

A method to produce crystalline lactose produced by a biotechnological production process

The present invention relates to methods to produce crystalline lactose, more specifically lactose obtained from a biotechnological production process.

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Lactose as a disaccharide knows a wide variety of applications, in the food and pharmaceutical industry (Hebbink GA, Dickhoff BHJ. Chapter 5 - Application of lactose in the pharmaceutical industry. In: Paques M, Lindner CBT-L, editors. Academic Press; 2019. p. 175–229. Available from: <https://www.sciencedirect.com/science/article/pii/B9780128117200000052>, Hettinga KA. Chapter 6 - Lactose in the dairy production chain. In: Paques M, Lindner CBT-L, editors. Academic Press; 2019. p. 231–66. Available from: <https://www.sciencedirect.com/science/article/pii/B9780128117200000064>). Today it is isolated from mammalian milk, mainly cow's milk. Due to the environmental impact of this production process, there is a need for alternative synthesis methods that can compete with the well-established extraction methods from milk at low production cost.

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Mammals synthesize lactose by means of a lactose synthase, or beta-1,4 galactosyltransferase, transferring a galactose to glucose with UDP-galactose as a donor substrate and glucose as an acceptor substrate. This principle has been engineered in microbes, copying the mammalian biosynthesis pathway (Mao Z, Shin H-D, Chen RR. Engineering the E. coli UDP-glucose synthesis pathway for oligosaccharide synthesis. Biotechnol Prog [Internet]. Jan [cited 2016 Mar 13];22(2):369–74. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/16599548>) and the synthesis enzymes have been applied in vitro before to synthesize lactose by the addition of the expensive UDP-galactose (Lau K, Thon V, Yu H, Ding L, Chen Y, Muthana MM, et al. Highly efficient chemoenzymatic synthesis of [small beta]1-4-linked galactosides with promiscuous bacterial [small beta]1-4-galactosyltransferases. Chem Commun [Internet]. 2010;46(33):6066–8. Available from: <http://dx.doi.org/10.1039/C0CC01381A>) as donor.

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A challenge one encounters with these methodologies is, is the energy requirement for lactose synthesis. The synthesis of UDP-galactose over UDP-glucose requires UTP, which either has to be added to the reaction mixture or be synthesized by the microbial host. After lactose synthesis UDP is released which is either lost as a substrate or may be recycled into UTP by means of ATP, another costly biochemical intermediate. Furthermore, the addition or synthesis of galactose is required, which is currently still derived from lactose and no alternative synthesis methods have been identified.

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A challenge one encounters is to purify lactose from a biotechnological synthesis environment, which encompasses products (such as e.g. medium component, buffer component, substrates, precursors, cells, proteins, DNA, lipids, cell debris, enzymes, chemicals, antifoam agents,...) that are not naturally present in lactose isolated from mammalian milk. These are amongst others sugars, such as glucose, fructose,

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UDP-glucose, UDP-galactose, galactose, sucrose; salts, such as phosphate buffers, sulphates, ammonia, magnesium, calcium...; proteins, cells or cellular components, which typically interfere with the purification of the biotechnologically produced lactose. These impurities can inhibit nucleation and crystal growth, and impurities themselves can potentially be incorporated into crystals, thus affecting the purity of the final product. It is therefore far from obvious to purify lactose from a biotechnological process at purities that are acceptable for the market.

Purely from an energy point and cost point of view, the current state of the art would not be able to compete with extractive production of lactose, even if it is more environmentally friendly.

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Summary of the invention

It is an object of the present invention to provide for methods to produce a crystalline lactose, biotechnologically produced, which solves the problem of the isolation of such lactose at high purity and at a reasonable production cost. The present invention provides a new method for efficiently producing lactose wherein said lactose is preferably first separated from its biotechnological production environment and then crystallized. According to this invention this and other objects are achieved by providing a method to produce a crystalline lactose, wherein said lactose is produced via a biotechnological production process wherein said method comprises a step of drying and/or crystallization of said lactose.

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Further benefits of the teachings of this invention will be apparent to one skilled in the art from reading this invention.

Definitions

The words used in this specification to describe the invention and its various embodiments are to be understood not only in the sense of their commonly defined meanings, but to include by special definition in this specification structure, material or acts beyond the scope of the commonly defined meanings. Thus, if an element can be understood in the context of this specification as including more than one meaning, then its use in a claim must be understood as being generic to all possible meanings supported by the specification and by the word itself.

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The various aspects and embodiments of the invention disclosed herein are to be understood not only in the order and context specifically described in this specification, but to include any order and any combination thereof. Each embodiment as identified herein may be combined together unless otherwise indicated. All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent

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application was specifically and individually indicated to be incorporated by reference in its entirety. Unless specifically stated otherwise, all words used in the singular number shall be deemed to include the plural and vice versa. Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described herein are those well-known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications.

In the specification, there have been disclosed embodiments of the invention, and although specific terms are employed, the terms are used in a descriptive sense only and not for purposes of limitation, the scope of the invention being set forth in the following claims. It must be understood that the illustrated embodiments have been set forth only for the purposes of example and that it should not be taken as limiting the invention. It will be apparent to those skilled in the art that alterations, other embodiments, improvements, details and uses can be made consistent with the letter and spirit of the disclosure herein and within the scope of this disclosure, which is limited only by the claims, construed in accordance with the patent law, including the doctrine of equivalents. In the claims that follow, reference characters used to designate claim steps are provided for convenience of description only, and are not intended to imply any particular order for performing the steps, unless specifically stated otherwise.

Throughout the application, unless explicitly stated otherwise, the features "synthesize", "synthesized" and "synthesis" are interchangeably used with the features "produce", "produced" and "production", respectively. Throughout the application, unless explicitly stated otherwise, the expressions "capable of...<verb>" and "capable to...<verb>" are preferably replaced with the active voice of said verb and *vice versa*. For example, the expression "capable of expressing" is preferably replaced with "expresses" and *vice versa*, i.e., "expresses" is preferably replaced with "capable of expressing". Throughout this document and in its claims, the verbs "to comprise", "to have" and "to contain" and their conjugations are used in their non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. The verb "to consist essentially of" means that a solution or a composition as defined herein may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristic of the invention. Said additional compound(s) might be inevitable by-product(s), for example, generated during production of lactose of present invention as well as compound(s) that were introduced into a process stream from which lactose is recovered but which could not have been removed therefrom. The term "consisting essentially of" with respect to spray-dried powders includes spray-dried powders containing with respect to the dry matter

of the spray-dried powder at least 80 %-wt., at least 85 %-wt., at least 90 %-wt., at least 93 %-wt., at least 95 %-wt. or at least 98 %-wt. of lactose. The term "consisting essentially of" is used likewise with respect to spray-dried powders, process streams and solutions containing lactose. Throughout this document and in its claims, unless specifically stated otherwise, the verbs "to comprise", "to have" and "to contain", and their conjugations, may be preferably replaced by "to consist of" (and its conjugations) or "to consist essentially of" (and its conjugations) and vice versa. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the elements is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one". Throughout the document and in the claims, unless explicitly stated otherwise, the articles "a" and "an" are preferably replaced by "at least one", more preferably "at least two", even more preferably by "at least three", even more preferably by "at least four", even more preferably by "at least five", even more preferably by "at least six", most preferably by "at least two". The word "about" or "approximately" when used in association with a numerical value (e.g., "about 10") or with a range (e.g., "about x to approximately y") preferably means that the value or range is interpreted as being as accurate as the method used to measure it. If no error margins are specified, the expression "about" or "approximately" when used in association with a numerical value is interpreted as having the same round-off as the given value. Throughout this document and its claims, unless otherwise stated, the expression "from x to y", wherein x and y represent numerical values, refers to a range of numerical values wherein x is the lower value of the range and y is the upper value of the range. Herein, x and y are also included in the range.

Throughout the application, unless explicitly stated otherwise, the term "beta 1,4-galactosyltransferase" is to be understood to refer to an enzyme being able to transfer a galactose from UDP-galactose to glucose to form lactose. Such beta-1,4- galactosyltransferase is preferably a lactose synthase. An example of such galactosyltransferase are the N-acetylglucosamine beta-1,4-galactosyltransferase GalT (Uniprot ID Q51116, sequence version 2023_02 of 03 May 2023) from *Neisseria meningitidis*.

The term "bioconversion" is to be understood as a conversion of a product by resting or permeabilized cells.

Throughout the application, unless explicitly stated otherwise, the terms "UDP-glucose-4-epimerase" and "UDP-galactose-4-epimerase" are used interchangeably.

The terms "glucose isomerase", "xylose isomerase" and "fructose isomerase" are used in the art to refer to an enzyme converting fructose to glucose and vice versa.

Protein or polypeptide sequence information and functional information can be provided by a comprehensive resource for protein sequence and annotation data like e.g., the Universal Protein Resource (UniProt) (www.uniprot.org) (Nucleic Acids Res. 2021, 49(D1), D480-D489). UniProt comprises

the expertly and richly curated protein database called the UniProt Knowledgebase (UniProtKB), together with the UniProt Reference Clusters (UniRef) and the UniProt Archive (UniParc). The UniProt identifiers (UniProt ID) are unique for each protein present in the database. Throughout the application, the sequence of a polypeptide is represented by an UniProt ID. Unless stated otherwise, the UniProt IDs of the proteins described correspond to their sequence version 01 as present in the UniProt Database (www.uniprot.org) version release 2023_02 of 03 May 2023.

It should be understood for those skilled in the art that for the databases used herein, the content of each database is fixed at each release and is not to be changed. When the content of a specific database is changed, this specific database receives a new release version with a new release date. All release versions for each database with their corresponding release dates and specific content as annotated at these specific release dates are available and known to those skilled in the art.

Brief description of the drawings

Figure 1 shows production of lactose using sucrose as raw material or carbon source.

Figure 2 shows production of lactose using trehalose as carbon source or raw material.

Figure 3 shows production of lactose using sucrose and glucose as raw materials or carbon source.

Detailed description of the invention

In a first aspect, the present invention provides a method to produce a crystalline lactose, wherein said lactose is produced via a biotechnological production process wherein said method comprises a step of drying and/or crystallisation of said lactose.

In a preferred embodiment of a process for biotechnological production of lactose the process is a fermentation process, a bioconversion process, or an enzymatic process. The fermentation process used in the present invention can be a microbial fermentation process, but also a cell culture fermentation process.

Cells used in such fermentation or bioconversion process are selected from the list consisting of a microorganism, a plant cell, an animal cell, an insect cell or a protozoan cell, more preferably said cell is a microorganism, more preferably said cell is a bacterium or a yeast, even more preferably said cell is a bacterium, even more preferably said cell is a bacterium belonging to the genus of *Escherichia* or *Bacillus*, even more preferably said cell is a bacterium belonging to the genus of *Escherichia*, even more preferably said cell is *Escherichia coli*, even more preferably said cell is an *Escherichia coli* K-12 strain, most preferably said cell is *Escherichia coli* MG1655.

Throughout the application and claims, unless specifically stated otherwise, a microorganism is preferably a bacterium, a yeast or a fungus, more preferably a bacterium or a yeast, most preferably a bacterium.

Throughout the application and claims, unless specifically stated otherwise, a bacterium preferably belongs to the phylum of the Proteobacteria or the phylum of the Firmicutes or the phylum of the Cyanobacteria or the phylum Deinococcus-Thermus. Said bacterium belonging to the phylum Proteobacteria belongs preferably to the family *Enterobacteriaceae*, preferably to the species *Escherichia coli*.
5 *coli*. Said bacterium preferably relates to any strain belonging to the species *Escherichia coli* such as but not limited to *Escherichia coli* B, *Escherichia coli* C, *Escherichia coli* W, *Escherichia coli* K12, *Escherichia coli* Nissle. More specifically, said bacterium relates to cultivated *Escherichia coli* strains – designated as *E. coli* K12 strains – which are well-adapted to the laboratory environment, and, unlike wild type strains, have lost their ability to thrive in the intestine. Well-known examples of the *E. coli* K12 strains are K12 Wild
10 type, W3110, MG1655, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, preferably the present invention specifically relates to an *E. coli* K12 strain, more preferably an *E. coli* MG1655 strain. Said bacterium belonging to the phylum Firmicutes belongs preferably to the Bacilli, preferably from the species *Bacillus*, such as *Bacillus subtilis* or, *B. amyloliquefaciens*. Said bacterium belonging to the phylum Actinobacteria, preferably belonging to the family of the Corynebacteriaceae,
15 with members *Corynebacterium glutamicum* or *C. afermentans*, or belonging to the family of the Streptomycetaceae with members *Streptomyces griseus* or *S. fradiae*.

Throughout the application and claims, unless specifically stated otherwise, a yeast cell preferably belongs to the phylum of the Ascomycota or the phylum of the Basidiomycota or the phylum of the Deuteromycota or the phylum of the Zygomycetes. Said yeast cell belongs preferably to the genus
20 *Saccharomyces* (with members like e.g. *Saccharomyces cerevisiae*, *S. bayanus*, *S. boulardii*), *Pichia* (with members like e.g. *Pichia pastoris*, *P. anomala*, *P. kluyveri*), *Komagataella*, *Hansunella*, *Kluyveromyces* (with members like e.g. *Kluyveromyces lactis*, *K. marxianus*, *K. thermotolerans*), *Yarrowia* (like e.g. *Yarrowia lipolytica*), *Eremothecium*, *Zygosaccharomyces*, *Starmerella* (like e.g. *Starmerella bombicola*) or *Debaromyces*. Said yeast cell is more preferably selected from *Pichia pastoris*, *Yarrowia lipolytica*,
25 *Saccharomyces cerevisiae* and *Kluyveromyces lactis*.

Throughout the application and claims, unless specifically stated otherwise, a fungus preferably belongs to the genus *Rhizopus*, *Dictyostelium*, *Penicillium*, *Mucor* or *Aspergillus*.

Throughout the application and claims, unless specifically stated otherwise, a plant cell includes cells of flowering and non-flowering plants, as well as algal cells, for example *Chlamydomonas*, *Chlorella*, etc.
30 Preferably, said plant cell is a tobacco, alfalfa, rice, cotton, rapeseed, tomato, corn, maize or soybean cell.

Throughout the application and claims, unless specifically stated otherwise, an animal cell is preferably derived from non-human mammals (e.g. cattle, buffalo, pig, sheep, mouse, rat), birds (e.g. chicken, duck, ostrich, turkey, pheasant), fish (e.g. swordfish, salmon, tuna, sea bass, trout, catfish), invertebrates (e.g. lobster, crab, shrimp, clams, oyster, mussel, sea urchin), reptiles (e.g. snake, alligator, turtle), amphibians
35 (e.g. frogs) or insects (e.g. fly, nematode) or is a genetically modified cell line derived from human cells excluding embryonic stem cells. Both human and non-human mammalian cells are preferably chosen from

the list consisting of an epithelial cell like e.g. a mammary epithelial cell, an embryonic kidney cell (e.g. HEK293 or HEK 293T cell), a fibroblast cell, a COS cell, a Chinese hamster ovary (CHO) cell, a murine myeloma cell like e.g. an N20, SP2/0 or YB2/0 cell, an NIH-3T3 cell, a non-mammary adult stem cell or derivatives thereof such as described in WO21067641.

