PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference
31687WO-wp

FOR FURTHER ACTION

See Form PCT/PEA/416

International application No.
PCT/FI2019/050199

International filing date (day/month/year)
11.03.2019

Priority date (day/month/year)
19.03.2018

International Patent Classification (IPC) or national classification and IPC
INV. C12N15/63

Applicant
Teknologian tutkimuskeskus VTT Oy

1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.
2. This REPORT consists of a total of 5 sheets, including this cover sheet.
3. This report is also accompanied by ANNEXES, comprising:
   a. ☑ (sent to the applicant and to the International Bureau) a total of 5 sheets, as follows:
      ☑ sheets of the description, claims and/or drawings which have been amended and/or sheets containing
         rectifications authorized by this Authority, unless those sheets were superseded or cancelled, and any
         accompanying letters (see Rules 46.5, 66.8, 70.16, 91.2, and Section 607 of the Administrative
         Instructions).
      ☐ sheets containing rectifications, where the decision was made by this Authority not to take them into account
         because they were not authorized by or notified to this Authority at the time when this Authority began to
         draw up this report, and any accompanying letters (Rules 66.4bis, 70.2(e), 70.16 and 91.2).
      ☐ superseded sheets and any accompanying letters, where this Authority either considers that the
         superseding sheets contain an amendment that goes beyond the disclosure in the international application
         as filed, or the superseding sheets were not accompanied by a letter indicating the basis for the
         amendments in the application as filed, as indicated in item 4 of Box No. I and the Supplemental Box (see
         Rule 70.16(b)).
   b. ☐ (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a
      sequence listing, in the form of an Annex CST.25 text file, as indicated in the Supplemental Box Relating to
      Sequence Listing (see paragraph 3ter of Annex C of the Administrative Instructions).

4. This report contains indications relating to the following items:
   ☑ Box No. I  Basis of the report
   ☐ Box No. II  Priority
   ☐ Box No. III  Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   ☐ Box No. IV  Lack of unity of invention
   ☑ Box No. V  Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial
                applicability; citations and explanations supporting such statement
   ☐ Box No. VI  Certain documents cited
   ☐ Box No. VII  Certain defects in the international application
   ☑ Box No. VIII  Certain observations on the international application

Date of submission of the demand
25.11.2019

Date of completion of this report
23.06.2020

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0
Fax: +49 89 2399 - 4465

Authorized officer
Weinberg, Suzanna
Telephone No. +49 89 2399-7603

Form PCT/PEA/409 (cover sheet) (January 2015)
Box No. 1  Basis of the report

1. With regard to the language, this report is based on

☑ the international application in the language in which it was filed

☐ a translation of the international application into , which is the language of a translation furnished for the purposes of:

☐ international search (under Rules 12.3(a) and 23.1(b))

☐ publication of the international application (under Rule 12.4(a))

☐ international preliminary examination (under Rules 55.2(a) and/or 55.3(a) and (b))

2. With regard to the elements* of the international application, this report is based on (replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as “originally filed” and are not annexed to this report):

Description, Pages
1-47 as originally filed

Sequence listings, SEQ ID NO
1-25 as originally filed

Claims, Numbers
1-16 filed with the letter of 25-11-2019

Drawings, Sheets
1/16-16/16 as originally filed

☑ a sequence listing - see Supplemental Box Relating to Sequence Listing.

3. ☐ The amendments have resulted in the cancellation of:

☐ the description, pages

☐ the claims, Nos.

☐ the drawings, sheets/figs

☐ the sequence listing (specify):

4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since either they are considered to go beyond the disclosure as filed, or they were not accompanied by a letter indicating the basis for the amendments in the application as filed, as indicated in the Supplemental Box (Rules 70.2(c) and (c-bis)):

☐ the description, pages

☐ the claims, Nos.

☐ the drawings, sheets/figs

☐ the sequence listing (specify):
5. □ This report has been established:
   □ taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rules 66.1(d-bis) and 70.2(e)).
   □ without taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rules 66.4bis and 70.2(e)).

