

Metabolically engineered organisms for the production of added value bio-products

Technical field of invention

The present invention relates to genetically engineered organisms, especially microorganisms
5 such as bacteria and yeasts, for the production of added value bio-products such as specialty
saccharides, glycolipids and glycoproteins. More specifically, the present invention relates to
host cells that are metabolically engineered so that they can produce said valuable specialty
products in large quantities and at a high rate by bypassing classical technical problems that
occur in biocatalytical or fermentative production processes.

10 Background art

The increasing cost of petroleum resources contributes to a growing awareness of the potential
of biological production processes. This has intensified the research efforts of companies and
research centres towards the development of economically viable and environmentally benign
technologies for the production of an increasing number of bio-products, e.g., bio-fuels, bio-
15 chemicals and bio-polymers. These are easily degradable and produced with minimal energy
requirements and waste streams. In spite of the favourable context for production processes
based on industrial biotechnology, the development of alternatives for well-established
chemical synthesis routes often is too time intensive and too expensive to be economically
viable. Consequently, there is a clear demand for a faster and cheaper development of new
20 production strains.

Nowadays oligosaccharides are typically synthesized via bioconversion processes. Isolated and
purified enzymes (so called *in vitro* bioconversions) and whole cell biocatalysts are commonly
used. In essence, they convert one or more precursors into a desired bio-product.

However, the application of the *in vitro* bioconversions is often hampered because the
25 synthesis of the product may require multiple enzymatic steps or because additional cofactors
are required (NADH, NADPH, UTP,...), which are expensive.

Another drawback of *in vitro* synthesis is the fact that the expression and purification of many
enzymes is laborious and their purification process may result in a decreased enzymatic activity.
Furthermore, each enzyme in such a multi-enzyme bioconversion process has its own optimal

process parameters, resulting in very complicated optimization schemes. In such a process, the reaction equilibria also play an important role. For instance, when using a phosphorylase, a set substrate/product ratio that limits product yield will be at hand. This leads to complicated downstream processing schemes to separate the product from the substrate (33, 35).

5 Metabolic engineering is another approach to optimize the production of value added bio-products such as specialty carbohydrates. Commonly, whole cells have been metabolically engineered to produce added value bio-products starting from a supplied precursor. In this context, the cells are engineered as such that all the metabolic pathways involved in the degradation of the precursor(s) are eliminated (3, 45, 70, 77, 100). By doing so, the precursor(s)
10 is (are) efficiently and directly converted into the desired product.

A major drawback of the latter approach is the fact that the biomass synthesis and the envisaged bio-product biosynthesis require different starting metabolites. For example, *E. coli* was metabolically engineered for the efficient production of 2-deoxy-scylo-inosose starting from glucose. This strategy renders the metabolically engineered *E. coli* unfit to grow on
15 glucose, requiring the addition of other substrates, e.g., glycerol to allow for biomass synthesis (45).

A second drawback of whole cell production systems is that there is a need for two phases, a growth phase, in which biomass is formed (or biomass synthesis), followed by a production phase of the envisaged product. This means that the growth phase and the production phase
20 are separated in the time (consecutive phases). This results in very low overall production rates of the desired product(s). In addition, this type of process is hard to optimize. Indeed, fermentation processes have been developed making use of metabolically engineered cells which over-express production pathway genes. A large amount of the substrate is converted into biomass, resulting in only a minor flux of the substrate towards the product (13).

25 The present invention overcomes the above-described disadvantages as it provides metabolically engineered organisms which are capable to produce desired products with a high productivity and a guaranteed high yield (Figure 1). This is accomplished by clearly splitting the metabolism of the organism in two parts: 1) a so-called 'production part' or 'production pathway', and 2) a 'biomass and cofactor supplementation' part or 'biomass and/or bio-
30 catalytic enzyme formation pathway'. Said two parts are created by splitting a sugar into: a) an

activated saccharide, and b) a (non-activated) saccharide. Each of said saccharides a) or b) are - or can be- the first precursors of either the production pathway a) or biomass and/or bio-catalytic enzyme formation pathways b), allowing a pull/push mechanism in the cell.

Indeed, biomass synthesis, which is the main goal of the cell, converts the activated saccharide or the saccharide into biomass and shifts the equilibrium of the reaction that splits the sugar towards the activated saccharide and saccharide. In this way, the life maintaining drive of the cell acts as a pulling mechanism for the product pathway. This pulling effect is created by biomass synthesis as it ensures the accumulation of the first substrate molecule of the production pathway, which, as such and in turn, also pushes the production pathway. This strategy solves the production rate problem which occurs in the two phase production strategies as described in the prior art. Moreover, by catabolising one part of the sugar moiety, the cell is always supplied with the necessary cofactors and the needed energy requirements for production of the specialty bio-product. The current strategy thus solves also the problem of co-factor supplementation that is needed in biocatalytic production as described in the prior art. In addition, the necessary enzymes in the production pathway are always synthesized efficiently and easily maintained via the engineering strategy of the current invention.

In addition, the present invention discloses the usage of a 2-fucosyltransferase originating from *Dictyostellium discoideum* to produce 2-fucosyllactose by the metabolically engineered organisms of the present invention.

Brief description of figures

Figure 1: (a) A normal equilibrium reaction, which occurs in the current production technologies (b) pull/push principle: the equilibrium is shifted towards the saccharide and activated saccharide. The main goal of a cell is to grow, hence it pulls at the saccharide (by means of example), to form biomass ("biomass and/or bio-catalytic enzyme formation pathway") and supplements the necessary co-factors for the production pathway next to life maintenance. Due to this pulling effect, the activated saccharide will accumulate in the cell and pushes the production pathway.

Figure 2: Growth rate of *E. coli* transformants on minimal medium carrying a plasmid encoding for a sucrose phosphorylase. (Abbreviations are given in Table 2)

Figure 3: Projected carbon flow in the wild type strain. Small arrow: Indicating reactions in the metabolism Bold arrow: Indicating enhanced or novel introduced reactions Cross: indicates the knocking-out of a gene or rendering it less functional

Figure 4: Projected carbon flow in Base strain 1. The α glucose-1-phosphate (α G1P) pool in Base strain 1 increases because the main reactions that convert α G1P into cellular components are eliminated. Small arrow: Indicating reactions in the metabolism Bold arrow: Indicating enhanced or novel introduced reactions Cross: indicates the knocking-out of a gene or rendering it less functional

Figure 5: The α glucose-1-phosphate pool in the wild type *E. coli* MG1655 (WT) grown on glucose, *E. coli* MG1655 P22-BaSP grown on sucrose, and in the plug strain MG1655 Δ glgC Δ pgm Δ lacZ P22-BaSP grown on sucrose: shake flask experiments and 1.5 L batch experiments

Figure 6: Evolution of sucrose, acetate and the biomass concentration during a culture of Δ pgm Δ lacZ Δ glgC (3KO) P22-BaSP on buffered LB medium.

Figure 7: Evolution of α Glucose-1-phosphate, fructose, glucose, and pyruvate concentration during a culture of Δ pgm Δ lacZ Δ glgC (3KO) P22-BaSP on buffered LB medium.

Figure 8: Schematic view of the cellobiose producer Δ pgm Δ lacZ Δ glgC (3KO) P22-BaSP-CuCP.

Figure 9: Cellobiose production and yield of Δ pgm Δ lacZ Δ glgC Δ agp (4KO) P22-BaSP-CuCP. High phosphate indicates a phosphate concentration of 0.2 M phosphate, low phosphate indicates a concentration of 13 mM phosphate

Figure 10: Starting from base strain 5, fucosylated sugar derivatives such as fucosyllactose and more specifically 1,2-fucosyllactose can be produced. The strain is modified to force the cell to produce fructose-6-phosphate which is an intermediate in the synthesis of GDP-fucose. Glucose or glucose-1-phosphate (if the starting enzyme is either a sucrase or a sucrose phosphorylase) is then fed to the central carbon metabolism via the pentose phosphate pathway. Fig 10 shows the route towards product and biomass and the needed knock outs to achieve this, the left pathway indicates the optimal route with a sucrose phosphorylase and the right pathway indicates the optimal route with an invertase combined with glucokinase.