- 5 Throughout the application and claims, unless specifically stated otherwise, an insect cell is preferably derived from *Spodoptera frugiperda* like e.g. Sf9 or Sf21 cells, *Bombyx mori*, *Mamestra brassicae*, *Trichoplusia ni* like e.g. BTI-TN-5B1-4 cells or *Drosophila melanogaster* like e.g. Drosophila S2 cells.

Throughout the application and claims, unless specifically stated otherwise, a protozoan cell is preferably a *Leishmania tarentolae* cell.

- 10 In another preferred embodiment, said cell used in a biotechnological production process according to the invention is a single cell. It is further preferred that said cell used in a biotechnological production process according to the invention is an isolated cell.

- In a preferred embodiment, when the process is a fermentation process or a bioconversion process the
15 fermentation or bioconversion is followed by a biomass removal step. Preferably, such biomass removal step comprises at least one of centrifugation, microfiltration and/or ultrafiltration.

- The lactose produced via a biotechnological production process can be produced by a method which comprises use of i) a synthase and ii) Uridine Diphosphate (UDP), for forming UDP-glucose. In such process
20 UDP is preferably added in catalytic amounts, more preferably said UDP is provided in a concentration between 0,1 mM and 10 mM. In an alternative embodiment, UDP is preferably added in stoichiometric amounts. Preferably, such process further comprises use of UDP-glucose-4-epimerase to convert said UDP-glucose to UDP-galactose. Alternatively or preferably, said UDP-galactose is a galactose donor for a beta 1,4-galactosyltransferase to form lactose.

- 25 Alternatively or preferably, the process can further comprise use of a beta 1,4-galactosyltransferase to convert glucose with UDP-galactose to form lactose and UDP. Preferably, said beta 1,4-galactosyltransferase uses glucose as an acceptor and UDP-galactose as a donor. In such process, preferably, the UDP released by the beta 1,4-galactosyltransferase is recycled to be used by the synthase to form UDP-glucose. The UDP generated can thus in turn be used in sucrose synthase catalyzed reactions.

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In a preferred embodiment of the process for biotechnological production of lactose, the synthase used also forms either a glucose or fructose. Preferably, in the process for biotechnological production of lactose wherein the synthase also forms a fructose, said fructose is converted to glucose.

- In a preferred embodiment of the process for biotechnological production of lactose, the synthase is a
35 sucrose synthase or a trehalose synthase.

Preferably said sucrose synthase is chosen from the GT4 glycosyltransferase subfamily, or chosen from KEGG Enzyme class EC 2.4.1.13. Alternatively, said sucrose synthase is chosen from the list of the sucrose synthase (Uniprot ID P13708, sequence version 2023_02 of 03 May 2023) from *Glycine max*, the sucrose synthase (Uniprot ID P49040, sequence version 2023_02 of 03 May 2023) from *Arabidopsis thaliana*, the sucrose synthase (Uniprot ID Q9ZEV2, sequence version 2023_02 of 03 May 2023) from *Anabaena* sp. PCC 7119 (ATCC no. 29151), the sucrose synthase (Uniprot ID A0A059ZV61, sequence version 2023_02 of 03 May 2023) from *Acidithiobacillus caldus* ATCC 51756 or any protein sequence comprising a sequence identity of 80% or more to any one of the sucrose synthase (Uniprot ID P13708, sequence version 2023_02 of 03 May 2023) from *Glycine max*, the sucrose synthase (Uniprot ID P49040, sequence version 2023_02 of 03 May 2023) from *Arabidopsis thaliana*, the sucrose synthase (Uniprot ID Q9ZEV2, sequence version 2023_02 of 03 May 2023) from *Anabaena* sp. PCC 7119 (ATCC no. 29151), the sucrose synthase (Uniprot ID A0A059ZV61, sequence version 2023_02 of 03 May 2023) from *Acidithiobacillus caldus* ATCC 51756 and having sucrose synthase activity.

Preferably said trehalose synthase is chosen from GT4 glycosyltransferase subfamily, or chosen from KEGG Enzyme class EC 2.4.1.245. Alternatively, said trehalose synthase is chosen from the list of trehalose synthase (Uniprot ID Q7LYW5, sequence version 2023_02 of 03 May 2023) from *Thermococcus bergensis* (strain T7324) and trehalose synthase (Uniprot ID O58762, sequence version 2023_02 of 03 May 2023) from *Pyrococcus horikoshii* (strain ATCC 700860 / DSM 12428 / JCM 9974 / NBRC 100139 / OT-3) or any protein sequence comprising a sequence identity of 80% or more to any one of the trehalose synthase (Uniprot ID Q7LYW5, sequence version 2023_02 of 03 May 2023) from *Thermococcus bergensis* (strain T7324), or trehalose synthase (Uniprot ID O58762, sequence version 2023_02 of 03 May 2023) from *Pyrococcus horikoshii* (strain ATCC 700860 / DSM 12428 / JCM 9974 / NBRC 100139 / OT-3) and having trehalose synthase activity.

Preferably said UDP-glucose-4-epimerase is chosen from enzyme class EC 5.1.3.2, or chosen from the list of GalE (Uniprot ID P09147, sequence version 2023_02 of 03 May 2023) from *Escherichia coli* K-12 MG1655, GalE (Uniprot ID E8MF10, sequence version 2023_02 of 03 May 2023) from *Bifidobacterium longum* subsp. *longum*, GalE (Uniprot ID A0A0K1E1Q5, sequence version 2023_02 of 03 May 2023) from *Pyrobaculum* sp. WP30, GalE (Uniprot ID Q0P9C3, sequence version 2023_02 of 03 May 2023) from *Campylobacter jejuni* subsp. *jejuni* serotype O:2 or any protein sequence comprising a sequence identity of 80% or more to any one of GalE (Uniprot ID P09147, sequence version 2023_02 of 03 May 2023) from *Escherichia coli* K-12 MG1655, GalE (Uniprot ID E8MF10, sequence version 2023_02 of 03 May 2023) from *Bifidobacterium longum* subsp. *longum*, GalE (Uniprot ID A0A0K1E1Q5, sequence version 2023_02 of 03 May 2023) from *Pyrobaculum* sp. WP30, GalE (Uniprot ID Q0P9C3, sequence version 2023_02 of 03 May 2023) from *Campylobacter jejuni* subsp. *jejuni* serotype O:2 and having UDP-glucose-4-epimerase activity.

Preferably said beta 1,4-galactosyltransferase is a lactose synthase or selected from Enzyme Class EC 2.4.1.87, GalT (Uniprot ID Q51116, sequence version 2023_02 of 03 May 2023) from *Neisseria meningitidis*, or GalT (UniProt ID D0EAD4 sequence version 2023_02 of 03 May 2023) of *Pasteurella multocida* M1404 or any protein with a sequence comprising a sequence identity of 80% or more to said GalT (Uniprot ID Q51116, sequence version 2023_02 of 03 May 2023) from *Neisseria meningitidis* or to said GalT (UniProt ID D0EAD4 sequence version 2023_02 of 03 May 2023) of *Pasteurella multocida* M1404.

10 Preferably said glucose isomerase is selected from the list of XylA (Uniprot ID P37031, sequence version 2023_02 of 03 May 2023) from *Streptomyces murinus*, XylA (Uniprot ID P12070, sequence version 2023_02 of 03 May 2023) from *Arthrobacter* sp. (strain NRRL B3728), XylA (Uniprot ID D2DK62, sequence version 2023_02 of 03 May 2023) from *Thermoanaerobacter ethanolicus* or any protein with a sequence comprising a sequence identity of 80% or more to any one of said XylA (Uniprot ID P37031, sequence version 2023_02 of 03 May 2023) from *Streptomyces murinus*, XylA (Uniprot ID P12070, sequence version 2023_02 of 03 May 2023) from *Arthrobacter* sp. (strain NRRL B3728), XylA (Uniprot ID D2DK62, sequence version 2023_02 of 03 May 2023) from *Thermoanaerobacter ethanolicus* and having glucose isomerase activity.

20 In a preferred embodiment of the process for biotechnological production of lactose the carbon source used for conversion by the synthase into lactose is chosen from the list of sucrose, trehalose, combination of sucrose and glucose, and even starch.

In a preferred embodiment of the process for biotechnological production of lactose, the process uses a synthase which is a sucrose synthase and the process uses sucrose to start the conversion from. Said sucrose is converted with said sucrose synthase into UDP-glucose and fructose. The formed UDP-glucose is converted into UDP-galactose. Said fructose is converted into glucose and said UDP-galactose and said glucose are then converted into lactose. An exemplary embodiment hereof can be found in figure 1.

30 In an alternative preferred embodiment of the process for biotechnological production of lactose, the process uses a synthase which is a trehalose synthase and the process uses trehalose to start the conversion from. Said trehalose is converted with said trehalose synthase into UDP-glucose and glucose. The formed UDP-glucose is converted into UDP-galactose and said UDP-galactose is then together with glucose converted into lactose. An exemplary embodiment hereof can be found in figure 2.

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In another alternative preferred embodiment of the process for biotechnological production of lactose,

the process uses a synthase which is a sucrose synthase and the process uses sucrose and glucose to start the conversion from. Said sucrose is converted with said sucrose synthase into UDP-glucose and fructose. The formed UDP-glucose is converted into UDP-galactose. No need to convert the fructose into glucose, because glucose is also present as carbon source. Said UDP-galactose and said glucose are then converted
5 into lactose. An exemplary embodiment hereof can be found in figure 3 wherein the carbon sources are underlined.

In a further preferred embodiment of the process for biotechnological production of lactose, the process is an enzymatic production of lactose and uses i) sucrose, ii) trehalose or iii) sucrose and glucose as a raw
10 material or carbon source. Lactose is then produced by using i) sucrose, ii) trehalose or iii) sucrose and glucose as a carbon source or raw material, and the reaction thereof is catalysed by a plurality of enzyme combinations. In one preferred embodiment all enzymatic reaction are performed in one pot. In another preferred embodiment, some enzymatic reactions are performed separate from the other reactions in the method.

15 Another process for biotechnological production of lactose can be found in WO2020048927, WO23121564, CN116496963, CN116064346 or CN117551713.

In a more preferred embodiment of the process for biotechnological production of lactose, when said
20 process is an enzymatic process at least one of said enzymes are immobilized. Preferably said at least one enzymes are immobilised on a carrier or immobilised by entrapping, adsorption or covalent binding. Said enzymes are immobilized by well known technologies such as by entrapping the enzymes ('Entrapped immobilized enzymes') wherein said enzyme is entrapped in for instance collagen, gelatin, cellulose triacetate, polyacrylamide, and/or j-carrageenan, by binding (Bound immobilized enzymes), by adsorption
25 ('Adsorbed bound immobilized enzymes') wherein said enzyme is adsorbed to polysaccharide derivatives, synthetic polymers, and porous glass, by covalent binding ('Covalently bound immobilized enzymes') wherein said enzyme is bound with glutaraldehyde, bisdiazoben-zidine, and hexamethylene, diisocyanate (see e.g. Rafiq Khan M, Bulletin of the National Research Centre 45, 207 (2021)).

30 In an alternative or more preferred embodiment of the process for biotechnological production of lactose, when said process is an enzymatic process as described herein at least one reaction is performed between 50°C and 80°C, preferably between 55°C and 70°C.

In a more preferred embodiment of the process for biotechnological production of lactose, when said
35 process is a fermentation process or a bioconversion, the enzymatic conversions are performed by at least one enzyme produced by a cell synthesizing said enzyme. Preferably, such process is performed at a

temperature between about 25°C and about 50°C, preferably between about 25°C and about 40°C.

In an alternative more preferred embodiment of the process for biotechnological production of lactose, when said process is a fermentation process, said lactose is produced by a cell genetically engineered to produce all enzymes needed for production of lactose.

According to a preferred embodiment of the present invention, the step of crystallisation of said lactose is preferably followed by a step of drying. The lactose crystals can then be dried by band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, vacuum drum drying, roller drying, vacuum roller drying and other types of drying.

According to a preferred embodiment of the present invention, the method as described herein comprises a step of drying and/or crystallisation of said lactose, preferably said step of drying comprises any one or more of spray drying, lyophilization, evaporation, precipitation, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying, vacuum roller drying, and agitated thin film drying.

According to a preferred embodiment of the present invention, the process for biotechnological production of lactose further comprises a step of purification of said lactose.

In a preferred aspect, the present invention provides a method to produce a crystalline lactose, wherein said lactose is produced via a biotechnological production process wherein said lactose is purified by at least one of the following purification steps: microfiltration, centrifugation, ultrafiltration, nanofiltration, ion exchange, Simulated Moving Bed (SMB), colour removal, removal of the carbon source, removal of sucrose, glucose or trehalose by addition of yeast, removal of trehalose by addition of trehalase, removal of sucrose by addition of an invertase, and preferably further comprises a concentration step, a drying step and/or a crystallization step as described herein. Preferably said ion exchange step is a cation and/or anion exchange step. In a preferred embodiment, said lactose is purified from the biotechnological production process resulting liquid comprising sucrose, glucose, trehalose, fructose, and/or glycerol.

According to a preferred embodiment of the present invention, the lactose purity before purification is < 70%, < 60%, < 50%, < 40%, < 30%, < 20%, < 10% on total solids, and/or the purity at the end of said method comprising purification is higher than > 80% on dry solids, preferably > 85% on dry solids, more preferably > 90% on dry solids, even more preferably > 95% on dry solids, even more preferably > 97% on dry solids, even more preferably > 98% on dry solids, most preferably > 99% on dry solids.

In a further preferred embodiment, purification of an enzymatically produced lactose comprises a step of a microfiltration or ultrafiltration to recover the used enzymes.

In a further preferred embodiment, cells obtained from said fermentation or bioconversion upon purification of said fermentation or bioconversion medium are reused in a further fermentation or a bioconversion process.

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In a further preferred embodiment of the present invention, the biotechnological production process as described herein can comprise i) a step of nanofiltration to remove/reduce monosaccharides and/or salts; and/or ii) a step of ion exchange to remove charged materials.

10 According to the invention as described herein the purified lactose preferably has an ash content below 1% on total solid, more preferably below 0,5% on total solid. According to an alternative or further preferred embodiment, the purified lactose has one or more of:

- a) Lead content lower than 0,1 mg/kg solid, preferably lower than 0,02 mg/kg solid;
- b) Arsenic content lower than 0,2 mg/kg solid, preferably lower than 0,02 mg/kg solid;
- 15 c) Cadmium content lower than 0,1 mg/kg solid, preferably lower than 0,01 mg/kg solid; or
- d) Mercury content was lower than 0,5 mg/kg solid, preferably lower than 0,1 mg/kg solid.

In another preferred embodiment according to the present invention, the purified lactose has at least one or more of: i) a protein content below 100 mg per kg dry solid, ii) DNA content below 10 ng per gram dry
20 solid and iii) endotoxin content below 10000 EU per gram dry solid.

The present invention thus provides a crystalline lactose product wherein said lactose product is derived from a biotechnological production process. Preferably the lactose is produced by any of the biotechnological production processes as described herein. More preferably, the biotechnological
25 production process is an enzymatic, bioconversion of fermentation process.

In a preferred embodiment, the lactose product as described herein is lactose monohydrate.

In a preferred embodiment, the lactose product as described herein has a purity of at least 90% on dry mass, preferably at least 94% on dry mass.

