



(51) International Patent Classification:

C12P 19/56 (2006.01) C12N 9/26 (2006.01)

C12N 1/16 (2006.01) C12N 9/10 (2006.01)

C12N 15/52 (2006.01)

Published:

— with international search report (Art. 21(3))

(21) International Application Number:

PCT/EP2017/083618

(22) International Filing Date:

19 December 2017 (19.12.2017)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

16206221.0 22 December 2016 (22.12.2016) EP

62/437,929 22 December 2016 (22.12.2016) US

(71) Applicant: DSM IP ASSETS B.V. [NL/NL]; Het Overloon 1, 6411 TE HEERLEN (NL).

(72) Inventors: BROERS, Nicolette, Jasmijn; P.O. Box 4, 6100 AA ECHT (NL). JANSEN, Mickel, Leonardus, August; P.O. Box 4, 6100 AA ECHT (NL).

(74) Agent: DSM INTELLECTUAL PROPERTY; P.O. Box 4, 6100 AA ECHT (NL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) Title: FERMENTATION PROCESS FOR PRODUCING STEVIOL GLYCOSIDES

(57) Abstract: The invention relates to a process for the production of a steviol glycoside, which process comprises fermenting a first cell capable of producing a steviol glycoside in a suitable fermentation medium under conditions which allow for production of the steviol glycoside, wherein the fermentation medium comprises oligosaccharides which are not completely fermentable by the first cell; and wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the oligosaccharides; and, optionally, recovering the steviol glycoside from the reaction medium. The invention also relates to a fermentation broth obtainable by such a process.



FERMENTATION PROCESS FOR PRODUCING STEVIOL GLYCOSIDES

Field

5 The present disclosure relates to a process for the production of a steviol glycoside by fermentation. The disclosure also relates to an end-of-fermentation broth, for example one obtainable by a process of the disclosure.

Background

10 The leaves of the perennial herb, *Stevia rebaudiana* Bert., accumulate quantities of intensely sweet compounds known as steviol glycosides. Whilst the biological function of these compounds is unclear, they have commercial significance as alternative high potency sweeteners.

15 These sweet steviol glycosides have functional and sensory properties that appear to be superior to those of many high potency sweeteners. In addition, studies suggest that stevioside can reduce blood glucose levels in Type II diabetics and can reduce blood pressure in mildly hypertensive patients.

20 Steviol glycosides accumulate in *Stevia* leaves where they may comprise from 10 to 20% of the leaf dry weight. Stevioside and rebaudioside A are both heat and pH stable and suitable for use in carbonated beverages and can be applied in many other foods. Stevioside is between 110 and 270 times sweeter than sucrose, rebaudioside A between 150 and 320 times sweeter than sucrose. In addition, rebaudioside D is also a high-potency diterpene glycoside sweetener which accumulates in *Stevia* leaves. It may be
25 about 200 times sweeter than sucrose. Rebaudioside M is a further high-potency diterpene glycoside sweetener. It is present in trace amounts in certain *stevia* variety leaves, but has been suggested to have a superior taste profile.

30 Steviol glycosides have traditionally been extracted from the *Stevia* plant. In *Stevia*, (-)-kaurenoic acid, an intermediate in gibberellic acid (GA) biosynthesis, is converted into the tetracyclic diterpene steviol, which then proceeds through a multi-step glycosylation pathway to form the various steviol glycosides. However, yields may be variable and affected by agriculture and environmental conditions. Also, *Stevia* cultivation requires substantial land area, a long time prior to harvest, intensive labour and additional costs for the extraction and purification of the glycosides.

35 More recently, interest has grown in producing steviol glycosides using fermentative processes. WO2013/110673 and WO2015/007748 describe microorganisms that may be used to produce at least the steviol glycosides rebaudioside A, rebaudioside D and rebaudioside M.

In order to realize an economically viable fermentation process, fast and efficient production of steviol glycosides is required. State of the art fermentation-based production of steviol glycosides will need to make use of industrial feed-stocks. Typical industrial feed-stocks used are starch-hydrolysates which contain oligosaccharides, the quantity of such oligosaccharides being dependent on the grade of hydrolysate. Some of these oligosaccharides cannot be consumed by the micro-organisms used in fermentation processes and thereby limit the potential fermentation yield. More importantly, oligosaccharides present in the process following fermentation have a negative effect on the recovery yield and product quality.

Summary

The present disclosure relates to a process for the production of a steviol glycoside. The process is a fermentation process, for example a process for the fermentative production of a steviol glycoside such as rebaudioside A, rebaudioside D or rebaudioside M. In the process of the disclosure, fermentation is carried out in the presence of an enzyme, so as to reduce the amount of residual sugar present at the end of fermentation.

The use of an enzyme in the process enables non-fermentable oligosaccharide present in the feedstock, for example a DE-95 type of glucose syrup, to be hydrolyzed during fermentation towards fermentable sugars. Such fermentable sugars may in turn be converted towards a desired end-product, for example a steviol glycoside. In this way, a higher product yield may be achieved in fermentation. In addition, a higher yield in downstream processing and/or a higher product quality may be realized.

Accordingly, the disclosure relates to a process for the production of a steviol glycoside, which process comprises fermenting a first cell capable of producing a steviol glycoside in a suitable fermentation medium under conditions which allow for production of the steviol glycoside,

wherein the fermentation medium comprises oligosaccharides which are not completely fermentable by the first cell; and

wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the oligosaccharides;

and, optionally, recovering the steviol glycoside from the reaction medium or broth.

The disclosure also relates to:

- an end-of-fermentation broth comprising a steviol glycoside obtainable by a process according to such a method; and
- an end-of-fermentation broth comprising a steviol glycoside and a lower amount of non-fermentable oligosaccharides than were added during fermentation.

The steviol glycoside may be any steviol glycoside, such as rebaudioside A, rebaudioside D or rebaudioside M.

Brief description of the drawings

Figure 1 sets out a schematic diagram of the potential pathways leading to biosynthesis of steviol glycosides.

Figure 2 sets the effect of the addition of Amigase Mega to fermentation on glucose, maltose and isomaltose supernatant concentrations.

Figure 3 sets out the effect of the addition of Amigase Mega to fermentation on disaccharides, saccharides larger than disaccharides and total oligosaccharides supernatant concentrations.

Detailed description

Throughout the present specification and the accompanying claims, the words "comprise", "include" and "having" and variations such as "comprises", "comprising", "includes" and "including" are to be interpreted inclusively. That is, these words are intended to convey the possible inclusion of other elements or integers not specifically recited, where the context allows.

The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to one or at least one) of the grammatical object of the article. By way of example, "an element" may mean one element or more than one element.

Herein "rebaudioside" may be abbreviated to "reb" or "Reb" or the like.

The disclosure relates to a process for the production of a steviol glycoside, which process comprises fermenting a first cell capable of producing the steviol glycoside in a suitable fermentation medium under conditions which allow for production of the steviol glycoside,

wherein, the fermentation medium comprises oligosaccharides which are not completely fermentable by the first cell; and

wherein the fermentation is carried out, at least in part, in the presence of an enzyme which is capable of hydrolyzing, at least in part, the oligosaccharides;

and, optionally, recovering the steviol glycoside from the reaction medium or broth.

The disclosure relates to a process for the production of any steviol glycoside. The process comprises fermenting a first cell capable of producing the steviol glycoside in

a suitable fermentation medium under conditions which allow for production of the steviol glycoside.

The fermentation process according to the disclosure can be performed according to methods known to those skilled in the art.

5 The cultivation medium conveniently contains a carbon source, a nitrogen source as well as additional compounds required for growth of the microorganism and/or the formation of the product. For instance, additional compounds may be necessary for inducing the production of the product such as a steviol glycoside.

10 Examples of suitable carbon sources known in the art include glucose, maltose, maltodextrins, sucrose, hydrolysed starch, starch, molasses, oils, glycerol. Examples of nitrogen sources known in the art include soy bean meal, corn steep liquor, yeast extract, ammonia, ammonium salts, nitrate salts, urea. Examples of additional compounds include phosphate, sulphate, trace elements and/or vitamins. Antifoaming compounds may also be added to the cultivation medium.

15 The total amount of carbon and nitrogen source to be added to the cultivation process according to the disclosure may vary depending on e.g. the needs of the microorganism and/or the length of the fermentation process.

20 The ratio between carbon and nitrogen source in a cultivation process may vary considerably, whereby one determinant for an optimal ratio between carbon and nitrogen source is the elemental composition of the product to be formed.

25 Additional compounds required for growth of a microorganism and/or for product formation, like phosphate, sulphate or trace elements, may be added in amounts that may vary between different classes of microorganisms, i.e. between fungi, yeasts and bacteria. In addition, the amount of additional compound to be added may be determined by the type of product that is formed.

Typically, the amount of medium components necessary for growth of a microorganism may be determined in relation to the amount of carbon source used in the cultivation, since the amount of biomass formed will be primarily determined by the amount of carbon source used.

30 The fermentation medium is one which comprises oligosaccharides which are not completely fermentable by the first cell. That is to say, the first cell is a cell capable of producing the steviol glycoside, but typically not capable of fermenting all of the oligosaccharides in the fermentation medium. However, the cell may be modified as described herein, so that it is capable of fermenting a greater fraction of the oligosaccharides than it otherwise would be.

35 A typical fermentation process in which a feedstock is used which comprises non-fermentable oligosaccharides will result in an amount of residual sugar being present in the fermentation broth at the end of fermentation. That is to say, residual sugar is typically present in the form of non-fermentable oligosaccharides.