Figure 11: Partial genome sequence of wild type chromosome at the location of the *pgm* gene. The *pgm* gene sequence is marked in bold/italic

Figure 12: Partial genome sequence of a mutant strain in which only a scar remains at the location of the *pgm* gene. The scar sequence is marked in bold/italic

5 Figure 13: Partial genome sequence of a *pgm* mutant strain in which the *pgm* gene is replaced with a part of a GFP protein sequence. The newly introduced sequence is marked in bold/italic

Figure 14: Partial genome sequence of a *pgm* mutant strain in which the *pgm* gene is replaced with kanamycine cassette. The newly introduced sequence is marked in bold/italic

10 Figure 15: The amount of glucose that was released after polysaccharide hydrolysis for the wild type strains transformed with a heterologous sucrose phosphorylase originating from *Bifidobacterium adolescentis* and a mutant strain $\Delta pgm \Delta agp \Delta glgC \Delta lacZ \Delta glk \Delta ptsG$ with a heterologous sucrose phosphorylase originating from *Bifidobacterium adolescentis* and a heterologous gene *tts* originating from *Streptococcus pneumoniae*

15 Figure 16: Kojibiose, maltose and optical density evolution in time of a kojibiose producing strain with the genotype $\Delta lacZ \Delta glgC \Delta agp \Delta ptsG \Delta malPQ \Delta ycjU$ pCXp22MPp22KP.

Figure 17: Growth profile and F6P accumulation of a $\Delta pgi \Delta pfkA \Delta pfkB$ mutant strain in a sucrose based medium.

Figure 18: Phylogenetic tree of the fucosyltransferases from the different glycosyltransferase families, the tree was constructed with MCoffee (58)

20 Figure 19: Alignment of *Dictyostelium discoideum* and *Helicobacter pylori* fucosyltransferase, the amino acids marked in colour are conserved motives found in the 2-fucosyltransferases of GT family 11. Red indicates motif 1, blue, motif 2, and green, motif 3 (67).

Figure 20: LC-MS results of the fucosyltransferase assay with phenol red. The upper chromatogram is the blanc without GDP-fucose, the lower is a sample of the *D. discoideum* fucosyltransferase assay. A 13.5 min a peak of 2FL appeared in the chromatogram.

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Figure 21: 2-fucosyllactose LC MSMS analysis of the partial fucosyltransferase enzyme. The upper chromatogram shows a 100mg/l standard of 2-fucosyllactose, the lower chromatogram

shows the 2-fucosyllactose peak of the enzyme assay. In this analysis only the mass of 2-fucosyllactose was scanned with the mass spectrometer.

Description of invention

The present invention discloses metabolically engineered organisms, especially microorganisms, which are capable to produce added value bio-products with a high productivity and a guaranteed high yield. The organisms of the present invention are metabolically engineered so that two distinct routes are constructed leading to product and to growth/biomass. This is achieved by reducing or eliminating the activity of enzymes catalyzing reactions converting metabolites from the 'biomass and/or bio-catalytic enzyme and/or cofactor supplementation part' into metabolites from the 'production pathway' part and vice versa, e.g. by reducing or eliminating/knocking-out at least one, some or all the genes encoding for enzymes performing reactions which convert the production pathway intermediates into biomass precursors, and/or reducing or eliminating/knocking out at least one, some or all the genes coding for enzymes performing the reactions which degrade the production pathway intermediates. Moreover, these metabolic/genetic changes do not impair the growth of the engineered cells. For example: carbohydrate hydrolases in combination with carbohydrate kinases, carbohydrate synthases, and, carbohydrate phosphorylases can be introduced into the cell. The latter enzymes convert the substrates comprising a saccharide, an oligosaccharide, a polysaccharide or a mixture thereof in a sugar moiety and an activated sugar moiety (e.g. a phosphorylated saccharide moiety, UDP, CMP, GDP, ADP, TDP or dTDP... activated sugar moiety). Additional metabolic engineering of the cell involves blocking of the pathway starting from the activated sugar moiety towards the biomass constituents. In this way, the (non-activated) sugar moiety is used as 'fuel' (or energy-source) and building block for the synthesis of biomass, of the numerous bio-catalytic enzymes that will perform the conversion of the activated sugar moiety to the desired product (= e.g. a specialty carbohydrate) and of the necessary cofactors (NADH, ATP, UTP ...). Conversely, the activated sugar moiety can also be used as 'fuel', while the sugar moiety is activated by a carbohydrate kinase and 'pushed' into the production route of the desired specialty product.

Using the engineered organisms of the present invention, product formation through the conversion of the activated sugar can be linked to growth which is fuelled by the other sugar moiety (or vice versa). In this way, the cell's natural drive for multiplication is used as an asset

to push the production of the desired bio-product. This means that the former drawback of having to produce biomass before the actual production of the bio-product can start, is now turned into a benefit. This methodology results in high production rates, without the inherent problems that come with multi-enzymes systems and two phase fermentation systems. In addition, the organisms of the present invention may use the same substrate(s) as indicated above for both growth or biomass production and production of the desired product at a high rate, the overall principle behind this metabolic engineering strategy is thus a pull/push principle as is also explained above. The central carbon metabolism that leads to biomass and cofactors pulls at one part of the sugar moiety for growth while the other part accumulates in the cell, pushing the production pathway.

The latter approach cannot only be used to produce desired specialty carbohydrates or activated carbohydrates but can also be applied for the synthesis of a wide variety of glycosylated compounds, e.g., saccharides, nucleosides, glycosylphosphates, glycoproteins and glycolipids.

Multiple starting enzymes can be introduced into a cell to split the metabolism into two parts, in combination with gene knock outs. Non-limiting examples of enzymes that can be used to split sugars into an activated saccharide and a saccharide are sucrose phosphorylases, sucrose synthases, sucrases (invertases) combined with a glucokinase and/or fructokinase, a trehalase combined with a glucokinase, a maltase combined with a glucokinase, a sucrose-6-phosphate hydrolase combined with a fructokinase, a maltose phosphorylase, a maltose synthase, a amylase combined with a phosphorylase or synthase or hydrolase, a lactose synthase, a lactose phosphorylase, a lactase (or beta-galactosidase) combined with a galactokinase and/or a glucokinase.

The present invention relates to a metabolically engineered organism for the production of at least one specialty product chosen from the group consisting of a saccharide, an activated saccharide, a nucleoside, a glycoside, a glycolipid and a glycoprotein, characterized in that:

a) said organism is genetically modified with the introduction of at least: i) a gene encoding for a carbohydrate hydrolase in combination with a gene encoding for a carbohydrate kinase, ii) a gene encoding for a carbohydrate synthase, or, iii) a gene encoding for a carbohydrate phosphorylase, so that said organism is capable to split a disaccharide,

oligosaccharide, polysaccharide or a mixture thereof into an activated saccharide and a saccharide, and

b) said organism is further genetically modified so that at least one other gene than any of the introduced genes of step a) of said organism is rendered less- functional or non-functional and wherein said other gene encodes for an enzyme which converts said
5 activated saccharide into biomass and/or bio-catalytic enzymes.

The term 'saccharide' relates to monosaccharides such as, but not limited to, aldoses, ketoses, pentoses, methylpentoses, hexoses, polyols with or without either carbonyl, carboxyl, amino groups or in which a hydroxylgroup is replaced by, but not limited to a hydrogen, amino, thiol,
10 phosphate and/or similar group or a derivative of these groups. The term 'saccharide' also relates to di-, oligo-, and polysaccharide which are made up of one or more monosaccharides as described above, linked to each other by a glycosidic bond.

The term 'nucleoside' relates to each monosaccharide that is substituted with a nucleotide which is for instance, but not limited to, UDP, GDP, ADP, TDP, CMP, or dTDP.

15 The term 'glycoside' relates to a saccharide which forms a glycosidic bond with other chemical compounds, such as, but not limited to sterols, phenols, fatty acids, phosphatidylinositols, vitamine C, cartenoides and artimisinine.

The term 'glycolipid' relates to a saccharide which forms a glycosidic bond with a fatty acid or lipid.

20 The term 'glycoprotein' relates to a saccharide which forms a glycosidic bond with a protein.

The term 'glycosylphosphate' relates to a phosphorylated saccharide.