30 In an additional or alternative preferred embodiment, the lactose product as described herein is free of milk protein. Such milk protein can be milk protein originating from bovine, horse, sheep, goat, buffalo, ...

Based on the description, the embodiments and the examples in the present invention, all other
35 embodiments obtained by those skilled in the art without inventive effort fall within the scope of protection of the present invention.

In summary, the process for producing lactose of the present invention is completely different from the known processes for producing lactose, and the final product lactose can be used in industries such as food products.

- 5 In an embodiment, the crystalline lactose product obtained as described herein can be used in medicine, preferably used in prophylaxis or therapy of a gastrointestinal disorder.

The present invention provides for use of the crystalline lactose obtained as described herein in a food or feed preparation, in a dietary supplement, in a cosmetic ingredient or in a pharmaceutical ingredient.

- 10 Furthermore, the present invention provides for use of the crystalline lactose as described herein in a food or feed preparation, in a dietary supplement, in a cosmetic ingredient or in a pharmaceutical ingredient.

The present invention provides for use as described herein, wherein the food is a human food, preferably infant food and/or an infant formula or an infant supplement.

- 15 Furthermore, the present invention provides for use of a crystalline lactose as described herein as additive in food, preferably as additive in human food and/or pet food, more preferably as additive in human baby food.

The present invention provides for use as described herein wherein the feed is a pet food, animal milk replacer, veterinary product, post weaning feed, or creep feed.

- 20 Further advantages follow from the specific embodiments and the examples. It goes without saying that the abovementioned features and the features which are still to be explained below can be used not only in the respectively specified combinations, but also in other combinations or on their own, without departing from the scope of the present invention.

- 25 The present invention relates to following specific embodiments:

1. A method for the production of lactose, characterised in that said method comprises use of i) a synthase and ii) UDP, for forming UDP-glucose.
2. The method according to embodiment 1 wherein UDP is provided in catalytic amounts, preferably UDP is provided in a concentration between 0,1mM and 10mM.
- 30 3. The method according to embodiment 1, said method further comprising use of an UDP-glucose-4-epimerase to convert said UDP-glucose to UDP-galactose.
4. The method according to any one of embodiment 1 or 2 wherein said UDP-galactose is a galactose donor for a beta 1,4-galactosyltransferase to form lactose.
5. The method according to any one of embodiment 1 to 3, said method further comprising use of a
- 35 beta 1,4-galactosyltransferase to convert glucose with UDP-galactose to form lactose and UDP.
6. The method according to any one of embodiment 1 to 5, wherein the UDP released by the beta 1,4-

galactosyltransferase is recycled to be used by the synthase to form UDP-glucose.

7. The method according to any one of embodiment 1 to 6, wherein said synthase further forms either glucose or fructose.
8. The method according to any one of embodiment 1 to 7, wherein said synthase is a sucrose synthase or a trehalose synthase.
9. The method according to any one of embodiment 1 to 8, wherein said beta 1,4-galactosyltransferase uses glucose as an acceptor and UDP-galactose as a donor.
10. The method according to any one of embodiment 8 to 9, wherein said fructose is converted to glucose.
11. The method according to any one of embodiment 1 to 10, wherein said method is a microbial fermentation process, a bioconversion, or an enzymatic process.
12. The method according to any one of embodiment 1 to 11, wherein said method is an enzymatic process and wherein at least one of said enzymes are immobilised, preferably said enzymes are immobilised on a carrier or immobilised by entrapping, adsorption or covalent binding.
13. The method according to any one of embodiment 1 to 12, wherein said method is an enzymatic process and wherein the reaction is performed between 50°C and 80°C, preferably between 55°C and 70°C.
14. The method according to any one of embodiment 1 to 11, wherein said method is a microbial fermentation process or a bioconversion characterised in that the enzymatic conversions are performed by at least one enzyme produced by a cell synthesizing said enzyme.
15. The method according to any one of embodiment 1 to 11, wherein said method is a microbial fermentation process characterised in that said lactose is produced by a cell genetically engineered to produce all enzymes needed for production of lactose.
16. The method according to any one of the previous embodiments, wherein the carbon source used for conversion by the synthase is chosen from the list of sucrose, trehalose, combination of sucrose and glucose.
17. The method according to any one of embodiment 11 to 13, for enzymatic production of lactose using i) sucrose, ii) trehalose or iii) sucrose and glucose as a raw material or carbon source, characterized in that lactose is produced by using i) sucrose, ii) trehalose or iii) sucrose and glucose as a raw material or carbon source, and the reaction thereof is catalysed by a plurality of enzyme combinations.
18. The method according to any one of embodiment 1 to 17, wherein said synthase is a trehalose synthase and wherein i) trehalose is converted with said trehalose synthase into UDP-glucose and glucose, and ii) said UDP-glucose is converted into UDP-galactose and iii) glucose and UDP-galactose are converted into lactose.
19. The method according to any one of embodiment 1 to 17, wherein said synthase is a sucrose synthase and wherein i) sucrose is converted with said sucrose synthase into UDP-glucose and fructose, ii) said

UDP-glucose is converted into UDP-galactose and iii) fructose is converted into glucose and iv) said UDP-galactose from step ii) and said glucose from step iii) are converted into lactose.

20. The method according to any one of embodiment 1 to 17, wherein said synthase is a sucrose synthase and the carbon source is sucrose with glucose, wherein i) sucrose is converted with said sucrose synthase into UDP-glucose and fructose, ii) said UDP-glucose is converted into UDP-galactose and iii) said UDP-galactose from step ii) and said glucose which was provided together with the sucrose are converted into lactose.
21. The method according to any one of embodiments 4, 5 or 9 wherein said beta 1,4-galactosyltransferase is a lactose synthase.
22. The method according to any one of the previous embodiments, wherein said method further comprises a step of separating said lactose.
23. The method according to any one of the previous embodiments, wherein said method further comprises a step of purification of said lactose.
24. A method to purify an enzymatically or biotechnologically synthesised lactose wherein said lactose is purified by purification steps selected from a microfiltration step, a centrifugation step, an ultrafiltration step, a nanofiltration step, an ion exchange step, preferable a cation and/or anion exchange step, a Simulated Moving Bed step, a colour removal step, a step of removal of sucrose, glucose or trehalose by addition of yeast, a step of removal of trehalose by addition of trehalase, a step of removal of sucrose by addition of an invertase, and preferably further comprises a concentration step, a spray drying step and/or a crystallization step.
25. The method according to any one of the previous embodiments, wherein said method comprises a step of drying or crystallisation of said lactose, preferably said step of drying comprises any one or more of spray drying, lyophilization, evaporation, precipitation, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying, vacuum roller drying, and agitated thin film drying.
26. The method according to any one embodiment 23 to 25, wherein the lactose purity before purification is < 70%, < 60%, < 50%, < 40%, < 30%, < 20%, < 10% on total dry solids, and/or the purity at the end of said method comprising purification is higher than > 80% on total dry solids, preferably > 85% on total dry solids, more preferably > 90% on total dry solids, even more preferably > 95% on total dry solids, even more preferably > 97% on total dry solids, even more preferably > 98% on total dry solids, most preferably > 99% on total dry solids.
27. The method according to any one of embodiment 23 to 26, wherein said purification of an enzymatically synthesised lactose comprises a step of a microfiltration or ultrafiltration to recover the used enzymes.
28. The method according to any one of embodiment 23 to 27, wherein said purification of a biotechnologically synthesised lactose comprises a step of centrifugation, microfiltration and/or

ultrafiltration to remove biomass.

29. A method according to any of one of embodiment 23 to 28, comprising i) a step of nanofiltration to remove/reduce monosaccharides and/or salts; and/or ii) a step of ion exchange to remove charged materials.
- 5 30. The method according to any one of embodiment 23 to 29, wherein said purified lactose has an ash content below 1% on total dry solid, preferably below 0,5% on total dry solid, preferably with one or more of:
- 10 a) Lead content lower than 0,1 mg/kg dry solid, preferably lower than 0,02 mg/kg dry solid;
b) Arsenic content lower than 0,2 mg/kg dry solid, preferably lower than 0,02 mg/kg dry solid;
c) Cadmium content lower than 0,1 mg/kg dry solid, preferably lower than 0,01 mg/kg dry solid; or
d) Mercury content was lower than 0,5 mg/kg dry solid, preferably lower than 0,1 mg/kg dry solid.
31. The method according to any one of embodiment 23 to 30, wherein said purified lactose has at least one or more of: i) a protein content below 100 mg per kg dry solid, ii) DNA content below 10 ng per gram dry solid and iii) endotoxin content below 10000 EU per gram dry solid.

15

The present invention relates to following specific preferred embodiments:

1. A method to produce a crystalline lactose, wherein said lactose is produced via a biotechnological production process wherein said method comprises a step of drying and/or crystallisation of said lactose.
- 20 2. The method according to preferred embodiment 1, wherein said lactose is purified by purification steps selected from a microfiltration step, a centrifugation step, an ultrafiltration step, a nanofiltration step, an ion exchange step, a Simulated Moving Bed step, a colour removal step, a step of removal of sucrose, glucose or trehalose by addition of yeast, a step of removal of trehalose by addition of trehalase, a step of removal of sucrose by addition of an invertase; and preferably further
- 25 comprises a concentration step; preferable said ion exchange step is a cation and/or anion exchange step, and/or a mixed bed ion exchange step.
3. The method according to any one of the preceding preferred embodiments, wherein said biotechnological production process is a fermentation process, a bioconversion process or an enzymatic process.
- 30 4. The method according to preferred embodiment 3, wherein said fermentation or bioconversion is followed by a biomass removal step.
5. The method according to preferred embodiment 3, wherein said biotechnological production process is an enzymatic process and wherein at least one enzyme is immobilised.
6. The method according to any one of preferred embodiment 1 to 3 or 5, wherein said biotechnological
- 35 production process is an enzymatic process and wherein the reaction is performed between 50°C and 80°C, preferably between 55°C and 70°C.

7. The method according to any one of preferred embodiment 1 to 4, wherein said biotechnological production process is a fermentation process or a bioconversion process characterised in that enzymatic conversions are performed by at least one enzyme produced by a cell synthesizing said enzyme.
- 5 8. The method according to preferred embodiment 7, wherein said fermentation process or bioconversion process is performed at a temperature between about 25°C and about 50°C, preferably between about 25°C and about 40°C.
9. The method according to any one of preferred embodiment 1 to 4, 7 or 8, wherein said biotechnological production process is a fermentation process characterised in that said lactose is
10 produced by a cell genetically engineered to produce all enzymes necessary for production of lactose.
10. The method according to any one of the previous preferred embodiments, wherein said lactose is purified from the biotechnological production process resulting liquid comprising sucrose, glucose, trehalose, fructose, and/or glycerol.
11. The method according to any one preferred embodiment 2 to 10, wherein the lactose purity before
15 purification is < 70%, < 60%, < 50%, < 40%, < 30%, < 20%, < 10% on total solids, and/or the purity at the end of said method comprising purification is higher than > 80% on dry solids, preferably > 85% on dry solids, more preferably > 90% on dry solids, even more preferably > 95% on dry solids, even more preferably > 97% on dry solids, even more preferably > 98% on dry solids, most preferably > 99% on dry solids.
- 20 12. The method according to any one of preferred embodiment 2, 3, 5, 6, 10 or 11, wherein said purification of an enzymatically produced lactose comprises a step of a microfiltration or ultrafiltration to recover the used enzymes.
13. The method according to any one of preferred embodiment 2 to 4, 7 to 10, wherein said purification of said lactose produced by a fermentation or a bioconversion process comprises a step of
25 centrifugation, microfiltration and/or ultrafiltration to remove biomass.
14. The method according to any one of preferred embodiment 2 to 4, 7 to 11 or 13 wherein cells removed from said fermentation or bioconversion are reused in a further fermentation or a bioconversion process.
15. A method according to any of one of preferred embodiment 1 to 14, wherein said biotechnological
30 production process comprises i) a step of nanofiltration to remove/reduce monosaccharides and/or salts; and/or ii) a step of ion exchange to remove charged materials.
16. The method according to any one of preferred embodiment 1 to 15, wherein said lactose has an ash content below 1% on total solid, preferably below 0,5% on total solid, preferably with one or more of:
35 a) Lead content lower than 0,1 mg/kg solid, preferably lower than 0,02 mg/kg solid;
b) Arsenic content lower than 0,2 mg/kg solid, preferably lower than 0,02 mg/kg solid;

- c) Cadmium content lower than 0,1 mg/kg solid, preferably lower than 0,01 mg/kg solid; or
d) Mercury content was lower than 0,5 mg/kg solid, preferably lower than 0,1 mg/kg solid.
17. The method according to any one of preferred embodiment 1 to 16, wherein said lactose has at least one or more of: i) a protein content below 100 mg per kg dry solid, ii) DNA content below 10 ng per gram dry solid and iii) endotoxin content below 10000 EU per gram dry solid.
18. The method according to any one of preferred embodiment 1 to 17, wherein said lactose crystals are dried by band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, vacuum drum drying, roller drying, vacuum roller drying and other types of drying.
19. The method according to any one of preferred embodiment 1 to 17, said step of drying comprises any one or more of spray drying, lyophilization, evaporation, precipitation, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying, vacuum roller drying, and agitated thin film drying.
20. A crystalline lactose product wherein said lactose product is derived from a biotechnological production process, preferably produced by any of preferred embodiments 1 to 19.
21. The lactose product of preferred embodiments 20, wherein said lactose is lactose monohydrate.
22. The lactose product of any one of preferred embodiments 20 or 21, wherein said lactose has a purity of at least 90% on dry mass, preferably at least 94% on dry mass.
23. The lactose product of any one of preferred embodiments 20 to 22, wherein said lactose is free of milk protein.
24. The lactose product of any one of preferred embodiments 20 to 23, wherein said lactose has at least one or more of: i) a protein content below 100 mg per kg dry solid, ii) DNA content below 10 ng per gram dry solid and iii) endotoxin content below 10000 EU per gram dry solid.
25. The lactose according to any one of preferred embodiment 20 to 24, wherein said lactose has an ash content below 1% on total solid, preferably below 0,5% on total solid, preferably with one or more of:
- a) Lead content lower than 0,1 mg/kg solid, preferably lower than 0,02 mg/kg solid;
b) Arsenic content lower than 0,2 mg/kg solid, preferably lower than 0,02 mg/kg solid;
c) Cadmium content lower than 0,1 mg/kg solid, preferably lower than 0,01 mg/kg solid; or
d) Mercury content was lower than 0,5 mg/kg solid, preferably lower than 0,1 mg/kg solid.
26. Crystalline lactose product according to any one of preferred embodiment 20 to 25 for use in medicine, preferably for use in prophylaxis or therapy of a gastrointestinal disorder.
27. Use of the crystalline lactose obtained according to the process of any one of preferred embodiment 1 to 19 in a food or feed preparation, in a dietary supplement, in a cosmetic ingredient or in a pharmaceutical ingredient.
28. Use of the crystalline lactose according to any one of preferred embodiment 20 to 25 in a food or feed preparation, in a dietary supplement, in a cosmetic ingredient or in a pharmaceutical ingredient.