6. ☑ With regard to top-up searches (Rules 66.1ter and 70.2(f)):
   ☑ A top-up search was carried out by this Authority on **15.06.2020** (all discovered documents are listed in the Supplemental Box Relating to Top-up Search).
   □ Additional relevant documents have been discovered during the top-up search.
   □ No top-up search was carried out by this Authority because it would serve no useful purpose.

7. □ Supplementary international search report(s) from Authority(ies) has/have been received and taken into account in establishing this report (Rule 45bis.8(b) and (c)).

* If item 4 applies, some or all of those sheets may be marked "superseded".

---

**Box No. V** Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

   **Novelty (N)**
   - Yes: Claims 1-16
   - No: Claims

   **Inventive step (IS)**
   - Yes: Claims 1-10, 13, 14, 16
   - No: Claims 11, 12, 15

   **Industrial applicability (IA)**
   - Yes: Claims 1-16
   - No: Claims

2. Citations and explanations (Rule 70.7):

   *see separate sheet*

---

**Box No. VIII** Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

*see separate sheet*
Supplemental Box relating to Sequence Listing

Continuation of Box I, item 2:

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed:
      ☑ in the form of an Annex C/ST.25 text file.
      ☐ on paper or in the form of an image file.
   b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. ☐ furnished subsequent to the international filing date for the purposes of international search and/or examination:
      ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
      ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
   d. ☐ furnished to this Authority as an amendment* under PCT Article 34 on :
      ☐ in the form of an Annex C/ST.25 text file, and preferably identified as "Amended" at the first line of text.
      ☐ on paper or in the form of an image file.

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

* If item 4 in Box No. 1 applies, the sequence listing, which forms part of the basis of the report, may be marked "superseded."
1 The present application is directed to the heterologous expression of the psilocybin biosynthetic enzymes PsiD, PsiH, PsiK and PsiM in a host cell, and production of psilocybin by the host cell.

2 Reference is made to the following document:


3 **Re Item V**

3.1 **Novelty**

The present application meets the criteria of Article 33(2) PCT, because the subject-matter of **Claims 1-16** appears to be new.

3.2 None of the cited prior art discloses heterologous expression of the psilocybin biosynthetic enzymes PsiD, PsiH, PsiK and PsiM in a host cell, and hence the subject-matter of the independent **Claims 1, 11 and 16**, as well as their dependent claims, is new.

**Inventive step**

3.3 The present application does not meet the criteria of Article 33(3) PCT, because the subject-matter of **Claims 11-12 and 15** does not involve an inventive step.

3.4 **Claim 1** is directed to a recombinant host cell comprising and capable of expressing heterologous polynucleotides encoding PsiD, PsiH, PsiK and PsiM, the recombinant host cell further comprising a further genetic element encoding Trp2 and Trp3 arranged to increase biosynthetic production of L-tryptophan in the host cell, wherein the cell is capable of producing psilocybin.

**D1** discloses that the four enzymes for psilocybin biosynthesis are PsiD, PsiH, PsiK and PsiM (abstract). **D1** discloses the *in vitro* production of psilocybin using the enzymes and with tryptophan as a starting substrate (Figures 3 and 4). **D1** teaches that this information sets the stage for the heterologous production of psilocybin with engineered microbial hosts (page 12355, left column, last sentence).
The host cell of Claim 1 differs from D1 in that it defines the host cell suggested by D1, and additionally in that it comprises a genetic element encoding Trp2 and Trp3 arranged to increase biosynthetic production of L-tryptophan in the host cell.

Since the host cell of Claim 1 is clearly suggested by D1, the skilled person could, and would, consider providing a recombinant host cell expressing the PsiD, PsiH, PsiK and PsiM enzymes, based on the teaching of D1.

The skilled person would also understand from D1 that tryptophan is required as a starting substrate, and would consider how this could be provided to the recombinant host cell. D1 however provides no hint that the tryptophan production could be suitably achieved by the recombinant host cell further comprising a genetic element encoding Trp2 and Trp3 arranged to increase biosynthetic production of L-tryptophan.

The yeast enzymes for tryptophan biosynthesis are well known in the art. The initial step of chorismate biosynthesis can be catalyzed by two isoenzymes, ARO3 and ARO4. Tryptophan synthesis from chorismate requires the enzymes Trp1-Trp5.

