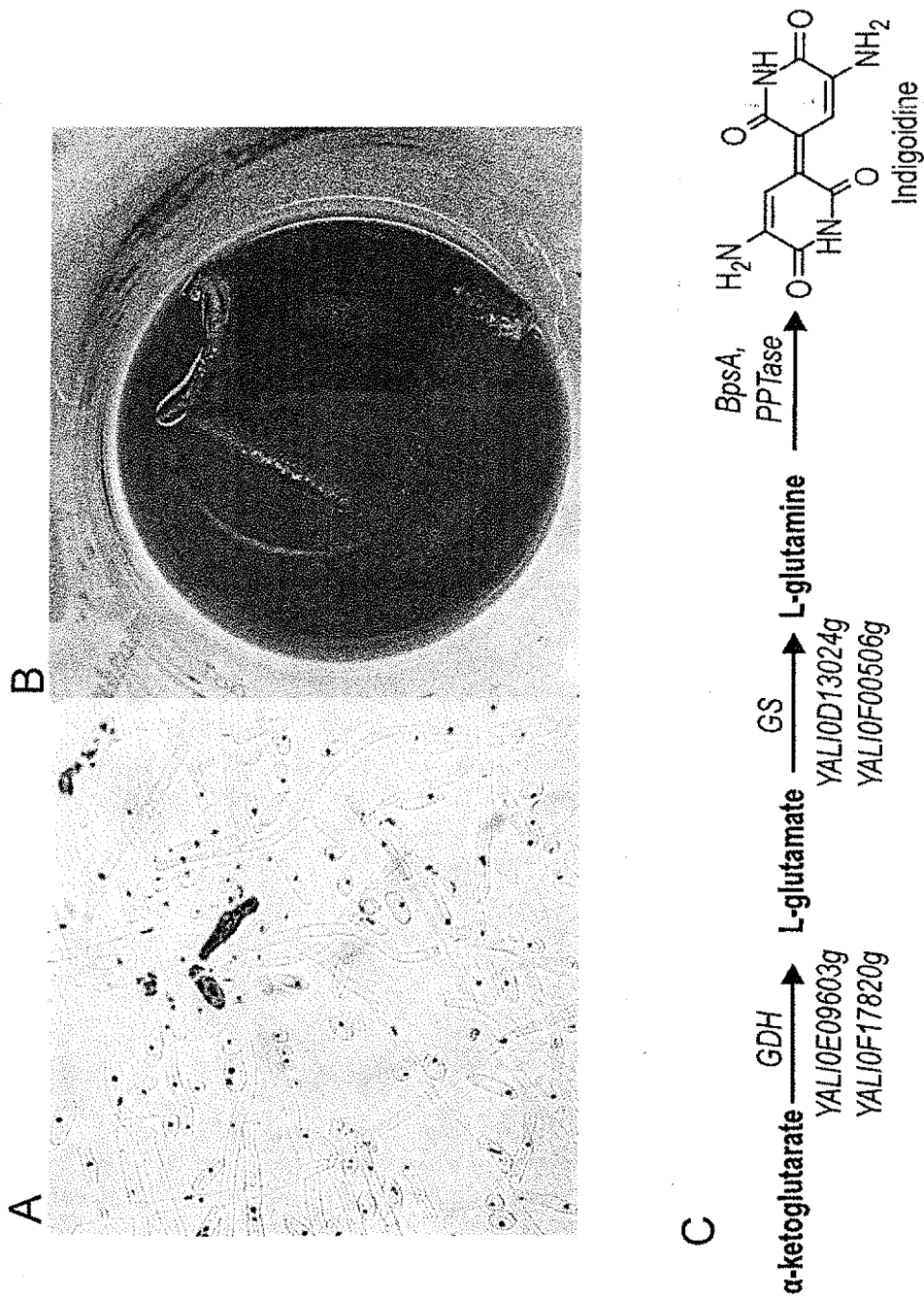


Figure 1

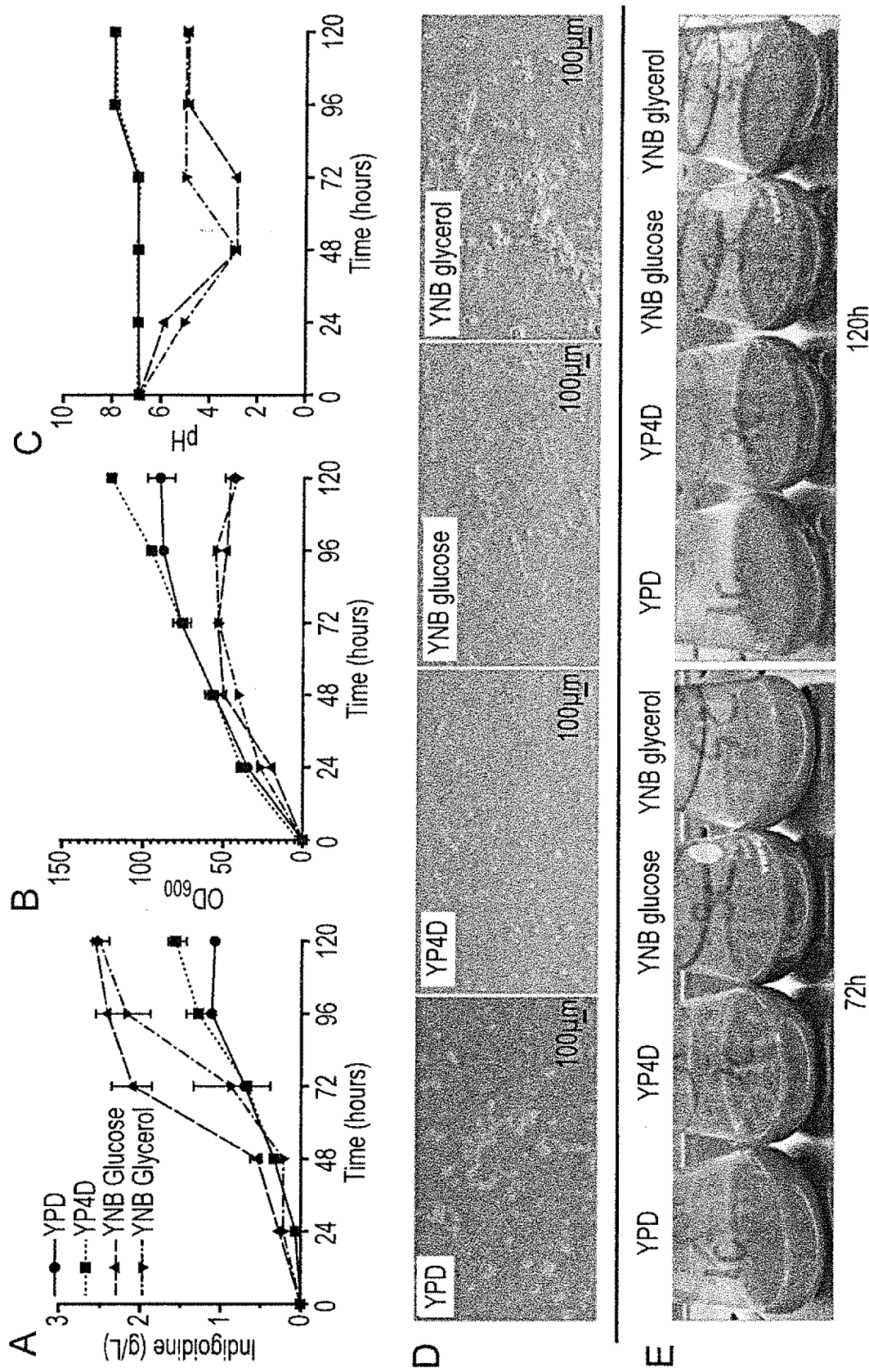


Figure 2

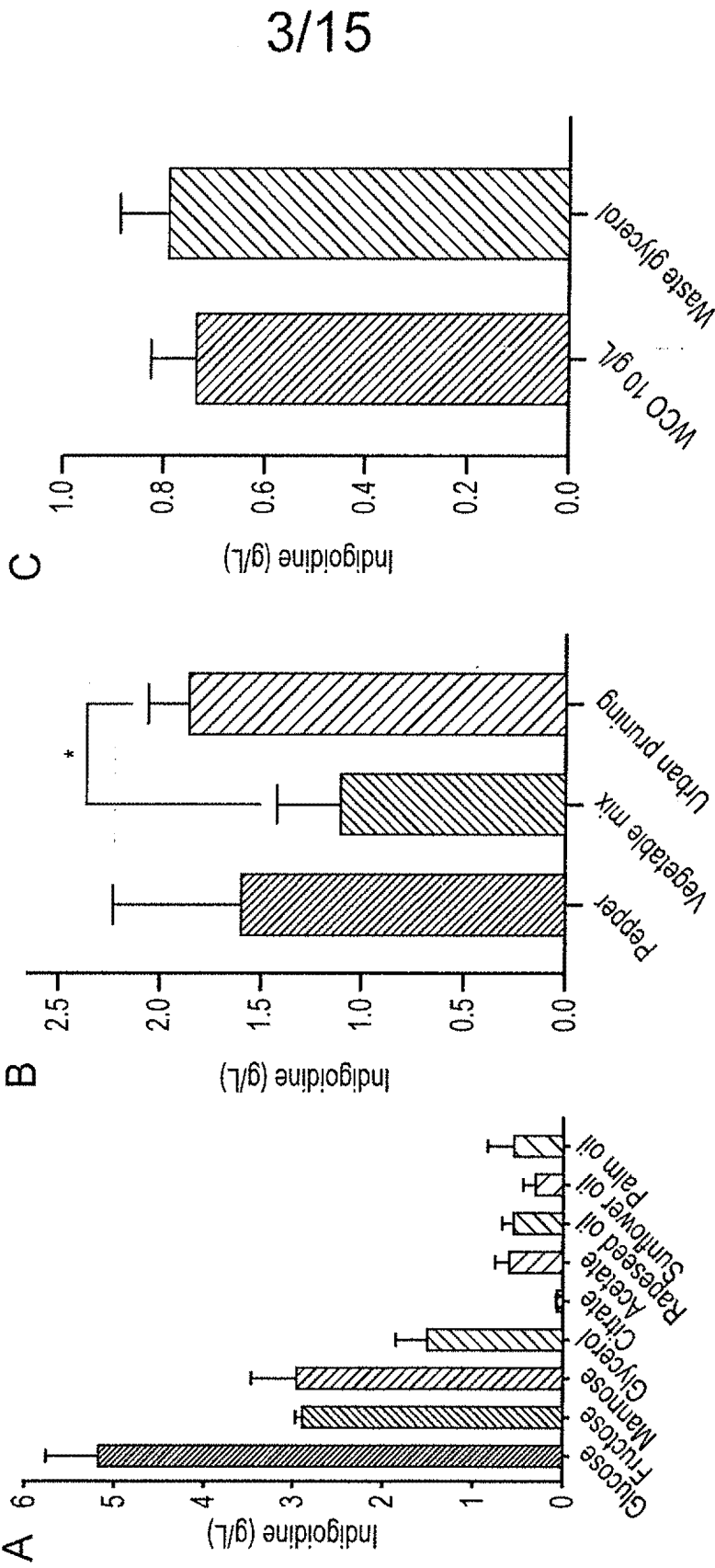