29. Use according to any one of preferred embodiment 27 or 28, wherein the food is a human food, preferably infant food and/or an infant formula or an infant supplement.
30. Use of a crystalline lactose according to any one of preferred embodiment 20 to 25 as additive in food, preferably as additive in human food and/or pet food, more preferably as additive in human baby food.
31. Use according to any one of preferred embodiment 27 or 28, wherein the feed is a pet food, animal milk replacer, veterinary product, post weaning feed, or creep feed.

Examples

10 **Example 1: Materials and Methods**

Analytical analysis

Standards such as but not limited to sucrose, lactose, glucose, fructose, UDP-glucose, UDP-galactose were purchased from Sigma, Carbosynth (UK), Elicityl (France) and IsoSep (Sweden). Other compounds were analyzed with in-house made standards.

- 15 The carbohydrates were analyzed on a Waters Acquity H-class UPLC with Evaporative Light Scattering Detector (ELSD) or a Refractive Index (RI) detection. A volume of 0.7 μ L sample was injected on a Waters Acquity UPLC BEH Amide column (2.1 x 100 mm; 130 Å; 1.7 μ m) column with an Acquity UPLC BEH Amide VanGuard column, 130 Å, 2.1x 5 mm. The column temperature was 50 °C. The mobile phase consisted of a ¼ water and ¾ acetonitrile solution to which 0.2 % triethylamine was added. The method was isocratic
- 20 with a flow of 0.130 mL/min. The ELS detector had a drift tube temperature of 50 °C and the N₂ gas pressure was 50 psi, the gain 200 and the data rate 10 pps. The temperature of the RI detector was set at 35 °C.

Ash content

- 25 The ash content is a measure of the total amount of minerals present within a food or ingredients such as oligosaccharides, whereas the mineral content is a measure of the amount of specific inorganic components present within a food, such as Ca, Na, K, Mg, phosphate, sulphate and Cl. Determination of the ash and mineral content of foods or oligosaccharides is important for a number of reasons: Nutritional labeling. The concentration and type of minerals present must often be stipulated on the label of a food
- 30 or ingredient such as oligosaccharides. The quality of many foods depends on the concentration and type of minerals they contain, including their taste, appearance, texture and stability. Microbiological stability. High mineral contents are sometimes used to retard the growth of certain microorganisms. Nutrition. Some minerals are essential to a healthy diet (*e.g.*, calcium, phosphorous, potassium and sodium) whereas others can be toxic (*e.g.*, lead, mercury, cadmium and aluminum). Processing. It is often important to
- 35 know the mineral content of foods/products during processing because this affects the physicochemical properties of foods or ingredient such as oligosaccharides.

Ash is the inorganic residue remaining after the water and organic matter have been removed by heating in the presence of oxidizing agents, which provides a measure of the total amount of minerals within a food. Analytical techniques for providing information about the total mineral content are based on the fact that the minerals (the analyte) can be distinguished from all the other components (the matrix) within a food or ingredient in some measurable way. The most widely used methods are based on the fact that minerals are not destroyed by heating, and that they have a low volatility compared to other food components. The three main types of analytical procedure used to determine the ash content of foods are based on this principle: *dry ashing*, *wet ashing* and *low temperature plasma dry ashing*. The method chosen for a particular analysis depends on the reason for carrying out the analysis, the type of food or ingredient analyzed and the equipment available. Ashing may also be used as the first step in preparing samples for analysis of specific minerals, by atomic spectroscopy or the various traditional methods described below.

For the sample preparation a sample whose composition represents that of the ingredient is selected to ensure that its composition does not change significantly prior to analysis. For instance a dry oligosaccharide sample is generally hygroscopic and the selected sample should be kept under dry conditions avoiding the absorption of water. Typically, samples of 1-10g are used in the analysis of ash content. Solid ingredients are finely ground and then carefully mixed to facilitate the choice of a representative sample. Before carrying out an ash analysis, samples that are high in moisture or in solution are generally dried to prevent spattering during ashing. Other possible problems include contamination of samples by minerals in grinders, glassware or crucibles which come into contact with the sample during the analysis. For the same reason, deionized water is used when preparing samples and the same is used in the blank sample.

Dry ashing procedures use a high temperature muffle furnace capable of maintaining temperatures of between 500 and 600 °C. Water and other volatile materials are vaporized and organic substances are burned in the presence of the oxygen in air to CO₂, H₂O and N₂. Most minerals are converted to oxides, sulfates, phosphates, chlorides or silicates. Although most minerals have fairly low volatility at these high temperatures, some are volatile and may be partially lost, *e.g.*, iron, lead and mercury, for these minerals ICP-MS analysis of the product is more appropriate for quantification.

The food sample is weighed before and after ashing to determine the concentration of ash present. The ash content can be expressed on dry basis is calculated by dividing the mass of the ashed material, ingredient, or food by the mass of the dry material, ingredient, or food before ashing. Multiplied with 100, this gives the percentage of ash in the the material, ingredient, or food. In a similar way the wet ash

percentage can be determined for liquid products, wherein the mass of the liquid before and after ashing is used instead of the mass of the dry material, ingredient, or food.

Heavy metal determination

5 A robust general inductively coupled plasma-mass spectrometry (ICP-MS) based method was used for the detection and quantitation for each of the following elements: arsenic (As), selenium (Se), cadmium (Cd), tin (Sn), lead (Pb), silver (Ag), palladium (Pd), platinum (Pt), mercury (Hg), molybdenum (Mo), sodium (Na), potassium (K), Calcium (Ca), Magnesium (Mg), Iron (Fe), zinc (Zn), manganese (Mn), Phosphorus (P), selenium (Se).

10

Nitric acid ($\geq 65\%$, Sigma-Aldrich) was used for microwave digestion and standard/sample preparation. All dilutions were done using 18.2 M Ω -cm (Millipore, Bedford, MA, USA) de-ionized water (DIW). About 0.2 g of each oligosaccharide, ingredient, sample were digested in 5 mL of HNO₃ using the microwave digestion (CEM, Mars 6) program 15 minutes (min) ramping time and 15 min holding time at 100W and 50°C followed by 15 min ramping time and 20 min holding time at 1800 W and 210°C. The samples were cooled after digestion for 30 minutes. 1. The fully digested samples were then diluted to 50 mL with DIW.

Analyses were carried out using a standard Agilent 7800 ICP-MS, which includes the fourth-generation ORS cell system for effective control of polyatomic interferences using helium collision mode (He mode). The ORS controls polyatomic interferences using He to reduce the transmission of all common matrix-based polyatomic interferences. Smaller, faster analyte ions are separated from larger, slower interference-ions using kinetic energy discrimination (KED). All elements, except Se, were measured in He mode with a flow rate of 5 mL/min. Se was measured in High Energy He (HEHe) mode, using a cell gas flow rate of 10 mL/min. The 7800 ICP-MS was configured with the standard sample introduction system consisting of a MicroMist glass concentric nebulizer, quartz spray chamber, quartz torch with 2.5 mm i.d. injector, and nickel interface cones. The ICP-MS operating conditions are: 1550 W RF power, 8mm sampling depth, 1.16 l/min nebulizing gas, autotuned lens tuning, 5 or 10 ml/min helium gas flow, 5 V KED.

Dry matter and moisture content quantification

Sartorius MA150 Infrared Moisture Analyzer is used to determine the dry matter content of the oligosaccharides. 0.5g of oligosaccharide is weighed on an analytical balance and is dried in the infrared moisture analyzer until the weight of the sample is stable. The mass of the dried sample divided by the mass of the sample before drying gives the dry matter content (in percent) of the oligosaccharides or sample including oligosaccharides. In a similar way a liquid sample is weighed, however, the amount of liquid weighed is adapted to the expected amount of dry matter in the liquid, so the mass of the dry

matter is properly measurable on an analytical balance.

A moisture analyser measures the dry matter, but not the water content. Karl Fisher titration is used to determine the amount of water present in a powder, ingredient of food. The KF titration is carried out with a Karl Fischer titrator DL31 from Mettler Toledo using the two-component technique with Hydra-Point Solvent G and Hydra-Point titrant (5 mg H₂O/ml), both purchased from J.T. Baker (Deventer, Holland). The polarising current for bipotentiometric end-point determination was 20 microA and the stop voltage 100 mV. The end-point criterion was the drift stabilisation (15 micro gram H₂O min⁻¹) or maximum titration time (10 min).

The moisture content (MC) of sample was calculated using the following equation:

$$MC = V_{KF} W_{eq} 100 / W_{sample}$$
 ; where V_{KF} is the consumption of titrant in mL, W_{eq} the titer of titrant in mg H₂O/mL and W_{sample} the weight of sample in mg.

Protein quantification

For protein quantification a method is used that is compatible with reducing agents, such as reducing sugars or oligosaccharides with a reducing end. To this end, a Bradford assay (Thermo Scientific, Pierce) was used with a linear range between 1 and 1500 µg/ml. The assay was calibrated with a standard curve of BSA. The protein content of dried oligosaccharide products was quantified by dissolving a pre-weighed quantify in 18.2 MΩ·cm (Millipore, Bedford, MA, USA) de-ionized water (DIW) up to a quantity of 50% (m/v). The amount of protein is measured at 595 nm and converted to concentration with the calibration curve based on BSA.

DNA quantification

Production host specific DNA residue is quantified by RT-qPCR, for which specific primers on the host are designed so that residual DNA of the production host is amplified. The RT-qPCR was performed according to the standard protocol of a kit obtained from Sigma and was based on SYBR Green detection.

Total DNA is measured by means of a Threshold assay (Molecular Devices), based on an immunoassay allowing to measure as low as 2 pg of DNA in a sample in solution. Double stranded DNA is measured by means of SpectraMax® Quant™ AccuBlue™ Pico dsDNA Assay Kit (Molecular Devices) having a linear range between 5 pg and 3 ng of dsDNA.

Endotoxin measurement

Endotoxin in the liquid was measured by means of a LAL test.

A. *Escherichia coli*Media, cultivation and cell lysis

The Luria Broth (LB) medium consisted of 1% tryptone peptone (Difco, Erembodegem, Belgium), 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). The minimal medium used in

5 cultivation experiments in 96-well plates or in shake flasks contained 2.00 g/L NH_4Cl , 5.00 g/L $(\text{NH}_4)_2\text{SO}_4$, 2.993 g/L KH_2PO_4 , 7.315 g/L K_2HPO_4 , 8.372 g/L MOPS, 0.5 g/L NaCl, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 g/L sucrose or 30 g/L glycerol, 1 ml/L vitamin solution, 100 μL /L molybdate solution, and 1 mL/L selenium solution. The minimal medium was set to a pH of 7 with 1M KOH. Vitamin solution consisted of 3.6 g/L $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 5.0 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.3 g/L $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.38 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.5 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.94 g/L ZnCl_2 , 0.0311 g/L

10 H_3BO_4 , 0.4 g/L $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 1.01 g/L thiamine.HCl. The molybdate solution contained 0.967 g/L $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$. The selenium solution contained 42 g/L SeO_2 . The minimal medium for fermentations contained 6.75 g/L NH_4Cl , 1.25 g/L $(\text{NH}_4)_2\text{SO}_4$, 2.93 g/L KH_2PO_4 and 7.31 g/L KH_2PO_4 , 0.5 g/L NaCl, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 30 g/L sucrose or 30 g/L glycerol, 1 mL/L vitamin solution, 100 μL /L molybdate solution, and 1 mL/L selenium solution with the same composition as described above. Complex medium was sterilized

15 by autoclaving (121°C, 21 min) and minimal medium by filtration (0.22 μm Sartorius). When necessary, the medium was made selective by adding an antibiotic: e.g., chloramphenicol (20 mg/L), carbenicillin (100 mg/L), spectinomycin (40 mg/L) and/or kanamycin (50 mg/L). When necessary, inducer was added.

For fermentative production, a preculture of 96-well microtiter plate experiments was started from a

20 cryovial, in 150 μL LB and was incubated overnight at 37 °C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96well square microtiter plate, with 400 μL minimal medium by diluting 400x. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm for 72h, or shorter, or longer. To measure sugar concentrations at the end of the cultivation experiment whole broth samples were taken from each well by boiling the culture broth for 15 min at 60°C before spinning down

25 the cells (= average of intra- and extracellular sugar concentrations).

A preculture for the bioreactor was started from an entire 1 mL cryovial of a certain strain, inoculated in 250 mL or 500 mL minimal medium in a 1 L or 2.5 L shake flask and incubated for 24 h at 37°C on an orbital shaker at 200 rpm. A 5 L bioreactor was then inoculated (250 mL inoculum in 2 L batch medium); the

30 process was controlled by MFCS control software (Sartorius Stedim Biotech, Melsungen, Germany). Culturing condition were set to 37 °C, and maximal stirring; pressure gas flow rates were dependent on the strain and bioreactor. The pH was controlled at 6.8 using 0.5 M H_2SO_4 and 20% NH_4OH . The exhaust gas was cooled. 10% solution of silicone antifoaming agent was added when foaming raised during the fermentation.

35

For heterologous expression of enzyme, a preculture of 96-well microtiter plate experiments was started

- from a cryovial, in 175 μ L LB and was incubated overnight at 37°C on an orbital shaker at 800 rpm. This culture was used as inoculum for a 96well deep well microtiter plate, with 1 mL LB medium by diluting 100x. These final 96-well culture plates were then incubated at 37°C on an orbital shaker at 800 rpm, induced if necessary, and further grown at 16-37°C for 5-24h. After expression, DW plates were
- 5 centrifuged for 30 min at 4200 rpm and 4°C, the supernatant was discarded and the pellets were frozen at -80°C for at least one hour. Pellets were subsequently resuspended in buffer containing 1 mg/ml of lysozyme, 10 U/mL DNase and 1 mM protease inhibitor and incubated for 30 min at 37°C. Cell lysates were optionally cleared by centrifuging the plates (30 min at 4200 rpm and 4°C) and transferring the supernatant to new plates.
- 10 A preculture of a shake flask experiment was started from a cryovial in 5 mL LB medium and was incubated overnight at 37°C on an orbital shaker at 200 rpm. This culture was used as inoculum for a shake flask with 50-250 mL LB medium by diluting 50x. These final cultures were then incubated at 37°C on an orbital shaker at 200 rpm, induced if necessary, and further grown at 16-37°C for 5-24h, or longer or shorter. After expression, cells were harvest by centrifuging for 30 min at 4200 rpm and 4°C. The supernatant was
- 15 discarded and the pellets were frozen at -20°C for at least one hour. Pellets were resuspended and lysed by sonication. Cell lysates were optionally cleared by centrifuging (30 min at 4200 rpm and 4°C) and the supernatant was transferred to a new recipient.
- A preculture for the bioreactor was started from an entire 1 mL cryovial of a certain strain, inoculated in
- 20 250 mL or 500 mL LB medium in a 1 L or 2.5 L shake flask and incubated for 24 h at 37°C on an orbital shaker at 200 rpm. A 5 L bioreactor was then inoculated (250 mL inoculum in 2 L batch medium); the process was controlled by MFCS control software (Sartorius Stedim Biotech, Melsungen, Germany). Culturing condition were set to 25-37°C, and maximal stirring; pressure gas flow rates were dependent on the strain and bioreactor. The pH was controlled at 6.8 using 0.5 M H₂SO₄ and 20% NH₄OH. The exhaust
- 25 gas was cooled. 10% solution of silicone antifoaming agent was added when foaming raised during the fermentation. A soft release of the product was established by physically disrupting cells by means of sonication. Other commonly used methods known in the art are methods such as, freeze thawing and/or shear stress through mixing, a homogenizer and/or French press.
- 30 Cells for whole cell biotransformation were obtained by centrifuging overnight cultures (produced as described above) for 30 min at 4200 rpm and 4°C. The obtained pellets were subsequently resuspended in 0.9% NaCl solution and transferred to a 50 mL tube and centrifuged for 30 min at 4200 rpm and 4°C. The supernatant was removed and 1000 mg of each pellet was taken and resuspended in 2 mL of 0,9% NaCl (cell concentration is 0.5 g/mL). Optionally, the cells could be permeabilized (e.g. xylene).