The present invention further relates to an organism as indicated above wherein said organism is further genetically modified with the introduction of at least one other gene which converts said activated saccharide into a specialty product, or, wherein at least one other
25 endogenous gene of said organism which converts said activated saccharide into a specialty product is over-expressed.

In addition, the present invention relates to an organism as indicated above wherein said organism is capable to grow on a disaccharide, oligosaccharide, polysaccharide or a mixture

thereof as the main carbon source. With the term 'main' is meant the most important carbon source for biomass formation, i.e. 75, 80, 85, 90, 95, 98, 99 % of all the required carbon is derived from the above-indicated carbon source. In one embodiment of the invention, said carbon source is the sole carbon source for said organism, i.e. 100 % of all the required carbon is derived from the above-indicated carbon source.

The term 'metabolic engineering' refers to the practice of optimizing genetic and regulatory processes within said organism to increase the organism's production of a certain desired substance or product. The latter product is hereby denominated as a 'specialty product' and specifically relates to a desired saccharide (activated or non-activated), a nucleoside, a glycoside, a glycolipid or a glycoprotein. Some non-limiting examples of such products are sugar derivatives such as 1,2-fucosyllactose, 1,3-fucosyllactose, 1,4-fucosyllactose, 1,6-fucosyllactose, galactinol, stachyose, globotriose, galactose(beta1-4)rhamnose, sophorose, cellobiose, UDP-glucose, sophorolipids, *myo*-inositol, L-arabinose, *scyllo*-inosose, glycosylphosphatidylinositol, lacto-N-biose, lacto-N-tetraose, lactosamine, fucosylated galactosyloligosaccharides, L-fucose, N-Ac glucosamine, sialic acid, sialyllactose, chitosan and chitin.

The term 'engineering' relates to any well-known technique which can be used to genetically modify an organism as is for example described in (9, 17, 19, 21, 22, 42).

The terms 'an organism being capable to grow on a disaccharide, oligosaccharide, polysaccharide or a mixture thereof as the main carbon source' means that organisms of the present invention may use the same disaccharide, oligosaccharide, polysaccharide or a mixture thereof for both growth (or biomass production) and production of the desired product, and, that they can use the latter saccharides as the only carbon source in order to multiply and/or to be metabolically active. In short, the organisms of the present invention are capable to multiply and metabolize in or on a medium comprising said saccharides as the only carbon source.

With the terms 'splitting (or conversion) into an activated saccharide and a saccharide' is meant that the latter saccharides which are used as carbon source will be split (or converted) by the organism of the present invention into an activated sugar moiety -some non-limiting examples of activated sugar moieties are sugars moieties bearing a phosphate, UDP, GDP, ADP, TDP or dTDP group- and a non-activated sugar moiety which does not bear or is not bound to the latter groups.

The terms 'biocatalytic enzymes' refers to all enzymes needed for the production of the specialty carbohydrate.

5 The term 'biomass' refers to all cellular components (i.e. proteins, DNA, RNA, phosphatidylserine, phosphatidylethanolamine, cardiolipin, phosphatidylglycerol, putrescine, spermidine, peptidoglycan, glycogen and/or lipopolysaccharide (63) that can be synthesized in the modified specialty carbohydrate production strain from the sugar moiety that is not used in the specialty carbohydrate (and other added value products) production route.

10 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, ...) 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 (2, 4, 5, 7, 8, 14, 19, 37, 40, 47, 73, 79, 80, 85, 93, 98).

The term '(gene) knockout' thus refers to a gene which is rendered non-functional.

15 The term 'polysaccharide' refers to a saccharide which contains 6 or more monosaccharide subunits.

The present invention further relates to a metabolically engineered organism as indicated above wherein the genetic modification of step a) is optional, or, is replaced by over-expressing at least: i) an endogenous gene encoding for a carbohydrate hydrolase in combination with an endogenous or heterologous gene encoding for carbohydrate kinase, 20 ii) an endogenous gene encoding for a carbohydrate synthase, or, iii) an endogenous gene encoding for a carbohydrate phosphorylase, and wherein said organism is capable to split a disaccharide, oligosaccharide, polysaccharide or a mixture thereof into an activated saccharide and a saccharide.

25 A preferred carbohydrate hydrolase of the present invention is a lactase, invertase, sucrose, trehalase, sucrose-6-phosphate hydrolase, maltase or amylase. A preferred carbohydrate kinase of the present invention is galactokinase, a fructokinase, a glucokinase or a mannokinase.

The present invention further relates to a metabolically engineered organism as indicated above wherein said activated saccharide in step b) is replaced by said saccharide. Hence, the 'activated sugar moiety' in this embodiment is used as 'fuel', whereas the 'sugar moiety' is activated by a kinase and is pushed into the production route of the desired specialty product.

- 5 The present invention also relates to a metabolically engineered organism as indicated above wherein said gene in step a) splits a disaccharide, oligosaccharide, or polysaccharide in two similar or different activated saccharides or in two similar or different saccharides.

The term 'organism' as indicated above refers to a microorganism chosen from the list consisting of a bacterium, a yeast or a fungus cell, or, refers to a plant or animal cell. The latter
10 bacterium preferably belongs to the species *Escherichia coli*. The latter yeast preferably belongs to the species *Saccharomyces cerevisiae*.

More specifically, the present invention relates to a metabolically engineered organism as indicated above, wherein said activated saccharide is selected from the group consisting of alpha glucose-1-phosphate, alpha galactose-1-phosphate, beta glucose-1-phosphate, beta
15 galactose-1-phosphate, fructose-6-phosphate, glucose-6-phosphate, UDP-glucose and UDP-galactose and wherein said saccharide is selected from the group consisting of fructose, glucose and /or galactose.

The present invention further relates, as indicated above, to a metabolically engineered organism as indicated above, wherein said carbohydrate hydrolase is a lactase, invertase,
20 sucrase, maltase, trehalase, sucrose-6-phosphate hydrolase and/or amylase, and, wherein said carbohydrate kinase is a galactokinase, a fructokinase, a glucokinase and/or mannokinase.

Even more specifically, the present invention relates to a metabolically engineered organism as indicated above wherein:

- 25 - said gene in step a) is encoding for a sucrose phosphorylase, and/or
- the following genes in step b) are rendered non-functional: a gene encoding for a beta-galactosidase, and/or, a gene encoding for a phosphoglucomutase, and/or, a gene encoding for a glucose-1-phosphate adenylyltransferase, and/or, a gene encoding for a phosphatase (such as but not limited to a glucose-1-phosphatase) and/or, a gene

encoding for a glucose-1-phosphate uridylyltransferase, and/or, a gene encoding for a UDP-glucose-4-epimerase, and/or, a gene encoding for UDP-glucose:galactose-1-phosphate uridylyltransferase, and/or, a gene encoding for UDP-galactopyranose mutase, and/or, a gene encoding for UDP-galactose: (glucosyl)lipopolysaccharide-1,6-galactosyltransferase, and/or, a gene encoding for a UDP-galactosyltransferase, and/or, a gene encoding for a UDP-glucosyltransferase, and/or, a gene encoding for an UDP-glucuronate transferase, and/or, a gene encoding for an UDP-glucose lipid carrier transferase, and/or, a gene encoding for an UDP-sugar hydrolase, and/or, a gene encoding for an invertase, and/or, a gene encoding for a maltase, and/or, and/or a gene encoding for a trehalase, and/or, a gene encoding for a sugar transporting phosphotransferase, and/or, a gene encoding for a hexokinase.

An example of the latter metabolically engineered organism is an organism wherein:

- said gene in step a) is the gene encoding for a sucrose phosphorylase possibly (but not solely) originating from a Lactic acid bacterium such as *Bifidobacterium adolescentis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, and/or *Streptococcus mutans*, and/or
- said genes in step b) are: gene *lacZ*, the gene *pgm*, the gene *ycjU*, the gene *glgC*, the gene *agp*, the gene *ptsG*, the gene *glk*, the gene *glf*, the gene *waaB*, the gene *ushA*, the gene *wcaA*, the gene *wcaC*, the gene *wcaE*, the gene *wcaI*, the gene *wcaL*, the gene *wcaJ*, the gene *galU*, the gene *galF*, the gene *galE*, the gene *malP*, the gene *malQ*, and/or, the gene *galT* (20, 25, 27, 28, 32, 46, 48, 49, 52, 54, 62, 82, 86, 87, 96, 97).