Figure 3

4/15

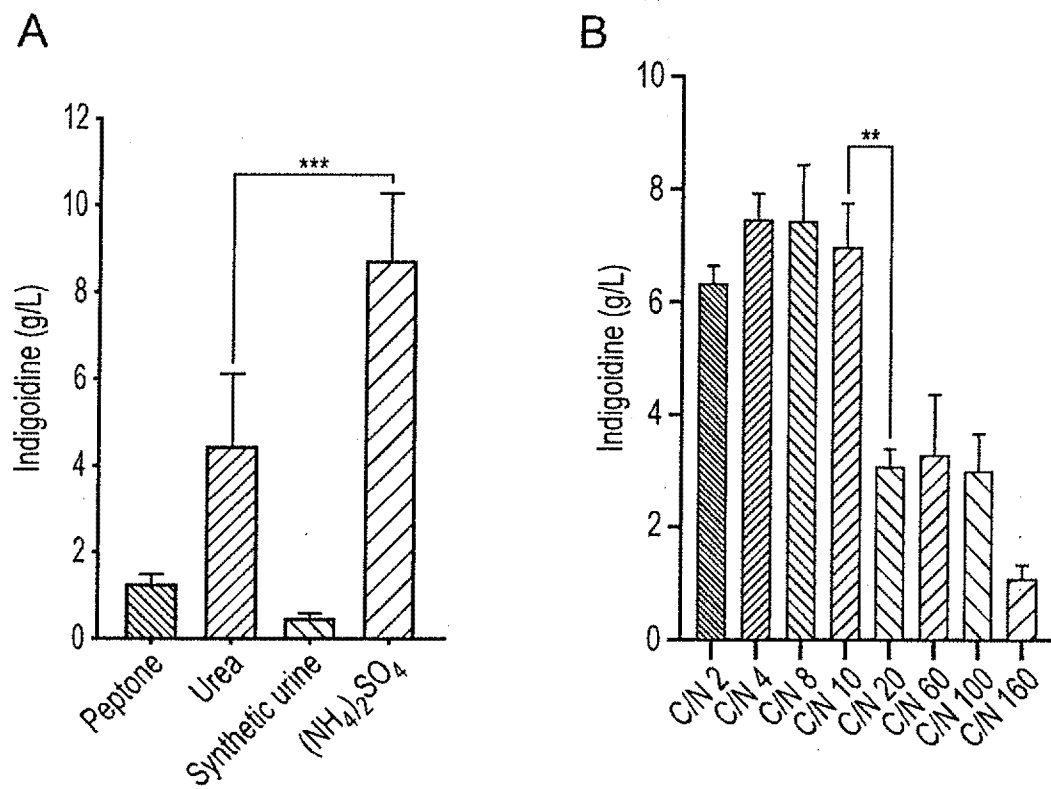


Figure 4

5/15

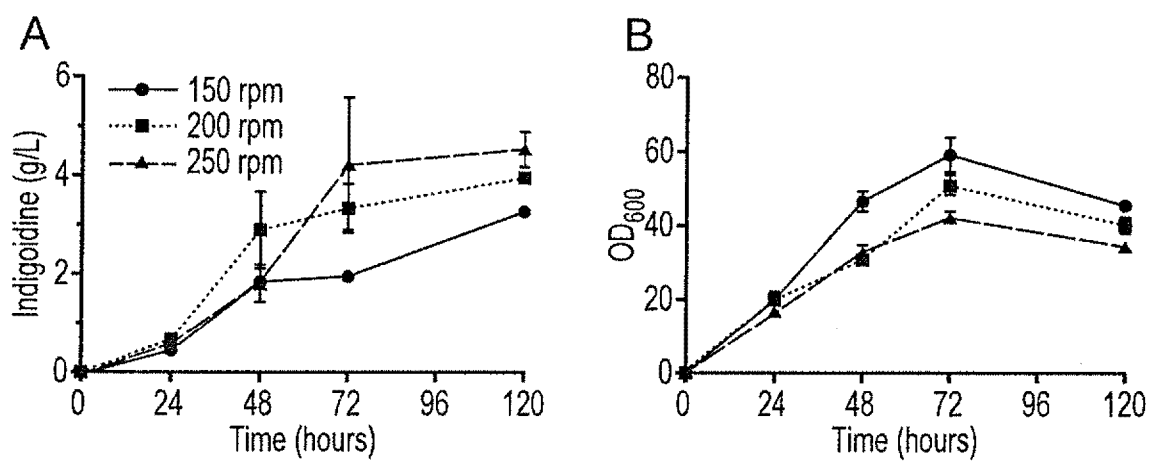