In the process of the disclosure, the fermentation medium comprises oligosaccharides which are not completely fermentable by the first cell. The fermentation medium may not necessarily comprise such oligosaccharides at the beginning of fermentation. For example, they may be produced in the fermentation process by the cells or sterilisation conditions. Further, oligosaccharides may originate from, i.e. be added, with a feed added during the fermentation. However, the term "the fermentation medium comprises oligosaccharides" is intended to cover any medium in which at some point oligosaccharides are or become present which are not completely fermentable.

Herein, an oligosaccharide is a saccharide polymer comprising a small number, typically from two to about 10 or more of monosaccharides. Oligosaccharides are non-fermentable in the sense intended in the disclosure if (in the absence of enzyme according to the disclosure) they are not available to the first cell in a form in which that cell can convert the sugars to the steviol glycoside.

According to the disclosure, the process is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the non-fermentable oligosaccharides.

Thus, in a process of the disclosure, the fermentation broth at the end of fermentation will comprise less residual sugar than would be present for an equivalent process carried out in the absence of the enzyme.

The enzyme may be introduced into the fermentation in any convenient fashion.

In the disclosure, therefore, enzyme *per se* is added to the fermentation medium. That is to say, the enzyme, for example in the form of a suitably formulated composition may be added directly to the fermentation medium.

Alternatively, or in addition, the fermentation process of the disclosure may be carried out in the presence of a second cell, different to the first cell, which is capable of expressing the enzyme. In other words, the fermentation process of the disclosure may be carried out in the presence, in the fermentation broth, of a second cell, different to the first cell, which second cell expresses the enzyme under the (fermentation) process conditions.

Alternatively, or in addition, the fermentation process of the disclosure may be carried out wherein the first cell is modified so that it is capable of expressing the enzyme to a greater degree than an unmodified form of the cell.

Any combination of these approaches could be used. Expression of the enzyme by a second cell or by a modified first cell may be inducible.

In the process of the disclosure, the enzyme may be present for only a portion of the fermentation period or for the entire duration of fermentation.

In the process of the disclosure, the production phase may preferably be preceded by a biomass formation phase for optimal biomass production. When such a process is carried out, the enzyme may be present in the biomass formation phase and/or the production phase.

If the process of the disclosure is carried out where enzyme is present only for a portion of the fermentation period, the enzyme may be preferably present for up to about the final 20% of the fermentation period, for example up to about the final 15% of the fermentation period, such as up to about the final 10% of the fermentation period (the percentage expressed being in terms of the total fermentation period, for example the total production period).

In the process of the disclosure, the enzyme may be any enzyme capable of hydrolysing an oligosaccharide. Typically, a suitable enzyme may be an amylase such as an alpha-amylase, a beta-amylase or a gluco-amylase, a pullulanase, an alpha-glucosidase, a beta-glucosidase, a trehalase or a transglucosidase.

Amylase is any glycoside hydrolase which is capable of acting on α -1,4-glycosidic bonds. The amylase may be an α -amylase (EC 3.2.1.1), a β -amylase (EC 3.2.1.2) or a glucoamylase (EC 3.2.1.3).

A suitable pullulanase (EC 3.1.41) may be a type I pullulanase, which specifically attacks α -1,6 linkages, or a type II pullulanase, which is also able to hydrolyze α -1,4 linkages.

A transglucosidase (EC 2.4.1.24) may be any enzyme which is capable of converting at least in part an α 1-6 linked backbone (eg. isomaltose) into glucose.

A combination of two or more of any such enzymes may be used.

Typically, the enzyme or enzymes used should not be ones which are capable of removing sugars from a steviol glycoside.

A suitable enzyme may be added in any suitable amount. The skilled person will readily be able to determine an appropriate dosage of enzyme in order to reduce the amount of residual sugar, for example dependent on the specific enzyme being used.

The fermentation process as herein disclosed may be performed on any scale. Typically, it may be performed on an industrial scale. An industrial scale process is understood to encompass a cultivation process in a fermenter volume which is $\geq 0.01 \text{ m}^3$, or $\geq 0.1 \text{ m}^3$, or $\geq 0.5 \text{ m}^3$, or $\geq 5 \text{ m}^3$, preferably $\geq 10 \text{ m}^3$, more or $\geq 25 \text{ m}^3$, more or $\geq 50 \text{ m}^3$, or $\geq 100 \text{ m}^3$, or $\geq 200 \text{ m}^3$.

Typically, a microbial fermentation process such as a fermentation process according to the present disclosure may be divided in a growth phase directed to formation of biomass and a production phase (also indicated as main fermentation) directed to production of a product by the microorganism, such as, e.g. steviol glycosides. The skilled person will comprehend that the two phases of fermentation may not be strictly separated in time, but may overlap to some extent. Moreover, the skilled person will comprehend that during any phase of the cultivation of the microorganism, the product will be produced to some extent.

The growth phase in the fermentation process typically comprises the preparation of the inoculum and the seed fermentation.

The preparation of the inoculum, may occur by transferring aseptically the production microorganism from culture vials into the fermentation medium and can be performed in various ways, e.g. using shake flask(s), a bubble column or stirred fermenter

The typical fermentation temperature in the inoculum phase may range between
5 20°-40° C, such as 20°-30° C or 30°-40°C or 25°-35° C.

Typically, in this phase of the fermentation the pH is usually below 8, below 7.5, or at most 7. In other embodiment, the pH in this phase may be at most 6.5, at most 6, at most 5.5 or at most 5.

Typically, the fermentation during the inoculum phase may be growth-rate limited
10 followed by an oxygen limitation or carbon limitation phase until the fermentation process is upscaled to a seed fermentation. This first inoculum preparation step may be repeated at larger scale with the smaller scale as an inoculum until enough biomass has been produced.

Therefore after sufficient growth, the biomass may be transferred to a seed
15 fermentor, where further growth (i.e. the seed fermentation) may take place. The seed fermentation may typically occur under agitation and typically in the presence of oxygen. This process step can be performed in various settings, such as continuous fermentation, batch fermentation or fed-batch fermentation, preferably batch or fed-batch fermentation. This phase of the fermentation can take place in a bubble column, or stirred fermenter for
20 example. Conditions of oxygen- or carbon limitation can be applied. The medium may comprise any suitable nutrient as discussed herein before, including defined salts and/or vitamins such as thiamine. Further the medium may comprise an antifoam. Typically, the medium used in this phase of the fermentation may be sterilized in the fermenter prior to use in the absence of carbon source, e.g. in the absence of glucose, at a pH preferably
25 below 5 or lower and afterwards adjusted to the pH of the fermentation with any suitable base, e.g. such as using gaseous or liquid ammonia. The fermenter can also be sterilized empty and previously sterilised medium (e.g. by heat shock) can be added. The pH in the seed fermentation may be maintained below 8, or below 7, or below 6 or below 5 or below 4. The amount of carbon source such as glucose or any other carbon source as mentioned
30 herein before, may be between 0 – 100 g/kg, or at most 50, 60, 70, 80, 90 g/kg, or at least 5, 10, 20, 30, 40 g/kg. The fermentation temperature and pH in this phase may be in the same range as the temperature and/or pH used for the inoculum preparation. When the batch carbon source such as glucose is consumed or almost consumed, glucose or other carbon source may be added or the fermentation can proceed to the main phase (or
35 production phase).

In case further carbon source is added, the latter can occur either in continuous or repeated batch modus. Typically, in the seed fermentation the first phase may be a growth limited phase followed by oxygen limitation or carbon limitation. An antifoam may be applied to control foaming. This seed preparation step may be repeated at larger scale
40 with the smaller scale as an inoculum until enough biomass has been produced. The main

fermentation will typically be the phase wherein the product, such as steviol-glucosides, is produced. This phase may typically be performed in continuous or fed-batch process. Typically, this phase may be performed in a bubble column or stirred fermenter. Typically, the main fermentation will comprise more phases characterised by different limitations. Typically, a first phase may be a batch process growth rate limited followed by an optional oxygen limited phase. The oxygen limited phase may be dispensed with. The second or third phase may typically be a carbon-limited such as glucose-limited production phase. The medium may have a composition as described for the seed fermentation phase. Prior to use, the medium may be sterilized and the pH adjusted as disclosed for the seed fermentation phase. After sterilization of the medium, carbon source may be added in an amount which may be as previously described for the seed fermentation phase.

According to the disclosure, the process is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the non-fermentable oligosaccharides.

An enzyme which is capable of hydrolyzing, at least in part, the non-fermentable oligosaccharides as described herein above may be added to the fermentation broth. The enzyme may for example be added at the beginning or during the seed fermentation or at the beginning or during the main fermentation. Conditions and temperature are typically those mentioned above. Optionally however, pH and temperature may be adjusted during the main fermentation to increase production. During the main fermentation, dissolved oxygen levels may preferably be above 2% in the production phase (calibrated at 100% starting conditions) with an OUR between 5 and 250 mmol/kg/h and adjusted if necessary. The OUR (oxygen uptake rate) is defined herewith as the rate with which the microorganism consumes oxygen fed to the fermentation broth. Carbon dioxide levels may be controlled as well depending on the need of the microorganism. An antifoam feed can be applied to control foaming.

The disclosure also relates to an end-of-fermentation broth comprising a steviol glycoside obtainable by a process of the disclosure.

A broth according to the disclosure may comprise a recombinant host cell of the disclosure. Alternatively, a broth of the disclosure may be one from which all host cells of the disclosure are absent or substantially absent, for example a supernatant or fermentation medium.