The Examples disclose data relating to cells expressing one or some of these enzymes, namely ARO4, Trp2 and Trp3. The present application demonstrates that in a strain expressing only ARO4 (the A4M strain), no clear increase in tryptophan production can be identified on day 1 or 2.

Furthermore, as can be seen in Figures 9A, 9B, 10A and 10B, all strains comprising the A4M designation (i.e. comprising the ARO4 gene) produce less psilocybin than the background strain, where the background strain comprises only the genes for psilocybin production.

Hence it appears that even if the skilled person were to consider providing tryptophan by the biosynthetic route in a recombinant host cell expressing the PsiD, PsiH, PsiK and PsiM enzymes, the selection of the correct enzymes to achieve that would have to be made.

It appears that merely adding any gene involved in tryptophan production is not suitable for increasing tryptophan production in a host cell, such that psilocybin production is increased. Rather, the specific genes are an essential feature of solving the technical problem of how to increase biosynthetic production of L-tryptophan in the cell, such that psilocybin production is increased.
Subject-matter relating to the psilocybin-producing cell in which tryptophan production is increased, wherein the increase in tryptophan production is achieved by expression of the Trp2 and Trp3 genes, appears to represent a solution to the technical problem, which solution is not taught or suggested by the prior art and hence is considered to involve an inventive step.

Since **Claims 1-10** are restricted to this subject-matter, inventive step can be acknowledged over **D1** alone.

3.5 **Claim 11** is directed to a method for producing metabolites, in which a recombinant host cell comprising heterologous polynucleotides encoding PsiD, PsiH, PsiK and PsiM and which recombinant host cell is capable of producing psilocybin is provided, and wherein the recombinant host cells are supplemented with L-tryptophan during cultivation.

Unlike Claim 1, knowledge of the specific enzymes of the tryptophan biosynthetic pathway, which enzymes contribute to an increase in psilocybin production, is not required.

The skilled person is motivated to provide a recombinant host cell expressing the PsiD, PsiH, PsiK and PsiM enzymes for the enzymatic synthesis of psilocybin, based on the teaching of **D1**.

The skilled person would also understand from **D1** that tryptophan is required as a starting substrate, and would consider how this could be provided to the recombinant host cell.

Supplementation of growth media with required amino acids is a standard approach in yeast cell culture, and is the simplest approach (when compared with further genetic modification of a recombinant cell). The skilled person would therefore, and could, take this approach, and in so doing would arrive at the subject-matter of Claim 11.

For this reason, no inventive step can be acknowledged for the method of Claim 11, and dependent **Claims 12 and 15**.

3.6 **Dependent Claims 13 and 14** specify that recombinant host cell of Claim 11 is one according to Claim 1. Since provision of the recombinant host cell of Claim 1 is considered to involve an inventive step, the same view applies to Claims 13 and 14 as for Claim 1, and an inventive step is acknowledged.

3.7 **Claim 16** requires the recombinant host cell of Claim 1, and hence an inventive step can be acknowledged for the same reason as set out for Claim 1.
Re Item VIII

4 Certain observations on the international application

4.1 Claim 2 depends on Claim 1 refers to the "at least one heterologous polynucleotide". Claim 1 however lacks a precedent for this term.

4.2 Claim 5 specifies that the "further genetic element encoding Trp2 is genetically modified to inhibit its allosteric regulation". Claim 8 refers to the genetic modification of the recombinant host cell of Claim 5, but specifies that the genetic modification comprises at least one of a modification of a polynucleotide encoding Trp2 with a S76 mutation and a modification of a polynucleotide encoding Aro4 with a K229 mutation.

It is unclear how the genetic modification of Trp2 in Claim 5 can be further defined by reference to a polynucleotide encoding Aro4 in Claim 8.

4.3 A similar point applies to Claim 10, which refers to the further genetic element (in the recombinant host cell of Claim 5 where the further genetic element is defined as being a genetically modified Trp2), yet Claim 10 specifies that the further genetic element may encode Aro4.
Claims

1. A recombinant host cell comprising:
   heterologous polynucleotides encoding PsiD, PsiH, PsiK, and PsiM;
   wherein the heterologous polynucleotides are operably linked to at least one
   promoter which is capable of directing expression of said heterologous
   polynucleotides in the host cell;
   wherein the recombinant host cell further comprises at least one further
   genetic element encoding Trp2 and Trp3 arranged to increase biosynthetic
   production of L-tryptophan in the host cell, wherein the further genetic element
   is operably linked to at least one promoter which is capable of directing
   expression of said further genetic element in the host cell; and wherein the
   recombinant host cell is capable of producing psilocybin.