Strains and mutations

Escherichia coli K12 MG1655 [λ -, F-, rph-1] was obtained from the Coli Genetic Stock Center (US), CGSC Strain#: 7740, in March 2007 and *Escherichia coli* NiCo21 (DE3) from New England Biolabs, in November 2017. Gene disruptions, gene introductions and gene replacements were performed using the technique published by Datsenko and Wanner (PNAS 97 (2000), 6640-6645). All constitutive promoters, UTRs and terminator sequences originated from the libraries described by Cambray et al. (Nucleic Acids Res. 2013, 41(9), 5139-5148), Dunn et al. (Nucleic Acids Res. 1980, 8, 2119-2132), Edens et al. (Nucleic Acids Res. 1975, 2, 1811-1820), Kim and Lee (FEBS Letters 1997, 407, 353-356) and Mutalik et al. (Nat. Methods 2013, No. 10, 354-360). Genes were ordered synthetically at Twist Bioscience (twistbioscience.com) or IDT (eu.idtdna.com) and the codon usage was adapted using the tools of the supplier. All strains were stored in cryovials at -80°C (overnight LB culture mixed in a 1:1 ratio with 70% glycerol).

E. coli K12 MG1655 or NiCo21(DE3) was modified with a knock-out of the *E. coli* gene coding for beta galactosidase, e.g. *lacZ*, in case of lactose production through whole cell biotransformation, cell extract(s), enzymatic synthesis with partially purified enzymes or fermentative production. Knock out of *lacZ* might not be a requirement in case of enzymatic synthesis of lactose with purified enzymes. For fermentative production the strain is further modified with an appropriate sugar importer, e.g. *cscB* for sucrose. The strain could additionally be modified by for instance knocking out proteases (e.g. *lon*, *OmpT*), nucleotide sugar degrading enzymes (e.g. *ushA*) or glucose consuming enzymes (e.g. *glk*). Optionally, the strain could also be modified by overexpressing an exporter (e.g. *setA*) to excrete the lactose from the cell.

B. *Saccharomyces cerevisiae*

Media, cultivation and cell lysis

Strains were grown on Synthetic Defined yeast medium with Complete Supplement Mixture (SD CSM) or CSM drop-out (SD CSM-Ura, SD CSM-Trp, SD CSM-His) containing 6.7 g/L Yeast Nitrogen Base without amino acids (YNB w/o AA, Difco), 20 g/L agar (Difco) (solid cultures), 22 g/L sucrose monohydrate or glucose, or fructose and 0.79 g/L CSM or 0.77 g/L CSM-Ura, 0.77 g/L CSM-Trp, or 0.77 g/L CSM-His (MP Biomedicals). In general, yeast strains were initially grown on SD CSM plates to obtain single colonies. These plates were grown for 2-3 days at 30°C. Starting from a single colony, a preculture was grown overnight in 5 mL at 30°C, shaking at 200 rpm. Subsequent 125 mL shake flask experiments were inoculated with 2% of this preculture, in 25 mL media. These shake flasks were incubated at 30°C with an orbital shaking of 200 rpm. A soft release of the product was established by physically disrupting cells by means of sonication. Other commonly used methods known in the art are methods such as, freeze thawing and/or shear stress through mixing, a homogenizer and/or French press.

Strains, plasmids and mutations

S. cerevisiae BY4742 created by Brachmann et al. (Yeast (1998) 14:115-32) was used, available in the Euroscarf culture collection. All mutant strains were created by homologous recombination or plasmid transformation using the method of Gietz (Yeast 11:355-360, 1995). Genes were expressed using synthetic constitutive promoters, as described by e.g., Blazeck (Biotechnology and Bioengineering, Vol. 109, No. 11, 2012), Redden and Alper (Nat. Commun. 2015, 6, 7810), Liu et al. (Microb. Cell Fact. 2020, 19, 38), Xu et al. (Microb. Cell Fact. 2021, 20, 148) and Lee et al. (ACS Synth. Biol. 2015, 4(9), 975-986).

C. *Bacillus subtilis*Media, cultivation and cell lysis

Two media are used to cultivate *B. subtilis*: i.e., a complex medium like a rich Luria Broth (LB) and a minimal medium for shake flask cultures. The LB medium consisted of 1% tryptone peptone (Difco), 0.5% yeast extract (Difco) and 0.5% sodium chloride (VWR). Luria Broth agar (LBA) plates consisted of the LB media, with 12 g/L agar (Difco) added. The minimal medium contained 2.00 g/L $(\text{NH}_4)_2\text{SO}_4$, 7.5 g/L KH_2PO_4 , 17.5 g/L K_2HPO_4 , 1.25 g/L Na-citrate, 0.25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 g/L tryptophan, from 10 up to 30 g/L glucose (or another carbon source including but not limited to fructose, maltose, sucrose, glycerol and maltotriose), 10 mL/L trace element mix and 10 mL/L Fe-citrate solution. The medium was set to a pH of 7 with 1 M KOH. The trace element mix consisted of 0.735 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1 g/L $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 0.033 g/L $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.06 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.17 g/L ZnCl_2 , 0.0311 g/L H_3BO_3 , 0.4 g/L $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ and 0.06 g/L Na_2MoO_4 . The Fe-citrate solution contained 0.135 g/L $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1 g/L Na-citrate (Hoch 1973 PMC1212887). Complex medium, e.g., LB, was sterilized by autoclaving (121°C, 21 min) and minimal medium by filtration (0.22 µm Sartorius). When necessary, the medium was made selective by adding an antibiotic. *B. subtilis* strains were initially grown on LB agar to obtain single colonies. These plates were grown over night at 37°C. Starting from a single colony, a preculture was grown over night in 5 mL at 37°C, shaking at 200 rpm. Subsequent 125 mL shake flask experiments were inoculated with 2% of this preculture, in 25 mL media. These shake flasks were incubated at 37°C with an orbital shaking of 200 rpm for 72h, or shorter or longer. At the end of the cultivation experiment samples were taken to measure the supernatant concentration (extracellular sugar concentrations, after 5 min. spinning down the cells), or by boiling the culture broth for 15 min at 90°C or for 60 min at 60°C before spinning down the cells (= whole broth concentration, i.e., intra- and extracellular sugar concentrations). A soft release of the product was established by physically disrupting cells by means of sonication. Other commonly used methods known in the art are methods such as, freeze thawing and/or shear stress through mixing, a homogenizer and/or French press.

Strains, plasmids and mutations

B. subtilis 168 is used as available at the Bacillus Genetic Stock Center (Ohio, USA). Plasmids for gene

deletion via Cre/lox are constructed as described by Yan et al. (Appl & Environm microbial, Sept 2008, p5556-5562). Gene disruption is done via homologous recombination with linear DNA and transformation via the electroporation as described by Xue et al. (J. microb. Meth. 34 (1999) 183-191). The method of gene knockouts is described by Liu et al. (Metab. Engine. 24 (2014) 61-69). Integrative vectors as described by Popp et al. (Sci. Rep., 2017, 7, 15158) are used as expression vector and could be further used for genomic integrations if necessary. A suitable promoter for expression can be derived from the part repository (iGEM): sequence id: BBa_K143012, BBa_K823000, BBa_K823002 or BBa_K823003. Cloning can be performed using Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation.

D. Corynebacterium glutamicum

Media, cultivation and cell lysis

Two different media are used, namely complex medium like e.g., a rich tryptone-yeast extract (TY) medium, and a minimal medium for shake flask (MMsf). The minimal medium uses a 1000x stock trace element mix. Trace element mix consisted of 10 g/L CaCl₂, 10 g/L FeSO₄·7H₂O, 10 g/L MnSO₄·H₂O, 1 g/L ZnSO₄·7H₂O, 0.2 g/L CuSO₄, 0.02 g/L NiCl₂·6H₂O, 0.2 g/L biotin (pH 7) and 0.03 g/L protocatechuic acid. The minimal medium for the shake flasks (MMsf) experiments contained 20 g/L (NH₄)₂SO₄, 5 g/L urea, 1 g/L KH₂PO₄, 1 g/L K₂HPO₄, 0.25 g/L MgSO₄·7H₂O, 42 g/L MOPS, from 10 up to 30 g/L glucose or another carbon source including but not limited to fructose, maltose, sucrose, glycerol and maltotriose when specified in the examples and 1 ml/L trace element mix. The TY medium consisted of 1.6% tryptone (Difco, Erembodegem, Belgium), 1% yeast extract (Difco) and 0.5% sodium chloride (VWR, Leuven, Belgium). TY agar (TYA) plates consisted of the TY media, with 12 g/L agar (Difco, Erembodegem, Belgium) added. Complex medium, e.g., TY, was sterilized by autoclaving (121°C, 21 min) and minimal medium by filtration (0.22 µm Sartorius). When necessary, the medium was made selective by adding an antibiotic.

A preculture was started from a cryovial or a single colony from a TY plate, in 6 mL TY and was incubated overnight at 37 °C on an orbital shaker at 200 rpm. Subsequent 125 mL shake flask experiments were inoculated with 2% of this preculture, in 25 mL MMsf medium. These shake flasks were incubated at 37°C with an orbital shaking of 200 rpm for 72h, or shorter or longer. At the end of the cultivation experiment samples were taken to measure the supernatant concentration (extracellular sugar concentrations, after 5 min. spinning down the cells), or by boiling the culture broth for 15 min at 90°C or for 60 min at 60°C before spinning down the cells (= whole broth concentration, i.e., intra- and extracellular sugar concentrations). A soft release of the product was established by physically disrupting cells by means of sonication. Other commonly used methods known in the art are methods such as, freeze thawing and/or shear stress through mixing, a homogenizer and/or French press.

Strains and mutations

Corynebacterium glutamicum was used as available at the American Type Culture Collection (ATCC 13032). Integrative plasmid vectors were made using the Cre/loxP technique as described by Suzuki et al.

(Appl. Microbiol. Biotechnol., 2005 Apr, 67(2):225-33) and temperature-sensitive shuttle vectors as described by Okibe et al. (Journal of Microbiological Methods 85, 2011, 155-163) are constructed for gene deletions, mutations and insertions. Suitable promoters for (heterologous) gene expression can be derived from Yim et al. (Biotechnol. Bioeng., 2013 Nov, 110(11):2959-69). Cloning can be performed using
 5 Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation.

E. Optical density

Cell density of the cultures was frequently monitored by measuring optical density at 600 nm (Implen Nanophotometer NP80, Westburg, Belgium or with a Spark 10M microplate reader, Tecan, Switzerland).
 10 The maximum growth speed (μ_{max}) was calculated based on the observed optical densities at 600nm using the R package grofit.

F. Growth rate/speed measurement

The maximal growth rate (μ_{max}) was calculated based on the observed optical densities at 600 nm using
 15 the R package grofit.

G. Heterologous and homologous expression

Genes that needed to be expressed, be it from a plasmid or from the genome were synthetically synthesized with one of the following companies: IDT or Twist Bioscience. Proteins described in present
 20 disclosure are summarized in Table 1. Unless stated otherwise, the UniProt IDs of the proteins described correspond to their sequence version 01 as present in the UniProt Database version release 2023_02 of 03 May 2023. Expression could be further facilitated by optimizing the codon usage to the codon usage of the expression host. Genes were optimized using the tools of the supplier.

25 *Table 1 Overview of genes and their UniProtID described in the present invention*

Enzyme	UniProt ID	Organism – Origin – Country of origin of digital sequence information
SuSy	P13708	<i>Glycine max</i> – Synthetic - unknown
SuSy	P49040	<i>Arabidopsis thaliana</i> – Synthetic - USA
SuSy	Q9ZEV2	<i>Anabaena</i> sp. PCC 7119 (ATCC no. 29151) – Synthetic - USA
SuSy	A0A059ZV61	<i>Acidithiobacillus caldus</i> ATCC 51756 – Synthetic - UK
GalE	P09147	<i>Escherichia coli</i> K-12 MG1655 – Synthetic - USA
GalE	E8MF10	<i>Bifidobacterium longum</i> subsp. <i>longum</i> (strain ATCC 15707 / DSM 20219 / JCM 1217 / NCTC 11818 / E194b) – Synthetic – unknown

GalE	A0A0K1E1Q5	<i>Pyrobaculum</i> sp. WP30 – Synthetic - USA
GalE	Q0P9C3	<i>Campylobacter jejuni</i> subsp. <i>jejuni</i> serotype O:2 (strain ATCC 700819 / NCTC 11168) – Synthetic - UK
GalT	Q51116	<i>Neisseria meningitidis</i> MC58 – Synthetic – UK
XylA	P37031	<i>Streptomyces murinus</i> – Synthetic – unknown
XylA	P12070	<i>Arthrobacter</i> sp. (strain NRRL B3728) – Synthetic - unknown
XylA	D2DK62	<i>Thermoanaerobacter ethanolicus</i> JW 200 – Synthetic - USA
treT	Q7LYW5 (GenPept ID WP_0040687 20)	<i>Thermococcus bergensis</i> strain T7324 – Synthetic - Norway
treT	O58762	<i>Pyrococcus horikoshii</i> (strain ATCC 700860 / DSM 12428 / JCM 9974 / NBRC 100139 / OT-3) – Synthetic – Japan
GalT	D0EAD4 (GenPept ID WP_0217343 30)	<i>Pasteurella multocida</i> M1404 – Synthetic - Canada

H. Analysis

To determine carbohydrates HPAEC-PAD system (Dionex) is used with a Dionex CarboPac PA1 Guard, 2 × 50 mm and Dionex CarboPac PA1 Separation, 2 × 250 mm is used with gradient of sodium acetate (1M) (A), sodium hydroxide (200mM) (B), water (C) and sodium acetate (25mM) (D), wherein the gradient from 0-10 min is 5% B, 88% C and 7% D, from 10-15 min changing B from 5% to 17% and C from 88% to 76%, from 15-25 min changing B from 17% to 93% and C from 76% to 0%, from 25-28.1 min changing A from 0% to 20% and B from 93% to 73%, from 28.1 min to 32 min maintaining the percentages of A, B C and D, from 32 to 32.1 min changing A from 20 to 0%, B from 73 to 0%, C from 0% to 88%, from 32.1 to 50 min maintaining the percentages of A, B, C, and D.