Another example of a metabolically engineered organism as indicated above is an organism wherein:

- said gene in step a) is encoding for a sucrose phosphorylase possibly (but not solely) originating from a Lactic acid bacterium such as *Bifidobacterium adolescentis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, and/or *Streptococcus mutans*, and/or
- said genes in step b) are: the gene PGM1, the gene PGM2, the gene GLG1, the gene GLG2, the gene INM1, the gene INM2, the gene GLK1, the gene HXK1, the gene HXK2, the gene GAL10, the gene GAL7, the gene YHL012W, the gene UGP1, the gene GSY1, the gene GSY2, the gene DIE2, the gene ALG8, the gene ATG26, the gene SUC2, the gene MAL32, the gene

MAL12, the gene YJL216C, and/or, the gene YGR287C, and/or, FEN1, and/or, FKS1, and/or, GSC2, and/or, TPS1 (10, 12, 15, 16, 18, 24, 30, 31, 36, 41, 51, 56, 57, 59, 61, 66, 76, 81, 84, 89-91, 99).

5 The latter engineered organisms can, for example but not limited to, be used to produce cellobiose, kojibiose, trehalose, L-arabinose, *myo*-inositol, raffinose, stachyose, L-rhamnose or L-ribose as a specialty product.

A further aspect of the present invention relates to a metabolically engineered organism as indicated above wherein said sucrose phosphorylase of step a) is replaced by a sucrose synthase, a maltose phosphorylase or a maltose synthase, or, wherein said sucrose
10 phosphorylase of step a) is replaced by a lactose phosphorylase or a lactose synthase.

The latter organisms can, for example but limited to, be used to produce sophorose, UDP-glucose, glycolipids, flavone 3-O- β -D-glucoside (sucrose synthase in step a), galactose(β 1-4)rhamnose (lactose phosphorylase in step a), or, UDP-galactose, galactinol, stachyose or globotriose, psychosine (lactose synthase in step a) as specialty products.

15 The present invention further relates to a metabolically engineered organism as indicated above wherein said activated saccharide in step b) is replaced by said saccharide and wherein:

- said gene in step a) is encoding for a sucrose phosphorylase, and/or
- the following genes in step b) are rendered non-functional: a gene encoding for a beta-galactosidase, and/or, a gene encoding for a glucose-6-phosphate isomerase, and/or, a gene
20 encoding for a glucose-1-phosphate adenylyltransferase, and/or, a gene encoding for a phosphatase (for example, glucose-1-phosphatase), and/or, a gene encoding for a phosphofructokinase A, and/or, a gene encoding for a phosphofructokinase B, and/or, a gene encoding for phosphogluconate dehydratase, and/or, a gene encoding for 2-keto-3-deoxygluconate-6-phosphate aldolase, and/or, a gene encoding for a glucose-1-phosphate
25 uridylyltransferase, and/or, a gene encoding for an UDP-glucose-4-epimerase, and/or, a gene encoding for an UDP-glucose:galactose-1-phosphate uridylyltransferase, and/or, a gene encoding for an UDP-galactopyranose mutase, and/or, a gene encoding for an UDP-galactose:(glucosyl)lipopolysaccharide-1,6-galactosyltransferase, and/or, a gene encoding for an UDP-galactosyltransferase, and/or, a gene encoding for an UDP-glycosyltransferase, and/or, a gene

encoding for an UDP-glucuronate transferase, and/or, a gene encoding for an UDP-glucose lipid carrier transferase, and/or, a gene encoding for GDP-mannose hydrolase, and/or, a gene encoding for an UDP-sugar hydrolase, and/or, a gene encoding for a mannose-6-phosphate isomerase, and/or, a gene encoding for an UDP-N-acetylglucosamine enoylpyruvoyl transferase, and/or, a gene encoding for an UDP-N acetylglucosamine acetyltransferase, and/or, a gene encoding for an UDP-N-acetylglucosamine-2-epimerase, and/or, a gene encoding for an undecaprenyl-phosphate-alfa-N-acetylglucosaminyl transferase , and/or, glucose-6-phosphate-1-dehydrogenase, and/or, a gene encoding for a L-glutamine:D-fructose-6-phosphate aminotransferase, and/or, a gene encoding for a mannose-6-phosphate isomerase, and/or a gene encoding for a sorbitol-6-phosphate dehydrogenase, and/or, a gene encoding for a mannitol-1-phosphate 5-dehydrogenase, and/or a gene encoding for a allulose-6-phosphate 3-epimerase, and/or, a gene encoding for an invertase, and/or, a gene incoding for a maltase, and/or, and/or a gene encoding for a trehalase, and/or, a gene encoding for a sugar transporting phosphotransferase, and/or, a gene encoding for a hexokinase.

More specifically, the present invention relates to a metabolically engineered organism as indicated above wherein:

- said gene in step a) is the sucrose phosphorylase originating from a Lactic acid bacterium such as *Bifidobacterium adolescentis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, or *Streptococcus mutans*, and/or

- said genes in step b) are: the gene *lacZ*, the gene *pgi*, the gene *glgC*, the gene *agp*, the gene *pfkA*, the gene *pfkB*, the gene *waaB*, the gene *ushA*, the gene *eda*, the gene *edd*, the gene *wcaA*, the gene *wcaC*, the gene *wcaE*, the gene *wcaI*, the gene *wcaL*, the gene *wcaJ*, the gene *wcaB*, the gene *wcaF*, the gene *wcaK*, the gene *wcaD*, the gene *galU*, the gene *galF*, the gene *galE*, the gene *gmm*, the gene *galT*, the gene *manA*, the gene *murA*, the gene *lpxA*, the gene *rffE*, and/or, the gene *rfe*, the gene *glmS*, the gene *srlD*, the gene *mtlD*, the gene *alsE* and/or, *zwf* (6, 20, 25, 28, 29, 32, 43, 44, 46, 49, 52-55, 62, 65, 75, 78, 82, 86, 87, 96, 97, 101).

Another metabolically engineered organism according to the present invention is an organism wherein:

-said gene in step a) is a gene encoding for a sucrose phosphorylase originating from a Lactic acid bacterium such as *Bifidobacterium adolescentis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, or *Streptococcus mutans*, and/or

5 -said genes in step b) are: the gene PGI1, the gene PFK1, the gene PFK2, the gene PFK26, the gene PFK26, the gene PFK27, the gene GND1, the gene GND2, the gene PMI40, the gene ZWF1, the gene GFA1, the gene GLG1, the gene GLG2, the gene INM1, the gene INM2, the gene GLK1, the gene HXK1, the gene HXK2, the gene GAL10, the gene GAL7, the gene YHL012W, the gene UGP1, the gene GSY1, the gene GSY2, the gene DIE2, the gene ALG8, the gene ATG26, the gene SUC2, the gene MAL32, the gene MAL12, the gene YJL216C, and/or,
10 the gene YGR287C, and/or, FEN1, and/or, FKS1, and/or, GSC2, and/or, TPS1 (12, 15, 16, 24, 26, 30, 31, 34, 36, 38, 39, 41, 51, 56, 57, 59-61, 64, 66, 74, 76, 83, 84, 89, 90, 95, 99).

The latter engineered organism can, for example, be used to produce fucosylated sugar derivates such as fucosyllactose, and more specifically α -1,2-fucosyllactose, α -1,3-fucosyllactose, α -1,4-fucosyllactose, α -1,6-fucosyllactose as specialty products with specific
15 fucosyltransferases originating from for example but not limited to *Helicobacter pylori*, *Bacteroides sp.*, *Homo sapiens*, *Mus musculus*, *Bos taurus* and, *Dictyostelium discoideum*. In addition, said engineered organism can be used to produce chitosans by a chitine synthase and chitine deacetylase or to produce *myo*-inositol by introducing inositol-1-phosphate synthase in combination with inositol monophosphatase.