2. The host cell of claim 1 wherein the at least one promoter provides production
   of the at least one heterologous polynucleotide.

3. The host cell of claim 1 or 2 wherein the at least one heterologous polynucleotide
   is operably linked to a single promoter, which controls the expression of each of
   PsiD, PsiH, PsiK, and PsiM.

4. The recombinant host cell of claims 1-3 wherein the promoter is controlled by a
   synthetic transcription factor.

5. The host cell of claim 1, wherein the further genetic element encoding Trp2 is
   genetically modified to inhibit its allosteric regulation.

6. The recombinant host cell of claim 5 comprising at least two further genetic
   elements that are controlled by a single synthetic transcription factor.

7. The recombinant host cell of claim 6 wherein the synthetic transcription factor is
   the same synthetic transcription factor which controls expression of the
   heterologous polynucleotides encoding PsiD, PsiH, PsiK and/or PsiM.

8. The host cell of claims 5-7 wherein the genetic modification comprises at least
   one of:
   a modification of a polynucleotide encoding Trp2 with a S76 mutation, wherein
   the residue numbering corresponds to that of SEQ ID NO: 18 (S. cerevisiae

AMENDED SHEET
Trp2), and
a modification of a polynucleotide encoding Aro4 with a K229 mutation, wherein the residue numbering corresponds to that of SEQ ID NO: 17 (S. cerevisiae Aro4).

9. The recombinant host cell of claims 1-8, wherein:

PsiD has at least 60% amino acid sequence identity with the protein sequence deposited in GenBank accession number ASU62239.1 or the GenBank accession number ASU62242.1, or with the amino acid sequence encoded by polynucleotide SEQ ID NO: 1 or 2 or 9 or 10;

PsiH has at least 60% amino acid sequence identity with the protein sequence deposited in GenBank accession number ASU62246.1 or the GenBank accession number ASU62250.1, or with the amino acid sequence encoded by polynucleotide SEQ ID NO: 5 or 6 or 13 or 14;

PsiK has at least 60% amino acid sequence identity with the protein sequence deposited in GenBank accession number ASU62237.1 or the GenBank accession number ASU62240.1, or with the amino acid sequence encoded by polynucleotide SEQ ID NO: 7 or 8 or 15 or 16;

PsiM has at least 60% amino acid sequence identity with the protein sequence deposited in GenBank accession number ASU62238.1 or the GenBank accession number ASU62241.1, or with the amino acid sequence encoded by polynucleotide SEQ ID NO: 3 or 4 or 11 or 12.

10. The recombinant host cell of claims 5-9, wherein the further genetic element encodes at least one of:

Aro4 which has at least 60% amino acid sequence identity with SEQ ID NO: 17 or 19;

Trp2 which has at least 60% amino acid sequence identity with SEQ ID NO: 18 or 20; and

Trp3 which has at least 60% amino acid sequence identity with the sequence corresponding to the GenBank accession number CAA82056.1 or the GenBank accession number OWW28508.1.
11. A method for producing metabolites comprising
   a. providing a recombinant host cell comprising
      heterologous polynucleotides encoding PsiD, PsiH, PsiK, and PsiM;
      wherein the heterologous polynucleotides are operably linked to at least one
      promoter which is capable of directing expression of said heterologous
      polynucleotides in the host cell; and
      wherein the recombinant host cell is capable of producing psilocybin;
   b. cultivating the recombinant host cell in conditions allowing growth and
      propagation of the host cell, and wherein the recombinant host cells are
      supplemented with L-tryptophan;
   c. continuing cultivating the recombinant host cells to synthesize the
      metabolites; and
   d. recovering at least one metabolite synthesised by an enzyme encoded by
      the heterologous polynucleotide of the host cell.

