

Mutant microorganisms resistant to lactose killing

Technical field of invention

The present invention relates to a method to produce mutated microorganisms which resist the phenomenon of lactose killing and to the microorganisms obtainable via said method. Such engineered microorganisms can be applied for the production of specialty products, such as but not limited to specialty carbohydrates, glycolipids and galactosylated compounds.

Background art

Lactose killing is a well-known and well-studied principle that hampers growth of many organisms in the presence of lactose with another carbon source. The exact mechanism behind this phenomenon is however not known, although the features needed to induce the phenomenon are quite clear. Lactose killing occurs when lactose is added to a microbial culture that grows on another carbon source, such as but not limited to glycerol or sucrose. It furthermore occurs when lactose transport gene is either inducible or constitutively expressed (33, 39, 75). Lactose killing was first observed for *E. coli* where the expression of lactose permease was modulated with IPTG and lactose in chemostat conditions (28). Lactose killing was later also observed for *Rhizobium meliloti*, *Kluyveromyces lactis* and *Zymomonas mobilis* (55, 70, 77). One of the potential reasons for this phenomenon was ascribed to the so-called “cost” of lactose transporter activity for the cell, this “cost” results in a reduction or inhibition of growth and is also related to the extracellular lactose concentration (34), which is mostly kept high in industrial processes to acquire high enough product titers and yields. However, the art states that as long as there is lactose transport under these conditions, lactose killing should occur. To solve the problem of lactose killing the deletion of lactose permease or severe impairment of lactose uptake has been proposed (34). For instance Lodi *et al* (55) deleted (knocked out) lactose permease in *K. lactis* and found that lactose killing did not occur anymore. Spontaneous mutations during their experiments further showed that the selected lactose killing negative strains are severely impaired in their lactose uptake. However, lactose uptake is essential in order to synthesize specialty products or bioproducts efficiently. Hence a deletion of lactose permease or severe impairment in lactose permease activity is clearly not a solution as the production of such bioproducts requires 1) an efficient lactose uptake and 2) an expression cassette that does not lead to ‘the lactose-killing phenotype’. Lactose permease for the production of lactose-based bioproducts has been previously used but without solving ‘the

lactose-killing phenotype'. In the past this problem was solved by either reducing the uptake of lactose severely (and hence little to none specialty product is produced based on lactose) or by decoupling the growth phase and the production phase in order to obtain first enough biomass. After this growth phase lactose is added in a second phase to produce a specialty product. In this second phase no or strongly reduced growth occurs (59).

Thus, current consensus is that, in order to avoid lactose killing, lactose uptake has to be eliminated or severely impaired as normal lactose uptake would always lead to lactose killing. In contrast, the present invention discloses a screening methodology to find expression cassettes of lactose permease that allow efficient uptake of lactose without undergoing lactose killing!.

Lactose is a building block for many bioproducts, more particularly specialty carbohydrates (14), glycolipids, and galactosylated compounds such as galactosyllipids, galactosylceramides, and galactosylated aglycons. In many cases the galactose moiety is used for organ targeting of pharmaceuticals (28). The use of lactose as a substrate in combination with other substrates is however not as evident as it might seem due to the above-described phenomenon of 'lactose-killing'. Mostly multi-phase production systems, non-growing coupled cell systems are needed to avoid lactose killing (32, 48).

The basic structural backbone of many specialty carbohydrates consist of lactose or galactose units. More specifically, human milk oligosaccharides and by extension mothers milk oligosaccharides, a broad group of saccharides and oligosaccharides, are build up from galactose and lactose units (15). These carbohydrates are further modified with sugar moieties such as for example N-acetylglucosamine, N-acetylgalactosamine, sialic acids (such as N-acetylneuraminate, N-glycoylneuraminate, 2-keto-3-deoxy-D-glycero-galacto-nonulosonic acid, ...) (21), L-fucose,... . The synthesis of these compounds requires then activated carbohydrates such as UDP-N-acetylglucosamine, UDP-N-acetylgalactosamine, CMP-sialic acid, GDP-fucose, ... , which are very expensive and hard to synthesize molecules and are optimally produced by living, growing cells due to the energy their biosynthesis requires.

The oligosaccharide components of human/mothers milk have anti-inflammatory and prebiotic effects and/or have applications in therapeutics as nutraceutical, anti-inflammatory agent, prebiotic, or, pharmaceuticals (15, 24, 68). However, an efficient method to produce the latter high-value compounds is still needed.

The present invention describes synthetic expression systems for lactose transporters that do not result in lactose killing, even at high lactose concentrations. The mutated organisms

obtainable via said expression systems are thus useful to produce the above-described bioproducts.

Brief description of figures

5 Figure 1: Effect of lactose on an *E. coli* wild type strain. At the arrow lactose was added to one of the cultures and growth of this culture stopped immediately, while the other strain continued to grow in the other culture.

Figure 2: Effect of lactose on an *E. coli* lactose transporter mutant strain in which lactose transporter expression is altered by means of a synthetic constitutive promoter. The arrow
10 indicates the moment on which lactose was added to the medium in one of the cultures. In this case no effect was observed on growth. Hence, these mutant strains can be selected in this manner.

Figure 3: Example of a promoter, RBS and lactose transporter sequence combination (SEQ ID N° 1) that does not result in lactose killing when introduced into an *E. coli* mutant strain.

15 Figure 4: Example of a sequence of the lactose permease gene translational coupled to the lacZ gene (SEQ ID N° 2).

Figure 5: Example of a sequence of the lactose permease gene *lacY* translational coupled to the cat gene (SEQ ID N° 3).

Figure 6: Example of a sequence of the *K. maxianus* lactose permease gene translational
20 coupled to the aph 1 gene (SEQ ID N° 4).

Figure 7: Example of a sequence of the lactose permease gene *lacy* coupled with an aptamer that binds (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one (SEQ ID N° 5) and allows the detection of lactose permease expression.

Figure 8: Chloramphenicol resistance of a reference strain containing a reference plasmid
25 pSC101 without a lactose permease translational coupled to a chloramphenicol resistance gene and a mutant strain with a lactose permease translational coupled to a chloramphenicol resistance gene. The X-axis shows the different chloramphenicol concentrations tested, the Y-axis shows the optical density of the culture after 48 hours of incubation. The reference strain shows growth retardation at a lower chloramphenicol screening than the mutant strain, which
30 proves that expression of lactose permease can be screened via translational coupling with an antibiotic resistance gene. Lactose permease expressing organisms can hence be selected from a mixture of non-expressing and expressing organisms in this manner.

Figure 9: Chloramphenicol resistance of a reference strain containing a reference plasmid pSC101 without a lactose permease translational coupled to a chloramphenicol resistance gene and a mutant strain with a lactose permease translational coupled to a chloramphenicol resistance gene. The X-axis shows the different chloramphenicol concentrations tested, the Y-axis shows the optical density of the culture after 92 hours of incubation. The reference strain shows growth retardation at a lower chloramphenicol screening than the mutant strain, which proves that expression of lactose permease can be screened via translational coupling with an antibiotic resistance gene. Lactose permease expressing organisms can hence be selected from a mixture of non-expressing and expressing organisms in this manner.

Figure 10: pCXP14-FT_H. pylori (SEQ ID N° 6).

Figure 11: Effect of lactose on a yeast wild type strain (*Kluyveromyces marxianus lactis*). At the arrow lactose was added to one of the cultures and growth of this culture stopped immediately, while the other strain continued to grow in the other culture.

Figure 12: Effect of lactose on yeast lactose transporter mutant strains (*Saccharomyces cerevisiae*) in which lactose transporter expression is altered by means of a synthetic constitutive promoter. The arrow indicates the moment on which lactose was added to the medium in one of the cultures. In this case no effect was observed on growth. Hence, these mutant strains can be selected in this manner.

Figure 13: Example of a promoter (p1), Kozak, *K. marxianus* lactose permease coding sequence and terminator combination (SEQ ID N° 7) that does not result in lactose killing when introduced into a yeast mutant strain.

Figure 14: Example of a promoter (p2), Kozak, *K. marxianus* lactose permease coding sequence and terminator combination (SEQ ID N° 8) that does not result in lactose killing when introduced into a yeast mutant strain.

Figure 15: Example of a promoter, Kozak, *K. marxianus* β -galactosidase coding sequence and terminator combination (SEQ ID N° 9).

Figure 16: HR1 rDNA (SEQ ID N° 10).

Figure 17: HR2 rDNA (SEQ ID N° 11).

Figure 18: Examples of selected sequences that originate from the lactose killing screening methodology as describe in the examples (SEQ ID N° 12 to 57).

Figure 19: Relative growth rate of the lactose permease expression cassettes, relative to the wild type. The error bars are standard deviations of at least 3 repeated measurements. The sequences correlating with the sequence numbers in the X-axis are shown in Figure 18.

Figure 20: The sequence of the lacIQ_lacY expression cassette that was tested on lactose killing in example 16 (SEQ ID N° 58).

Figure 21: Effect of lactose on an *E. coli* wild type strain and a placIQ_lacY mutant strain. Both strains were grown with and without the addition of lactose mid exponential phase. The arrow indicates the moment of lactose addition. The growth of both strains was severely impaired by addition of lactose.

Summary of the invention

The present invention relates to a method to produce microorganisms which resist the phenomenon of lactose killing when grown in an environment in which lactose is combined with another carbon source, wherein said method comprises:

- a. mutating the expression of lactose transporters within microorganisms, wherein said mutation results in an expressed lactose transporter,
- b. growing said mutated microorganisms on a medium comprising a carbon-source which is not lactose,
- c. adding lactose to said medium during growth of said mutated microorganisms, and
- d. selecting the microorganisms which resist the phenomenon of lactose killing growing on said medium comprising lactose.