Figure 5

6/15

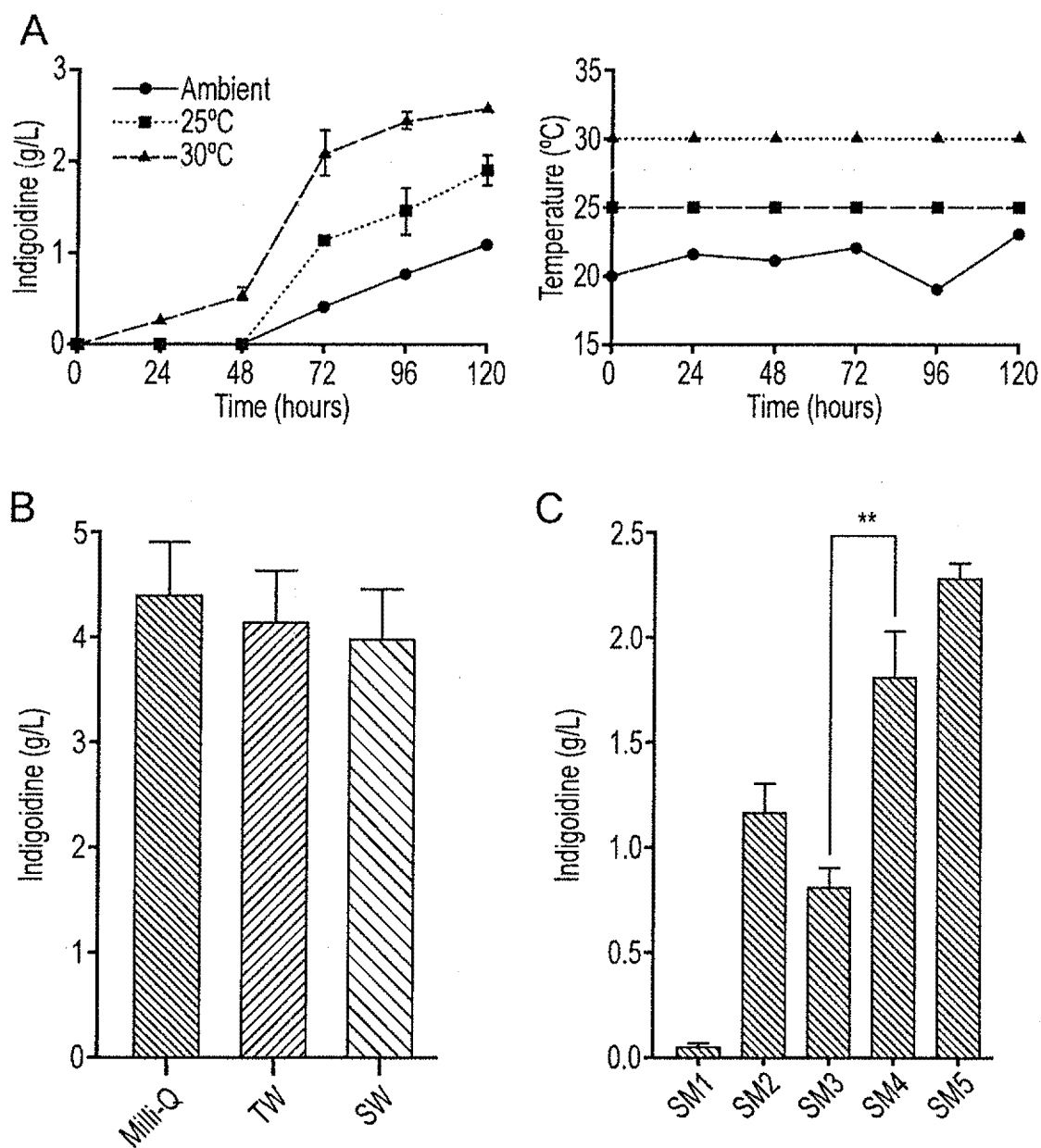


Figure 6

7/15

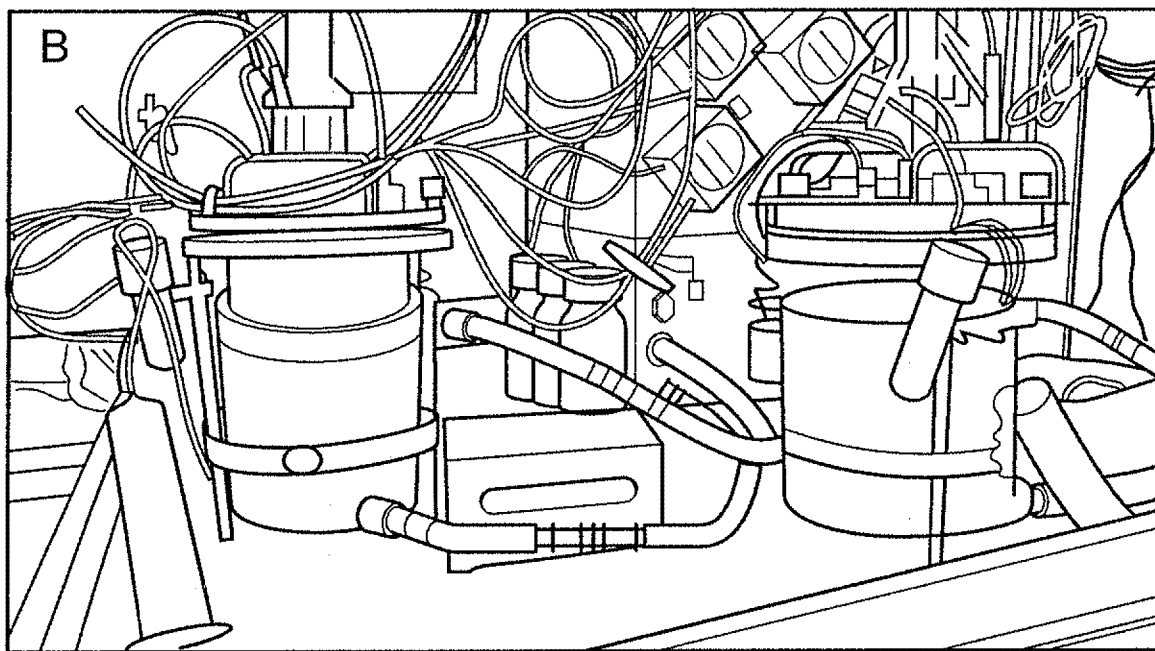