The disclosure also relates to an end-of-fermentation broth comprising a steviol glycoside and a lower amount of non-fermentable oligosaccharides than were added during fermentation, for example an end-of-fermentation broth as obtained by a process of the disclosure. The disclosure also relates to an end-of-fermentation broth comprising a steviol glycoside and a lower amount of non-fermentable oligosaccharides than would be present had no enzyme as described herein been added during fermentation.

Such an end-of-fermentation broth may comprise an amount of non-fermentable oligosaccharides of less than about 22 or 21 g/L oligosaccharides, such as less than

about 15 g/L oligosaccharides, for example less than about 5g/L oligosaccharides, less than about 2g/L oligosaccharides or less.

The steviol glycoside in the method according to the disclosure can be any steviol glycoside, for example steviol-13-monoside, steviol-19-monoside, 13-[(β -D-Glucopyranosyl)oxy]kaur-16-en-18-oic acid 2-O- β -D-glucopyranosyl- β -D-glucopyranosyl ester, rubusoside, stevioside, steviol-19-diside, steviolbioside, rebaudiosideA, rebaudioside E, rebaudioside D or rebaudioside M.

A suitable first cell for use in the method of the disclosure may be any cell capable of producing a steviol glycoside. Typically, the cell will be a recombinant cell provided with one or more homologous or heterologous expression constructs that encode a polypeptide involved in the production of the steviol glycoside.

The person skilled in the art will be aware of methods for modification of a cell, such as a microbial cell, such that it is capable of production of the polypeptides involved in the production of the steviol glycoside, for example as described in WO2013/110673 and WO2015/007748.

As used herein, a recombinant cell for use in a method according to the present disclosure is defined as a cell which contains, or is transformed or genetically modified with a nucleotide sequence or polypeptide that does not naturally occur in the cell, or it contains additional copy or copies of an endogenous nucleic acid sequence, or it contains a deletion or disruption of an endogenous or homologous nucleotide sequence. A wild-type eukaryotic cell is herein defined as the parental cell of the recombinant cell.

The term "homologous" when used to indicate the relation between a given (recombinant) nucleic acid or polypeptide molecule and a given host organism or host cell, is understood to mean that in nature the nucleic acid or polypeptide molecule is produced by a host cell or organism of the same species, preferably of the same variety or strain.

The term "heterologous" when used with respect to a nucleic acid (DNA or RNA) or protein refers to a nucleic acid or protein that does not occur naturally as part of the organism, cell, genome or DNA or RNA sequence in which it is present, or that is found in a cell or location or locations in the genome or DNA or RNA sequence that differ from that in which it is found in nature. Heterologous nucleic acids or proteins are not endogenous to the cell into which they are introduced, but have been obtained from another cell or synthetically or recombinantly produced.

A suitable recombinant cell for use in the disclosure may comprise one or more recombinant nucleic acid sequences encoding one or more polypeptides having UDP-glycosyltransferase (UGT) activity.

For the purposes of this disclosure, a polypeptide having UGT activity is one which has glycosyltransferase activity (EC 2.4), i.e. that can act as a catalyst for the transfer of a monosaccharide unit from an activated nucleotide sugar (also known as the "glycosyl donor") to a glycosyl acceptor molecule, usually an alcohol. The glycosyl donor

for a UGT is typically the nucleotide sugar uridine diphosphate glucose (uracil-diphosphate glucose, UDP-glucose).

Such additional UGTs may be selected so as to produce a desired steviol glycoside. Schematic diagrams of steviol glycoside formation are set out in Humphrey *et al.*, Plant Molecular Biology (2006) 61: 47-62 and Mohamed *et al.*, J. Plant Physiology 168 (2011) 1136-1141. In addition, Figure 2 sets out a schematic diagram of steviol glycoside formation.

A recombinant cell may thus comprise one or more recombinant nucleic acid sequences encoding one or more of:

- (i) a polypeptide having UGT74G1 activity;
- (ii) a polypeptide having UGT2 activity;
- (ii) a polypeptide having UGT85C2 activity; and
- (iii) a polypeptide having UGT76G1 activity.

A recombinant cell may comprise a nucleotide sequence encoding a polypeptide capable of catalyzing the addition of a glucose moiety to the C-13 position of steviol. That is to say, a recombinant yeast suitable for use in a method of the disclosure may comprise a UGT which is capable of catalyzing a reaction in which steviol is converted to steviolmonoside.

Such a recombinant cell may comprise a nucleotide sequence encoding a polypeptide having the activity shown by UDP-glycosyltransferase (UGT) UGT85C2, whereby the nucleotide sequence upon transformation of the cell confers on that cell the ability to convert steviol to steviolmonoside.

UGT85C2 activity is transfer of a glucose unit to the 13-OH of steviol. Thus, a suitable UGT85C2 may function as a uridine 5'-diphospho glucosyl: steviol 13-OH transferase, and a uridine 5'-diphospho glucosyl: steviol- 19-O- glucoside 13-OH transferase. A functional UGT85C2 polypeptide may also catalyze glucosyl transferase reactions that utilize steviol glycoside substrates other than steviol and steviol- 19-O-glucoside. Such sequences may be referred to as UGT1 sequences herein.

A recombinant cell may comprise a nucleotide sequence encoding a polypeptide which has UGT2 activity.

A polypeptide having UGT2 activity is one which functions as a uridine 5'-diphospho glucosyl: steviol- 13-O-glucoside transferase (also referred to as a steviol-13- monoglucoside 1,2-glucosylase), transferring a glucose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, steviol- 13-O-glucoside. Typically, a suitable UGT2 polypeptide also functions as a uridine 5'-diphospho glucosyl: rubusoside transferase transferring a glucose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, rubusoside.

A polypeptide having UGT2 activity may also catalyze reactions that utilize steviol glycoside substrates other than steviol- 13-O-glucoside and rubusoside, e.g., functional UGT2 polypeptides may utilize stevioside as a substrate, transferring a glucose moiety to

the C-2' of the 19-O-glucose residue to produce rebaudioside E. A functional UGT2 polypeptides may also utilize rebaudioside A as a substrate, transferring a glucose moiety to the C-2' of the 19-O-glucose residue to produce rebaudioside D. However, a functional UGT2 polypeptide typically does not transfer a glucose moiety to steviol compounds having a 1,3-bound glucose at the C- 13 position, i.e., transfer of a glucose moiety to steviol 1,3-bioside and 1,3-stevioside typically does not occur.

A polypeptide having UGT2 activity may also transfer sugar moieties from donors other than uridine diphosphate glucose. For example, a polypeptide having UGT2 activity act as a uridine 5'-diphospho D-xylosyl: steviol- 13 -O-glucoside transferase, transferring a xylose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, steviol- 13 -O-glucoside. As another example, a polypeptide having UGT2 activity may act as a uridine 5'-diphospho L-rhamnosyl: steviol- 13-O- glucoside transferase, transferring a rhamnose moiety to the C-2' of the 13-O-glucose of the acceptor molecule, steviol.

A recombinant cell may comprise a nucleotide sequence encoding a polypeptide having UGT activity capable of catalyzing the addition of a C-19-glucose to steviolbioside. That is to say, a recombinant cell may comprise a UGT which is capable of catalyzing a reaction in which steviolbioside is converted to stevioside. Accordingly, such a recombinant cell may be capable of converting steviolbioside to stevioside. Expression of such a nucleotide sequence may confer on the recombinant cell the ability to produce at least stevioside.

A recombinant cell may thus also comprise a nucleotide sequence encoding a polypeptide having the activity shown by UDP-glycosyltransferase (UGT) UGT74G1, whereby the nucleotide sequence upon transformation of the cell confers on the cell the ability to convert steviolbioside to stevioside.

Suitable UGT74G1 polypeptides may be capable of transferring a glucose unit to the 13-OH or the 19-COOH, respectively, of steviol. A suitable UGT74G1 polypeptide may function as a uridine 5'-diphospho glucosyl: steviol 19-COOH transferase and a uridine 5'-diphospho glucosyl: steviol- 13-O-glucoside 19-COOH transferase. Functional UGT74G1 polypeptides also may catalyze glycosyl transferase reactions that utilize steviol glycoside substrates other than steviol and steviol- 13-O-glucoside, or that transfer sugar moieties from donors other than uridine diphosphate glucose. Such sequences may be referred to herein as UGT3 sequences.

A recombinant cell may comprise a nucleotide sequence encoding a polypeptide capable of catalyzing glucosylation of the C-3' of the glucose at the C-13 position of stevioside. That is to say, a recombinant yeast suitable for use in a method of the disclosure may comprise a UGT which is capable of catalyzing a reaction in which stevioside is converted to rebaudioside A. Accordingly, such a recombinant cell may be capable of converting stevioside to rebaudioside A. Expression of such a nucleotide sequence may confer on the cell the ability to produce at least rebaudioside A.

A recombinant cell may thus also comprise a nucleotide sequence encoding a polypeptide having the activity shown by UDP-glycosyltransferase (UGT) UGT76G1, whereby the nucleotide sequence upon transformation of a cell confers on that cell the ability to convert stevioside to rebaudioside A.

5 A suitable UGT76G1 adds a glucose moiety to the C-3' of the C-13-O-glucose of the acceptor molecule, a steviol 1,2 glycoside. Thus, UGT76G1 functions, for example, as a uridine 5'-diphospho glucosyl: steviol 13-O-1,2 glucoside C-3 ' glucosyl transferase and a uridine 5'-diphospho glucosyl: steviol- 19-O-glucose, 13-O-1,2 bioside C-3' glucosyl transferase. Functional UGT76G1 polypeptides may also catalyze glucosyl transferase reactions that utilize
10 steviol glycoside substrates that contain sugars other than glucose, e.g., steviol rhamnosides and steviol xylosides. Such sequences may be referred to herein as UGT4 sequences. A UGT4 may alternatively or in addition be capable of converting RebD to RebM.