More specifically, the present invention relates to a method to produce microorganisms which resist the phenomenon of lactose killing when grown in an environment in which lactose is combined with another carbon source, wherein said method comprises:

- a. mutating the expression of lactose transporters within microorganisms, wherein said mutation results in the expression of said lactose transporter
- b. growing said mutated microorganisms on a medium comprising a carbon-source which is not lactose,
- c. adding lactose to said medium during growth of said mutated microorganisms, and
- d. selecting the microorganisms which resist the phenomenon of lactose killing growing on said medium comprising lactose and which retain at least 50% of the lactose influx obtained with the wild type expression cassette of said lactose transporter.

The present invention further relates to a method as indicated above wherein step a) is undertaken by introducing a heterologous promoter in front of an endogenous or exogenous lactose transporter gene, and/or by mutating the untranslated region in front of the coding sequence that contains the ribosome binding or Kozak sequences and/or by modifying the codon usage of the endogenous lactose transporter gene.

The present invention also relates to a method as described wherein said introduction of a heterologous promoter in front of an endogenous or exogenous lactose transporter gene is undertaken by: a) deleting the endogenous lactose transporters from the genome and reintroducing them at another location within the genome of said microorganism, or, b) by introducing a heterologous promoter in front of the endogenous lactose transporters, or, c) by knocking out the endogenous lactose promoter and introducing a heterologous promoter at the same location in the genome of said microorganism.

The present invention further relates to the method described above wherein the expression of lactose permease in step b) is detected by means of translational coupling with a reporter gene and/or via aptamer coupling.

The present invention relates to the method described above wherein the expressed lactose transporter is detected via genetic constructs as given by SEQ ID N° 2, 3, 4 and/or 5.

The present invention further relates to a method as described above wherein said lactose transporter is a lactose permease.

The present invention further relates to a method as described above wherein said microorganism is a bacterium, a yeast or a fungus.

The present invention relates also to promoter sequences, untranslated regions in front of the coding sequence that contain ribosome binding sequences or Kozak sequences and/or lactose permease sequences that lead to the expression of a lactose transporter, that does not result into lactose killing phenotype when microorganism containing such sequence is grown in an

environment in which lactose is combined with (an)other carbon source(s), and, that is obtainable by the a method as described above.

The present invention also relates to a microorganism which resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with another carbon source, and, is obtainable by a method as described above.

More specifically the present invention relates to a microorganism as described above, and, having a heterologous sequence in front of a lactose transporter gene as given by SEQ ID N° 1, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 or 57.

The present invention relates also to a microorganism which resists the phenomenon of lactose killing in which the genes coding for the enzymes from the lactose and/or galactose degradation pathways are rendered less functional or non-functional.

The present invention further relates to the usage of a microorganism as described above for the production of lactose-based specialty products such as specialty carbohydrates, glycolipids and galactosylated compounds.

The present invention also relates to the usage a microorganism as described above wherein said specialty carbohydrates are 2-fucosyllactose or 2'-fucosyllactose or 3-fucosyllactose or 2',3-difucosyllactose or lacto/Ntriose or lacto-N-tetraose, or lacto-nN-tetraose or 3'sialyllactose or 6'sialyllactose.

Description of invention

The present invention describes a novel way to avoid lactose killing by changing the expression of a lactose transporter via genetic engineering in an organism, resulting in a mutant lactose transporter expressing organism. To this end, an exogenous and/or an endogenous lactose transporter gene is expressed by a heterologous promoter that does not lead to a lactose killing phenotype and/or the codon usage of the lactose transporter is modified to create an altered expression of the lactose transporter that does not lead to a lactose killing phenotype. To this end the natural expression control of lactose transporters has to be removed and/or replaced in

such a way that lactose killing does not occur. For example, the naturally occurring lactose transporter expression cassette is deleted from the genome and reintroduced at another location, and/or, a heterologous promoter is introduced in front of lactose transporter at its original location, and/or, lactose transporter is knocked out first and reintroduced with a heterologous promoter at the same and/or another location in the genome, and/or, lactose transporter is introduced into an operon that is expressed via a heterologous promoter.

The present invention thus relates to a method to produce microorganisms which resist the phenomenon of lactose killing when grown in an environment in which lactose is combined with another carbon source, wherein said method comprises: 1) mutating the expression of lactose transporters within microorganisms, wherein said mutation results in the expression of said lactose transporter, 2) growing said mutated microorganisms on a medium comprising a carbon-source which is not lactose, 3) adding lactose to said medium during growth of said mutated microorganisms, and 4) selecting the microorganisms which resist the phenomenon of lactose killing growing on said medium comprising lactose and which retain at least 50% of the lactose influx obtained with the wild type expression cassette of said lactose transporter.

The term 'lactose killing' refers to the phenomenon of growth retardation or growth arrest of an organism that is grown in an environment in which lactose or galactoside is combined with (an)other carbon source(s). These carbon sources are, non-limiting, glycerol, maltose, glucose, fructose, sucrose, fucose, mannose, sialic acid, starch, cellulose, polyols (such as mannitol, xylitol, sorbitol), organic acids (lactate, succinate, acetate,...), and/or, pentoses (xylose, arabinose,...).

The present invention describes a method to identify lactose permease expression systems that do not result in lactose killing. This method encompasses a growth analysis of the mutant strain to which lactose is added mid exponential phase. This method can be performed in a high throughput manner in micro-titre plates or with cell sorters, enabling the screening of multiple promoters, ribosome binding sites, codon usage, and other factors that can influence the expression of lactose permease in the mutant micro-organism. The present invention describes a method to detect lactose permease expression via translational coupling and/or aptamer coupling and the selection of sequences that lead to expression via a reporter gene, such as but not limited to an antibiotic resistance gene (for instance but not limited to chloramphenicol, Geneticin G418), a fluorescent protein, a hydrolase (for instance but not limited to

galactosidase, xylanase) and, or an aptamer sequence. Sequences that lead to the expression of lactose permease are further selected based on the reporter gene, by growth in a medium with an antibiotic, a colorimetric assay (such as X-gal) and/or by means of a fluorescence-activated cell sorter that sorts out fluorescent cells and/or an aptamer assay for instance, but not limited to, based on (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one (37, 64). The present invention further describes a procedure to screen for lactose transporter expressing mutant organisms that do not undergo lactose killing. In addition the present invention describes how libraries of lactose permease expression cassettes can be created via promoter libraries, RBS or Kozak sequence libraries, transcription terminator libraries, and/or codon usages variants of the lactose permease gene. These libraries are further created via methods such as but not limited to Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation (25, 36, 50, 79).

The term 'which retain at least 50% of the lactose influx obtained with the wild type expression cassette of said lactose transporter' relates to the fact that the mutated microorganisms of the present invention should –although they resist lactose killing- be still capable of retaining at least 50% (i.e. 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 99 %) of the lactose influx which is obtained when using the wild type expression cassette of said lactose transporter.

The term 'expression cassette' relates to any sequence in which a promoter sequence, untranslated region sequence (containing either a ribozyme binding sequence or Kozak sequence), a coding sequence (for instance a lactose permease gene sequence) and optionally a transcription terminator (18) is present.

The term 'lactose transporter' refers to any protein expressed in a microorganism that is capable to translocate (transport) lactose across the cytoplasmic membrane. Such proteins are for instance, lactose permeases (transporters from the superfamily MFS or Major Facilitator Superfamily).

The term 'heterologous promoter' refers to any promoter that does not naturally occur in front of a coding sequence. A "promoter" is the whole of the RNA polymerase binding sequence that is located before the transcription start site and the untranslated region in front of the coding sequence. A "heterologous promoter" sequence is thus: 1) a variant of the naturally occurring

promoter sequence containing at least 1 (i.e. 1, 2, 3, 4, ...) mutation, and/or 2) a native promoter from the mutant lactose transporter expressing micro-organism that does not naturally occur in front of the coding sequence of said transporter, and/or 3) a sequence that does not occur naturally in the lactose transporter expressing micro-organism, and/or 4) an artificial promoter which is an *in silico* designed promoter. These promoters can be derived from libraries such as, but not limited to, described by Alper *et al.* (2005), and/or HXT7p, Hammer *et al.* (2006), De Mey *et al.* (2007), Coussement *et al.* (2014) or, Mutalik *et al.* (2013) (3, 25, 29, 41, 60) (66), or Blount *et al.* (2012), promoters such as but not limited to ADH1p, TEF1p, TEF2p, GPDp, PDC1p, FBA1p, PGK1p, PGI1p, TDH2p, PYK1p, ENO2p, GPM1p, TPI1p (13), or designed as described, for example, by Rhodius *et al.* (2012). The term 'artificial promoter' also refers to promoters with DNA sequences that are combinations of the native (autologous) promoter sequence with parts of different (autologous or heterologous) promoter sequences. Sequences of such 'artificial promoters' can be found in databases such as for example partsregistry.org (19). The heterologous promoters lead either to constitutive expression or regulated expression via a transcription factor.

The term 'constitutive expression' is defined as expression that is not regulated by transcription factors other than the subunits of RNA polymerase (e.g. the bacterial sigma factors) under certain growth conditions. None limiting examples of such transcription factors are CRP, LacI, ArcA, Cra, IclR, ... in *E. coli*, or, Aft2p, Crz1p, Skn7, ... in *Saccharomyces cerevisiae*, or, DeoR, GntR, Fur, ... in *B. subtilis*. These transcription factors bind on a specific sequence and may block or enhance expression in certain growth conditions. RNA polymerase binds a specific sequence to initiate transcription, for instance via a sigma factor in prokaryotic hosts.

The term 'regulated expression' is defined as expression that is regulated by transcription factors other than the subunits of RNA polymerase (e.g. bacterial sigma factors) under certain growth conditions. Examples of such transcription factors are described above.