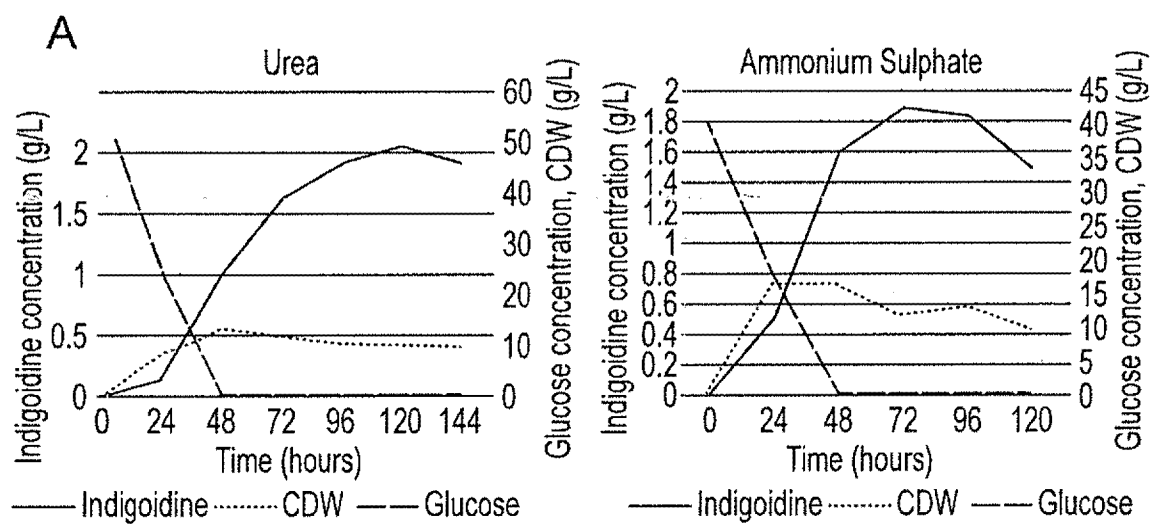
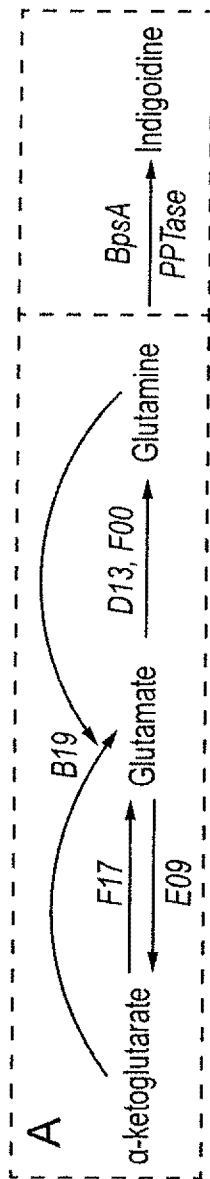
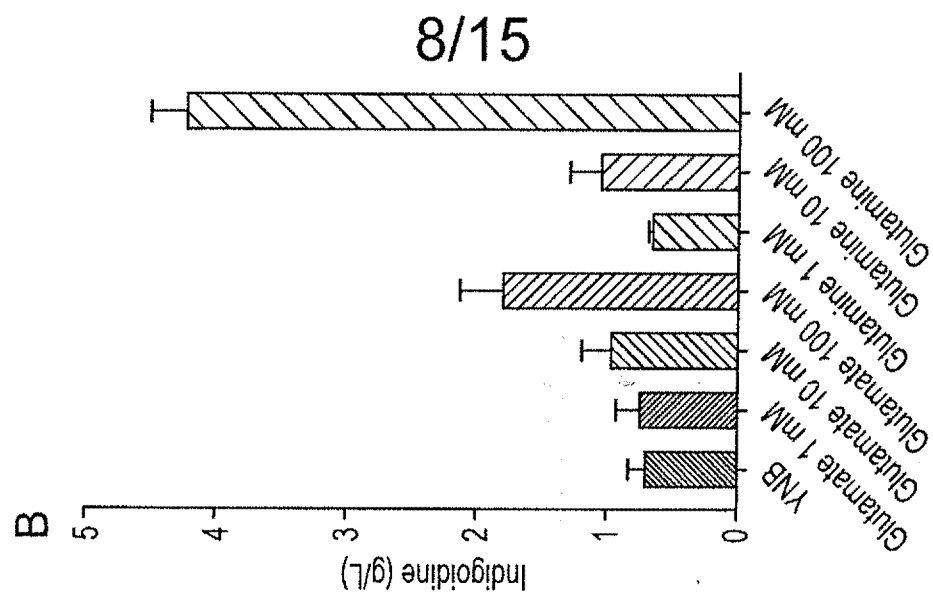