20 Specific examples of genes which convert said activated saccharide into a specialty product are genes coding for an epimerase, a transferase, a reductase, a (pyro)phosphorylase, a (de)carboxylase, a dehydratase, a permease, a synthase and/or an isomerase. Therefore the present invention relates to a metabolically engineered organism as indicated above wherein the genes which convert said activated saccharide into a specialty product code for an
25 epimerase, transferase, reductase, dehydrogenase, oxidase, pyrophosphorylase, (de)carboxylase, dehydratase, permease, synthase and/or isomerase. The present invention even more specifically relates to the latter metabolically engineered organisms wherein said epimerase is UDP-galactose-4-epimerase or UDP-N-acetylglucosamine epimerase, and/or, wherein said transferase is a glycosyltransferase, a sialyltransferase or a sulfotransferase.

The invention further relates to a metabolically engineered organism as indicated above wherein said specialty product is a monosaccharide, a disaccharide, a trisaccharide, a tetrasaccharide, a pentasaccharide, an oligosaccharide, a polysaccharide, a nucleoside, an O-glycoside, an S-glycoside, an N-glycoside, a C-glycoside, a glycoprotein, a glycolipid or an activated carbohydrate such as but not limited to *myo*-inositol, L-arabinose, *Scyllo*-inosose, Glycosylphosphatidylinositol, Lacto-N-biose, Lacto-N-tetraose, Lactosamine, Fucosylated galactosyloligosaccharides (GOS), L-Fucose, N-Ac glucosamine, Sialic acid, Sialyllactose, chitosan, chitin, 1,2-fucosyllactose, 1,3-fucosyllactose, 1,4-fucosyllactose, 1,6-fucosyllactose, galactinol, stachyose, globotriose, galactose(beta1-4)ramnose, sophorose, cellobiose, UDP-glucose and sophorolipids.

The present invention further relates to a method to produce a specialty product as described above comprising:

- i) metabolically engineering a microorganism as described above, and
- ii) cultivating said genetically engineered microorganism, and
- 15 iii) extracting and purifying said specialty product.

It is clear that any methodology known in the art to cultivate micro-organisms, and, to extract and purify specialty products from said cultivation can be employed in the present invention.

In addition, the present invention relates to the usage of a 2-fucosyltransferase originating from *Dictyostelium discoideum* and having an amino acid sequence given by SEQ ID N° 1, or, a fragment thereof having 2-fucosyltransferase activity, or, a variant thereof having a sequence identity of at least 75 % and having 2-fucosyltransferase activity to produce 2-fucosyllactose (α 1,2-fucosyllactose). A specific fragment having 2-fucosyltransferase activity as indicated above is given by SEQ ID N° 4.

Also the usage of a nucleic acid encoding for a 2-fucosyltransferase as indicated above, and specifically wherein said nucleic acid is given by SEQ ID N° 2 or SEQ ID N° 3 (which both encode for SEQ ID N° 1), to produce fucosyllactose is part of present invention. Nucleic acids encoding for SEQ ID N° 4 are given by SEQ ID N° 5 and SEQ ID N° 6 and are also part of the present invention.

The term 'fragment' refers to a protein (or peptide or polypeptide) containing fewer amino acids than the amino acid sequence as depicted by SEQ ID N° 1 and that retains said 2-fucosyltransferase activity. Such fragment can -for example- be a protein with a deletion of 10% or less of the total number of amino acids at the C- and/or N-terminus or can correspond to SEQ ID N° 4. The term "variant" refers to a protein having at least 75 % sequence identity, preferably having at least 76-85 % sequence identity, more preferably having at least 86-90% sequence identity or most preferably having at least 91, 92, 93, 94, 95, 96, 97, 98 or 99 % sequence identity with SEQ ID N° 1 or with a fragment thereof, and, that encodes for a protein retaining said 2-fucosyltransferase activity.

Hence, orthologues, or genes in other genera and species (than *Dictyostellium discoideum* the from which SEQ ID N° 1 is derived) with at least 75% identity at amino acid level, and having the described function are part of the present invention. The percentage of amino acid sequence identity is determined by alignment of the two sequences and identification of the number of positions with identical amino acids divided by the number of amino acids in the shorter of the sequences x 100. The latter 'variant' may also differ from the protein as depicted by SEQ ID N° 1 only in conservative substitutions and/or modifications, such that the ability of the protein to have 2-fucosyltransferase activity is retained. A "conservative substitution" is one in which an amino acid is substituted for another amino acid that has similar properties, such that one skilled in the art of protein chemistry would expect the nature of the protein to be substantially unchanged. In general, the following groups of amino acids represent conservative changes: (1) ala, pro, gly, glu, asp, gln, asn, ser, thr; (2) cys, ser, tyr, thr; (3) val, ile, leu, met, ala, phe; (4) lys, arg, his; and (5) phe, tyr, trp, his.

Variants may also (or alternatively) be proteins as described herein modified by, for example, by the deletion or addition of amino acids that have minimal influence on the 2-fucosyltransferase activity as defined above, secondary structure and hydrophobic nature of the enzyme.

- 5 The following specific sequences, as indicated above, are part of the present invention:

SEQ ID N° 1: the complete amino acid sequence of the 2-fucosyltransferase of *Dictyostellium discoideum*:

MNDSPIIISVVLFPFLIKDNDKSLNYQGNNLIISIDSIIEQTFKEWELILVDDGSNNEILEQLL
SKRYSTDNRIKFIINKENKGIVKSLNDAILNHCSPTSKYIARMDSDDISHPTRLQSQLKYLQSN
10 ETIDILGCPKMFNNKLEIELNNNNNNNNNNNNVKELINIINNEESFKFIQHPDKDILMWSMF
FNCCIVHPSVIFKRSIFTIEHCYEENNQFPFIEDYLFWLKSLIMKGLNISNIQSSTPLLYLRKH
NNSISFKNIEKQKSTANASCYLNILFKRFNIDSEIIQNSSLSMKEIIQFFQLSPSSLSKINN
ISIELFEFAFKYLELIEKSTKQQPNYSNSIKDAANEKMGELVSLCLSNYPNNQKSLLWEKWL
SRNPTSQLLSLLSNLNVKSSTTIINNINNNNNNNNNNNNNNNNNNNNNNNNNNNNSILNFISG
15 INSNKINTPKSNNKFKENGIRIICFSKDRAFQLKEYLRTFFKYLKNDNGNDKFEIIVDVLFT
YSNEKFKNSYQLVIESFPQVNFIEENFTDQLINLVQKTNKLEYVMFSVDDILYYNEFNLKEYC
LSLNSEPLALGFYMKLNKNITYCHTCNQDITIPLNSNTISRTENNFKYLNKWRNDNDCKKDWNY
PWDLCSTIYRCNDIDSIIINGIVKYYGIRNGINHPNRFEFNGNRPIIQKQIYQNKPYCLCLSDHY
SPMSVVTINRVQDVYDNPIYDQTLSLDDLDQLLYSNKSLNDEKYKENSLSLNFKSVHIGELFIS
20

SEQ ID N°2: the codon optimized nucleotide sequence encoding SEQ ID N°1 for expression in *E. Coli*:

ATGAACGATAGCCCGATTATTAGCGTTGTTCTGCCGTTTCTGATCAAAGATAACGATGATAAAA
GCCTGAACTACCAGGGCATTAAACAACCTGATTATTAGCATCGATAGCATCATCGAGCAGACCTT
25 TAAAGAATGGGAACTGATTCTGGTTGATGATGGCAGCAATAACGAAATTCTGGAACAGCTGCTG
AGCAAACGTTATAGCACCGATAACCGCATCAAATTTATTATTAATAAAGAAAATAAAGGCATTG
TGAAAAGCCTGAATGATGCCATTCTGAATCATTGTAGCCCGACCAGCAAATATATTGCACGTAT
GGATAGCGACGATATTAGCCATCCGACCCGTCTGCAGAGCCAGCTGAAATATCTGCAGAGCAAT
GAAACCATTGATATTCTGGGTTGCCCGATCAAATGTTTAATAATAATAAACTGATTGAAATTC