The term 'untranslated region' in front of the coding sequence that contains the ribosome binding sites or Kozak sequences relates to the sequence between the RNA polymerase binding sequence and the coding sequence. This untranslated region is also the sequence naturally occurring in front of the coding sequence, and/or, a sequence that naturally occurs in the lactose transporter expressing micro-organism, and/or, a sequence that is derived from other organisms

(either prokaryotes or eukaryotes), and/or, a sequence that is artificially designed, which relates to non-natural or *in silico* designed ribosome binding sites with known or measurable translation rates, these sequences can be derived from libraries as described by Mutalik et al (2013) (60) or designed via algorithms for example as described by Salis *et al* (2009) (67) or
5 can be found in databases such as partsregistry.org (19).

The term ‘modified codon usage’ relates to the altering of the codons used within a DNA coding sequence, either to codons used more frequently by the organism or used rarely by the organism. Codon usage is defined in databases such as the codon usage database (61) and codon
10 usage is optimized via codon usage design algorithms (73).

The term ‘translational coupling’ refers to the coupled expression of a gene of interest and a reporter gene such as a green fluorescent proteins, antibiotic resistance genes, toxic genes, (49, 53, 58, 63). The term ‘translational sensor’ refers to any mechanism that is an indicator for
15 expression and translation of a gene, e.g. fluorescent tags and split tags as described in the following references (17, 72).

The term ‘aptamer coupling’ refers to the introduction of an aptamer sequence into the messenger RNA of the lactose transporter that can be detected by a fluorophore such as but not
20 limited to (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one (37, 64).

The term ‘growth analysis’ refers to the analysis of the growth curve of an organism. This organism is cultivated in a growth medium in a growth environment. The term growth
25 environment relates to all environmental parameters such as pH, temperature, dissolved oxygen. The pH is set by means of a pH buffer or by pH control and is for example but not limited to pH 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, or 8. The temperature is set at for example but not limited to 25, 28, 30, 32, 34, 37, 40, 42, 45°C. The dissolved oxygen is either anaerobic, micro-aerobic (with dissolved oxygen below 1.5 mg/l) or aerobic conditions.

30 The term ‘medium’ or ‘growth medium’ relates to any solution containing the necessary substrates for an organism to grow. These substrates are, but not limited to, nitrogen sources such as ammonium salts, nitrate salts, yeast extract, pepton, casamino, and/or amino acids,

phosphor sources such as but not limited to phosphate salts, sulphur sources such as but not limited to sulphate salts, elements such as but not limited to copper, cobalt, iron, selenium, iodine, molybdate, magnesium, calcium, potassium, sodium, zinc, nickel, manganese, and/or boric acid and/or vitamins such as but not limited to thiamine, pantothenic acid, and/or niacin and/or a carbon source such as but non-limiting, glycerol, maltose, glucose, fructose, sucrose, fucose, mannose, sialic acid, starch, cellulose, polyols (such as mannitol, xylitol, sorbitol), organic acids (lactate, succinate, acetate,...), and/or, pentoses (xylose, arabinose,...).

The term 'organism' or 'cell' as indicated above refers to a microorganism chosen from the list consisting of a bacterium, a yeast or a fungus, or, refers to a plant or animal cell. The latter 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. The latter bacterium belonging to the phylum Proteobacteria belongs preferably to the family *Enterobacteriaceae*, preferably to the species *Escherichia coli*. The latter 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, the latter term 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 type, W3110, MG1655, M182, MC1000, MC1060, MC1061, MC4100, JM101, NZN111 and AA200. Hence, the present invention specifically relates to a mutated and/or transformed *Escherichia coli* strain as indicated above wherein said *E. coli* strain is a K12 strain. More specifically, the present invention relates to a mutated and/or transformed *Escherichia coli* strain as indicated above wherein said K12 strain is *E. coli* MG1655. The latter bacterium belonging to the phylum Firmicutes belongs preferably to the Bacilli, preferably from the species *Bacillus*. The latter yeast 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. The latter yeast belongs preferably to the genus *Saccharomyces*, *Pichia*, *Hansenella*, *Kluyveromyces*, *Yarrowia* or *Starmerella*. The latter fungus belongs preferably to the genus *Rhizopus*, *Dictyostelium* or *Aspergillus*.

The present invention describes organisms that are able to take up lactose without undergoing lactose killing and are able to convert lactose or its galactose moiety into a specialty product.

More particularly said specialty products or bioproducts are specialty carbohydrates, glycolipids, such as but not limited to galactolipids and/or lactolipids, and/or, galactosylated compounds such as galactosyllipids, galactosylceramides and/or galactosylated aglycons.

5 The present invention describes organisms that are able to take up lactose without undergoing lactose killing and convert of said lactose into a human milk oligosaccharide, such as but not limited to 3-fucosyllactose, 2'-fucosyllactose, 6-fucosyllactose, 2',3-difucosyllactose, 2',2-difucosyllactose, 3,4-difucosyllactose, 6'-sialyllactose, 3'-sialyllactose, 3,6-disialyllactose, 6,6'-disialyllactose, 3,6-disialyllacto-N-tetraose, lactodifucotetraose, lacto-N-tetraose, lacto-
 10 N-neotetraose, lacto-N-fucopentaose II, lacto-N-fucopentaose I, lacto-N-fucopentaose III, sialyllacto-N-tetraose c, sialyllacto-N-tetraose b, sialyllacto-N-tetraose a, lacto-N-difucohexaose I, lacto-N-difucohexaose II, lacto-N-hexaose, lacto-N-neohexaose, para-lacto-N-hexaose, monofucosylmonosialyllacto-N-tetraose c, monofucosyl para-lacto-N-hexaose, monofucosyllacto-N-hexaose III, isomeric fucosylated lacto-N-hexaose III, isomeric
 15 fucosylated lacto-N-hexaose I, sialyllacto-N-hexaose, sialyllacto-N-neohexaose II, difucosyl-para-lacto-N-hexaose, difucosyllacto-N-hexaose, difucosyllacto-N-hexaose a, difucosyllacto-N-hexaose c, galactosylated chitosan, fucosylated oligosaccharides, and/or sialylated oligosaccharides....

20 The present invention further describes organisms that do not undergo lactose killing and that synthesize nucleotide sugars such as but not limited to GDP-L-fucose, GDP-mannose, GDP-glucose, CMP-sialic acid, UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine, UDP-N-acetylmannosamine, UDP-N-acetylgalactosamine, UDP-glucuronic acid, UDP-galacturonic acid, UDP-xylose, UDP-arabinose, and/or, dTDP-rhamnose. The term "sialic acid" is the group
 25 name for compounds such as, but not limited to neuramic acid, N-acetylneuramic acid, or N-glycoylneuramic acid as defined by Varki (1992) (71). The intracellular GDP-fucose concentration or pool is enhanced by upregulation by either the de novo pathway and/or the salvage pathway. The *de novo* pathway consists of a GDP-4-keto-6-deoxymannose-3,5-epimerase-4-reductase or GDP-fucose synthase, a GDP-mannose 4,6-dehydratase, a GDP-D-
 30 mannose pyrophosphorylase, a phosphomannose isomerase and/or a phosphomannomutase, of which the expression is enhanced individually via genetic elements such as but not limited to promoters and/or ribosome binding sites and/or altered codon usage in a mono-cistron or polycistron (operon structure); and/or via the regulators *arcA* *iclR* in *Enterobacteriaceae* and/or

via the transcriptional regulator RcsA in *Enterobacteriaceae* or homologous genes in bacteria, or, *Xbp1*, *Spt20*, *Sfp1*, *Rpd3*, *Rap1*, *Gcr1*, *Gcn5*, *Cst6*, *Abf1*, *Hsf1*, *Reb1*, *Cad1*, *Sin4*, *Ash1*, *Ixr1*, *Met32*, *Pho2*, *Rgr1*, *Spt7*, *Swi4* in *Saccharomyces cerevisiae* or homologous genes in yeasts or fungi. The salvage pathway consists of a L-fucose kinase and/or a GDP-L-fucose pyrophosphorylase. The GDP-mannose pool is enhanced by upregulating genes coding for a GDP-D-mannose pyrophosphorylase, a phosphomannose isomerase and/or a phosphomannomutase; and/or, genes coding for a mannokinase and a GDP-D-mannose pyrophosphorylase. The UDP-galactose pool is enhanced by upregulating the genes coding for a galactokinase and/or galactose 1-phosphate uridyl transferase, and/or, a UDP-galactose-4-epimerase, and/or, a UDP-galactose/glucose pyrophosphorylase, and/or a lactose synthase, and/or a lactose phosphorylase and/or a sucrose phosphorylase. The UDP-glucose pool is enhanced by upregulating the genes coding for a glucokinase, and/or, a UDP-glucose pyrophosphorylase, and/or a sucrose phosphorylase and/or a phosphoglucosmutase, and/or a sucrose synthase. The CMP-sialic acid pool is enhanced by upregulating genes coding for L-glutamine:D-fructose-6-phosphate aminotransferase, and/or, phosphoglucosamine mutase, and/or, glucosamine-1-phosphate acetyltransferase and/or N-acetylglucosamine-1-phosphate uridyltransferase, and/or UDP-N-acetylglucosamine 2-epimerase and/or N-acetylneuraminate synthase and/or cytidine 5'-monophosphate N-acetylneuraminate synthetase. The UDP-N-acetylglucosamine pool is enhanced by upregulating genes coding for L-glutamine:D-fructose-6-phosphate aminotransferase, and/or, phosphoglucosamine mutase, and/or, glucosamine-1-phosphate acetyltransferase and/or N-acetylglucosamine-1-phosphate uridyltransferase and/or glucosamine-6-phosphate N-acetyltransferase and/or, phosphoacetylglucosamine mutase, and/or, UDP-N-acetylglucosamine pyrophosphorylase. The UDP-N-acetylmannosamine pool is enhanced by upregulating genes coding for the UDP- N-acetylglucosamine pool enhancement and/or a UDP-N-acetylglucosamine 2-epimerase. The UDP-N-acetylglactosamine pool is enhanced by upregulating genes coding for the UDP- N-acetylglucosamine pool enhancement and/or a UDP-N-acetylglucosamine C4-epimerase. The UDP-glucuronic acid pool is enhanced by upregulating genes coding for the UDP-glucose pool enhancement and/or UDP-glucose dehydrogenase. The UDP-xylose pool is enhanced by upregulating genes coding for the UDP-glucuronic pool enhancement and/or UDP-D-xylose synthase. The UDP-galacturonic acid pool is enhanced by upregulating genes coding for the UDP-glucuronic acid pool enhancement and/or UDP-D-glucuronic acid 4-epimerase. The UDP-arabinose pool is enhanced by upregulating genes coding for the UDP-glucuronic pool enhancement and/or UDP-D-xylose 4-