Figure 7



Gene	Gene ID	Function
F17	YALI0F17820g	Glutamate dehydrogenase (NADP ⁺)
E09	YALI0E09603g	Glutamate dehydrogenase (NAD ⁺)
D13	YALI0D13024g	Glutamine synthetase
F00	YALI0F00506g	Glutamine synthetase
B19	YALI0B19998g	Glutamate synthase (NAD ⁺)

Figure 8

9/15

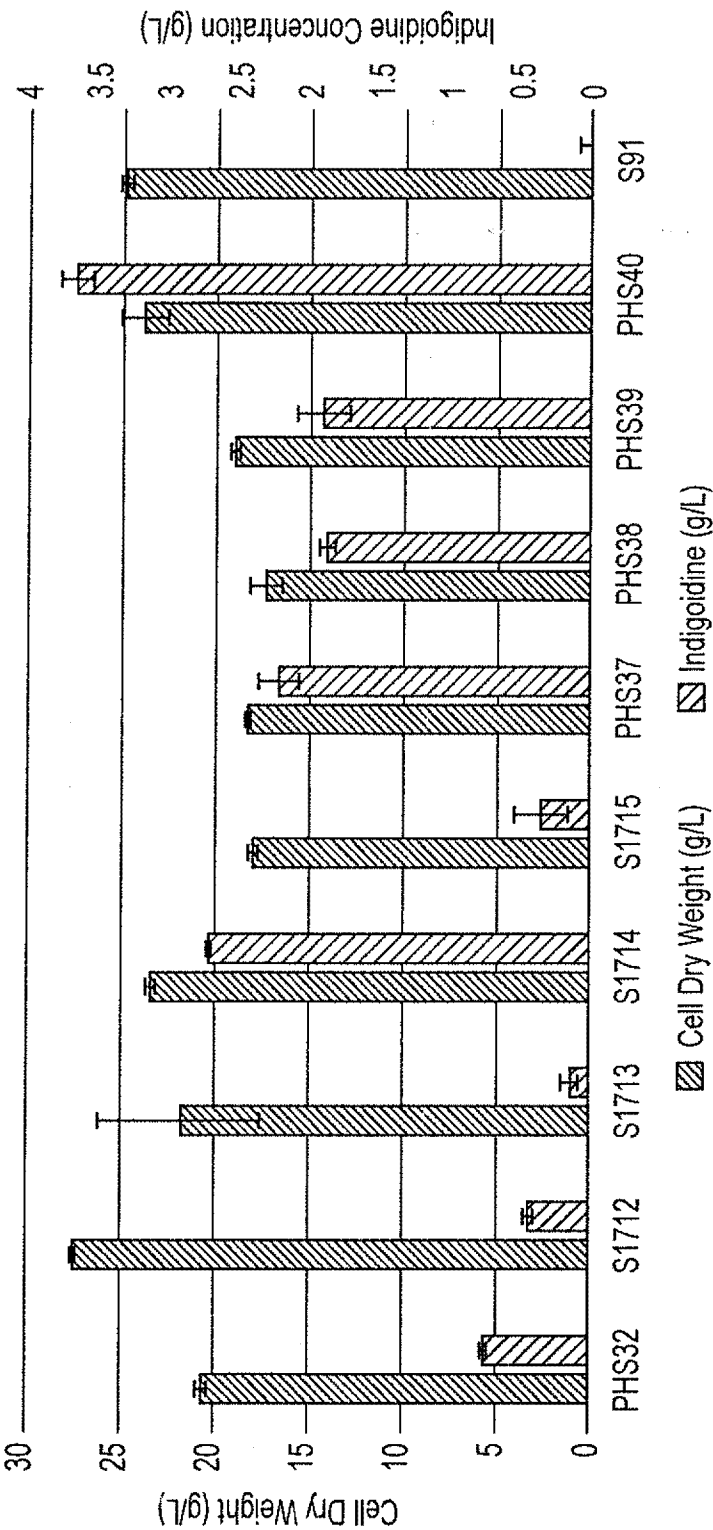


Figure 9

10/15

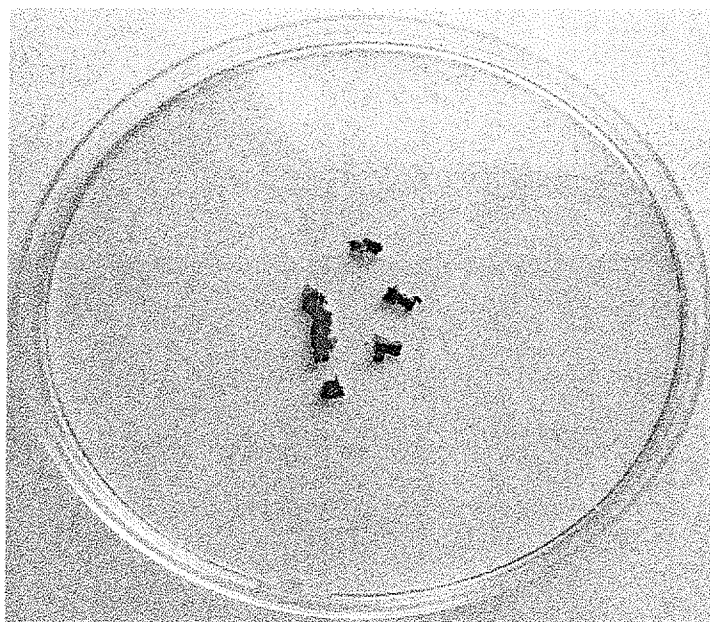


Figure 10

11/15

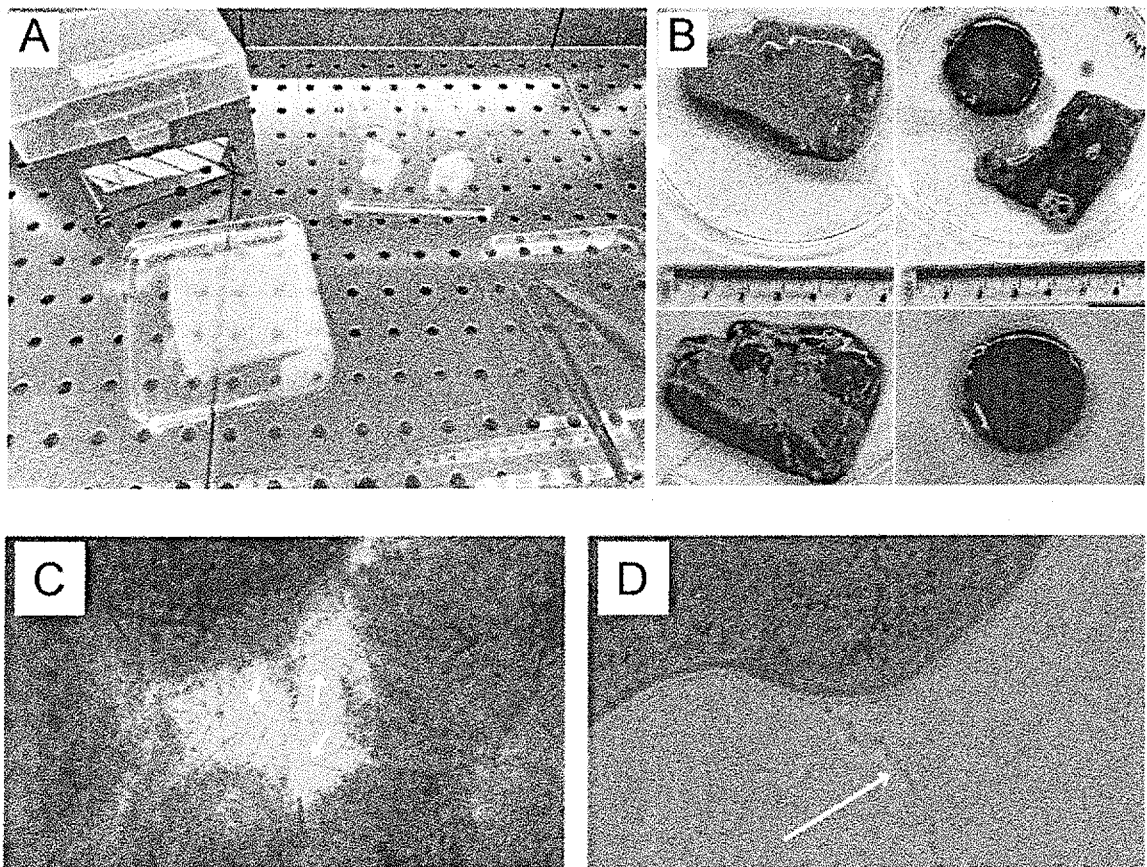


Figure 11

12/15

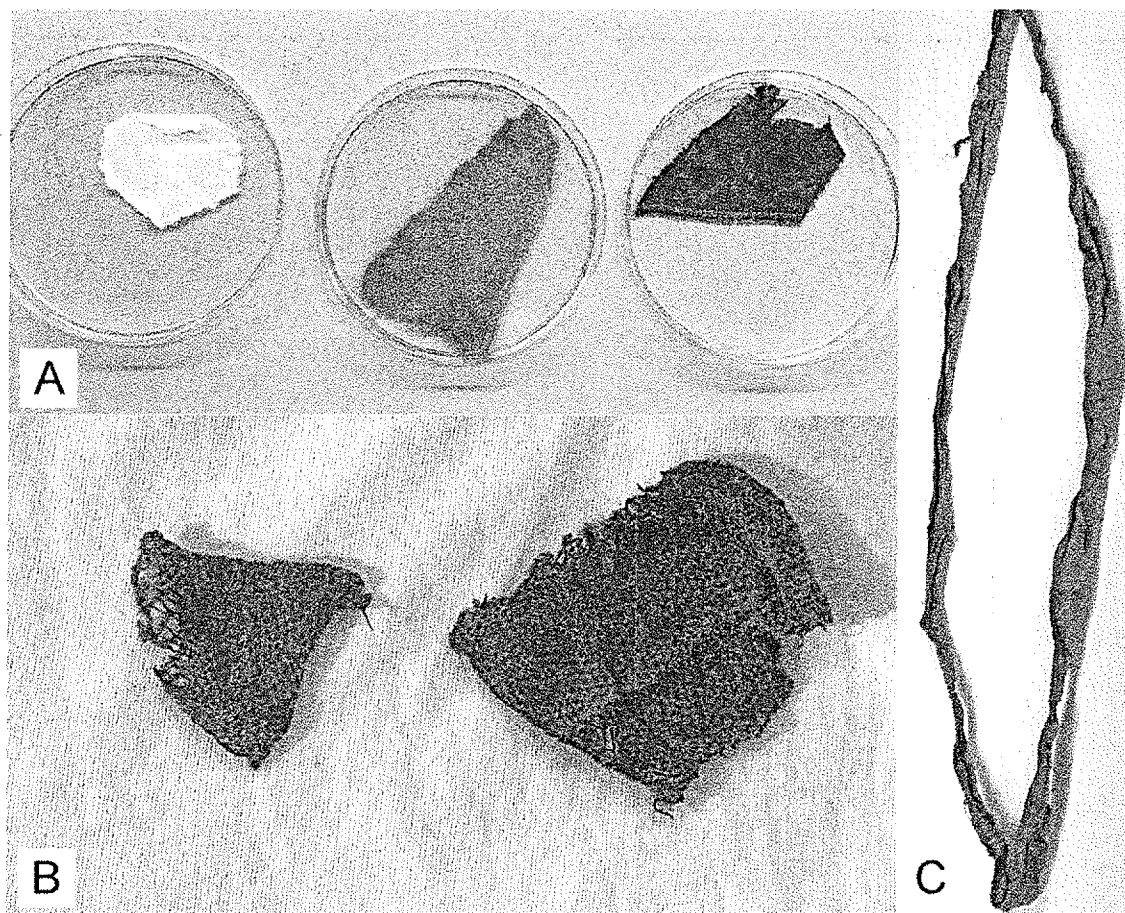


Figure 12

A

	651	660	670	680	690	700	710	720	730	740	750	760	770	780
BpsA_X5	EKAATGRPTAHIFIGKRGGIEPVYKNNIQEVLETETGHVGLFEQVLPAYGLDTRDLAVEPAVRDILLDPPEEDFVLGTIFELVPHT-GRREDHAEIVWQTHGSIVANILPEGQVRYADGTLTRFSDDIVLK													
BpsA_X5-UNC	ALPEDCAHCFNIHFIGKRSATIEHNVYKNNVLEVEFEAGHMLGLEEVLPRLGLEVRPDGFTPAKSRDLDAEEDHYLGTFAVAPRHGGTRPEEVELFVQAGDRVDGLPGGLYRYREGSLEPLGEQVDR													
Consensus	ataadcahcanIHFIGKRGaIEHNVYNNI;qEVLEIEaGhiLgILE#VLPaIGL#IRdda#ePaardrILDVAEDHYLGTaFap.HgGrReReaEL%QaHGDvIaNPeGqYRYa#GSLerlg##!Vdr													
	781	790	800	810	820	830	840	850	860	870	880	890	900	910