TGAATAATAATAACAATAACAACAATATTAATAATAATGTGAAAGAACTGATTAATATTATTAA
TAATGAAGAAAGCTTTAAATTTATTCAGCATCCGGATAAAGATATTCTGATGTGGTCCATGTTC
TTCAATTGCTGTATTGTTTCATCCGAGCGTGATTTTTTAAACGCAGCATTTTTTACCATCGAGCACT
GCTATGAAGAGAATAATCAGTTTCCGTTCATCGAGGATTACCTGTTTTGGCTGAAATCCCTGAT
5 TATGAAAGGCCTGAACATTAGCAATATCCAGAGCAGCACACCGCTGCTGTATCTGCGTAAACAT
AATAACAGCATTAGCTTTAAAAATATTGAAAAACAGAAAGATAGCACCGCCAATGCCAGCTGTT
ATTATCTGAACATTCTGTTCAAACGCTTTAACATCGACAGCGAAATTATTCAGAATAGCAGCCT
GAGCATGAAAGAAATCATCCAGTTTTTTTCAGCTGAGCCCGAGCAGCCTGTCCAAAATTAATAAC
ATTAGCATCGAACTGTTTGAATTTGCCTTTAAATATCTGGAAGCTGATCGAGAAAAGCTGTACCA
10 AACAGCAGCCGAATTATAGCAACAGCATTAAAGATGCAGCCAACGAAAAAATGGGTGAACTGGT
TAGCCTGTGTCTGAGCAATTATCCGAATAATCAGAAAAGCAGTCTGCTGTGGGAAAAATGGCTG
AGCCGTAATCCGACCAGCCAGCTGCTGAGTCTGCTGAGCAATCTGAATGTTAAAAGCAGCACCA
CCATTATTAATAACAATATTAACAACAACAATAATAACAACAATAATAACAATAACAATAA
CAATAATAACAACAACAACAATAATAATAATAACAACAACAGCATTCTGAATTTTATTAGCGGC
15 ATTAATAGCAATAAAATTAATACCCCGAAAAGCAACAATAACAAATTTAAAGAGAATGGCATT
GCATTATTTGCTTCAGCAAAGATCGTGCATTCCAGCTGAAAGAATATCTGCGCACCTTCTTCAA
ATATCTGAAAAATGATGATAATGGCAATGATAAATTTGAAATTATTGTGGATGTGCTGTTTACC
TATAGCAATGAAAAATTCAAAAATAGCTATCAGCTGGTGATCGAAAGCTTTCCGCAGGTAACT
TTATTAAAGAAGAAAACTTTACCGATCAGCTGATTAACCTGGTGCAGAAAACCAACAACTGGA
20 ATATGTGATGTTTCAGCGTGGATGATATCCTGTATTACAACGAGTTCAATCTGAAAGAGTATTGC
CTGAGCCTGAATAGCGAACCGCTGGCACTGGGTTTTTATATGAACTGAATAAAAATATTACCT
ATTGCCATACCTGCAACCAGGATATTACCATTCCGCTGAATAGCAATACCATTAGCCGCACCGA
AAATAACTTTAAATACCTGAAATGGAATCGCAACGATAATGATTGCAAAAAAGACTGGAACAT
CCGTGGGATCTGTGTAGCACCATTTATCGTTGCAACGACATTGACAGCATCATTAATGGTATTG
25 TGAAATATTATGGTATTCGCAACGGCATTAAATCATCCGAATCGCTTTGAATTTAATGGCAACCG
TCCGATTATTCAGAAACAAATCTACCAGAACAAACCGTATTGTCTGTGCCTGAGCGATCATTAT
TCACCGATGAGCGTTGTTACCATTAAATCGTGTTTCAGGATGTGTATGATAACCCGATTTATGATC
AGACCCTGAGCCTGGATGATCTGGATCAACTGCTGTATAGCAATAAATCCCTGAACGATGAAAA
ATATAAAGAAAACAGCCTGAGTCTGAACTTCAAAGCGTTCATATTGGCGAACTGTTTCATCAGC
30 TAA

SEQ ID N°3: the native nucleotide sequence encoding SEQ ID N°1: the 2-fucosyltransferase of
Dictyostelium discoideum:

ATGAATGATTACCAATAATAAGTGTAGTTTTACCTTTTTTAATAAAGGACAATGACGATAAATCATTAA
ATTACCAAGGAATAAATAATTTAATAATATCAATAGATAGCATTATTGAACAACTTTTAAAGAATGGGA
ATTAATTTTAGTTGATGATGGATCAAATAATGAAATTTTGGAGCAATTACTTTCAAAAAGATATAGTACA
GATAATAGAATTAAATTCATAATAAATAAAGAGAATAAAGGTATTGTTAAAAAGTTTAAATGATGCAATTT
5 TAAATCATTTGTTACCAACTTCAAAATATATTGCTCGTATGGATTTCAGATGATATTTCTCATCCAACAAG
ATTACAATCTCAACTTAAATATCTTCAATCAAAATGAAACAATTGATATATTAGGTTGTCCAATTAAAAATG
TTTAATAATAATAAATTAATTGAAATTTTAAATAATAATAATAATAATAATAATAATATTAATAATAATGTGA
AAGAGTTAATTAATATAATTAATAATGAAGAATCTTTTAAATTTATTCAACATCCTGATAAAGATATTTT
AATGTGGTCAATGTTTTTCAATTGTTGTATTGTTACCCCTTCTGTAATATTTAAAAGATCGATATTTCACT
10 ATTGAACATTGTTATGAAGAAAACAACCAATTTCCATTCAATTGAAGATTACTTATTTTGGTTAAAATCCT
TAATAATGAAAGGTTTAAATATTTCAAATATCCAATCATCAACACCATTACTATATTTAAGAAAACATAA
TAACTCTATATCTTTTAAAAATATTGAAAAACAAAAAGATTCCACTGCTAATGCATCTTGTTATTATCTA
AATATACTTTTTTAAAAGATTTAATATTGATTCTGAAATTATTCAAAATTCTTCACTCTCAATGAAAGAAA
TTATTCAAGTTCTTCAACTTTTACCATCATCTTTATCAAAAATCAATAATATTTCAATTGAATTATTTGA
15 ATTTGCATTTAAATATCTAGAATTAATTGAAAAATCATGTACAAAACAACAACCAAACTATTCAAACAGT
ATAAAAAGATGCAGCAAATGAAAAAATGGGTGAATTAGTATCTTTATGTTTATCAAATTATCCAAATAATC
AAAAATCATCATTACTTTTGGGAAAAATGGTTATCAAGAAATCCAACCTCACAATTACTATCACTTTTATC
AAATTTAAATGTAAAATCTTCAACTACTATAATTAATAATAATATTAATAATAATAATAATAATAATAAT
AATTTCAATTTTAA
20 ATTTTATATCTGGCATTAAATAGTAATAAAATAAATACTCCAAAATCTAATAATAATAAAATTTAAAGAAAA
TGGAATTAGAATAATTTGTTTCTCAAAGATAGAGCATTTCATTTAAAGAATATCTTAGAACATTTTTT
AAATATTTAAAAAATGATGATAATGGAAATGATAAATTTGAAATTATTGTTGATGTATTATTTACATATT
CAAATGAGAAATTCAAAACCTCTTATCAATTAGTTATTGAAAGTTTTCCACAAGTTAATTTTATTAAAGA
AGAGAATTTCACTGATCAATTAATTAATTTAGTTCAAAAACAAATAAACTTGAATATGTCATGTTTTCA
25 GTTGATGATATTCTTTATTATAATGAATTCATCTCAAGAATATTGTTTATCTTTGAATAGTGAGCCAT
TGGCATTAGGTTTCTATATGAAGTTAAATAAAAAATATTACCTATTGTCATACTTGTAAATCAAGATATAAC
AATACCATTAAATTCAAATACTATTAGTAGAACAGAGAATAATTTTAAATATTTAAAATGGAATAGAAAT
GATAATGATTGTAAAAAGGATTGGAATTATCCATGGGATTTATGTTCAACCATTTATAGATGTAATGATA
TTGATTCAATCATTAATGGTATAGTTAAATATTATGGAATTAGAAATGGTATTAATCATCCAAATAGATT
30 CGAATTCAATGGTAATAGACCAATCATTCAAAAGCAAATCTATCAAAATAAACCTACTGTTTATGTTTA
TCAGATCACTATTCTCCAATGTCTGTTGTAAGTATTAATAGAGTTCAAGATGTCTATGATAATCCAATTT
ATGACCAAACCTATCTTTAGATGATTTAGATCAATTACTTTATTCAAACAAATCATTAATGATGAAAA
ATATAAAGAAAATAGTTTATCTTTAAATTTTAAAGTGTTCATATTGGTGAACTTTTTATTTCTTAA