epimerase and/or arabinose kinase and/or UDP-L-arabinose pyrophosphorylase. The dTDP-rhamnose pool is enhanced by upregulating genes coding for a dTDP-glucose pyrophosphorylase and/or dTDP-glucose 4,6-dehydratase and/or dTDP-4-dehydrorhamnose 3,5-epimerase and/or dTDP-4-dehydrorhamnose reductase and or a glucose-1-phosphate thymidyltransferase and/or a nucleotide rhamnose synthase.

The term “pool” further relates to concentrations of metabolites that naturally occur in the wild type organism, e.g. the concentration of a nucleotide sugar pool. The term “enhanced pool” relates to a significantly increased concentration of said metabolite pool, higher than the metabolite pool in the wild type organism.

The term “upregulating a gene” relates to each genetic modification that leads to the enhanced expression of the gene and/or activity of the product of said gene. Said genetic modification is either a modification in the promoter, untranslated region, ribosome binding site, the coding sequence, the gene location, the intron/exon structure and/or the transcriptional terminator, leading to said increased expression and/or activity.

In addition, the present invention describes genetically modified organisms that can transfer these nucleotide sugars onto a mono-, di- or oligosaccharide, such as, but not limited to, galactose, lactose, lactoNbiose, lactoNtriose, lactoNtetraose, lacto-N-neotetraose, globotriose, 2’fucosyllactose, 3-fucosyllactose, 3-sialyllactose, 6-sialyllactose, human milk oligosaccharides, heparosans, chitosans, nod-factors, glycolipids, and/or aglycons, ... by means of a glycosyltransferase enzyme.

The present invention also describes organisms that do not undergo lactose killing and that can further modify said lactose with enzymes such as but not limited to carbohydrate hydrolases, carbohydrate transferases, carbohydrate synthases, acetylases, acyltransferases, carbohydrate phosphatases, polysaccharide Lyases, kinases, pyruvylases and/or sulfotransferase.

The present invention further describes organisms that do not undergo lactose killing and does not degrade lactose anymore by rendering the lactose hydrolase gene less-functional or non-functional.

The terms 'genes which are rendered less-functional or non-functional' refer to the well-known technologies for a skilled person such as the usage of siRNA, RNAi, miRNA, asRNA, mutating genes, knocking-out genes, transposon mutagenesis, CrispR/CAS etc... which are used to change the genes in such a way that they are less able (i.e. statistically significantly 'less able' compared to a functional wild-type gene) or completely unable (such as knocked-out genes) to produce functional final products. The term '(gene) knockout' thus refers to a gene which is rendered non-functional. The term 'deleted gene' or 'gene deletion' also refers to a gene which is rendered non-functional (2, 4-9, 22, 27, 30, 43, 45, 46, 51, 65, 74).

The present invention describes organisms in which genes are introduced/knocked in/upregulated to produce bioproducts as described above. These genes are found in gene databases such as but not limited to genbank or protein databases such as but not limited to uniprot, or enzyme databases such as but not limited to Brenda enzyme database (16, 23, 38) and pathways towards bioproducts are found in databases such as but not limited to KEGG, Biocyc, Metacyc (20, 44, 47). The pathway towards a certain bioproduct described above can be determined by several mathematical tools described in the art (35, 54, 57, 76).

Examples

1. Material and methods *Escherichia coli*

Strains and plasmids

Escherichia coli MG1655 [λ , F⁻, *rph*-1] and JM109 were obtained from the Netherlands Culture Collection of Bacteria (NCCB). All mutant strains were created using the method of Datsenko & Wanner (27).

Media

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).

Shake flask medium contained 2 g/l NH₄Cl, 5 g/l (NH₄)₂SO₄, 2.993 g/l KH₂PO₄, 7.315 g/l K₂HPO₄, 8.372 g/l MOPS, 0.5 g/l NaCl, 0.5 g/l MgSO₄·7H₂O, 15 g/l glycerol (unless stated otherwise), 1 ml/l vitamin solution, 100 µl/l molybdate solution, and 1 ml/l selenium solution. The medium was set to a pH of 7 with 1M KOH.

Vitamin solution consisted of 3.6 g/l FeCl₂ · 4H₂O, 5 g/l CaCl₂ · 2H₂O, 1.3 g/l MnCl₂ · 2H₂O, 0.38 g/l CuCl₂ · 2H₂O, 0.5 g/l CoCl₂ · 6H₂O, 0.94 g/l ZnCl₂, 0.0311 g/l H₃BO₄, 0.4 g/l Na₂EDTA · 2H₂O and 1.01 g/l thiamine · HCl. The molybdate solution contained 0.967 g/l Na₂MoO₄ · 2H₂O. The selenium solution contained 42 g/l SeO₂.

The minimal medium for fermentations contained 6.75 g/l NH₄Cl, 1.25 g/l (NH₄)₂SO₄, 1.15 g/l KH₂PO₄, 0.5 g/l NaCl, 0.5 g/l MgSO₄·7H₂O, 30 g/l lactose and 20 g/l sucrose (or different concentrations if stated otherwise), 1 ml/l vitamin solution, 100 µl/l molybdate solution, and 1 ml/l selenium solution with the same composition as described above.

Cultivation conditions

A preculture, from a single colony on a LB-plate, in 5 ml LB medium was incubated during 8 hours at 37 °C on an orbital shaker at 200 rpm. From this culture, 2 ml was transferred to 100 ml minimal medium in a 500 ml shake flask and incubated for 16 hours at 37 °C on an orbital shaker at 200 rpm. 4 % inoculum was used in a 2 or 5 l Biostat B Plus culture vessel with 1.5 l or 4 L working volume (Sartorius Stedim Biotech, Melsungen, Germany). The culture conditions were: 37 °C, stirring at 800 rpm, and a gas flow rate of 1.5 l/min. Aerobic conditions were maintained by sparging with air. The pH was maintained at 7 with 0.5 M H₂SO₄ and 35% M ammonia solution. The exhaust gas was cooled down to 4 °C by an exhaust cooler (Frigomix

1000, Sartorius Stedim Biotech, Melsungen, Germany). 10 % solution of silicone antifoaming agent (BDH 331512K, VWR Int Ltd., Poole, England) was added when foaming raised during the fermentation (approximately 10 μ l). The off-gas was measured with an EL3020 off-gas analyser (ABB Automation GmbH, 60488 Frankfurt am Main, Germany).

- 5 All data was logged with the Sartorius MFCS/win v3.0 system (Sartorius Stedim Biotech, Melsungen, Germany).

Sampling methodology

The bioreactor contains in its interior a harvest pipe (BD Spinal Needle, 1.2x152 mm (BDMedical Systems, Franklin Lakes, NJ - USA) connected to a reactor port, linked outside to
10 a Masterflex-14 tubing (Cole-Parmer, Antwerpen, Belgium) followed by a harvest port with a septum for sampling. The other side of this harvest port is connected back to the reactor vessel with a Masterflex-16 tubing. This system is referred to as rapid sampling loop. During sampling, reactor broth is pumped around in the sampling loop. It has been estimated that, at a flow rate of 150 ml/min, the reactor broth needs 0.04 s to reach the harvest port and 3.2 s to re-
15 enter the reactor. At a pO₂ level of 50 %, there is around 3 mg/l of oxygen in the liquid at 37 °C. The pO₂ level should never drop below 20 % to avoid micro-aerobic conditions. Thus 1.8 mg/l of oxygen may be consumed during transit through the harvesting loop. Assuming an oxygen uptake rate of 0.4 g oxygen/g biomass/h (the maximal oxygen uptake rate found at $\mu_{\max}$), this gives for 5 g/l biomass, an oxygen uptake rate of 2 g/l/h or 0.56 mg/l/s, which
20 multiplied by 3.2 s (residence time in the loop) gives 1.8 mg/l oxygen consumption.

In order to quench the metabolism of cells during the sampling, reactor broth was sucked through the harvest port in a syringe filled with 62 g stainless steel beads pre-cooled at -20 °C, to cool down 5 ml broth immediately to 4 °C. Sampling was immediately followed by cold centrifugation (15000 g, 5 min, 4 °C). During the batch experiments, a sample for OD_{600nm}
25 measurement was taken using the rapid sampling loop and the cold stainless bead sampling method.