BpsA_X5	KOVIAINOSVYQAASFGISVISRAPPEEMHYVTLGKLOHLMHNGLGLGFMSGYSSKTGNPLPASRRIDSVLQANGVESGP-SYFFVGGVRSDEQLGHEGMREDSVHMRGPAELTRDDLVSFLPDYHIP													
BpsA_X5-UNC	RHVIAINORVYERSDFGISNVCSNEDKSDSHVYHLGRALHRFOSNHQLLGHRS-----GHPLASLRDLDDLTRAGVPAAPASVYFFVGGPISAEQAEHEGMNEDAVHTKGAPELLKEELLEQQLPNYHIP													
Consensus	rqVIAINqrVY#aadFGISm!crae#edmhYVhLGralqr!qmNgqLGmrS.....GnPLPASrRiDdLLqaGVeaaP.SYFFVGGr!SaEQaeHEGNEdAVHmrGPAELIr##LeqLP#YHIP													
	911	920	930	940	950	960	970	980	990	1000	1010	1020	1030	1040
BpsA_X5	NRVWFERLPLSANGKIDAKALAAASDOVNAELVERPFVAPRTIETEKEIAEVWAKSLRRSVSVODDFESGNSLIAGVLTRELNSRLGVSLPLOSIVESPTVEKLSRRLEREVAOESSRLVRLHAETGK													
BpsA_X5-UNC	NKVLVLNNLPQTVNGKVDLAALNQLDALKSLDINREMPLCTEAERKIGDTWCRVMWESVSADDDFFESGNSLHVALVNRVNRFGTRLPLQTVFDTPMLADLAACIDGGAPGTASRMVRLNAE-GP													
Consensus	NrVLVlrLPqsanGK!DaaALaaLDalnaed!#RemValrTeaEkeIa#Iwars\$rrESVSaQDDFFESGNSLhaVal!rrLnRrLgtrLPQsVL#sPmladLaari#reaaqeaSR\$VRLNAE.GK													
	1041	1050	1060	1070	1080	1090	1100	1110	1120	1130	1140	1150	1160	1170