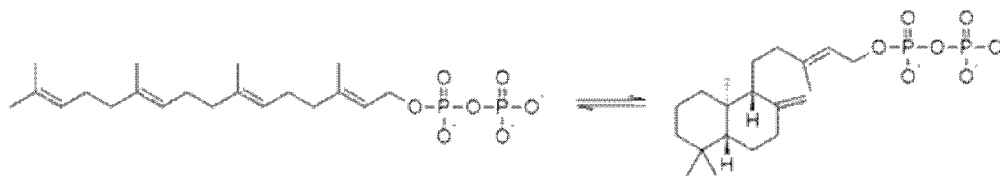
A recombinant cell typically comprises nucleotide sequences encoding at least one polypeptide having UGT1 activity, at least one polypeptide having UGT2 activity, least one
15 polypeptide having UGT3 activity and at least one polypeptide having UGT4 activity. One or more of these nucleic acid sequences may be recombinant. A given nucleic acid may encode a polypeptide having one or more of the above activities. For example, a nucleic acid encodes for a polypeptide which has two, three or four of the activities set out above. Preferably, a recombinant cell for use in the method of the disclosure comprises UGT1, UGT2 and UGT3
20 and UGT4 activity. Suitable UGT1, UGT2, UGT3 and UGT4 sequences are described in Table 1 of WO2015/007748.

A recombinant cell may comprise two or more nucleic acid sequences encoding a polypeptide having any one UGT activity, for example UGT1, 2, 3 or 4, activity. Where a recombinant cell comprises two or more nucleic acid sequences encoding a polypeptide
25 having any one UGT activity, those nucleic acid sequences may be the same or different and/or may encode the same or different polypeptides. In particular, a recombinant cell may comprise a nucleic acid sequence encoding two different UGT2 polypeptides.

A recombinant cell may comprise one or more recombinant nucleotide sequence(s) encoding one or more of:

- 30 a polypeptide having *ent*-copalyl pyrophosphate synthase activity;
a polypeptide having *ent*-Kaurene synthase activity;
a polypeptide having *ent*-Kaurene oxidase activity; and
a polypeptide having kaurenoic acid 13-hydroxylase activity.

For the purposes of this disclosure, a polypeptide having *ent*-copalyl pyrophosphate
35 synthase (EC 5.5.1.13) is capable of catalyzing the chemical reaction:



This enzyme has one substrate, geranylgeranyl pyrophosphate, and one product, *ent*-copalyl pyrophosphate. This enzyme participates in gibberellin biosynthesis. This enzyme belongs to the family of isomerases, specifically the class of intramolecular lyases. The systematic name of this enzyme class is *ent*-copalyl-diphosphate lyase (decyclizing). Other names in common use include *ent*-copalyl pyrophosphate synthase, *ent*-kaurene synthase A, and *ent*-kaurene synthetase A.

Suitable nucleic acid sequences encoding an *ent*-copalyl pyrophosphate synthase may for instance comprise a sequence as set out in SEQ ID. NO: 1, 3, 5, 7, 17, 19, 59, 61, 141, 142, 151, 152, 153, 154, 159, 160, 182 or 184 of WO2015/007748.

For the purposes of this disclosure, a polypeptide having *ent*-kaurene synthase activity (EC 4.2.3.19) is a polypeptide that is capable of catalyzing the chemical reaction:



Hence, this enzyme has one substrate, *ent*-copalyl diphosphate, and two products, *ent*-kaurene and diphosphate.

This enzyme belongs to the family of lyases, specifically those carbon-oxygen lyases acting on phosphates. The systematic name of this enzyme class is *ent*-copalyl-diphosphate diphosphate-lyase (cyclizing, *ent*-kaurene-forming). Other names in common use include *ent*-kaurene synthase B, *ent*-kaurene synthetase B, *ent*-copalyl-diphosphate diphosphate-lyase, and (cyclizing). This enzyme participates in diterpenoid biosynthesis.

Suitable nucleic acid sequences encoding an *ent*-Kaurene synthase may for instance comprise a sequence as set out in SEQ ID. NO: 9, 11, 13, 15, 17, 19, 63, 65, 143, 144, 155, 156, 157, 158, 159, 160, 183 or 184 of WO2015/007748.

ent-copalyl diphosphate synthases may also have a distinct *ent*-kaurene synthase activity associated with the same protein molecule. The reaction catalyzed by *ent*-kaurene synthase is the next step in the biosynthetic pathway to gibberellins. The two types of enzymic activity are distinct, and site-directed mutagenesis to suppress the *ent*-kaurene synthase activity of the protein leads to build up of *ent*-copalyl pyrophosphate.

Accordingly, a single nucleotide sequence used in a recombinant cell of the disclosure may encode a polypeptide having *ent*-copalyl pyrophosphate synthase activity and *ent*-kaurene synthase activity. Alternatively, the two activities may be encoded by two distinct, separate nucleotide sequences.

For the purposes of this disclosure, a polypeptide having *ent*-kaurene oxidase activity (EC 1.14.13.78) is a polypeptide which is capable of catalysing three successive oxidations of the 4-methyl group of *ent*-kaurene to give kaurenoic acid. Such activity typically requires the presence of a cytochrome P450.

5 Suitable nucleic acid sequences encoding an *ent*-kaurene oxidase may for instance comprise a sequence as set out in SEQ ID. NO: 21, 23, 25, 67, 85, 145, 161, 162, 163, 180 or 186 of WO2015/007748.

For the purposes of this disclosure, a polypeptide having kaurenoic acid 13-hydroxylase activity (EC 1.14.13) is one which is capable of catalyzing the formation of
10 steviol (*ent*-kaur-16-en-13-ol-19-oic acid) using NADPH and O₂. Such activity may also be referred to as *ent*-ka 13-hydroxylase activity.

Suitable nucleic acid sequences encoding a kaurenoic acid 13-hydroxylase may for instance comprise a sequence as set out in SEQ ID. NO: 27, 29, 31, 33, 69, 89, 91, 93, 95, 97, 146, 164, 165, 166, 167 or 185 of WO2015/007748.

15 A recombinant cell may comprise a recombinant nucleic acid sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity. That is to say, a recombinant cell for use in the process of the disclosure may be capable of expressing a nucleotide sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity. For the purposes of this disclosure, a polypeptide having NADPH-Cytochrome
20 P450 reductase activity (EC 1.6.2.4; also known as NADPH:ferrihaemoprotein oxidoreductase, NADPH:haemoprotein oxidoreductase, NADPH:P450 oxidoreductase, P450 reductase, POR, CPR, CYPOR) is typically one which is a membrane-bound enzyme allowing electron transfer to cytochrome P450 in the microsome of the eukaryotic cell from a FAD- and FMN-containing enzyme NADPH:cytochrome P450 reductase (POR; EC 1.6.2.4).
25

In a recombinant cell, the ability of the cell to produce geranylgeranyl diphosphate (GGPP) may be upregulated. Upregulated in the context of this disclosure implies that the recombinant cell produces more GGPP than an equivalent non-recombinant cell.

Accordingly, a recombinant cell may comprise one or more nucleotide
30 sequence(s) encoding hydroxymethylglutaryl-CoA reductase, farnesyl-pyrophosphate synthetase and geranylgeranyl diphosphate synthase, whereby the nucleotide sequence(s) upon transformation of a cell confer(s) on that cell the ability to produce elevated levels of GGPP. Thus, a recombinant cell for use in a method of the disclosure may comprise one or more recombinant nucleic acid sequence(s) encoding one or more of
35 hydroxymethylglutaryl-CoA reductase, farnesyl-pyrophosphate synthetase and geranylgeranyl diphosphate synthase.

Accordingly, a recombinant cell for use in a process of the disclosure may comprise nucleic acid sequences encoding one or more of:

a polypeptide having hydroxymethylglutaryl-CoA reductase activity;

a polypeptide having farnesyl-pyrophosphate synthetase activity; and
a polypeptide having geranylgeranyl diphosphate synthase activity.

A first cell as defined herein is an organism suitable for genetic manipulation and one which may be cultured at cell densities useful for industrial production of a target
5 product. A suitable cell may be a microorganism, for example one which may be maintained in a fermentation device. A cell may be a cell found in nature or a cell derived from a parent cell after genetic manipulation or classical mutagenesis.

As used herein, a recombinant cell is one which is genetically modified or transformed/transfected with one or more of the nucleotide sequences as defined herein.
10 The presence of the one or more such nucleotide sequences alters the ability of the microorganism to produce steviol or a steviol glycoside, in particular one or more steviol glycosides. A non-recombinant cell, i.e. one that is not transformed/transfected or genetically modified, typically does not comprise one or more of the nucleotide sequences enabling the cell to produce a steviol glycoside. Hence, a non-recombinant cell is typically
15 a cell that does not naturally produce a steviol glycoside, although a cell which naturally produces a steviol or a steviol glycoside and which has been modified as described herein (and which thus has an altered ability to produce a diterpene glycoside) is considered a recombinant cell for use in a process according to the disclosure.