Analytical methods

Cell density of the culture was frequently monitored by measuring optical density at 600 nm (Uvikom 922 spectrophotometer, BRS, Brussel, Belgium). Cell dry weight was obtained by
30 centrifugation (15 min, 5000 g, GSA rotor, Sorvall RC-5B, Goffin Meyvis, Kapellen, Belgium) of 20 g reactor broth in pre-dried and weighted falcons. The pellets were subsequently washed once with 20 ml physiological solution (9 g/l NaCl) and dried at 70 °C to a constant weight. To

be able to convert OD_{600nm} measurements to biomass concentrations, a correlation curve of the OD_{600nm} to the biomass concentration was made. The concentrations of glucose and organic acids were determined on a Varian Prostar HPLC system (Varian, Sint-Katelijne-Waver, Belgium), using an Aminex HPX-87H column (Bio-Rad, Eke, Belgium) heated at 65 °C, equipped with a 1 cm precolumn, using 5 mM H₂SO₄ (0.6 ml/min) as mobile phase. A dual-wave UV-VIS (210 nm and 265 nm) detector (Varian Prostar 325) and a differential refractive index detector (Merck LaChrom L-7490, Merck, Leuven, Belgium) was used for peak detection. By dividing the absorptions of the peaks in both 265 and 210 nm, the peaks could be identified. The division results in a constant value, typical for a certain compound (formula of Beer-Lambert).

Glucose, fructose, sucrose, oligosaccharides and glucose-1-phosphate were measured by HPLC with a Hypercarb column and were detected with an MSMS detector (Antonio et al., 2007; Nielsen et al., 2006).

Genetic methods

The methods used for mutant construction is described below.

Plasmids were maintained in the host *E. coli* DH5 α (F⁻, ϕ 80 Δ lacZ Δ M15, Δ (lacZYA-argF)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, λ^- , *thi-1*, *gyrA96*, *relA1*).

Plasmids. pKD46 (Red helper plasmid, Ampicillin resistance), pKD3 (contains an FRT-flanked chloramphenicol resistance (*cat*) gene), pKD4 (contains an FRT-flanked kanamycin resistance (*kan*) gene), and pCP20 (expresses FLP recombinase activity) plasmids were used for the mutant construction. The plasmid pBluescript (Fermentas, St. Leon-Rot, Germany) was used to construct the derivatives of pKD3 and pKD4 with a promoter library, or with alleles carrying a point mutation.

Mutations. The mutations consisted in gene disruption (knock-out, KO). They were introduced using the concept of Datsenko and Wanner (27).

Transformants carrying a Red helper plasmid were grown in 10 ml LB media with ampicillin (100 mg/l) and L-arabinose (10 mM) at 30 °C to an OD_{600nm} of 0.6. The cells were made electrocompetent by washing them with 50 ml of ice-cold water, a first time, and with 1 ml ice-cold water, a second time. Then, the cells were resuspended in 50 μ l of ice-cold water. Electroporation was done with 50 μ l of cells and 10–100 ng of linear double-stranded-DNA product by using a Gene PulserTM (BioRad) (600 Ω , 25 μ FD, and 250 volts).

After electroporation, cells were added to 1 ml LB media incubated 1 h at 37 °C, and finally spread onto LB-agar containing 25 mg/l of chloramphenicol or 50 mg/l of kanamycin to select antibiotic resistant transformants. The selected mutants were verified by PCR with primers upstream and downstream of the modified region and were grown in LB-agar at 42 °C for the loss of the helper plasmid. The mutants were tested for ampicillin sensitivity

Linear double-stranded-DNA. The linear ds-DNA amplicons were obtained by PCR using pKD3, pKD4 and their derivatives as template. The primers used had a part of the sequence complementary to the template and another part complementary to the side on the chromosomal DNA where the recombination has to take place. For the KO, the region of homology was designed 50-nt upstream and 50-nt downstream of the start and stop codon of the gene of interest. For the KI, the transcriptional starting point (+1) had to be respected. PCR products were PCR-purified, digested with *DpnI*, repurified from an agarose gel, and suspended in elution buffer (5 mM Tris, pH 8.0).

Elimination of the antibiotic resistance gene. The selected mutants (chloramphenicol or kanamycin resistant) were transformed with pCP20 plasmid, which is an ampicillin and chloramphenicol resistant plasmid that shows temperature-sensitive replication and thermal induction of FLP synthesis. The ampicillin-resistant transformants were selected at 30 °C, after which a few were colony purified in LB at 42 °C and then tested for loss of all antibiotic resistance and of the FLP helper plasmid. The gene knock outs and knock ins are checked with control primers (Fw/Rv-gene-out).

Transformation. Plasmids were transformed in CaCl₂ competent cells using the simplified procedure of Hanahan (42) or via electroporation as described above.

2. Material and methods yeast

2.1. Strains

Saccharomyces cerevisiae BY4742 was obtained from the Euroscarf culture collection. All mutant strains were created by homologous recombination or plasmid transformation using the method of Gietz (40). *Kluyveromyces marxianus lactis* was obtained from the culture collection of the *Laboratory of Industrial Biotechnology and Biocatalysis*.

2.2. Media

Strains are grown on Synthetic Defined yeast medium with Complete Supplement Mixture (SD CSM) or CSM drop-out (SD CSM-Ura) containing 6.7 g.L⁻¹ Yeast Nitrogen Base without

amino acids (YNB w/o AA, Difco), 20 g.L⁻¹ agar (Difco) (solid cultures), 22 g.L⁻¹ glucose monohydrate or 20 g.L⁻¹ lactose and 0.79 g.L⁻¹ CSM or 0.77 g.L⁻¹ CSM-Ura (MP Biomedicals).

2.3. Cultivation conditions

Yeast cultures are first inoculated in 5 mL of the appropriate medium and incubated overnight at 30 °C and 200 rpm. In order to obtain higher volume cultures, 2 % (or higher) of the pre-culture is inoculated in 50-200 mL medium. These cultures are again incubated at 30 °C and 200 rpm.

Growth experiments are conducted in 96-well plate or Erlenmeyer scale. In order to obtain single colonies as start material for the growth and production experiments, strains are plated on selective SD CSM plates and incubated for 2-3 days at 30 °C. One colony is then picked and transferred to 5 mL medium for the Erlenmeyer studies or to 1 mL medium for the microtiterplate experiments.

For the Erlenmeyer experiments, the pre-cultures are incubated overnight at 30 °C and 200 rpm and 2 % of these pre-cultures are added to 100 mL medium in order to start the growth experiments.

For the MTP growth experiments, colonies are added to 150 µL medium and incubated for 24 hours at 30°C. After incubation, 2 µL of the MTP pre-cultures are added to an MTP containing 150µL fresh media. OD is measured every fifteen minutes for 48 hours with the Infinite® 200 Pro Tecan.

2.4. Sampling methodology

Samples of both the OD (0.2 mL) and the cellular and supernatant fraction (1 mL) of the culture are taken every two hours until the stationary phase and every couple hours during the stationary phase. The 1 mL sample is first centrifuge (11) after which the cell pellet and the supernatant are separated and stored separately at -20 °C. Supernatant is stored for extracellular product analysis while the pellets are used for intracellular metabolite analysis.

2.5. Analytical methods

Cell density of the culture was frequently monitored by measuring optical density at 600 nm (Uvikom 922 spectrophotometer, BRS, Brussel, Belgium) or with the with the Biochrom Anthos Zenyth 340 Microtiterplate reader. Cell dry weight was obtained by centrifugation (15 min, 5000 g, GSA rotor, Sorvall RC-5B, Goffin Meyvis, Kapellen, Belgium) of 20 g reactor broth in pre-dried and weighted falcons. The pellets were subsequently washed once with 20 ml physiological solution (9 g/l NaCl) and dried at 70 °C to a constant weight. To be able to convert OD_{600nm} measurements to biomass concentrations, a correlation curve of the OD_{600nm}

to the biomass concentration was made. The concentrations of glucose and organic acids were determined on a Varian Prostar HPLC system (Varian, Sint-Katelijne-Waver, Belgium), using an Aminex HPX-87H column (Bio-Rad, Eke, Belgium) heated at 65 °C, equipped with a 1 cm precolumn, using 5 mM H₂SO₄ (0.6 ml/min) as mobile phase. A dual-wave UV-VIS (210 nm and 265 nm) detector (Varian Prostar 325) and a differential refractive index detector (Merck LaChrom L-7490, Merck, Leuven, Belgium) was used for peak detection. By dividing the absorptions of the peaks in both 265 and 210 nm, the peaks could be identified. The division results in a constant value, typical for a certain compound (formula of Beer-Lambert).

Glucose, fructose, sucrose, oligosaccharides and glucose-1-phosphate were measured by HPLC with a Hypercarb column and were detected with an MSMS detector (Antonio et al., 2007; Nielsen et al., 2006).

2.6. Genetic methods

The methods used for mutant construction is described below.

Plasmids were maintained in the host *E. coli* DH5 α (F⁻, ϕ 80dlacZ Δ M15, Δ (lacZYA-argF)U169, *deoR*, *recA1*, *endA1*, *hsdR17*(rk⁻, mk⁺), *phoA*, *supE44*, λ ⁻, *thi-1*, *gyrA96*, *relA1*).

Plasmids. Yeast expression plasmid p2a_2 μ _Lac4 available at the *Laboratory of Industrial Biotechnology and Biocatalysis* was used to enable growth of *Saccharomyces* on lactose as the sole C-source. This plasmid contains an ampicillin resistance gene and a bacterial origin of replication to allow for selection and maintenance in *E. coli*. The plasmid further contains the 2 μ yeast ori and the *Ura3* selection marker for selection and maintenance in yeast. Finally, the plasmid contains a β -galactosidase expression cassette (SEQ ID 9, Figure 15).

Mutations. The mutations consisted in plasmid introduction using p2a_2 μ _Lac4 (described above) and gene knock-in (KI) (KI at the rDNA locus) using double stranded linear DNA (described above). Transformants were plated on SD CSM-Ura after transformation with plasmid DNA or on SD CSM-Ura with lactose as the sole C-source after transformation with double stranded linear DNA. The selected plasmid bearing mutants were verified by PCR with primers matching p2a_2 μ _Lac4. The selected genomic knock-in mutants were verified by PCR with primers upstream and downstream of the modified region and confirmed by sequencing (performed at LGC Genomics (LGC group, Germany)).