35 SEQ ID N°4: the amino acid sequence of a fragment of SEQ ID N° 1 having fucosyltransferase activity :

MSILNFISGINSNKINTPKSNNKFKENGIRIICFSKDRAFQLKEYLRTEFFKYLKNDDNGNDKF
EIIIVDVLFTYSNEKFKNSYQLVIESFPQVNFKEENFTDQLINLVQKTNKLEYVMFSVDDILYY
NEFNLKEYCLSLNSEPLALGFYMKLNKNITYCHTCNQDITIPLNSNTISRTENNFKYLNWNRND
40 NDCKKDWNPWDLCSIIYRCNDIDSIIINGIVKYYGIRNGINHPNRFEFNGNRPIIQKQIYQNKP
YCLCLSDHYSPMSVVTINRVQDVYDNPIDYDQTLSDLDQLLYSNKSLNDEKYKENSLSLNFKS
VHIGELFIS

SEQ ID N° 5: the codon optimized nucleic acid sequence encoding SEQ ID N° 4 with an ATG
45 added:

ATGAGCATTCTGAATTTTATTAGCGGCATTAATAGCAATAAAATTAATACCCCGAAAAGCAACA
ATAACAAATTTAAAGAGAATGGCATTTCGCATTATTTGCTTCAGCAAAGATCGTGCATTCCAGCT
GAAAGAATATCTGCGCACCTTCTTCAAATATCTGAAAAATGATGATAATGGCAATGATAAATTT
GAAATTATTGTGGATGTGCTGTTTACCTATAGCAATGAAAAATTCAAAAATAGCTATCAGCTGG
5 TGATCGAAAGCTTTCCGCAGGTAACTTTATTAAAGAAGAAAACCTTACCGATCAGCTGATTAA
CCTGGTGCAGAAAACCAACAACTGGAATATGTGATGTTTCAGCGTGGATGATATCCTGTATTAC
AACGAGTTCAATCTGAAAGAGTATTGCCTGAGCCTGAATAGCGAACCGCTGGCACTGGGTTTTT
ATATGAACTGAATAAAAAATATTACCTATTGCCATACCTGCAACCAGGATATTACCATTCCGCT
GAATAGCAATACCATTAGCCGCACCGAAAATAACTTTAAATACCTGAAATGGAATCGCAACGAT
10 AATGATTGCAAAAAGACTGGAATATCCGTGGGATCTGTGTAGCACCATTTATCGTTGCAACG
ACATTGACAGCATCATTAATGGTATTGTGAAATATTATGGTATTTCGCAACGGCATTAATCATCC
GAATCGCTTTGAATTTAATGGCAACCGTCCGATTATTCAGAAACAAATCTACCAGAACAAACCG
TATTGTCTGTGCCTGAGCGATCATTATTCACCGATGAGCGTTGTTACCATTAATCGTGTTCAGG
ATGTGTATGATAACCCGATTTATGATCAGACCCTGAGCCTGGATGATCTGGATCAACTGCTGTA
15 TAGCAATAAATCCCTGAACGATGAAAAATATAAAGAAAACAGCCTGAGTCTGAACTTCAAAAGC
GTTTCATATTGGCGAACTGTTTCATCAGCTAA

SEQ ID N°6: the native nucleic acid sequence encoding SEQ ID N°4:

TCAATTTTAAATTTTATATCTGGCATTAATAGTAATAAAATAAATACTCCAAAATCTAATAATA
20 ATAAATTTAAAGAAAATGGAATTAGAATAATTTGTTTCTCAAAAGATAGAGCATTTCAATTAAA
AGAATATCTTAGAACATTTTTTAAATATTTAAAAAATGATGATAATGGAAATGATAAATTTGAA
ATTATTGTTGATGTATTATTTACATATTCAAATGAGAAATTCAAAACTCTTATCAATTAGTTA
TTGAAAGTTTTCCACAAGTTAATTTTATTAAAGAAGAGAATTTCACTGATCAATTAATTAATTT
AGTTCAAAAACAAATAAACTTGAATATGTCATGTTTTTCAGTTGATGATATTCTTTATTATAAT
25 GAATTCAATCTCAAAGAATATTGTTTATCTTTGAATAGTGAGCCATTGGCATTAGGTTTCTATA
TGAAGTTAAATAAAAAATATTACCTATTGTCATACTTGTAATCAAGATATAACAATACCATTAAA
TTCAAATACTATTAGTAGAACAGAGAATAATTTTAAATATTTAAAATGGAATAGAAATGATAAT
GATTGTAAAAAGGATTGGAATTATCCATGGGATTTATGTTCAACCATTTATAGATGTAATGATA
TTGATTCAATCATTAATGGTATAGTTAAATATTATGGAATTAGAAATGGTATTAATCATCCAAA
30 TAGATTCTGAATTCAATGGTAATAGACCAATCATTCAAAAGCAAATCTATCAAAATAAACCTAC
TGTTTATGTTTATCAGATCACTATTCTCCAATGTCTGTTGTAACCTATTAATAGAGTTCAAGATG
TCTATGATAATCCAATTTATGACCAAACCCTATCTTTAGATGATTTAGATCAATTACTTTATTC

AAACAAATCATTAATGATGAAAAATATAAAGAAAATAGTTTATCTTTAAATTTTAAAAGTGTT
CATATTGGTGAACTTTTTATTTCTTAA

The present invention is hereby following illustrated by specific working examples.

Examples

5 *Example 1. Materials and methods* 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). The medium for the shake flasks experiments contained 2.00 g/l NH₄Cl, 5.00 g/l (NH₄)₂SO₄, , 2.993
10 g/l KH₂PO₄, 7.315 g/l K₂HPO₄, 8.372 g/l MOPS, 0.5 g/l NaCl, 0.5 g/l MgSO₄·7H₂O, 14.26 g/l sucrose or another carbon source when specified in the examples, 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,
15 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 (low phosphate medium) or 2.93 g/l KH₂PO₄ and 7.31 g/l KH₂PO₄ (high phosphate
20 medium), 0.5 g/l NaCl, 0.5 g/l MgSO₄·7H₂O, 14.26 g/l sucrose, 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 by autoclaving (121 °C, 21') and minimal medium by filtration (0.22 µm Sartorius). If necessary the medium was made selective by adding an antibiotic
25 (ampicilin, chloramphenicol, kanamycin).

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 l Biostat B Plus culture vessel with 1.5 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 4 M KOH. 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 µl). The off-gas was measured with an EL3020 off-gas analyser (ABB Automation GmbH, 60488 Frankfurt am Main, Germany). All data was logged with the Sartorius MFCS/win v3.0 system (Sartorius Stedim Biotech, Melsungen, Germany).

All strains were cultivated at least twice and the given standard deviations on yields and rates are based on at least 10 data points taken during the repeated experiments.

Sampling

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 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-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 go 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 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 precooled 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, samples for OD_{600nm}, CDW, and extracellular metabolites were taken each hour using the rapid sampling loop and the cold stainless bead sampling method. When exponential growth was reached, the sampling frequency was increased to every 20 to 30 minutes.

5 Broth sampling

Using a rapid sampling, which was coupled to the fermentor, samples of 1 ml broth were withdrawn from the fermentor within 0.5 s. Samples were withdrawn directly into tubes containing 5 ml of quenching solution precooled at -40 °C that were immediately mixed after sampling by vortexing. The exact sample sizes were quantified gravimetrically by weighing the
10 tubes before and after sampling.

Filtrate sampling

Samples of extracellular culture fluid were obtained with syringe filtration (pore size 0.45 µm, cellulose acetate) at room temperature without beads—Direct filtration of the broth sample After removal of the cells, the obtained filtrate or supernatant was immediately mixed with 5
15 ml of quenching solution to process these samples in the same way as the broth samples. Also in this case, the exact amount of sample obtained was quantified gravimetrically.