In particular, it may be possible that the enzymes selected from the group
20 consisting of *ent*-copalyl pyrophosphate synthase, *ent*-kaurene synthase, *ent*-kaurene oxidase, and kaurenoic acid 13-hydroxylase, UGTs, hydroxymethylglutaryl-CoA reductase, farnesyl-pyrophosphate synthetase, geranylgeranyl diphosphate synthase and NADPH-cytochrome p450 reductase are native to the cell and that transformation with one or more of the nucleotide sequences encoding these enzymes may not be required to confer the
25 cell the ability to produce steviol or a steviol glycoside. A preferred cell for use in a process of the present disclosure may be a recombinant cell which is naturally capable of producing GGPP (i.e. in its non-recombinant form).

Further improvement of steviol or steviol glycoside production by the host microorganism may be obtained by classical strain improvement.

30 A recombinant cell may be any suitable host cell, for example, a multicellular organism or a cell thereof or a unicellular organism. A host may be a prokaryotic, archaeobacterial or eukaryotic host cell.

A prokaryotic host cell may, but is not limited to, a bacterial host cell. An eukaryotic host cell may be, but is not limited to, a yeast, a fungus, an amoeba, an algae,
35 an animal, an insect host cell.

An eukaryotic host cell may be a fungal host cell. "Fungi" include all species of the subdivision *Eumycotina* (Alexopoulos, C. J., 1962, In: Introductory Mycology, John Wiley & Sons, Inc., New York). The term fungus thus includes among others filamentous fungi and yeasts.

"Filamentous fungi" are herein defined as eukaryotic microorganisms that include all filamentous forms of the subdivision *Eumycotina* and *Oomycota* (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligatory aerobic. Filamentous fungal strains include, but are not limited to, strains of *Acremonium*, *Aspergillus*, *Agaricus*, *Aureobasidium*, *Cryptococcus*, *Corynascus*, *Chrysosporium*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Monascus*, *Mucor*, *Myceliophthora*, *Mortierella*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Phanerochaete*, *Podospora*, *Pycnoporus*, *Rhizopus*, *Schizophyllum*, *Sordaria*, *Talaromyces*, *Rasmosonia*, *Thermoascus*, *Thielavia*, *Tolypocladium*, *Trametes* and *Trichoderma*. Preferred filamentous fungal strains that may serve as host cells belong to the species *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Acremonium chrysogenum*, *Trichoderma reesei*, *Rasmosonia emersonii* (formerly known as *Talaromyces emersonii*), *Aspergillus sojae*, *Chrysosporium lucknowense*, *Myceliophthora thermophyla*. Reference host cells for the comparison of fermentation characteristics of transformed and untransformed cells, include e.g. *Aspergillus niger* CBS120.49, CBS 513.88, *Aspergillus oryzae* ATCC16868, ATCC 20423, IFO 4177, ATCC 1011, ATCC 9576, ATCC14488-14491, ATCC 11601, ATCC12892, *Aspergillus fumigatus* AF293 (CBS101355), *P. chrysogenum* CBS 455.95, *Penicillium citrinum* ATCC 38065, *Penicillium chrysogenum* P2, *Acremonium chrysogenum* ATCC 36225, ATCC 48272, *Trichoderma reesei* ATCC 26921, ATCC 56765, ATCC 26921, *Aspergillus sojae* ATCC11906, *Chrysosporium lucknowense* ATCC44006 and derivatives of all of these strains. Particularly preferred as filamentous fungal host cell are *Aspergillus niger* CBS 513.88 and derivatives thereof.

An eukaryotic host cell may be a yeast cell. Preferred yeast host cells may be selected from the genera: *Saccharomyces* (e.g., *S. cerevisiae*, *S. bayanus*, *S. pastorianus*, *S. carlsbergensis*), *Brettanomyces*, *Kluyveromyces*, *Candida* (e.g., *C. krusei*, *C. revkaui*, *C. pulcherrima*, *C. tropicalis*, *C. utilis*), *Issatchenkia* (e.g. *I. orientalis*) *Pichia* (e.g., *P. pastoris*), *Schizosaccharomyces*, *Hansenula*, *Kloeckera*, *Pachysolen*, *Schwanniomyces*, *Trichosporon*, *Yarrowia* (e.g., *Y. lipolytica* (formerly classified as *Candida lipolytica*)), *Yamadazyma*.

Prokaryotic host cells may be bacterial host cells. Bacterial host cell may be Gram negative or Gram positive bacteria. Examples of bacteria include, but are not limited to, bacteria belonging to the genus *Bacillus* (e.g., *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. megaterium*, *B. halodurans*, *B. pumilus*), *Acinetobacter*, *Nocardia*, *Xanthobacter*, *Escherichia* (e.g., *E. coli* (e.g., strains DH 1 OB, Stbl2, DH5-alpha, DB3, DB3.1), DB4, DB5, JDP682 and ccdA-over (e.g., U.S. application No. 09/518,188))), *Streptomyces*, *Erwinia*, *Klebsiella*, *Serratia* (e.g., *S. marcescens*),

Pseudomonas (e.g., *P. aeruginosa*), *Salmonella* (e.g., *S. typhimurium*, *S. typhi*). Bacteria also include, but are not limited to, photosynthetic bacteria (e.g., green non-sulfur bacteria (e.g., *Chloroflexus* bacteria (e.g., *C. aurantiacus*), *Chloronema* (e.g., *C. gigateum*)), green sulfur bacteria (e.g., *Chlorobium* bacteria (e.g., *C. limicola*), *Pelodictyon* (e.g., *P. luteolum*),
5 purple sulfur bacteria (e.g., *Chromatium* (e.g., *C. okenii*)), and purple non-sulfur bacteria (e.g., *Rhodospirillum* (e.g., *R. rubrum*), *Rhodobacter* (e.g. *R. sphaeroides*, *R. capsulatus*), and *Rhodomicrobium* bacteria (e.g., *R. vanellii*)).

Host cells may be host cells from non-microbial organisms. Examples of such cells, include, but are not limited to, insect cells (e.g., *Drosophila* (e.g., *D. melanogaster*),
10 *Spodoptera* (e.g., *S. frugiperda* Sf9 or Sf21 cells) and *Trichoplusia* (e.g., High-Five cells); nematode cells (e.g., *C. elegans* cells); avian cells; amphibian cells (e.g., *Xenopus laevis* cells); reptilian cells; and mammalian cells (e.g., NIH3T3, 293, CHO, COS, VERO, C127, BHK, Per-C6, Bowes melanoma and HeLa cells).

A recombinant cell for use in a process of the disclosure may be able to grow on
15 any suitable carbon source known in the art and convert it to a steviol glycoside. The recombinant cell may be able to convert directly plant biomass, celluloses, hemicelluloses, pectins, rhamnose, galactose, fucose, maltose, maltodextrins, ribose, ribulose, or starch, starch derivatives, sucrose, lactose and glycerol. Hence, a preferred cell expresses enzymes such as cellulases (endocellulases and exocellulases) and hemicellulases (e.g.
20 endo- and exo-xylanases, arabinases) necessary for the conversion of cellulose into glucose monomers and hemicellulose into xylose and arabinose monomers, pectinases able to convert pectins into glucuronic acid and galacturonic acid or amylases to convert starch into glucose monomers. Preferably, the cell is able to convert a carbon source selected from the group consisting of glucose, xylose, arabinose, sucrose, lactose and
25 glycerol. The cell may for instance be a eukaryotic cell as described in WO03/062430, WO06/009434, EP1499708B1, WO2006096130 or WO04/099381.

Standard genetic techniques, for the construction of such recombinant cells, such as overexpression of enzymes in the cells, genetic modification of cells, or hybridisation techniques, are known methods in the art, such as described in Sambrook and Russel
30 (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation, genetic modification etc of fungal cells are known from e.g. EP-A-0 635 574, WO 98/46772, WO 99/60102 and WO 00/37671, WO90/14423,
35 EP-A-0481008, EP-A-0635 574 and US 6,265,186.

In the method of the disclosure, the cells are cultivated in a reaction medium and under conditions suitable for production of the steviol glycoside. Typically, in the method of the disclosure the cells may be cultivated at a large-scale (including, for example, in the form of continuous, batch or fed-batch cultivations) in a suitable reaction medium and

under conditions allowing the steviol glycoside to be produced and isolated. The amount of reaction medium may be maintained at an approximately constant volume in the method of the disclosure. That is to say, evaporated reaction medium may be replenished so that the volume of reaction medium remains approximately constant.

5 The cultivation takes place in a suitable reaction medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, e. g., *Bennett, J. W. and LaSure, L., eds., More Gene Manipulations in Fungi, Academic Press, CA, 1991*). Suitable media are available from commercial suppliers or may be prepared using published compositions (e. g., in catalogues of the American Type Culture
10 Collection).

A process for producing a steviol glycoside may be carried out at any suitable temperature. A suitable temperature may for instance be between about 10 and about 40 degrees Celsius, for instance between about 15 and about 30 degrees Celsius, in particular at about 30°C.

15 If the steviol glycoside is secreted into the reaction medium, the compound can be isolated directly from the reaction medium. If the steviol glycoside is not secreted, it can be isolated from, for example, cell lysates. That is to say, the steviol glycoside may be isolated from the total broth including cells.

20 The steviol glycoside may be isolated by methods known in the art. For example, the steviol glycoside may be isolated from the reaction medium by procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation. The isolated steviol glycoside may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (e. g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic
25 procedures (e.g., preparative isoelectric focusing), differential solubility (e. g., ammonium sulfate precipitation), or extraction (see, e.g., *Protein Purification, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989*). In some applications, the steviol glycoside may be used without substantial isolation from the culture broth; separation of the reaction medium from the biomass may be adequate.

30 In a preferred embodiment the process for the production of a steviol glycoside further comprises recovering the steviol glycoside. Recovery of the steviol glycoside may be carried out by any suitable method.