Linear double-stranded-DNA. The linear ds-DNA amplicons were obtained by PCR using plasmid pJet_KI_p1_Lac12_t@rDNA or pJet_KI_p2_Lac12_t@rDNA. These plasmids contain 2 500 bp homology regions (HR1 (SEQ ID 10, Figure 16) and HR2 (SEQ ID 11, Figure 17)) flanking SEQ ID 7 and SEQ ID 8, respectively, at the multi-cloning site of the pJET

Cloning vector (Thermoscientific). The primers used are homologous to the 5' end of HR1 (forward primer) and the 3' end of HR2 (reverse primer). PCR products were PCR-purified prior to transformation.

Transformations. Plasmids and linear double stranded DNA were transformed using the method Gietz (40).

3. Results

Example 1: Construction of an *E. coli* lactose permease *lacY* knock in at the *agp* locus

First the strain MG1655 Δ lacY was constructed according to the method of Datsenko and Wanner as described above. To this end MG1655 was transformed with pKD46 and linear DNA was constructed from the base plasmids pKD3 and pKD4 with flanking homologies to the lacY gene. Successful recombinations were then screened with the appropriate antibiotics. To ensure no lactose could be taken up in this strain, the strain was grown on a minimal medium only containing lactose as carbon source. No growth was observed during this experiment, hence this cell could not transport lactose anymore over its membrane.

To further construct a synthetic expression system, a synthetic promoter and RBS were synthesized in combination with the *lacY* gene (ordered from IDT and Geneart). This sequence is shown in Figure 3. This sequence was also introduced into the genome at the *agp* gene locus via an adaptation of the Datsenko and Wanner methodology. Briefly, the lactose permease construct was first assembled with a screening cassette from the pKD3 plasmid, resulting into a novel plasmid, pCX_{lacY}-kan. From this plasmid linear DNA could be PCR amplified with homologies to the *agp* genomic region. This obtained linear DNA can then be transformed into an *E. coli* MG1655 Δ lacY, in which the pKD46 plasmid is present. This led to the recombination of the lactose transporter expression cassette into the genome, resulting into a lactose permease expression organism MG1655 Δ lacY Δ agp::*lacY*_{synthetic}. To ensure growth on lactose was restored, this strain was grown on a lactose minimal medium as described above. This resulted in full restoration of growth on lactose, with a growth rate similar to the wild type.

Example 2: The effect of lactose on the wild type *E. coli* strain and a mutant *E. coli* strain that does not undergo lactose killing

A shake flask experiment as described in materials and methods was set up with the wild type MG1655 and MG1655 Δ lacY Δ agp::*lacY*_{synthetic}. These strains were grown in a glycerol shake

flask medium (15 g/l glycerol, as described in the materials and methods) and lactose (200g/l stock solution was added resulting in a final concentration of 10 g/l) was added mid exponential phase (approximately at OD 0.8). As can be seen in [Figure 1](#) and [Figure 2](#), the mutant strain does not undergo lactose killing.

5

Example 3: The use of translational coupling or translational sensors to ensure lactose transporter expression

Because a full lactose permease knock out strain would also not undergo lactose killing and the goal is to obtain a functional, active, expressed lactose permease a screen is needed to ensure
 10 lactose permease expression. To this end, sequence variants of promoters, ribozyme binding sites, Kozak sequences, codon usage and transcription terminators can be created. However, these sequence variants may lead to null-expression constructs, hence leading to lactose transporter negative mutant strains. Therefore a system needs to be designed to detect the expression of the lactose transporter, preferably by a easy to screen reporter gene such as *lacZ*,
 15 fluorescent proteins or antibiotic resistance genes.

Construction of a translational coupling system that reinitiates translation of the reporter gene to detect lactose transporter expression

Two genes can be translationally coupled by introducing a translation reinitiation region 3' from the gene of interest and 5' from the reporter gene. Translation can be reinitiated by several
 20 codons, such as AUG, UUG, GUG or AUA (63). The sequence of such a construct, which couples the lactose permease gene and the *lacZ* gene translationally is shown in Figure 4. To create this sequence, the *lacY* and *lacZ* sequence are amplified from the *E. coli* genome with primers with golden gate restriction sites (BsaI, obtained from NEB). The intergenic region that
 25 allows the translational coupling can be ordered from any gene synthesis company, such as IDT or Geneart. All parts are then assembled via the Golden Gate method as described by Engler *et al.* (2013) (36) into a pUC54 plasmid together with a promoter and RBS sequence as shown in Figure 3 (for *E. coli*) or into pGK12 which replicates in *Bacillus* sp. with a *Bacillus* promoter and ribosome binding site.

30

Construction of a translational coupling system that initiates translation of the reporter gene to detect lactose transporter expression via the opening of a loop on the ribosome binding site

A second method to screen for expression via translational coupling is described by Mendez-Perez *et al* (2012) (58). This method was adapted for the screening of lactose permease expression with a chloramphenicol reporter gene. In this case the lactose transporter *lacY* is coupled via a HIS-tag and ribosome binding site to the chloramphenicol resistance gene. The sequence parts for this construct are also ordered at IDT or Geneart and assembled via Golden gate assembly. The resulting sequence is given in Figure 5.

Construction of a translational coupling system that couples the yeast lactose transporter with a reporter gene

Although yeasts do not use cistrons, it is still possible to screen for expression via translational coupling via viral internal ribosomal entry sites (so called IRES sequences) (56). An example of such a sequence is the T2A sequence (10), which allows fully independent (which means not as a protein fusion), yet coupled translation of two proteins in a cistron. This means that if the last protein of the cistron is expressed, the first protein is also expressed.

In yeasts, the lactose permease gene of for instance *Kluyveromyces marxianus* can be used to transport lactose in the cell. This gene can be coupled with the T2A sequence to the *aph 1* gene, encoding resistance to Geneticin. This sequence is analogously constructed as describe above. The final sequence is given in Figure 6.

Construction of an aptamer coupling system that introduces an aptamer into the messenger RNA of lactose permease

Lactose permease expression can also be detected on a messenger RNA level. To this end, a (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one binding aptamer is cloned after the lactose permease coding sequence as shown in figure 7. The expression of this construct is modulated further as described in example 6. After growth of the cells, (Z)-4-(3,5-difluoro-4-hydroxybenzylidene)-1,2-dimethyl-1H-imidazol-5(4H)-one is added to the medium as described by Pothoulakis *et al* (2013) (64) and the lactose permease expressing mutant strains are selected via a fluorescence-activated cell sorter (FACS).

Example 4: Detection of the expression of a lactose transporter translational coupled with a chloramphenicol resistance gene

Two strains were constructed in which lactose permease was knockout out from the genome. In both strains a pSC101 plasmid containing a kanamycin resistance gene was transformed,

with the difference that one of the plasmids contained a constitutively expressed lactose permease as described in Example 1 and 2, resulting in a reference strain MG1655 Δ lacY pSC101_kan and the lacY_cat translational coupled strain MG1655 Δ lacY pSC101_kan_lacY_{synthetic}_cat as described in Example 4 and Figure 5. Both strains were grown in a minimal medium as described above at different chloramphenicol concentrations (between 0 and 30 mg/l). Figures 8 and 9 show after 48 and 92 hours of growth that the growth of reference strain is inhibited at a lower chloramphenicol concentration than the mutant lacY_cat translational coupled strain, which makes such a system an excellent screen for lactose permease expressing genetic constructs.

Example 5: Screening procedure for lactose permease expressing mutants that do not undergo lactose killing

Similar to example 2, a mixture of two strains resistant to chloramphenicol were grown, one strain that does not undergo lactose killing and translational coupled to chloramphenicol and one strain with the chloramphenicol cassette but with the natural expression system of lactose permease. Both strains were grown in the medium as described in example 2 and mid exponential phase lactose was added as shown in example 2. The mutant strain that does not undergo lactose killing kept on growing while the other strain, that is lactose killing sensitive, stopped growing. At the end of the exponential phase, 0.1 ml of this culture was inoculated in a second shake flask with a similar medium as described above. Again, at OD 0.8 lactose was added arresting the growth of the lactose killing sensitive strain and further enriching the mutant strain that does not undergo lactose killing. After 5 repeats of this procedure a 99% enrichment of the mutant strain that does not undergo lactose killing was obtained, which is then easily isolated from the culture.

Example 6: Creation of mutants of lactose permease expression cassettes

Sequence variants of promoters, ribosome binding sites, Kozak sequences, transcription terminators and lactose permease gene variants (with different codon usages) are ordered at gene synthesis vendors such as IDT, Geneart, Genscript, Gen9, The design of a promoter library for bacteria are based on the consensus sequence of bacterial promoters, with two conserved regions at -10 and -35 bp of the transcription start. The bases in between, before and after these conserved regions are then varied randomly with A, T, G or C, leading to promoters with different expression strengths. Alternatively, the conserved regions are varied and the

surrounding sequence is kept fixed, which also leads to promoters with different strengths. These sequence mixtures are then cloned (via either Gibson Assembly, Golden Gate assembly, Cliva assembly, LCR or restriction ligation) (25, 36, 50, 79) in front of a translational coupled lactose permease leading to a library of expression cassettes of lactose permease that can be screened by means of the screening protocol described in Example 5.

An eukaryotic promoter library is created based on a core promoter(13). For *Saccharomyces cerevisiae* this promoter may be a heterologous TEF promoter pTEF1 which is enhanced with UAS sequences and is mutated to vary promoter strength (12). Such a promoter is ordered and cloned as described above in front of a translational coupled lactose permease and the final construct transformed into a yeast such as *Saccharomyces cerevisiae* on a plasmid or for integration into a chromosome.