BpsA_X5	DRPVLCPGLGGYPNNRLTAGEIGLGRSFYGIQAHGINEGEAPYATITENAKADIEATKELOPKGPTYLLNGYSFGARVAFETAYQLEQAGEKVDNLFLIAPGSPTVRAENGKVGREASFAINRAYTIL													
BpsA_X5-UNC	DTQVFAMPGLGGYPNNLRPLAALGTERPYHGVQAHGIMPGEFPYDDVRMAAADVEATREIQPHGPYLLCGYSFGARVAFETAAQLAEMGDEVKALYLLAPGAPVTQMEREKAYSHEARFDPVFLAIL													
Consensus	DrqVLawPGLGGYPNNLRpLAaaIGLeRpFhg!QAHGINEGeaPYad!raMaAd!EATrEiQhGPyLLcGYSFGARVAFETaaQLEaa#eVdaL%LIAPGapttraEreKaYgrEaFaihra%aiL													
	1171	1180	1190	1200	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
BpsA_X5	FSVFTIGTSGDLEKLESATDEESFAGFISE-LKGDVDLAKRTISWGOTYEFYSRELAERTLAAPVTIFKARGDDYSIENSNGYSAEPTVIDLDADHYSLRTPDQIGELVWHR---YLLGE													
BpsA_X5-UNC	FSVFAHRIEGHLLDRCLQCRTEDDFVSFCQRFTELENLVKRIIRVAVNTYGSYQFDLANRKLNTPTITLKAQGDHYSFLENSPPFSQHPKRIELNVGHYQVLKEAGIQELKQLQMEHDEESL													
Consensus	FSVFAgrIeGdLrCL#lardE#dFagFic#.lkei#e#LakRIrVaqTYeYqF#rELARkLaaPTITUKARGDdYSFIENSng%SaepPk!I#L#adhYqllreadIqELkqhir..hdLeE..													
	130301													
BpsA_X5	..													
BpsA_X5-UNC	TA													
Consensus	..													