In one embodiment, a steviol glycoside that is produced in a process as disclosed herein is recovered from the fermentation medium or total broth. Recovery of a steviol
35 glycoside may be carried out by any suitable method known in the art, for instance by precipitation, adsorbant chromatography, ion exchange technology, centrifugation or filtration, crystallization or any suitable combination of these methods.

In a preferred embodiment, the fermentation medium or total broth comprises an amount of at least one steviol glycoside of between 1 and 150 g/l, preferably between 5

and 100 g/l, more preferably between 10 and 80 g/l or between 15 and 60 g/l of the steviol glycoside.

Standard genetic techniques, such as overexpression of enzymes in the host cells, genetic modification of host cells, or hybridisation techniques, are known methods in the art, such as described in Sambrook and Russel (2001) "Molecular Cloning: A Laboratory Manual (3rd edition), Cold Spring Harbor Laboratory, *Cold Spring Harbor Laboratory Press*, or F. Ausubel et al, eds., "Current protocols in molecular biology", Green Publishing and Wiley Interscience, New York (1987). Methods for transformation, genetic modification etc of fungal host cells are known from e.g. EP-A-0 635 574, WO 98/46772, WO 99/60102 and WO 00/37671, WO90/14423, EP-A-0481008, EP-A-0635 574 and US 6,265,186.

Embodiments of the disclosure:

1. A process for the production of a steviol glycoside, which process comprises fermenting a first cell capable of producing a steviol glycoside in a suitable fermentation medium under conditions which allow for production of the steviol glycoside,
wherein the fermentation medium comprises oligosaccharides which are not completely fermentable by the first cell; and
wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the oligosaccharides;
and, optionally, recovering the steviol glycoside from the reaction medium or broth.
2. A process according to embodiment 1, wherein the enzyme is added directly to the fermentation medium.
3. A process according to embodiment 1 or 2, wherein the fermentation is carried out in the presence of a second cell, different to the first cell, which is capable of expressing the enzyme.
4. A process according to any one of the preceding embodiments, wherein the first cell is modified so that it is capable of expressing the enzyme to a greater degree than an unmodified form of the cell.
5. A process according to any one of the preceding embodiments, wherein the enzyme is present for a portion of the fermentation period or for the whole of the fermentation period.

6. A process according to any one of the preceding embodiments, wherein the enzyme is an amylase such as an alpha-amylase, a beta-amylase or a gluco-amylase, a pullulanase, an alpha-glucosidase, a beta-glucosidase, a trehalase or a transglucosidase.
- 5
7. A process according to any one of the preceding embodiments, wherein the first cell is a recombinant cell which comprises one or more recombinant nucleotide sequence(s) encoding:
- 10
- a polypeptide having *ent*-copalyl pyrophosphate synthase activity;
- a polypeptide having *ent*-kaurene synthase activity;
- a polypeptide having *ent*-kaurene oxidase activity; and
- a polypeptide having kaurenoic acid 13-hydroxylase activity.
8. A process according to embodiment 7, wherein the first cell comprises a recombinant nucleic acid sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity.
- 15
9. A process according embodiment 7 or 8, wherein the first cell comprises a recombinant nucleic acid sequence encoding one or more of:
- 20
- (i) a polypeptide having UGT74G1 activity;
- (ii) a polypeptide having UGT2 activity;
- (iii) a polypeptide having UGT85C2 activity; and
- (iv) a polypeptide having UGT76G1 activity.
- 25
10. A process according to any one of embodiments 7 to 9, wherein the first cell belongs to one of the genera *Saccharomyces*, *Aspergillus*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Humicola*, *Issatchenkia*, *Trichosporon*, *Brettanomyces*, *Pachysolen*, *Yarrowia*, *Yamadazyma* or *Escherichia*.
- 30
11. A process according to embodiment 10, wherein the first cell is a *Saccharomyces cerevisiae* cell, a *Yarrowia lipolytica* cell, a *Candida krusei* cell, an *Issatchenkia orientalis* cell or an *Escherichia coli* cell.
- 35
12. A process according to any one of embodiments 7 to 11, wherein the ability of the first cell to produce geranylgeranyl diphosphate (GGPP) is upregulated.
13. A process according to any one of embodiments 7 to 12 which comprises a nucleic acid sequence encoding one or more of:

a polypeptide having hydroxymethylglutaryl-CoA reductase activity;
a polypeptide having farnesyl-pyrophosphate synthetase activity;
a polypeptide having geranylgeranyl diphosphate synthase activity.

5

14. A process according to any one of the preceding embodiments, wherein the steviol glycoside is rebaudioside A, rebaudioside D or rebaudioside M.

10

15. An end-of-fermentation broth comprising a steviol glycoside obtainable by a process according to any one of the preceding embodiments.

16. An end-of-fermentation broth comprising a steviol glycoside and a lower amount of non-fermentable oligosaccharides than were added during fermentation.

15

17. An end-of-fermentation broth according to embodiment 15 or 16, wherein the amount of non-fermentable oligosaccharides is less than about 22g/L oligosaccharides.

20

18. An end-of-fermentation broth according to any one of embodiments 15 to 17 obtainable by a process according to any one of embodiments 1 to 14.

A reference herein to a patent document or other matter which is given as prior art is not to be taken as an admission that that document or matter was known or that the information it contains was part of the common general knowledge as at the priority date of any of the claims.

25

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

The present disclosure is further illustrated by the following Examples:

30

EXAMPLES

Example 1: Construction of *Yarrowia* strain STV2021

35

Yarrowia lipolytica strain STV2021 having the genotype set out in Table 1 was constructed using the approach described in WO2013/110673 and WO2015/007748.

Table 1. Genotype of strain STV2021. Between brackets indicates the gene copy number present in the strain

Strain name	Genotype
STV2021	MATB tHMG (2; SEQ ID NO: 23 from WO2016/151046) GGS (2; SEQ ID NO: 24 from WO2016/151046) CPS (3; SEQ ID NO: 182 from WO2013/110673) KS (2; SEQ ID NO: 183 from WO2013/110673) KO (2; SEQ ID NO: 186 from WO2013/110673) KAH4 (3; SEQ ID NO: 185 from WO2013/110673) CPR (2; SEQ ID NO: 188 from WO2013/110673) UGT1 (4; SEQ ID NO: 189 from WO2013/110673) UGT2 (2; SEQ ID NO: 190 from WO2013/110673) UGT3 (2; SEQ ID NO: 191 from WO2013/110673) UGT4 (4; SEQ ID NO: 192 from WO2013/110673)

Example 2:

5 The *Y. lipolytica* strain (*Y. lipolytica* STV2021) having the genotype set out in Table 1 above, was cultivated in shake-flasks (0.5 l with 100 ml medium) for 20 hours at 30°C and 280 rpm on YEPD medium (20 g/l Peptone, 10 g/l Yeast extract, 20 g/l dextrose). Subsequently, 8 ml of the content of the shake-flask was transferred into shake-flasks (2 l with 500 ml medium) for 30 hours at 30°C and 220 rpm. The medium of the second shake
10 flask step, seed and main fermentation was based on Verduyn et al. (Verduyn C, Postma E, Scheffers WA, Van Dijken JP. Yeast, 1992 Jul;8(7):501-517), with modifications in the carbon and nitrogen sources, as described in Tables 2.

Tables 2. Preculture medium composition

Raw material	Formula	Concentration (g/kg)
Glucose	C ₆ H ₁₂ O ₆	60
Urea	(NH ₂) ₂ CO	6.9
Potassium dihydrogen phosphate	KH ₂ PO ₄	9
Magnesium sulphate	MgSO ₄ · 7H ₂ O	1.5
Trace elements solution ^a		3
Vitamins solution ^b		3

15

^aTrace elements solution

Component	Formula	Concentration (g/kg)
EDTA	C ₁₀ H ₁₄ N ₂ Na ₂ O ₈ · 2H ₂ O	15.00
Zinc sulphate · 7H ₂ O	ZnSO ₄ · 7H ₂ O	4.50
Manganese chloride · 2H ₂ O	MnCl ₂ · 2H ₂ O	0.84
Copper (II) sulphate · 5H ₂ O	CuSO ₄ · 5H ₂ O	0.30
Sodium molybdenum · 2H ₂ O	Na ₂ MoO ₄ · 2H ₂ O	0.40

Calcium chloride . 2H ₂ O	CaCl ₂ . 2H ₂ O	4.50
Iron sulphate . 7H ₂ O	FeSO ₄ .7H ₂ O	3.00
Potassium iodide	KI	0.10

^bVitamin solution

Component	Formula	Concentration (g/kg)
Biotin (D-)	C ₁₀ H ₁₆ N ₂ O ₃ S	0.05
Ca D(+) panthothenate	C ₁₈ H ₃₂ CaN ₂ O ₁₀	1.00
Nicotinic acid	C ₆ H ₅ NO ₂	1.00
Myo-inositol	C ₆ H ₁₂ O ₆	25.00
Thiamine chloride hydrochloride	C ₁₂ H ₁₈ Cl ₂ N ₄ OS . xH ₂ O	1.00
Pyridoxal hydrochloride	C ₈ H ₁₂ ClNO ₃	1.00
p-aminobenzoic acid	C ₇ H ₇ NO ₂	0.20

Subsequently, 400ml of the content of the shake-flask was transferred into a fermenter (starting volume 4 L), which contained the medium as set out in Table 3. The pH was controlled at 5.0 by addition of ammonia (25 w/w%). Temperature was controlled at 30°C. pO₂ was controlled at 20% (relative to air saturation) by adjusting the stirrer speed.