To determine nucleotide sugars the same system was used, albeit with a different gradient: sodium hydroxide (1 mM) (A) and sodium acetate (1M) in sodium hydroxide (1 mM) (B), wherein the gradient from 0-10 min changing A from 80% to 45% and B from 20% to 55%, from 10-25 min is 45% A and 55% B, from 25-35 changing A from 45% to 20% and B from 55% to 80%, from 35-40 min changing A from 20% to 0% and B from 80% to 100%, from 40-50 min is 0% A and 100% B, from 50-50.001 min changing A from 0% to 80% and B from 100% to 20% and from 50.001-60 min is 80% A and 20% B.

I. Enzyme purification

Cleared lysates containing N-terminal histidine-tagged enzymes in 50 mM Tris pH 7.0, 250 mM NaCl and 10 mM imidazole were applied on an ÄKTA Pure Protein Purification System, equipped with a Ni-NTA column. Bound protein was washed with five column volumes of wash buffer (50mM Tris, pH 7.0, 250 mM NaCl, 20-50 mM imidazole and eluted with elution buffer (50 mM Tris, pH 7.0, 250 mM NaCl, 250 mM imidazole) and the protein of interest was collected. Buffer exchange was afterwards performed on a Amicon Centrifugal Filter with the appropriate MW cutoff. Other commonly used purification methods known in the art are methods such as salting out, size exclusion chromatography, affinity chromatography, ion-exchange chromatography and/or any combination of the aforementioned methods.

J. SDS-PAGE and Western Blot

Three microL Laemmli 4x buffer containing 2-mercaptoethanol (1:10) was added to 9 microL of protein sample (final protein concentration of 5 mg/mL for cell extract or 0.05-0.5 mg/mL for purified protein) and heated for 5 min at 95°C. The protein samples were loaded together with a protein ladder and run at 120-220 V. After electrophoresis, gels were either stained (1 h in Coomassie Brilliant Blue R-250) or transferred to a blot membrane for Western Blot. The membrane with transferred protein was blocked for at least 30 minutes with 5% milk powder and 0.2% Tween 20, prior to the application of the primary antibody (anti-His). After 2 hours of incubation, the membrane was washed and HRP-linked secondary antibody was applied for one hour. Finally, the membrane was washed again and the his-tagged proteins were visualised by means of chemiluminescence.

Example 2: Expression of active sucrose synthase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the sucrose synthase (SuSy) from either *G. max* UniProt ID P13708, *A. thaliana* UniProt ID P49040, *Anabaena* sp. UniProt ID Q9ZEV2 or *A. caldus* UniProt ID A0A059ZV61. Expression of the enzymes was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis on the cleared cell lysates was performed as described in Example 1 and confirmed the expression of the target enzymes. Activity was verified by incubating 100 mM sucrose, 10 mM UDP and 50% (v/v) SuSy cell extract in 50 mM MOPS buffer (pH 7.0) for 24 h at 37°C and analysing the formation of UDP-glucose and fructose as described in Example 1.

Example 3: Expression of active UDP-glucose-4-epimerase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the UDP-glucose-4-epimerase from either *E. coli* UniProt ID P09147, *B. longum* UniProt ID E8MF10, *P. sp. WP30* UniProt ID A0A0K1E1Q5 or *C. jejuni* UniProt ID Q0P9C3.

Expression of the enzymes was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis on the cleared cell lysates was performed as described in Example 1 and confirmed the expression of the target enzymes. Activity was verified by incubating 10 mM UDP-glucose and 50% (v/v) GalE cell extract in 50 mM MOPS buffer (pH 6.5) for 24 h at 37°C and analysing the formation of UDP-galactose as described in Example 1.

Example 4: Expression of active beta 1,4-galactosyltransferase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the beta 1,4-galactosyltransferase (GalT) from *N. meningitidis* with UniProt ID Q51116. Expression of the enzyme was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis on the cleared cell lysate was performed as described in Example 1 and confirmed the expression of the target enzyme. Activity was verified by incubating 10 mM UDP-galactose, 10 mM glucose, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) for 24 h at 37°C and analysing the formation of lactose as described in Example 1.

Example 5: Expression of active glucose isomerase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the glucose isomerase from *S. murinis* UniProt ID P37031, *Arthrobacter* UniProt ID P12070 or *T. ethanolicus* UniProt ID D2DK62. Expression of the enzymes was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis on the cleared cell lysates was performed as described in Example 1 and confirmed the expression of the target enzymes. Activity was verified by incubating 100 mM fructose, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 6.5) for 24 h at 37°C and analysing the formation of glucose as described in Example 1.

Example 6: Expression of active trehalose synthase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the trehalose synthase (treT) from *T. bergensis* UniProt ID Q7LYW5 or *P. horikoshii* with UniProt ID O58762. Expression of the enzyme was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis on the cleared cell lysates was performed as described in Example 1 and confirmed the expression of the target enzyme. Activity was verified by incubating 10 mM trehalose, 10 mM UDP, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 6.5) for 24 h at 37°C and analysing the formation of glucose as described in Example 1.

Example 7: Activity of sucrose synthase at elevated temperature

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the sucrose synthase (SuSy) from *A. caldus* with UniProt ID A0A059ZV61. Expression of the enzyme was performed in a shake flask and cells were harvested and lysed as described in Example 1. Activity was verified by incubating 100 mM sucrose, 10 mM UDP and 50% (v/v) SuSy cell extract in 50 mM MOPS buffer (pH 7.0) for 1 h at 60°C and analysing the formation of UDP-glucose and fructose as described in Example 1.

Example 8: Activity of UDP-glucose-4-epimerase at elevated temperature

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the UDP-glucose-4-epimerase from *P. sp. WP30* with UniProt ID A0A0K1E1Q5. Expression of the enzyme was performed in a shake flask and cells were harvested and lysed as described in Example 1. Activity was verified by incubating 10 mM UDP-glucose and 50% (v/v) GalE cell extract in 50 mM MOPS buffer (pH 6.5) for 1 h at 60°C and analysing the formation of UDP-galactose as described in Example 1.

Example 9: Activity of glucose isomerase at elevated temperature

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the glucose isomerase from *T. ethanolicus* with UniProt ID D2DK62. Expression of the enzyme was performed in a shake flask and cells were harvested and lysed as described in Example 1. Activity was verified by incubating 100 mM fructose, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 6.5) for 1 h at 60°C and analysing the formation of glucose as described in Example 1.

Example 10: Activity of trehalose synthase at elevated temperature

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an inducible expression plasmid containing the gene coding for the trehalose synthase (treT) from *T. bergensis* UniProt ID Q7LYW5 or *P. horikoshii* with UniProt ID O58762. Expression of the enzyme was performed in a shake flask and cells were harvested and lysed as described in Example 1. Activity was verified by incubating 10 mM trehalose, 10 mM UDP, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 6.5) for 1 h at 60°C and analysing the formation of glucose as described in Example 1.

Example 11: Co-expression sucrose synthase, UDP-glucose-4-epimerase and beta 1,4-galactosyltransferase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an expression plasmid containing a constitutive transcriptional unit for the sucrose synthase (SuSy) from *A. caldus* with UniProt

ID A0A059ZV61, a constitutive transcriptional unit for the UDP-glucose-4-epimerase (GalE) from *P. sp. WP30* with UniProt ID A0A0K1E1Q5, and a constitutive transcriptional unit for the beta 1,4-galactosyltransferase (GalT) from *N. meningitidis* with UniProt ID Q51116. Expression of the enzymes was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis was performed on the cleared cell lysates as described in Example 1 and confirmed the expression of the target enzymes.

Example 12: Co-expression sucrose synthase, UDP-glucose-4-epimerase, beta 1,4-galactosyltransferase and glucose isomerase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an expression plasmid containing a constitutive transcriptional unit for the sucrose synthase (SuSy) from *A. caldus* with UniProt ID A0A059ZV61, a constitutive transcriptional unit for the UDP-glucose-4-epimerase (GalE) from *P. sp. WP30* with UniProt ID A0A0K1E1Q5, a constitutive transcriptional unit for the beta 1,4-galactosyltransferase (GalT) from *N. meningitidis* with UniProt ID Q51116 and a constitutive transcriptional unit for the glucose isomerase (XylA) from *T. ethanolicus* with UniProt ID D2DK62. Expression of the enzymes was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis was performed on the cleared cell lysates as described in Example 1 and confirmed the expression of the target enzymes.

Example 13: Co-expression of trehalose synthase, UDP-glucose-4-epimerase and beta 1,4-galactosyltransferase

An *E. coli* NiCo21(DE3) strain as described in Example 1 was transformed with an expression plasmid containing a constitutive transcriptional unit for the trehalose synthase (treT) from *T. bergensis* UniProt ID Q7LYW5, a constitutive transcriptional unit for the UDP-glucose-4-epimerase (GalE) from *P. sp. WP30* with UniProt ID A0A0K1E1Q5 and a constitutive transcriptional unit for the beta 1,4-galactosyltransferase (GalT) from *N. meningitidis* with UniProt ID Q51116. Expression of the enzymes was performed in a shake flask and cells were harvested and lysed as described in Example 1. Western blot analysis was performed on the cleared cell lysates as described in Example 1 and confirmed the expression of the target enzymes.

Example 14: In vitro synthesis of lactose via cell extracts of individually expressed enzymes

Cell lysates from strains expressing a sucrose synthase, as described in Example 2, a UDP-glucose-4-epimerase, as described in Example 3, and a beta 1,4-galactosyltransferase, as described in Example 4, were mixed with substrate in a one-pot multi-enzyme reaction containing 100 mM sucrose, 100 mM glucose, 2 mM UDP, 10 mM MgCl₂ and three times 16.6% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

Example 15: In vitro synthesis of lactose via cell extracts of individually expressed enzymes

Cell lysates from strains expressing a sucrose synthase, as described in Example 2, a UDP-glucose-4-epimerase, as described in Example 3, a beta 1,4-galactosyltransferase, as described in Example 4, and a glucose isomerase, as described in Example 5, were mixed with substrate in a one-pot multi-enzyme reaction containing 100 mM sucrose, 2 mM UDP, 10 mM MgCl₂ and four times 12.5% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

Example 16: In vitro synthesis of lactose via cell extracts of individually expressed enzymes

Cell lysates from strains expressing a trehalose synthase, as described in Example 6, a UDP-glucose-4-epimerase, as described in Example 3, and a beta 1,4-galactosyltransferase, as described in Example 4, were mixed with substrate in a one-pot multi-enzyme reaction containing 100 mM trehalose, 2 mM UDP, 10 mM MgCl₂ and three times 16.6% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

Example 17: In vitro synthesis of lactose via cell extracts of co-expressed enzymes

Cell lysates from strains co-expressing the three enzymes, as described in Example 11, were mixed with substrate in a one-pot multi-enzyme reaction containing 100 mM sucrose, 100 mM glucose, 2 mM UDP, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

Example 18: In vitro synthesis of lactose via cell extracts of co-expressed enzymes

Cell lysates from strains co-expressing the four enzymes, as described in Example 12, were mixed with substrate in a one-pot multi-enzyme reaction containing 100 mM sucrose, 2 mM UDP, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

Example 19: In vitro synthesis of lactose via cell extracts of co-expressed enzymes

Cell lysates from strains co-expressing the three enzymes, as described in Example 13, were mixed with substrate in a one-pot multi-enzyme reaction containing 100 mM trehalose, 2 mM UDP, 10 mM MgCl₂ and 50% (v/v) cell extract in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

Example 20: In vitro synthesis of lactose via purified enzymes

Cell lysates from strains expressing a sucrose synthase, as described in Example 2, a glucose isomerase, as described in Example 3, a beta 1,4-galactosyltransferase, as described in Example 4, and a glucose

isomerase, as described in Example 5, were purified as described in Example 1. The purified enzymes were subsequently mixed in a one-pot multi-enzyme reaction containing 100 mM sucrose, 2 mM UDP, 10 mM MgCl₂ and 0.1 mg/ml purified enzyme in 50 mM MOPS buffer (pH 7.0) and incubated for 24 h at 37°C. The formation of lactose was analysed as described in Example 1.

5

Example 21: In vivo synthesis of lactose

An *E. coli* K-12 MG1655 strain modified for growth on sucrose and deficient in the breakdown of lactose, as described in Example 1, was transformed with an expression plasmid containing a constitutive transcriptional unit for the sucrose synthase (SuSy) from either *G. max*, UniProt ID P13708, *A. thaliana* UniProt ID P49040, *Anabaena* sp. UniProt ID Q9ZEV2 or *A. caldus* UniProt ID A0A059ZV61, a constitutive transcriptional unit for the UDP-glucose-4-epimerase (GalE) from either *E. coli* UniProt ID P09147, *B. longum* UniProt ID E8MF10, *P. sp. WP30* UniProt ID A0A0K1E1Q5 or *C. jejuni* UniProt ID Q0P9C3, a constitutive transcriptional unit for the beta 1,4-galactosyltransferase (GalT) from *N. meningitidis* with UniProt ID Q51116 and a constitutive transcriptional unit for the glucose isomerase (XylA) from either *S. murinus* UniProt ID P37031, *Arthrobacter* UniProt ID P12070 or *T. ethanolicus* UniProt ID D2DK62, as described in Example 6. The novel strains were evaluated in a 96-well plate for production of lactose according to the culture conditions provided in Example 1 in which the strains were cultivated in minimal medium with 30 g/l sucrose. After 72h of incubation, the culture broth was harvested, and the formation of lactose was analysed as described in Example 1.

20

Example 22: Whole cell biotransformation synthesis of lactose

An *E. coli* K-12 MG1655 strain as described in Example 1 was transformed with an expression plasmid containing a constitutive transcriptional unit for the sucrose synthase (SuSy) from *A. caldus* with UniProt ID A0A059ZV61, a constitutive transcriptional unit for the UDP-glucose-4-epimerase (GalE) from *P. sp. WP30* with UniProt ID A0A0K1E1Q5, a constitutive transcriptional unit for the beta 1,4-galactosyltransferase (GalT) from *N. meningitidis* with UniProt ID Q51116 and a constitutive transcriptional unit for the glucose isomerase (XylA) from *T. ethanolicus* with UniProt ID D2DK62. The strain was cultivated in a shake flask and cells were harvested and prepared for whole cell biotransformation as described in Example 1. A reaction vessel containing 100 mM sucrose, 50 mM UDP, 100 mM MOPS, 15 mM MgSO₄ and 50 mg/mL cells, pH 7 was incubated for 48 hours at 21 °C and 175 rpm. The formation of lactose was analysed as described in Example 1.

30

Example 23: Recovery on the synthesis enzymes

The enzymes may be recovered from the solution by means of ultrafiltration, wherein the molecular cut-off of the ultrafiltration membrane is smaller than the molecular weight of the enzymes (i.e. for sucrose synthase between 80 and 440 kDa, for UDP-glucose-4-epimerase between 30 and 80 kDa, for beta 1,4-

35

galactosyltransferase between 30 and 45 kDa, for glucose isomerase between 40 and 200 kDa and for trehalose synthase between 45 and 200 kDa). The retained enzymes are recycled to the reaction vessel of column, while the filtrate with the residual substrates, byproducts and salts are further purified to form highly purified lactose.

- 5 Typical ultrafiltration membranes are PES membranes (Synder), PAN membranes (synder), PVDF membranes (Synder), SPES membranes or ceramic membranes (Tami) with molecular weight cutoff ranging between 1kDa and 100kDa.

In a preferred production setup, the ultrafiltration recycling of the enzymes is performed in a continuous manner, enabling continuous lactose synthesis.