Figure 13 (continued)

15/15

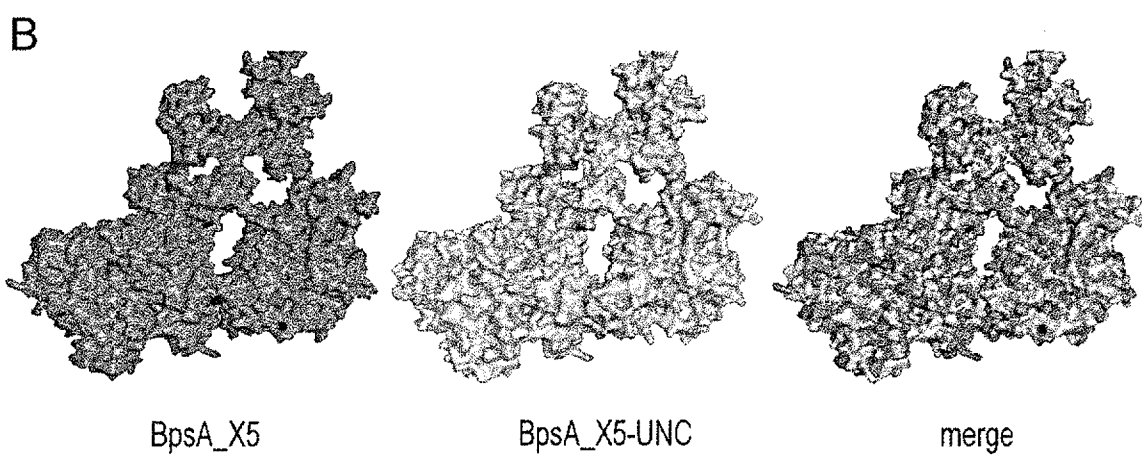


Figure 13 (continued)

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2024/051506

A. CLASSIFICATION OF SUBJECT MATTER					
INV.	C12N9/06	C12N9/12	C12N9/88	C12N9/00	C12P17/16
ADD.	C12R1/645				
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 C12R					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)					
EPO-Internal, WPI Data, BIOSIS, EMBASE					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category*	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	WO 2020/172438 A1 (UNIV CALIFORNIA [US]; NAT TECH & ENG SOLUTIONS SANDIA LLC [US]) 27 August 2020 (2020-08-27)				1-6, 9-21,25, 27
Y	the whole document				7,8,22, 23

	- / - -				
☒ Further documents are listed in the continuation of Box C.					
☒ See patent family annex.					
* Special categories of cited documents :					
"A" document defining the general state of the art which is not considered to be of particular relevance			"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		
"E" earlier application or patent but published on or after the international filing date			"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		
"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)			"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		
"O" document referring to an oral disclosure, use, exhibition or other means			"&" document member of the same patent family		
"P" document published prior to the international filing date but later than the priority date claimed					
Date of the actual completion of the international search			Date of mailing of the international search report		
25 September 2024			09/10/2024		
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016			Authorized officer Sonnerat, Isabelle		

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2024/051506

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WEHRS MAREN ET AL: "Sustainable bioproduction of the blue pigment indigoidine: Expanding the range of heterologous products in R. toruloides to include non-ribosomal peptides", GREEN CHEMISTRY, vol. 21, no. 12, 1 January 2019 (2019-01-01), pages 3394-3406, XP093185048, GB ISSN: 1463-9262, DOI: 10.1039/C9GC00920E Retrieved from the Internet: URL:http://pubs.rsc.org/en/content/article/pdf/2019/GC/C9GC00920E>	1-6, 9-21,25, 27
Y	the whole document	7,8,22, 23

X	GHIFFARY MOHAMMAD RIFQI ET AL: "High-Level Production of the Natural Blue Pigment Indigoidine from Metabolically Engineered Corynebacterium glutamicum for Sustainable Fabric Dyes", ACS SUSTAINABLE CHEMISTRY & ENGINEERING, vol. 9, no. 19, 19 April 2021 (2021-04-19), pages 6613-6622, XP093068119, US ISSN: 2168-0485, DOI: 10.1021/acssuschemeng.0c09341	25-27
Y	abstract	7,8
A	page 6614, left-hand column, paragraph 2 page 6616, right-hand column, paragraphs 1,2 page 6619, left-hand column, paragraph 2 - right-hand column, paragraph 1	1-6,9-21

X	XU FUCHAO ET AL: "Efficient production of indigoidine in Escherichia coli", JOURNAL OF INDUSTRIAL MICROBIOLOGY & BIOTECHNOLOGY, BASINGSTOKE, GB, vol. 42, no. 8, 25 June 2015 (2015-06-25), pages 1149-1155, XP035513232, ISSN: 1367-5435, DOI: 10.1007/S10295-015-1642-5 [retrieved on 2015-06-25]	25,27
Y	abstract	7,8
A	page 1152, left-hand column, paragraph 3 - right-hand column, paragraph 1; figure 3	1-6,9-21

X	Rainboweaver: "Engineering Success", , 1 January 2023 (2023-01-01), XP93205688, Retrieved from the Internet: URL:https://2023.igem.wiki/nanjing-china/engineering	24,25,27
Y	the whole document	22,23

-/-		

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2024/051506

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Yan Yiawei: "Registry of Standard Biological Parts", , 6 September 2022 (2022-09-06), XP93205692, Retrieved from the Internet: URL:https://parts.igem.org/Part:BBa_K43760 01	24,25,27
Y	the whole document -----	22,23

INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB2024/051506

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. ☒ 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/GB2024/051506

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2020172438 A1	27-08-2020	US 2022112531 A1	14-04-2022
		WO 2020172438 A1	27-08-2020