Table 3 Composition of seed medium composition

Raw material	Formula	Concentration (g/kg)
Glucose	C ₆ H ₁₂ O ₆	60
Ammonium sulphate	(NH ₄) ₂ SO ₄	1
Potassium dihydrogen phosphate	KH ₂ PO ₄	20
Magnesium sulphate	MgSO ₄ .7H ₂ O	10
Trace elements solution ^a		16
Vitamins solution ^b		16

Subsequently after 22 hours, 400ml of the content of the seed fermenter was transferred into a fermenter (starting volume 4 L), which contained the medium as set out in Table 4. The pH was controlled at 5.0 by addition of ammonia (25 w/w%). Temperature was controlled at 30°C. pO₂ was controlled at 20% (relative to air saturation) by adjusting the stirrer speed. Glucose concentration was kept limited by controlled feed to the fermenter as set out in Table 5.

Table 4. Composition main fermentation medium

Raw material		Final Concentration (g/kg)
Glucose	C ₆ H ₁₂ O ₆	60
Ammonium sulphate	(NH ₄) ₂ SO ₄	1
Potassium dihydrogen phosphate	KH ₂ PO ₄	20
Magnesium sulphate	MgSO ₄ .7H ₂ O	10

Trace element solution		16
Vitamin solution		16

Table 5. Fermentation feed composition used in this example

Compound	% on dry matter
Glucose	86
Fructose	1
Maltose	1.6
Isomaltose	2.3
Other DP2	4.1
DP>2	2.4
Other	2.6

Three main fermentations were started from the same seed fermentation. In one fermentation no formulated solution of Amigase Mega L (formulation in glycerol, containing approximately 89.5% of glucan 1,4- α -glucosidase; DSM Food Specialties, Delft, Netherlands was added). In a second fermentation 2.4 g of formulated solution of Amigase Mega was added to the fermentation 4 hours before end of fermentation (final concentration 0.22 mg Amigase Mega solution/ ml fermentation broth). For the third fermentation 2 g of formulated solution of Amigase Mega was added to the fermentation at the start of the main fermentation (final concentration 0.18 mg Amigase Mega solution/ ml fermentation broth).

Total sugar (after addition of strong acid to break all glycosidic bonds) and individual sugars were analyzed with HPLC or NMR.

The results are set out in Table 6 and Figures 2 and 3. The enzyme addition resulted in the total oligosaccharides end of fermentation being lowered from 24 g/l to 20.4 g/l when the enzyme was added 4 hours before end of fermentation and to 15.6 g/l when the enzyme was added at the start of the main fermentation. The Rebaudioside A concentration in the supernatant as determined by LCMS was increased from 1.4 g/l to 2.3 g/l. When the enzyme was added at the start of the main fermentation even to 2.8 g/l.

Table 6

End of fermentation concentration in supernatant	No enzyme addition	Enzyme added 4 hours before end of fermentation	Enzyme added at start of main fermentation
Glucose [g/l]	2.6	2.5	1.6
Maltose [g/l]	3.1	0.2	0.1
Isomaltose [g/l]	6.7	6.7	5.5
DP2 total [g/l]	17.9	15.3	13.8
DP>2 [g/l]	6.1	5.1	1.8
Total oligosaccharides [g/l]	24	20.4	15.6

Rebaudioside A	1.4	2.3	2.8
----------------	-----	-----	-----

CLAIMS

1. A process for the production of a steviol glycoside, which process comprises fermenting a first cell capable of producing a steviol glycoside in a suitable fermentation medium under conditions which allow for production of the steviol glycoside,
5 wherein the fermentation medium comprises oligosaccharides which are not completely fermentable by the first cell; and
wherein the fermentation is carried out in the presence of an enzyme which is capable of hydrolyzing, at least in part, the oligosaccharides;
10 and, optionally, recovering the steviol glycoside from the reaction medium or broth.
2. A process according to claim 1, wherein the enzyme is added directly to the fermentation medium.
15
3. A process according to claim 1 or 2, wherein the fermentation is carried out in the presence of a second cell, different to the first cell, which is capable of expressing the enzyme.
20
4. A process according to any one of the preceding claims, wherein the first cell is modified so that it is capable of expressing the enzyme to a greater degree than an unmodified form of the cell.
- 25 5. A process according to any one of the preceding claims, wherein the enzyme is present for a portion of the fermentation period or for the whole of the fermentation period.
6. A process according to any one of the preceding claims, wherein the enzyme is an amylase such as an alpha-amylase, a beta-amylase or a gluco-amylase, a pullulanase, an alpha-glucosidase, a beta-glucosidase, a trehalase or a transglucosidase.
30
7. A process according to any one of the preceding claims, wherein the first cell is a recombinant cell which comprises one or more recombinant nucleotide sequence(s) encoding:
35 a polypeptide having *ent*-copalyl pyrophosphate synthase activity;
a polypeptide having *ent*-kaurene synthase activity;
a polypeptide having *ent*-kaurene oxidase activity; and

a polypeptide having kaurenoic acid 13-hydroxylase activity.

8. A process according to claim 7, wherein the first cell comprises a recombinant nucleic acid sequence encoding a polypeptide having NADPH-cytochrome p450 reductase activity.

9. A process according claim7 or 8, wherein the first cell comprises a recombinant nucleic acid sequence encoding one or more of:

- (v) a polypeptide having UGT74G1 activity;
- (vi) a polypeptide having UGT2 activity;
- (vii) a polypeptide having UGT85C2 activity; and
- (viii) a polypeptide having UGT76G1 activity.

10. A process according to any one of claims 7 to 9, wherein the first cell belongs to one of the genera *Saccharomyces*, *Aspergillus*, *Pichia*, *Kluyveromyces*, *Candida*, *Hansenula*, *Humicola*, *Issatchenkia*, *Trichosporon*, *Brettanomyces*, *Pachysolen*, *Yarrowia*, *Yamadazyma* or *Escherichia*.

11. A process according to claim 10, wherein the first cell is a *Saccharomyces cerevisiae* cell, a *Yarrowia lipolitica* cell, a *Candida krusei* cell, an *Issatchenkia orientalis* cell or an *Escherichia coli* cell.

12. A process according to any one of claims 7 to 11, wherein the ability of the first cell to produce geranylgeranyl diphosphate (GGPP) is upregulated.

13. A process according to any one of claims 7 to 12 which comprises a nucleic acid sequence encoding one or more of:

a polypeptide having hydroxymethylglutaryl-CoA reductase activity;
a polypeptide having farnesyl-pyrophosphate synthetase activity;
a polypeptide having geranylgeranyl diphosphate synthase activity.

14. A process according to any one of the preceding claims, wherein the steviol glycoside is rebaudioside A, rebaudioside D or rebaudioside M.

15. An end-of-fermentation broth comprising a steviol glycoside obtainable by a process according to any one of the preceding claims.

16. An end-of-fermentation broth comprising a steviol glycoside and a lower amount of non-fermentable oligosaccharides than were added during fermentation.
17. An end-of-fermentation broth according to claim 15 or 16, wherein the amount of non-fermentable oligosaccharides is less than 22g/L oligosaccharides.
18. An end-of-fermentation broth according to any one of claims 15 to 17 obtainable by a process according to any one of claims 1 to 14.