10

Example 24: Removal of protein, cells, other carbohydrates and salts from the lactose solution

After the synthesis of lactose as described in the previous examples, typical impurities are sucrose, glucose, fructose, galactose, UDP-glucose, UDP-galactose, and buffer components (phosphate salts and cations such as magnesium).

- 15 These impurities need to be removed from the solution, yet separating sugars with from each other in a solution is not trivial. These sugars have similar properties and molecular weights and are hence not easily separated.

The use of a selective yeast to purify lactose

- 20 A solution containing lactose, sucrose, glucose, fructose, galactose, UDP-glucose, UDP-galactose, and buffer components and the synthesis enzymes was inoculated with bakers yeast (for instance Bruggeman yeast, *Saccharomyces cerevisiae*). This yeast converts sucrose, fructose, galactose, UDP-glucose, UDP-galactose to biomass and ethanol but leaves the lactose in solution.

- 25 After this conversion, the yeast was centrifuged off, leaving in the supernatant lactose, the buffer, and the metabolites formed by *Saccharomyces cerevisiae* (e.g. ethanol, acetate, glycerol) and cell debris. The formation of these metabolites and debris poses a new challenge, these need to be removed. An alternative method for the removal of the yeast is flocculation and decantation or microfiltration with pore size 0.1 to 10µm (ceramic, PES, PVDF membranes).

30

To this end, the cell debris is removed by ultrafiltration resulting in a solution with less than 100 mg protein per kg dry solid, a DNA content below 10 ng per gram dry solid and an endotoxin was below 10000 EU per gram dry solid. The ultrafiltration was followed by a nanofiltration step in which the buffer salts and yeast metabolites are separated from the lactose. For the nanofiltration a NFX membrane (Synder) with a

35 molecular weight cut-off of 300Da is used at 50°C, which has >99% rejection for lactose. The membrane has a salt rejection <40% which allows easy removal of salts and yeast metabolites while preforming

diafiltrations with water. The lactose yield after this step is higher than 95%. During the nanofiltration the solution is further concentrated to a solution of >15% dry matter.

An alternative nanofiltration membrane is NFW (Synder, with molecular weight cut-off <600Da). This membrane has a lactose rejection of >98.5%, salt rejection of <20.0%, which allows easy removal of the salts and yeast metabolites in a diafiltration step. The ash content on total solid after diafiltration is reduced to <5% on total solid.

The use of an invertase to selectively hydrolyse sucrose

10 A solution containing lactose, sucrose, glucose, fructose, galactose, UDP-glucose, UDP-galactose, and buffer components and the synthesis enzymes was treated with an invertase (Beta-fructofuranosidase, Novozymes, approx. 1 g/l, pH 5, 50°C). The residual sucrose is converted into glucose and fructose during this treatment, while leaving the lactose intact.

15 After the sucrose inversion, the enzymes are removed by ultrafiltration resulting in a solution with less than 100 mg protein per kg dry solid, a DNA content below 10 ng per gram dry solid and an endotoxin was below 10000 EU per gram dry solid. The ultrafiltration was followed by a nanofiltration step in which the buffer salts and monosaccharides are separated from the lactose. For the nanofiltration a DL series membrane (Suez) with a molecular weight cut-off of 300Da is used at 50°C, which has >99% rejection for lactose but a low rejection for monosaccharides (5-10%). By diafiltration the monosaccharides and the salts are removed in this step. During the nanofiltration the solution is further concentrated to a solution of >15% dry matter. The ash content on total solid after diafiltration is reduced to <5% on total solid.

Example 25: Removal of protein, cells, other carbohydrates and salts from the lactose solution

25 After the synthesis of lactose as described in the previous examples, typical impurities are trehalose, glucose, galactose, UDP-glucose, UDP-galactose, and buffer components (phosphate salts and cations such as magnesium).

30 These impurities need to be removed from the solution, yet separating sugars with from each other in a solution is not trivial. These sugars have similar properties and molecular weights and are hence not easily separated.

The use of a selective yeast to purify lactose

35 A solution containing lactose, trehalose, glucose, galactose, UDP-glucose, UDP-galactose, and buffer components and the synthesis enzymes was inoculated with bakers yeast (for instance Bruggeman yeast, *Saccharomyces cerevisiae*). This yeast converts trehalose, glucose, galactose, UDP-glucose, UDP-galactose

to biomass and ethanol but leaves the lactose in solution.

After this conversion, the yeast was centrifuged off, leaving in the supernatant lactose, the buffer, and the metabolites formed by *Saccharomyces cerevisiae* (e.g. ethanol, acetate, glycerol) and cell debris. The formation of these metabolites and debris poses a new challenge, these need to be removed. An
5 alternative method for the removal of the yeast is flocculation and decantation or microfiltration with pore size 0.1 to 10µm (ceramic, PES, PVDF membranes).

To this end, the cell debris is removed by ultrafiltration resulting in a solution with less than 100 mg protein per kg dry solid, a DNA content below 10 ng per gram dry solid and an endotoxin was below 10000 EU per
10 gram dry solid. The ultrafiltration was followed by a nanofiltration step in which the buffer salts and yeast metabolites are separated from the lactose. For the nanofiltration a NFX membrane (Synder) with a molecular weight cut-off of 300Da is used at 50°C, which has >99% rejection for lactose. The membrane has a salt rejection <40% which allows easy removal of salts and yeast metabolites while performing diafiltrations with water. The lactose yield after this step is higher than 95%. During the nanofiltration the
15 solution is further concentrated to a solution of >15% dry matter.

An alternative nanofiltration membrane is NFW (Synder, with molecular weight cut-off <600Da). This membrane has a lactose rejection of >98.5%, salt rejection of <20.0%, which allows easy removal of the salts and yeast metabolites in a diafiltration step. The ash content on total solid after diafiltration is
20 reduced to <5% on total solid.

The use of a trehalase to selectively hydrolyse trehalose

A solution containing lactose, trehalose glucose, fructose, galactose, UDP-glucose, UDP-galactose, and buffer components and the synthesis enzymes was treated with an trehalase (α,α -trehalase, Megazymes,
25 approx. 100 mg/l, 40°C, pH 5.5). The residual trehalose is converted into during this treatment, while leaving the lactose intact.

After the trehalose hydrolysis, the enzymes are removed by ultrafiltration resulting in a solution with less than 100 mg protein per kg dry solid, a DNA content below 10 ng per gram dry solid and an endotoxin was
30 below 10000 EU per gram dry solid. The ultrafiltration was followed by a nanofiltration step in which the buffer salts and monosaccharides are separated from the lactose. For the nanofiltration a DL series membrane (Suez) with a molecular weight cut-off of 300Da is used at 50°C, which has >99% rejection for lactose but a low rejection for monosaccharides (5-10%). By diafiltration the monosaccharides and the salts are removed in this step. During the nanofiltration the solution is further concentrated to a solution
35 of >15% dry matter. The ash content on total solid after diafiltration is reduced to <5% on total solid.

Example 26: Demineralization of the lactose solution

The solutions from any of examples 14 to 22 all contain residual salts and nucleotide sugars after treatment. To fully remove the salts from the solution, the solution is treated with a cation and anion exchange resin.

- 5 The solutions are first passed through a strong acid cation exchange resin containing column (1L of Amberlite IR120) in the proton form at a temperature of 10°C, resulting in exchange of all cations with a proton in the liquid. The liquid resulting from the cation exchange step was subjected to a weak base anion exchange resin containing column (1L of Amberlite IR400) in the hydroxide form at a temperature of 10°C, exchanging the anions in the liquid for hydroxide ions. After both cation and anion exchange, the
- 10 pH was set to a pH between 6 and 7. The lactose recovery is between 95 and 98%. The ash content on total solid after ion exchange is reduced to <1% on total solid.

- Alternative cation and anion exchange resins are Amberlite IR100, Amberlite IR120, Amberlite FPC22, Dowex 50WX, Finex CS16GC, Finex CS13GC, Finex CS12GC, Finex CS11GC, Lewatit S, Diaion SK, Diaion UBK,
- 15 Amberjet 1000, Amberjet 1200 and Amberjet 4200, Amberjet 4600, Amberlite IR400, Amberlite IR410, Amberlite IR458, Diaion SA, Diaion UBA120, Lewatit MonoPlus M, Lewatit S7468.

Example 27: color removal

- To achieve decolourization, several samples throughout the process were subjected to activated charcoal
- 20 treatment with Norit SX PLUS activated charcoal (0,5% m/v). Color removal was measured with a spectrophotometer at 420 nm. In all samples the color intensity at 420nm was reduced 50 to 100 fold. The activated charcoal is filtered off by means of a plate filter or chamber filter press preferably at elevated temperatures.

25 **Example 28: Concentration**

- Nanofiltration was carried out with an NF-2540 membrane (DOW) with a cut off of 200 Da to concentrate the de-ionized solutions after ion exchange, nanofiltration up to 25 Brix or the raw solution after ultrafiltration. During the filtration process a pressure across the membrane in the range of 20-25 bar was used and a process temperature of 45°C. The solution was continuously recirculated over the membrane
- 30 for concentration, leading to a dry matter content of the concentrate up to 25% Brix.

An alternative method of concentration is falling film evaporation or wiped film evaporation, wherein at high temperature the lactose concentration before drying or crystallization can be increased up to 80%. Typical temperatures are 50-100°C.

35 **Example 29: Spray drying of enzymatically produced lactose**

The lactose containing solutions from examples 14-22 are spray dried with pilot spray dry equipment. The

equipment has an evaporation capacity of 2 kg/h.

For spray drying the liquid was heated to a temperature between 50 and 100°C, to lower the viscosity. The pH of the liquid was set to a pH of 4 to 6. More preferably the pH is set to 4 to 5 and temperatures
5 are kept between 50 and 70°C.

The lactose concentration in the feed is between 5% and 80% brix, depending on if the solution was concentrated (example 14) or treated with nanofiltration (example 11). These concentrations were obtained by rotary evaporation or wiped film evaporation (example 14). The concentrated liquids were
10 fed to the spray dryer at a rate between 50 and 90%. The higher the percentage brix, the faster the feed rate.

The used inlet temperature ranged between 120 and 280°C. The outlet temperature ranged between 100°C and 180°C. The atomizer wheel rotation speed was set between 10000 and 28000 rpm. In one
15 specific test the inlet temperature was set at 184°C, outlet temperature was set at 110°C and atomizer rate was set at 21500 rpm.

The obtained spray dried powder after ultrafiltration, nanofiltration and active charcoal treatment had a white to off white colour and the pH after dissolving in water at a concentration of 10%, is between 4 and
20 6. The purity of the lactose was above 80% of oligosaccharides on dry solid. The spray dried oligosaccharide mixtures had about 3 to 10% of water content, the protein content was below 100 mg per kg dry solid, the DNA content below 10 ng per gram dry solid and the endotoxin was below 10000 EU per gram dry solid. No GMO DNA could be detected in the powder. The powder that was treated with ion exchange resins has an ash content below 1% (on total dry solid), the Lead content was lower than 0,1
25 mg/kg dry solid, Arsenic: lower than 0,2 mg/kg dry solid, Cadmium lower than 0,1 mg/kg dry solid and Mercury was lower than 0,5 mg/kg dry solid.

Example 30: Crystallization of lactose

The crystallization process is conducted in an agitated jacketed crystallizer. The concentrated lactose
30 solution from example 28 has a temperature above room temperature, and is cooled to between 20 °C and 25 °C to maximize lactose crystal yield over a period of 12 to 48 h. As the solution is cooled, the lactose crystals crystallized because of the increase in supersaturation. The yield of crystalline lactose in one cycle is about 80%. The mother liquor can be concentrated again by either passing the mother liquor through the nanofiltration step again (to remove concentrated impurities) or concentrated again to a dry matter
35 content of approx. 50%.

In another experiment, a biotechnologically produced lactose solution containing sucrose, glucose and

fructose was crystallized. The initial lactose concentration was 42% mass/total sugars mass and this solution contained 42% mass/total sugar mass sucrose, 8% fructose mass/total sugars mass and 8% glucose mass/total sugars mass. The solution was concentrated to 70 %m/m in water by evaporation in rotavap evaporator at 60°C and 70 mbar, but common alternative evaporation systems such as falling film evaporators, evacuated vessel evaporator, wiped film evaporators may also be used. After evaporation the solution was kept at 60°C and brought to atmospheric pressure after which the solution slowly cooled down to 45°C. At 45°C a fraction of the solution was seeded with lactose crystals; another fraction was not seeded. Both were slowly cooled down until turbidity occurred, indicating crystallization of both the seeded and unseeded solution. From the seeded solution 40% of the lactose was recovered, which was centrifuged and washed once with cold water (5°C) and dried. The final purity of the isolated lactose was 99% on dry mass.

In another experiment a biotechnologically produced lactose solution containing glucose and salts was crystallized. Said solution contained 20% mass on dry solid lactose, 15% mass on dry solid glucose and 10% mass on dry solid salts (which are phosphates, sulphates, ammonium and sodium). This solution was concentrated in the same way as described above to a 70% mass on dry matter solution at 60°C and brought to atmospheric pressure after which the solution was cooled down to 45°C. This solution at 45°C was seeded with lactose crystals and let to cool further to room temperature, whereby the solution became turbid. The crystals were isolated by centrifugation and washed once with cold water. The lactose recovery from the solution was 30% and the lactose purity was 95% on dry matter.

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Example 31: Separation of lactose from disaccharides by SMB

For SMB chromatography, a closed loop SMB system equipped with 12 glass columns with the dimensions can be used. Each glass column strong cation ion exchanger resin, typical resins used are Dowex Monosphere in the Ca²⁺ form. The SMB can be run at ambient temperature, but is preferably run above 50°C. The flow rates are adapted over the different zones in the SMB system with a constant feed rate to the SMB. As eluent typically water is used, but food grade ethanol may be used for better separation up to about 10% (v/v).

Example 32: Purification of biotechnologically produced lactose

Lactose produced by a fermentation process wherein cells and cell debris are present were purified by a first step, removing the cell debris and cells from the fermentation broth by microfiltration (.45µm ceramic membrane, Tami) at a temperature above 50°C. This step resulted into a clear solution that is then further purified. Said step can be replaced by centrifugation or direct ultrafiltration, leading to similar results.

The microfiltration step was followed by an ultrafiltration step (step 2), removing proteins and other high molecular weight molecules such as DNA. This ultrafiltration step was performed with a PES spiral wound

membrane (Synder) with a MWCO of 3 kDa. Said step can be replaced by other types of ultra or nanofiltration membranes of different sizes, such as membranes with MWCO ranging from 500 dalton and higher.

The filtrate of the ultrafiltration or nanofiltration step (step 2) is further treated in a nanofiltration step (step3) to remove salts and monosaccharides from the liquid. The membrane used was a PA TFC membrane with a MWCO of 150 to 300 Da, at a temperature above 40°C and pressure at about 20 bar. Its retentate was diafiltered once in a diafiltration step, leading to a reduction of 50% monosaccharides and salt reduction of 20%. The overall lactose yield of the steps 1 to 3 was 90%. Further, the removal of the monosaccharides and salts was enhanced by reducing the pH of the liquid with an acid (here as an example sulphuric acid, but other acids such as phosphoric acid, citric acid, acetic acid and lactic acid will have the same effect) resulting into the removal of 33% of the salts after one diafiltration, 50% of salts after 2 diafiltrations and 75% after 3 diafiltrations.