The untranslated region consists for prokaryotes of a ribosome binding site and for eukaryotes of a Kozak sequence. Both sequences are randomized and cloned in front of the coding sequence as described above leading to a library of expression cassettes with different translational efficiencies. The randomization can be rationalized with tools such as RBS calculator which calculates the sequence translation efficiency correlation, and reducing the number of variants that have to be included into the library (67).

The codon usage is changed by means of changing the coding sequence of the gene without changing the amino acid sequence of the protein. This is called codon adaptation. The codon usages through the codon sequence is changed in such a way that more or less rare codons are introduced in certain regions, leading to altered expression efficiencies and folding efficiencies. A permease with only rare codons in its sequence (determined on an organism basis by means of the codon usage database (61)) show lower translation rates than permeases with a fully codon optimized sequence (with only codons with high occurrence in the target organism). In addition, the first codons of the coding sequence also influence the Kozak or ribosome binding site efficiency (62, 69, 78).

The transcription terminator region is varied by means of endogenous or exogenous transcription terminator sequences found in database (18, 26). These transcription terminators are also cloned similar to the method described above.

Example 7: Enrichment of expression cassettes that express lactose permease and do not lead to lactose killing

The expression cassettes are created according to example 3 and 7. This leads to a library of expression cassettes of lactose permease. The expression cassettes that result in the expression of lactose permease are selected according to the methods of example 3 and 4. The expression cassettes, expression lactose permease, that do not lead to lactose killing are selected according to the methods described in Example 2 and 5. The selected expression cassettes are further analysed by sequencing and by the method described in example 2.

Example 8: Fermentative 2-fucosyllactose production with a fucosyltransferase originating from *Helicobacter pylori* with *E. coli*

The mutant strain in which the genes *lacZ*, *glgC*, *agp*, *pfkA*, *pfkB*, *pgi*, *arcA*, *iclR*, *wcaJ* are knocked out and *lacY* was expressed via constitutive expression as described in example 1 and example 2 to ensure expression under all culturing conditions, was transformed further with a fucosyltransferase originating from *Helicobacter pylori* and a sucrose phosphorylase originating from *Bifidobacterium adolescentis*, which were also constitutively expressed. The constitutive promoters originate from the promoter library described by De Mey *et al.* 2007. This strain was cultured in a medium as described in the materials and methods, however with 30 g/l of sucrose and 50 g/l of lactose. This resulted in the formation of up to 1.5 g/l 2'-fucosyllactose

Example 9: Fed batch production of 2-fucosyllactose with *E. coli*

A mutant strain was constructed via the genetic engineering methodologies described above with the following genotype:

ΔlacZYAΔglgCΔagpΔpgiΔpfkA-P22-baSPΔpfkBΔarcAΔiclR::slΔwcaJΔlonΔadhE-P14-frk + pCXP14-FT_*H. pylori* (a vector with sequence SEQ ID N° 6, see Figure 10) With lactose permease expression altered as described in example 1 and example 2. The promoter P22 and P14 originate from the promoter library constructed by De Mey *et al* (29) and was cloned similar to the methodology described by Aerts *et al* (1). “::sl” marks a scarless gene deletion, thus without a FRT site that remains in the chromosome.

This strain was cultured in a bioreactor as described above in materials and methods, in the mineral medium with 30 g/l of sucrose and 50 g/l of lactose. After the batch phase the bioreactor was fed with 500 g/l of sucrose, 50 g/l lactose and 1 g/l of magnesium sulphate heptahydrate. This led to the accumulation of 27.5 g/l of fucosyllactose in the supernatant.

Example 10: Production of lactoNtriose with *E. coli*

A mutant strain was constructed via genetic engineering with the methodologies described above expression a UDP-N-acetylglucosamine transferase, a sucrose phosphorylase and a L-glutamine:D-fructose-6-phosphate aminotransferase and a glucosamine uridyltransferase on a pBR322 plasmid with each a constitutive promoter from the promoter library of De Mey *et al* (29) and with a beta-lactamase selection marker. This vector was transformed in an *E. coli* mutant strain with genotype $\Delta lacZYA \Delta glgC \Delta agp::P14\text{-}frk\text{-}P22\text{-}BaSP \Delta pgi \Delta pfkA \Delta pfkB \Delta nagABCDEAmanAAnanATEKAmanXYZ$ expressing lactose permease constitutively as described above. This strain was cultivated in a shake flask as described above with lactose and sucrose as carbon sources, with or without additional glycerol. This production host did not undergo lactose killing and produced 62.5 and 55.3 mg/l lactoNtriose, respectively, from the added lactose.

Example 11: Construction of a yeast *K. marxianus* lactose permease (p1) knock in at the rDNA locus.

First, plasmid p2a_2 μ _Lac4 was used to transform into the *Saccharomyces cerevisiae* BY4742 wild type strain. Transformation was performed using a total of 4 μ g of plasmid using the Gietz protocol (40). The transformed yeast cells were plated out on SD-CSM drop-out plates (without uracil). After two days, growth was observed on the plates and the yeast colonies were tested for presence of the desired plasmid. Colony PCR was carried out on all 33 colonies. All colonies tested positive for presence of the plasmid p2a_2 μ _Lac4. Colony 5 was selected for further use. This colony was transformed with 2 μ g double stranded linear DNA obtained from pJet_KI_p1_Lac12_t@rDNA. The transformed cells were plated on SD-CSM –Ura plates with lactose as the sole C-source. Three days after transformation, several colonies were sufficiently grown on to be tested for the presence of the *Lac12* expression cassette in the *Saccharomyces* rDNA. Colony PCR was carried out on all colonies. All colonies tested positive for presence of the *Lac12* expression cassette. Colony 6 was selected for further use (*Saccharomyces* KI_p1_Lac12_t@rDNA).

Example 12: Construction of a yeast *K. marxianus* lactose permease (p2) knock in at the rDNA locus.

First, plasmid p2a_2 μ _Lac4 was used to transform into the *Saccharomyces cerevisiae* BY4742 wild type strain. Transformation was performed using a total of 4 μ g of plasmid using the Gietz

protocol (40). The transformed yeast cells were plated out on SD-CSM drop-out plates (without uracil). After two days, growth was observed on the plates and the yeast colonies were tested for presence of the desired plasmid. Colony PCR was carried out on all 33 colonies. All colonies tested positive for presence of the plasmid p2a_2 μ _Lac4. Colony 5 was selected for further use.

- 5 This colony was transformed with 2 μ g double stranded linear DNA obtained from pJet_KI_p2_Lac12_t@rDNA. The transformed cells were plated on SD-CSM –Ura plates with lactose as the sole C-source. Three days after transformation, several colonies were sufficiently grown on to be tested for the presence of the *Lac12* expression cassette in the *Saccharomyces* rDNA. Colony PCR was carried out on all colonies. All colonies tested positive for presence of
 10 the *Lac12* expression cassette. Colony 7 was selected for further use (*Saccharomyces* KI_p2_Lac12_t@rDNA).

Example 13: Growth on lactose of a wild type yeast strain and 2 mutant yeast strains

- A shake flask experiment as described in materials and methods was set up with the wild type
 15 *Kluyveromyces*, *Saccharomyces* KI_p1_Lac12_t@rDNA and *Saccharomyces* KI_p2_Lac12_t@rDNA. These strains were grown in a shake flask medium containing lactose as the sole C-source (20 g/L). As can be seen in Table 1, the mutant *Saccharomyces* strains grow as fast as the wild type *Kluyveromyces marxianus lactis*, which is known for fast growth on lactose (31). The constitutively expressed lactose permease thus ensures fast and efficient
 20 influx of lactose in the yeast cell.

Strain	$\mu_{\max}$
<i>Kluyveromyces marxianus lactis</i>	<u>0.14</u>
<i>Saccharomyces</i> KI_p1_Lac12_t@rDNA	<u>0.18</u>
<i>Saccharomyces</i> KI_p2_Lac12_t@rDNA	<u>0.18</u>

Table 1

Example 14: The effect of lactose on the wild type yeast strain and mutant yeast strains that do not undergo lactose killing

- 25 A shake flask experiment as described in materials and methods was set up with the wild type *Kluyveromyces*, *Saccharomyces* KI_p1_Lac12_t@rDNA and *Saccharomyces* KI_p2_Lac12_t@rDNA. These strains were grown in a glucose shake flask medium (20 g/L glucose, as described in the materials and methods) and lactose (200 g/L stock solution was added resulting in a final concentration of 10 g/L) was added mid exponential phase. As can be

seen in Figure 11 and Figure 12, the mutant strains do not undergo lactose killing. Yet, fast and efficient influx of lactose in these yeast cell was proven in example 13.

Example 15: Sequencing of lactose permease expression cassettes that do not undergo lactose killing

46 colonies originating from the screening described above were sequenced, resulting in SEQ ID N° 12 – 57 (Figure 18). These sequences are promotor and RBS variants that do not result into lactose killing when expressed in *E. coli*. Note that this is a selection of an enormous amount of colonies that has been sequenced, hence, the screening methodology has resulted in much more sequences than shown in Figure 18 and alternative library creation methodologies as described above will also lead other sequences than those shown in Figure 18.