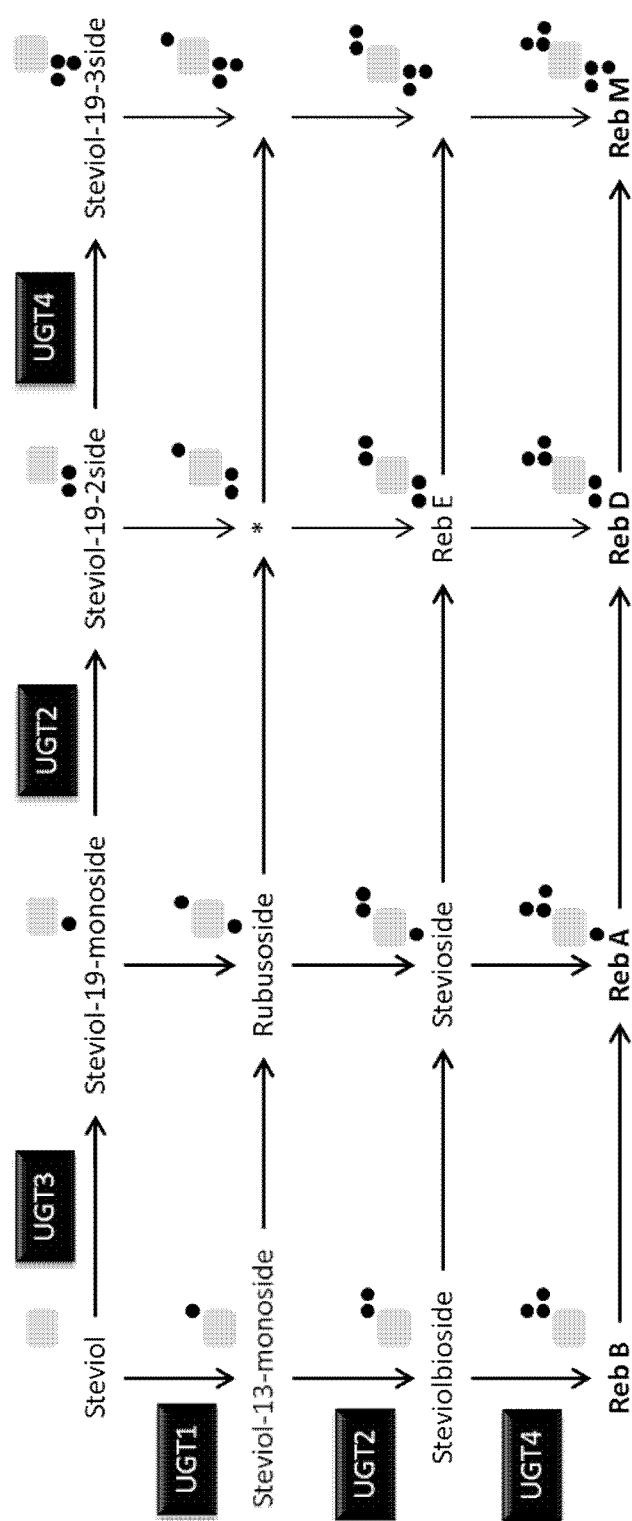


Fig. 1

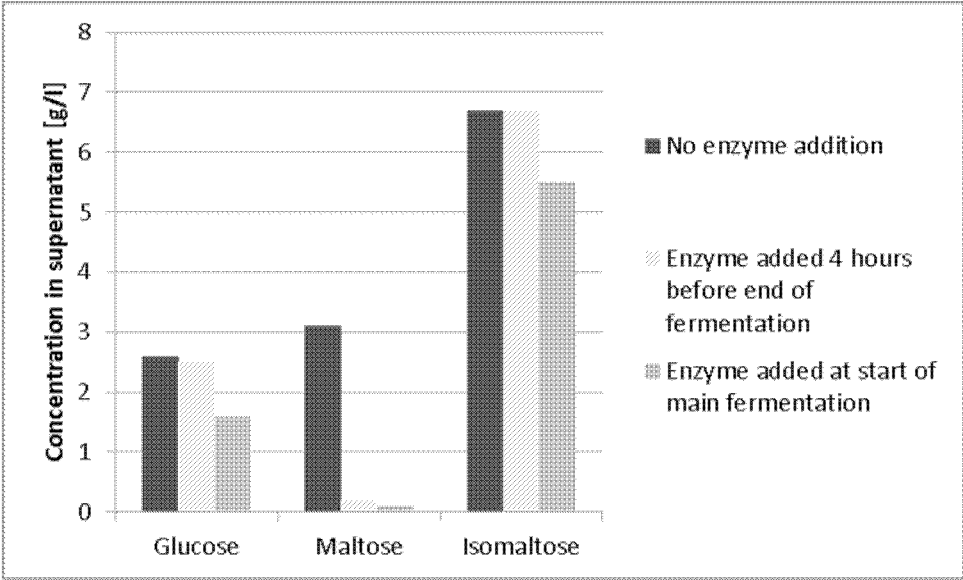


Fig. 2

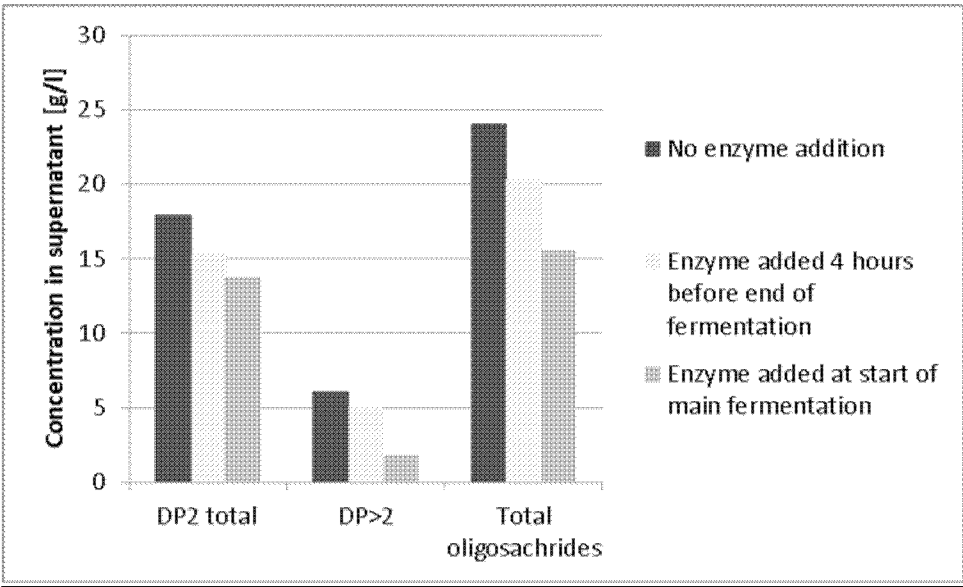


Fig. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/083618

A. CLASSIFICATION OF SUBJECT MATTER		
INV. C12P19/56	C12N1/16	C12N15/52 C12N9/26 C12N9/10
ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) C12P C12N		
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, CHEM ABS Data, EMBASE, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2017/024313 A1 (CARGILL INC [US]) 9 February 2017 (2017-02-09) claims 1-16 paragraph [0028] paragraph [0030] - paragraph [0034] paragraph [0040] paragraph [0094] - paragraph [0096] example 4	1,2, 4-16,18
X	----- WO 2016/196321 A1 (CARGILL INC [US]; EVOLVA [DK]; ANDERSON JAMES C [US]) 8 December 2016 (2016-12-08)	1,2,4-18
Y	claims 1-68 paragraph [0037] - paragraph [0044] paragraph [0053] ----- -/-	3
☒ 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 "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 17 January 2018		Date of mailing of the international search report 26/01/2018
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 Siatou, Evangelia

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/083618

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2009/134964 A2 (DANISCO US INC [US]; DUAN GANG [US]; RUAN ZHENHUA [US]; SHETTY JAYARAM) 5 November 2009 (2009-11-05) claims 1-32 paragraph [0004] - paragraph [0006] -----	3
Y	FUJII T ET AL: "Ethanol production from xylo-oligosaccharides by xylose-fermenting Saccharomyces cerevisiae expressing beta-xylosidase", BIOSCIENCE BIOTECHNOLOGY BIOCHEMISTRY, JAPAN SOCIETY FOR BIOSCIENCE, BIOTECHNOLOGY, AND AGROCHEMISTRY, TOKYO, JAPAN, vol. 75, no. 6, 1 January 2011 (2011-01-01), pages 1140-1146, XP002755002, ISSN: 0916-8451, DOI: 10.1271/BBB.110043 [retrieved on 2011-06-13] abstract page 1144, left-hand column - page 1145, right-hand column -----	3
Y	YU L ET AL: "Metabolic engineering of Clostridium tyrobutyricum for n-butanol production from maltose and soluble starch by overexpressing alpha-glucoside", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, SPRINGER, DE, vol. 99, no. 14, 1 July 2015 (2015-07-01), pages 6155-6165, XP002754889, ISSN: 0175-7598, DOI: 10.1007/S00253-015-6680-4 [retrieved on 2015-05-23] abstract page 6156, left-hand column, line 11 - line 32 page 6163, right-hand column - page 6164, left-hand column -----	3
A	WO 2013/110673 A1 (DSM IP ASSETS BV [NL]) 1 August 2013 (2013-08-01) cited in the application claims 1-32 paragraph [0004] - paragraph [0005] paragraph [0007] -----	1-18
A	WO 2015/007748 A1 (DSM IP ASSETS BV [NL]) 22 January 2015 (2015-01-22) cited in the application claims 1-20 ----- -/--	1-18

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/083618

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2017/060318 A2 (DSM IP ASSETS BV [NL]) 13 April 2017 (2017-04-13) claims 1-29 page 24, line 31 - page 25, line 2 page 25, line 28 - line 30 -----	1,2, 4-16,18
X,P	WO 2017/025648 A1 (DSM IP ASSETS BV [NL]) 16 February 2017 (2017-02-16) claims 1-17 page 19, line 12 - line 20 page 20, line 5 - line 7 -----	1,2, 4-16,18
X,P	WO 2017/025649 A1 (DSM IP ASSETS BV [NL]) 16 February 2017 (2017-02-16) claims 1-17 page 20, line 10 - line 18 page 21, line 3 - line 5 -----	1,2, 4-16,18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/083618

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2017024313	A1	09-02-2017	NONE
WO 2016196321	A1	08-12-2016	AU 2016271628 A1 21-12-2017 CA 2987585 A1 08-12-2016 WO 2016196321 A1 08-12-2016
WO 2009134964	A2	05-11-2009	BR PI0910539 A2 18-08-2015 CA 2723164 A1 05-11-2009 CN 102016056 A 13-04-2011 DK 2276848 T3 09-03-2015 EP 2276848 A2 26-01-2011 JP 5463349 B2 09-04-2014 JP 2011519559 A 14-07-2011 US 2011097765 A1 28-04-2011 US 2015140613 A1 21-05-2015 WO 2009134964 A2 05-11-2009
WO 2013110673	A1	01-08-2013	AU 2013211605 A1 17-07-2014 AU 2017202390 A1 27-04-2017 CA 2862980 A1 01-08-2013 CN 104203005 A 10-12-2014 EA 201400836 A1 30-01-2015 EP 2806754 A1 03-12-2014 HK 1204529 A1 27-11-2015 KR 20140127227 A 03-11-2014 US 2015031868 A1 29-01-2015 WO 2013110673 A1 01-08-2013
WO 2015007748	A1	22-01-2015	AU 2014292150 A1 28-01-2016 CA 2914900 A1 22-01-2015 EP 3021689 A1 25-05-2016 US 2016153017 A1 02-06-2016 WO 2015007748 A1 22-01-2015
WO 2017060318	A2	13-04-2017	NONE
WO 2017025648	A1	16-02-2017	NONE
WO 2017025649	A1	16-02-2017	NONE