The retentate of step 3 (obtained at neutral pH), was concentrated in a rotary evaporator to a dry matter concentration of 70% (step 4, 60°C and 70 mbar). Alternative methods of evaporation are falling film evaporators, evacuated vessel evaporator and wiped film evaporators and will result in similar outputs. The concentrate of step 4 is then further crystallized in accordance to example 30, wherein a similar yield was obtained in a single crystallization cycle.

Example 33: Purification of biotechnologically produced lactose

Lactose produced by a fermentation process wherein cells and cell debris are present were purified by a first step, removing the cell debris and cells from the fermentation broth by microfiltration (.45µm ceramic membrane, Tami) at a temperature above 50°C. This step resulted into a clear solution that is then further purified. Said step can be replaced by centrifugation or direct ultrafiltration, leading to similar results.

The microfiltration step was followed by an ultrafiltration step (step 2), removing proteins and other high molecular weight molecules such as DNA. This ultrafiltration step was performed with a PES spiral wound membrane (Synder) with a MWCO of 3 kDa. Said step can be replaced by other types of ultra or nanofiltration membranes of different sizes, such as membranes with MWCO ranging from 500 dalton and higher.

The filtrate of the ultrafiltration or nanofiltration step (step 2) is further treated in a nanofiltration step (step3) to remove salts and monosaccharides from the liquid. The membrane used was a PA TFC membrane with a MWCO of 150 to 300 Da, at a temperature above 40°C and pressure at about 20 bar. Its retentate was diafiltered once in a diafiltration step, leading to a reduction of 50% monosaccharides and salt reduction of 20%. The overall lactose yield of the steps 1 to 3 was 90%. Further, the removal of the monosaccharides and salts was enhanced by reducing the pH of the liquid with an acid (here as an example sulphuric acid, but other acids such as phosphoric acid, citric acid, acetic acid and lactic acid will

have the same effect) resulting into the removal of 33% of the salts after one diafiltration, 50% of salts after 2 diafiltrations and 75% after 3 diafiltrations.

The retentate of step 3 (obtained at neutral pH), was further treated in a 4th step with activated charcoal, removing colour from the solution. 4% weight to weight activated charcoal was added to the solution and incubated in a stirred tank for 1h. The activated charcoal was removed by a plate filter (0.45 µm).

A second portion of step 3 was treated with activated charcoal in a column, at least 5 bed volumes per volume of broth was used and the treatment was done at a flow rate of 3 bed volumes per hour.

The liquid obtained after activated charcoal treatment was concentrated in a rotary evaporator to a dry matter concentration of 70% (step 4, 60°C and 70 mbar). Alternative methods of evaporation are falling film evaporators, evacuated vessel evaporator and wiped film evaporators and will result in similar outputs.

The concentrate of step 4 is then further crystallized in accordance to example 30, wherein a similar yield was obtained in a single crystallization cycle.

Example 34: Whole cell biotransformation synthesis of lactose

An *E. coli* K-12 MG1655 strain as described in Example 1, natively expressing an UDP-glucose-4-epimerase (GalE) with UniProt ID P09147, was transformed with an expression plasmid containing a combination of a constitutive transcriptional unit for a sucrose synthase (SuSy) and a constitutive transcriptional unit for a beta-1,4-galactosyltransferase (GalT). The SuSy was either from *A. caldus* with UniProt ID A0A059ZV61 or *G. max* with UniProt ID P13708 combined with either the GalT from *N. meningitidis* with UniProt ID Q51116 or *P. multocida* with UniProt ID D0EAD4. For the SuSy from *G. max* and the GalT enzymes different transcriptional units (TUs) were evaluated, differing in their promotor, 5'-untranslated region and/or terminator sequence, as described in Example 1. Co-expression of the enzymes was performed in a 24-deepwell plate and cells were harvested and permeabilised by one freeze-thaw cycle, as described in Example 1. Whole cell biotransformation was performed by incubating 10 g/L cells, 100 mM sucrose, 25 mM glucose, 10 mM UDP and 5 mM MgCl₂ in 25 mM phosphate buffer (pH 7) and incubated for 48 h at 30°C. The formation of lactose was analysed as described in Example 1. Multiple combinations of the three co-expressed enzymes lead to the production of lactose in a whole cell biotransformation setup (Table 22).

Table 2 Lactose production in a whole cell biotransformation with a strain expressing different combinations of sucrose synthase, beta-1,4-galactosyltransferase and UDP-glucose-4-epimerase

(production normalised to the production of the *G. max* SuSy (TU variant 2) / *P. multocida* GalT (TU variant 1) combination)

Sucrose synthase	UDP-glucose-4-epimerase	Beta-,1,4-galactosyltransferase	Lactose production (%)
<i>G. max</i> (TU variant 1)	<i>E. coli</i>	<i>N. meningitidis</i> (TU variant 1)	222
<i>G. max</i> (TU variant 1)	<i>E. coli</i>	<i>N. meningitidis</i> (TU variant 2)	384
<i>G. max</i> (TU variant 1)	<i>E. coli</i>	<i>N. meningitidis</i> (TU variant 3)	203
<i>G. max</i> (TU variant 1)	<i>E. coli</i>	<i>P. multocida</i> (TU variant 1)	373
<i>G. max</i> (TU variant 1)	<i>E. coli</i>	<i>P. multocida</i> (TU variant 2)	151
<i>G. max</i> (TU variant 2)	<i>E. coli</i>	<i>P. multocida</i> (TU variant 1)	100
<i>G. max</i> (TU variant 2)	<i>E. coli</i>	<i>P. multocida</i> (TU variant 2)	152
<i>A. caldus</i>	<i>E. coli</i>	<i>N. meningitidis</i> (TU variant 2)	442
<i>A. caldus</i>	<i>E. coli</i>	<i>N. meningitidis</i> (TU variant 3)	379
<i>A. caldus</i>	<i>E. coli</i>	<i>P. multocida</i> (TU variant 1)	216
<i>A. caldus</i>	<i>E. coli</i>	<i>P. multocida</i> (TU variant 2)	287
<i>A. caldus</i>	<i>E. coli</i>	<i>P. multocida</i> (TU variant 3)	206

Example 35: Purification of biotechnologically produced lactose from a biotransformation process

Lactose produced by a bioconversion or whole cell biotransformation process wherein cells and cell debris are present were purified by a first step, removing the cell debris and cells from the bioconversion reaction mixture by microfiltration (.45µm ceramic membrane, Tami) at a temperature above 50°C. This step resulted into a clear solution that is then further purified. Said step can be replaced by centrifugation or direct ultrafiltration, leading to similar results.

The microfiltration step was followed by an ultrafiltration step (step 2), removing proteins and other high molecular weight molecules such as DNA. This ultrafiltration step was performed with a PES spiral wound membrane (Synder) with a MWCO of 3 kDa. Said step can be replaced by other types of ultra or nanofiltration membranes of different sizes, such as membranes with MWCO ranging from 500 dalton and higher.

The filtrate of the ultrafiltration or nanofiltration step (step 2) is further treated in a nanofiltration step (step3) to remove salts and monosaccharides from the liquid. The membrane used was a PA TFC membrane with a MWCO of 150 to 300 Da, at a temperature above 40°C and pressure at about 20 bar. Its retentate was diafiltered once in a diafiltration step, leading to a reduction of 50% monosaccharides and salt reduction of 20%. The overall lactose yield of the steps 1 to 3 was 90%. Further, the removal of the monosaccharides and salts was enhanced by reducing the pH of the liquid with an acid (here as an example sulphuric acid, but other acids such as phosphoric acid, citric acid, acetic acid and lactic acid will have the same effect) resulting into the removal of 33% of the salts after one diafiltration, 50% of salts

after 2 diafiltrations and 75% after 3 diafiltrations.

The retentate of step 3 (obtained at neutral pH), was further treated in a 4th step with activated charcoal, removing colour from the solution. 4% weight to weight activated charcoal was added to the solution and
5 incubated in a stirred tank for 1h. The activated charcoal was removed by a plate filter (0.45 µm).

A second portion of step 3 was treated with activated charcoal in a column, at least 5 bed volumes per volume of broth was used and the treatment was done at a flow rate of 3 bed volumes per hour.

The liquid obtained after activated charcoal treatment was concentrated in a rotary evaporator to a dry
10 matter concentration of 70% (step 4, 60°C and 70 mbar). Alternative methods of evaporation are falling film evaporators, evacuated vessel evaporator and wiped film evaporators and will result in similar outputs.

The concentrate of step 4 is then further crystallized in accordance to example 30, wherein a similar yield was obtained in a single crystallization cycle.

Claims

1. A method to produce a crystalline lactose, wherein said lactose is produced via a biotechnological production process wherein said method comprises a step of drying and/or crystallisation of said lactose.
- 5 2. The method according to claim 1, wherein said lactose is purified by purification steps selected from a microfiltration step, a centrifugation step, an ultrafiltration step, a nanofiltration step, an ion exchange step, a Simulated Moving Bed step, a colour removal step, a step of removal of sucrose, glucose or trehalose by addition of yeast, a step of removal of trehalose by addition of trehalase, a step of removal of sucrose by addition of an invertase; and preferably further comprises a concentration step; preferable said ion exchange step is a cation and/or anion exchange step, and/or a mixed bed ion exchange step.
- 10 3. The method according to any one of the preceding claims, wherein said biotechnological production process is a fermentation process, a bioconversion process or an enzymatic process.
4. The method according to claim 3, wherein said fermentation or bioconversion is followed by a biomass removal step, preferably said biomass removal step comprises a step of centrifugation, microfiltration and/or ultrafiltration.
- 15 5. The method according to claim 3, wherein said biotechnological production process is an enzymatic process and wherein at least one enzyme is immobilised.
6. The method according to any one of claim 1 to 3 or 5, wherein said biotechnological production process is an enzymatic process and wherein the reaction is performed between 50°C and 80°C, preferably between 55°C and 70°C.
- 20 7. The method according to any one of claim 1 to 4, wherein said biotechnological production process is a fermentation process or a bioconversion process characterised in that enzymatic conversions are performed by at least one enzyme produced by a cell synthesizing said enzyme.
- 25 8. The method according to claim 7, wherein said fermentation process or bioconversion process is performed at a temperature between about 25°C and about 50°C, preferably between about 25°C and about 40°C.
9. The method according to any one of claim 1 to 4, 7 or 8, wherein said biotechnological production process is a fermentation process characterised in that said lactose is produced by a cell genetically engineered to produce all enzymes necessary for production of lactose.
- 30 10. The method according to any one of the previous claims, wherein said lactose is purified from the biotechnological production process resulting liquid comprising sucrose, glucose, trehalose, fructose, and/or glycerol.
11. The method according to any one claim 2 to 10, wherein the lactose purity before purification is < 70%, < 60%, < 50%, < 40%, < 30%, < 20%, < 10% on total solids, and/or the purity at the end of said method comprising purification is higher than > 80% on dry solids, preferably > 85% on dry solids,
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more preferably > 90% on dry solids, even more preferably > 95% on dry solids, even more preferably > 97% on dry solids, even more preferably > 98% on dry solids, most preferably > 99% on dry solids.

12. The method according to any one of claim 2, 3, 5, 6, 10 or 11, wherein said purification of an enzymatically produced lactose comprises a step of a microfiltration or ultrafiltration to recover the used enzymes.
13. The method according to any one of claim 2 to 4, 7 to 11 wherein cells removed from said fermentation or bioconversion are reused in a further fermentation or bioconversion process.
14. A method according to any one of claim 1 to 13, wherein said biotechnological production process comprises i) a step of nanofiltration to remove/reduce monosaccharides and/or salts; and/or ii) a step of ion exchange to remove charged materials.
15. The method according to any one of claim 1 to 14, wherein said lactose has an ash content below 1% on total solid, preferably below 0,5% on total solid, preferably with one or more of:
- a) Lead content lower than 0,1 mg/kg solid, preferably lower than 0,02 mg/kg solid;
 - b) Arsenic content lower than 0,2 mg/kg solid, preferably lower than 0,02 mg/kg solid;
 - c) Cadmium content lower than 0,1 mg/kg solid, preferably lower than 0,01 mg/kg solid; or
 - d) Mercury content was lower than 0,5 mg/kg solid, preferably lower than 0,1 mg/kg solid.
16. The method according to any one of claim 1 to 15, wherein said lactose has at least one or more of:
- i) a protein content below 100 mg per kg dry solid, ii) DNA content below 10 ng per gram dry solid and iii) endotoxin content below 10000 EU per gram dry solid.
17. The method according to any one of claim 1 to 16, wherein said lactose crystals are dried by band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, vacuum drum drying, roller drying, vacuum roller drying and other types of drying.
18. The method according to any one of claim 1 to 16, said step of drying comprises any one or more of spray drying, lyophilization, evaporation, precipitation, spray freeze drying, freeze spray drying, band drying, belt drying, vacuum band drying, vacuum belt drying, drum drying, roller drying, vacuum drum drying, vacuum roller drying, and agitated thin film drying.
19. A crystalline lactose product wherein said lactose product is derived from a biotechnological production process, preferably produced by a method as described in any of claims 1 to 18.
20. The lactose product of claims 19, wherein said lactose is lactose monohydrate.
21. The lactose product of any one of claims 19 or 20, wherein said lactose has a purity of at least 90% on dry mass, preferably at least 94% on dry mass.
22. The lactose product of any one of claims 19 to 21, wherein said lactose is free of milk protein.
23. The lactose product of any one of claims 19 to 22, wherein said lactose has at least one or more of: i) a protein content below 100 mg per kg dry solid, ii) DNA content below 10 ng per gram dry solid and iii) endotoxin content below 10000 EU per gram dry solid.
24. The lactose according to any one of claim 19 to 23, wherein said lactose has an ash content below 1%

on total solid, preferably below 0,5% on total solid, preferably with one or more of:

- a) Lead content lower than 0,1 mg/kg solid, preferably lower than 0,02 mg/kg solid;
- b) Arsenic content lower than 0,2 mg/kg solid, preferably lower than 0,02 mg/kg solid;
- c) Cadmium content lower than 0,1 mg/kg solid, preferably lower than 0,01 mg/kg solid;
- d) Mercury content was lower than 0,5 mg/kg solid, preferably lower than 0,1 mg/kg solid.

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25. Crystalline lactose product according to any one of claim 19 to 24 for use in medicine, preferably for use in prophylaxis or therapy of a gastrointestinal disorder.

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26. Use of the crystalline lactose obtained according to the process of any one of claim 1 to 18 in a food or feed preparation, in a dietary supplement, in a cosmetic ingredient or in a pharmaceutical ingredient.

27. Use of the crystalline lactose according to any one of claim 19 to 24 i) in a food or feed preparation, in a dietary supplement, in a cosmetic ingredient or in a pharmaceutical ingredient, or ii) as additive in food, preferably as additive in human food and/or pet food, more preferably as additive in human baby food.

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28. Use according to any one of claim 26 or 27, wherein i) the food is a human food, preferably infant food and/or an infant formula or an infant supplement, or ii) the feed is a pet food, animal milk replacer, veterinary product, post weaning feed, or creep feed.