Example 16: Determination of μ_{MAX} of the lactose permease expression cassette mutant strains

All strains were either started from LB-agar plate or started from cryovial and inoculated in 5,0 mL Luria broth medium (10 g Tryptone; 5g yeast extract; 10 g NaCL). After growing o/n at 37 °C, 1 mL of this preculture was added to a 500 mL shaker flask containing 100 mL minimal Lactose media (2,0g/L NH₄Cl; 5,0g/L ; (NH₄)₂SO₄; 3,0g/L KH₂PO₄, 7,3g/L K₂HPO₄; 8,4 g/L MOPS; 0,5g/L MgSO₄ x 7H₂O; 0,5g/L NaCL; 10 g/L Lactose; 0,4 mg/L Na₂EDTA x 2H₂O; 0,03mg/L H₃BO₃; 1,01 mg/L Thiamine HCL; 0,94 mg/L ZnCL₂; 0,5 mg/L CoCL₂ x 6H₂O; 0,38 mg/L CuCL₂ x 2H₂O; 1,59 mg/L MnCL₂ x 4H₂O; 3,6 mg/L CaCL₂ and 0,096 mg/L Na₂MoO₄ x 2H₂O); pH 7.0. After growing o/n at 37 °C, both precultures were diluted with minimal lactose media to an OD₆₀₀ of 0,050. The suspension was then transferred to a 96 MTP plate (n=32), covered by an easyseal cover. OD measurements were performed every 10 minutes for 24 hours using the Infinite M200 pro (TECAN) under the following conditions: Temperature:37 °C+/- 0,5; shaking 597 seconds; 2mm shaking amplitude; 280 rpm; wavelength OD₆₀₀;Flash#10; settle time 150 ms).

Example 17: Characterization of lactose permease expression cassettes

One method to reduce or eliminate lactose killing is to significantly reduce or eliminate the activity of lactose permease (see Example 16). However, in light of the production of lactose or galactose based bioproducts, a high lactose influx into the cell is required. Here we proof that the lactose influx of the lactose killing resistant mutant strains is still comparable, equal or even higher than the lactose influx of the wild type organism that undergoes lactose killing. To

this end, the novel lactose permease expression cassettes were introduced into a MG1655 Δ lacY strain, which still expresses beta-galactosidase. The growth rate of these new strains are a measure for the lactose influx, because any strain that has a significant reduced expression in lactose permease expression will have a significantly reduced growth rate.

5

The results of this analysis is shown in Figure 19. Nearly all strains shown in this figure have a growth rate equal or higher than the wild type, indicating a lactose permease activity and expression which is equal or higher than the wild type, however, in contrast to the wild type expression system, these expression cassettes do not result into lactose killing. This method is however limited by the expression of the beta galactosidase gene which becomes the rate limiting step for growth. Therefore, the expression of the lactose permease gene was measured via the above described translational coupling system described above. The minimal inhibition concentration (MIC) for chloramphenicol is indicative for the expression of the lactose permease gene and this was determined for each of the cassettes. The lowest MIC that still resulted into the same growth rate compared to the wild type was used as an indicator for the expression of the wild type lactose permease expression.

The cassettes with the lowest MIC that have the same growth rate as the wild type strain have a MIC of approximately 20 mg/l chloramphenicol. Mutant strains with a slightly lower growth rate have a MIC that ranges between 15 and 20 mg/l and mutant strains with equal or higher growth rate have a MIC between 20 and 80 mg/l. 85% of the sequences fall in the latter category, which means most sequences that were identified as lactose killing negative expression cassettes have a higher lactose permease expression, contrary to what has been previously described in literature.

25 Example 18: Construction of a lacIq promoter expression cassette

Similar to the methodology described above a placIQ promoter was cloned in front of the lacY gene of *E. coli*. The final sequence of this construct is shown in Figure 20.

Example 19: Other used promoters in art that undergo lactose killing

30 The promoter placIQ is a promoter that has been used in the art for the expression of lactose permease in *E. coli*. The use of this promoter resulted into the uptake of lactose into the cell. However, the effect of such uptake was not tested. The growth rate on lactose of the wild type was not significantly different from the lacIq promoter ($\mu_{\max}$ of 0,1 and 0,11 h⁻¹ respectively),

hence indicating a similar lactose uptake rate. A lactose killing screen of this lacIQ expression cassette proofed that this promoter also led to lactose killing (see Figure 21). This proves that there are specific promoter sequences lead to lactose killing and other sequences that do not lead to lactose killing. Rationalization of the sequences is not possible and trial and error of individual sequences is a laborious job which would lead to enormous costs, hence the methodology described above to identity the expression cassettes that do not undergo lactose killing is the perfect way to avoid lengthy and costly screening work.

Example 20: Production of lactoNtetraose with *S. cerevisiae*

The mutant strains constructed in Example 11 and 12 were transformed further with a β -1,4-galactosyltransferase and a β 1,3-N-acetylglucosaminyltransferase originating from *Neisseria meningitidis*, which were also constitutively expressed, using a standard yeast expression vector, for example as described by Lee *et. al.* (52). The strains were cultured in a medium as described in the materials and methods, however with 20 g/l of sucrose and 20 g/l of lactose. This resulted in the formation of up to 30 mg/l lactoNtetraose.

Example 21: Production of 2-fucosyllactose with *S. cerevisiae*

The mutant strains constructed in Example 11 and 12 were transformed further with a GDP-fucose synthase and a GDP-mannose 4,6-dehydratase of *E. coli* and a fucosyltransferase originating from *Helicobacter pylori*, which were also constitutively expressed, using a standard yeast expression vector, for example as described by Lee *et. al.* (52). The strains were cultured in a medium as described in the materials and methods, however with 20 g/l of sucrose and 20 g/l of lactose. This resulted in the formation of up to 10 mg/l 2 fucosyllactose.

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Claims

1. A method to produce microorganisms which resist the phenomenon of lactose killing when grown in an environment in which lactose is combined with another carbon source, wherein said method comprises:
 - a. altering the expression of a lactose transporter within microorganisms by mutation comprising one or more of: introducing a heterologous promoter in front of an endogenous or exogenous lactose transporter gene; mutating the untranslated region in front of the coding sequence that contains the ribosome binding or Kozak sequences; and modifying the codon usage of the endogenous lactose transporter gene,
 - b. growing said mutated microorganisms on a medium comprising a carbon-source which is not lactose,
 - c. adding lactose to said medium during growth of said mutated microorganisms, and
 - d. selecting the microorganisms which resist the phenomenon of lactose killing growing on said medium comprising lactose and which retain at least 50% of the lactose influx obtained with the wild type expression cassette of said lactose transporter.
2. A method according to claim 1 wherein said introduction of a heterologous promoter in front of an endogenous or exogenous lactose transporter gene is undertaken by: a) deleting the endogenous lactose transporters from the genome and reintroducing them at another location within the genome of said microorganism, or, b) by introducing a heterologous promoter in front of the endogenous lactose transporters, or, c) by knocking out the endogenous lactose promoter and introducing a heterologous promoter at the same location in the genome of said microorganism.
3. A method according to claims 1-2 wherein said expressed lactose transporter is detected via translational coupling with a reporter gene and/or via aptamer coupling.
4. A method according to claims 1-3 wherein said expressed lactose transporter is detected via genetic constructs as given in by SEQ ID no 2, 3, 4 and /or 5.
5. A method according to claims 1-4 wherein said lactose transporter is a lactose permease.
6. A method according to claims 1-5 wherein said microorganism is a bacterium, a yeast or a fungus cell.

7. A lactose transporter expression cassette that leads to the expression of a lactose transporter and that does not result into lactose killing phenotype,, wherein said expression cassette comprises a sequence as given by SEQ ID no 1, 7, 8, 12, 13, 14, 15, 16, 17, 18, 19,20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 or 57.

8. A microorganism wherein the expression of a lactose transporter has been altered within said microorganism, which microorganism resists the phenomenon of lactose killing when grown in an environment in which lactose is combined with (an)other carbon source(s) and which retains at least 50% of the lactose influx obtained with the wild type expression cassette of said lactose transporter, and, is obtainable by a method according to claims 1-6, wherein said microorganism comprises a lactose transporter expression cassette comprising a sequence as given by SEQ ID no 1, 7, 8, 12, 13, 14, 15, 16, 17, 18, 19,20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 or 57.

9. A microorganism according to claim 8 in which the genes coding for the enzymes from the lactose and/or galactose degradation pathways are rendered less functional or non-functional.

10. Use of a microorganism and/or a lactose transporter expression cassette according to claims 8-9 and/or claim 7, respectively for the production of lactose- and/or galactose-based specialty products.

11. Use of a microorganism and/or a lactose transporter expression cassette according to claim 10 wherein said lactose-based specialty products are specialty carbohydrates, glycolipids and galactosylated compounds.

12. Use of a microorganism according to claim 11 wherein said specialty carbohydrates are 2-fucosyllactose or 2'-fucosyllactose or 3-fucosyllactose or 2',3-difucosyllactose or lactoNtriose or lacto-N-tetraose, or lacto-nN-tetraose or 3'sialyllactose or 6'sialyllactose.

13. A method for producing a lactose transporter expression cassette that does not result in lactose killing, comprising:

- creating mutants of a lactose transporter expression cassette;

- screening the mutant expression cassettes for expression cassettes that ensure lactose transporter expression in a microorganism transformed with the expression cassette;

- screening the mutant expression cassettes that ensure lactose transporter expression for expression cassettes that do not lead to lactose killing in a microorganism transformed with the expression cassette by growing microorganisms that have been transformed with the expression cassettes in a medium to which lactose is added during the mid exponential phase; and

isolating the expression cassette from the transformed microorganisms that resist the phenomenon of lactose killing.

14. The method according to claim 13, wherein lactose transporter expression from the expression cassette is detected via translational coupling with a reporter gene and/or via aptamer coupling.

15. Use of a lactose transporter expression cassette produced by the method according to claim 13 or 14, for the production of lactose- and/or galactose-based specialty products.

16. Use according to claim 15, wherein said lactose-based specialty products are specialty carbohydrates, glycolipids and galactosylated compounds.

17. Use according to claim 16, wherein said specialty carbohydrates are 2-fucosyllactose or 2'-fucosyllactose or 3-fucosyllactose or 2',3-difucosyllactose or lactoNtriose or lacto-N-tetraose, or lacto-nN-tetraose or 3'sialyllactose or 6'sialyllactose.