12. The method of claim 11 wherein L-tryptophan is supplemented by adding L-
    tryptophan in the growth medium wherein the recombinant host cells are cultivated.

13. The method of claim 11 wherein the recombinant host cell is a recombinant
    host cell of any one of claims 1-10.

14. The method of claims 11-13 wherein the recombinant host cell is the
    recombinant host cell of claims 5-10 and L-tryptophan is supplemented by initiating
    expression of Aro4, Trp2 and Trp3 to enhance production of L-tryptophan.

15. The method of claims 11-14 wherein the method is for producing psilocybin,
    and psilocybin is recovered in step d.

16. A psilocybin production system comprising:
   a production unit containing the recombinant host cell of claims 1-10; and
   a control unit comprising controlling means for operating the production unit.
RESPONSE TO WRITTEN OPINION

Title: Heterologous production of psilocybin
Applicant name: Teknologian tutkimuskeskus VTT Oy

Dear Sirs,

This is the applicants’ response to the Written Opinion dated 10 May 2019. Amended claims as a clean copy and as a copy showing the amendments are enclosed.

AMENDMENTS

The independent claim 1 is amended by inserting subject matter from claims 5 and 6. The amended claim 1 now requires that in addition to the psilocybin pathway genes, Trp2 and Trp3 are present and under the control of a promoter which initiates their expression to boost L-tryptophan production in the host cell.

Original claim 5 and 6 are deleted and claim 5 (originally 7) is amended to be consistent with the amended claim 1.

Method Claim 11 (originally 13) is amended by specifying the host cell with the enzymes required in the psilocybin biosynthesis, and that the host cells are supplemented with L-tryptophan. The amendment is based in the original claim 13 which referred directly to claim 1, and to claim 14 which referred directly to the original claim 13.

Claim 14 is deleted.

Claim 13 is inserted, and directed to the method of claim 11 wherein the recombinant host cell of claims 1-10 is used. The new claim has basis in the original claim 13 which referred to the host cell of the original claims 1-12.

Claim numbering is revised when necessary in view of the deleted claims.
NOVELTY AND INVENTIVE STEP

The written opinion states that specific genes are essential for solving the technical problem of increasing L-tryptophan production in the host cell, which in turn enhances production of psilocybin by the recombinant host cell.

The applicant has amended claim 1 by specifying that recombinant Trp2 and Trp3 genes are inserted in the host cell under the control of a promoter. Expression of these genes results into increased L-tryptophan production, which is used in the biosynthetic pathway by the claimed Psi enzymes to synthesize psilocybin. This is demonstrated in the application as filed in Example 1. Thus, the essential features needed for recombinant production of psilocybin are included in claim 1. Therefore, claims 1-10 as well as claim 16 are inventive.

Concerning the method claim 11 it is submitted that the amendments now introduced specify that the method uses recombinant host cells that are supplemented with L-tryptophan during cultivation. This has an effect of enhancing psilocybin production, as well as the production of other metabolites downstream of L-tryptophan in the psilocybin pathway. This is evidenced in Examples of the application that show that not only the production of psilocybin, but also the production of tryptamine, 4-hydroxytryptamine, and norbaecocystin is increased when L-tryptophan is provided (Table 2). Further, the application discloses (p. 15 line 21 – p. 16 line 3) that supplementation of L-tryptophan can be carried out by increasing its biosynthesis, by adding L-tryptophan directly in the growth medium, or by using the shikimate pathway (p. 24 line 31 – p. 25 line 2). Thus, the person having ordinary skill in the art can easily follow these instructions to supplement L-tryptophan and to reproduce the claimed invention. Thus, claims 11-15 are inventive.

An advantage of the invention over D1 is that by reconstructing the psilocybin biosynthetic pathway in a recombinant host cell and by supplementing the host cell with L-tryptophan, it is possible to produce psilocybin e.g. in a cell culture. This allows easier control and upscaling of the production compared to extraction of metabolites from biological sources or by carrying out enzymatic synthesis in a cell-free system.

Based on our analysis above, all claims are novel and inventive. The applicant awaits a positive opinion on patentability of the claims.

Yours faithfully

**Espatent Oy**

Jukka Taskinen
European Patent Attorney