Quenching procedure

The quenching solution used was a 60% (v/v) aqueous methanol. After quenching of broth samples in the quenching solution, precooled at -40 °C, the sample/quenching solution mixture
20 was centrifuged for 5 min at 8000g in a cooled centrifuge (-20 °C) using a rotor that was precooled at -40 °C. After decanting, the supernatant (QS) was stored at -40 °C until extraction. Subsequently, the cell pellets were resuspended in 5 ml of -40 °C quenching solution and again centrifuged. Also, this second supernatant (WS) was stored at -40 °C until extraction. For measurement of metabolites in total broth as well as in the culture filtrate, the
25 same quenching procedure was applied; however, the quenched total broth mixtures (B) or quenched culture filtrates (F) were not centrifuged, but after thorough vortexing, 500 µl of these mixtures was withdrawn for metabolite extraction.

Metabolite extraction procedure

Extraction of metabolites from the cell pellets as well as from the 500- μ l samples from the quenched total broth was performed with the hot ethanol method [34]. Metabolites were extracted in 75% boiling ethanol (3 min, 90 °C). After cooling the thus obtained extracts were evaporated to dryness in a RapidVap (Labconco Corporation, Kansas, Missouri, USA) during 110 min under vacuum. After resuspension of each residue in 500 μ L of H₂O, cell debris was removed by centrifugation during 5 min at 5000g. After decanting the supernatants were stored at -80 °C until further analysis.

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 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).

Carbohydrate measurements

Glucose, fructose, sucrose and glucose-1-phosphate were measured by HPLC with a Hypercarb column (100x4,6 mm; 5 μ m particle size) and were detected with an ELSD detector or mass spectrometer (Antonio et al., 2007; Nielsen et al., 2006). The LOQ of sucrose and G1P were 30 and 20 mg/l, respectively. All samples were diluted within the linear range of the detector, which is between the LOQ and approximately 100mg/l of the metabolite. When multiple phosphorylated and nucleotide sugars were present in the broth, an adaptation of the method

of Bucholz *et al* was applied (11). In this case a gradient of milliQ water (A) and 20mM ammonium acetate (B) was used to separate the analytes. The gradient started at 100% A with a flow of 1ml/min and changed to 100% B at 1ml/min over 10 minutes. The eluens composition of 100% B was then held for 4 minutes at 1ml/min and then changed to 100% A at 1ml/min over 1 minute, after which the flow was increased to 1.2 ml/min and held for 3 minutes to reduce the equilibration time of the column. After these three minutes the flow was reduced again in 2 minutes to 1ml/min. All analytes were detected with either an ELSD detector or mass spectrometer.

For the analysis of mono-, di-, and oligo-saccharides a Prevail Carbohydrate ES (5 μ ; 250 x 4.6 mm) column was used with a gradient of 100% acetone (A), 100% acetonitril (B) and 100% water (C). The gradient is initiated at 20% A, 60% B and 20% C. This is changed over 15 minutes to 15%A, 45%B and 40%C and then changed back to 20% A, 60% B and 20% C within 1 minute. The column is then equilibrated at its initial conditions for 6 minutes. All analytes were either measured with ELSD or mass spectrometer.

Measurement of cell dry weight

From a broth sample, 4 x 10 g was transferred to centrifuge tubes, the cells were spun down (5000g, 4 °C, 5 min), and the cells were washed twice with 0.9% NaCl solution. The centrifuge tubes containing the cell pellets were dried in an oven at 70 °C for 48 h until constant weight. The cell dry weight was obtained gravimetrically; the tubes were cooled in a desiccator prior to weighing.

Sophorose polysaccharide measurement

To determine the amount of sophorose polysaccharide that was produced by a mutant strain in which the heterologous *tts* gene (50) was expressed, a 100ml culture of this mutant and of the wild type strain at approximately OD 6 was centrifuged (5500 rpm, 4°C, 5 minutes, Heraeus Biofuge stratos). 80ml of the supernatant was then precipitated with 2 volumes of cold ethanol (100% at -20°C) en stored overnight at 6°C. The precipitate was separated from the supernatant by centrifugation (5500 rpm, 4°C, 5 min, Hereaus Biofuge stratos) en resuspended in 25ml distilled water (88). 2 ml of this polysaccharide solution was then hydrolyzed in pyrex boriumslicate tubes (26 x 100 mm) at 105°C with 2.25 M HCl (final concentration) for 4h. To neutralize the solution for glucose measurement, equimolar amounts of NaOH was added to

the solution after incubation and cooling. The amount of glucose in the solution was determined with an YSI biochemistry analyser (YSI (UK) Ltd.).

Strains and plasmids used for *Dictyostellium discoideum* α 1,2-fucosyltransferase characterization

- 5 A codon optimized α 1,2-fucosyltransferase originating from *Dictyostellium discoideum* was expressed heterologously in *E. coli* which has the genotype $\Delta lacZ \Delta glgC \Delta manA \Delta CA$ on a plasmid which was constructed as described by Aerts *et al.* (1). CA indicates all genes in the gene cluster that codes for the colanic acid biosynthetic pathway described by Stevenson *et al.* (86).

Enzyme isolation methodology

- 10 The strains were grown in LB (10 g/l tryptone, 5g/l yeast extract and 10 g/l NaCl) in an overnight culture (100ml in 500ml shake flask) at 37°C and 200 rpm. The cells were harvested by centrifugation (15 minutes at 7500 rpm and 4°C). This pellet was resuspended in 5ml PBS buffer and sonicated 3 times for 4 minutes on ice water (cycle 50%, intensity 3). The cell debris was centrifuged again 15 minutes at 7500 rpm and 4°C. The supernatant was used as crude cell
15 extract.

Protein determination

Protein content of the enzyme extract was measured with the Pierce BCA Protein Assay Kit (Thermo) as specified in the product manual.

Plasmid construction for the expression of heterologous and homologous genes

- 20 Plasmid which was constructed as described by Aerts *et al.* (1).

Genetic methods

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

- 25 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 obtained from Prof. Dr. J-P Hernalsteens (Vrije Universiteit Brussel, Belgium). 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), replacement of an endogenous promoter by an artificial promoter (knock-in, KI), respectively. They were introduced using the concept of Datsenko and Wanner (19).

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 electro competent 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 µl of ice-cold water. Electroporation was done with 50 µl of cells and 10–100 ng of linear double-stranded-DNA product by using a Gene PulserTM (BioRad) (600 Ω, 25 µFD, 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

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 resistances and of the FLP helper plasmid. The gene knock-outs and knock-ins are checked with control primers and sequenced.

The primers used to construct the various Knock-out and knock-in mutants are listed in Table 1.

Table 1: Primers used for the construction of the gene knock outs

gene	Fw-P1-H1	Rv-P2-H2
<i>lacZ</i>	CATAATGGATTTCCTTACGCGAAATACG GGCAGACATGGCCTGCCCCGGTTATTAg gtaggctggagctgcttc	GTATGTTGTGTGGAATTGTGAGCGGATAACAATTT CACACAGGAAACAGCTcatatgaatatacctcctta g
<i>glgC</i>	agaccgcccgtttttaagcagcgggaaca tctctgaacatacatgtaaacctgcag ttaggctggagctgcttc	gtctggcaggacctgcacacggattgtgtgtgtt ccagagatgataaaaaaggagtttagtccatatgaa tatacctccttag
<i>agp</i>	CATATTTCTGTCACACTCTTTAGTGATT GATAACAAAAGAGGTGCCAGGAgtgtag gctggagctgcttc	TAAAAACGTTTAACCAGCGACTCCCCGCTTCTCG CGGGGGAGTTTTCTGcatatgaatatacctccttag
<i>pgi</i>	GGCGCTACAATCTTCCAAAGTCACAATT CTCAAAATCAGAAGAGTATTGCgtgtag gctggagctgcttc	GGTTGCCGGATGCGGCGTGAACGCCTTATCCGGCC TACATATCGACGATGcatatgaatatacctccttag
<i>pfkA</i>	GACTTCCGGCAACAGATTTTCATTTTGCA TTCCAAAGTTCAGAGGTAGTCgtgtagg ctggagctgcttc	GCTTCTGTCATCGGTTTCAGGGTAAAGGAATCTGC CTTTTCCGAAATCcatatgaatatacctccttag
<i>pfkB</i>	CACTTTCGGCTGATTCCGTGCCAGACTG AAATCAGCCTATAGGAGGAAATGgtgta ggctggagctgcttc	GTTGCCGACAGGTTGGTGATGATTCCTTCAATGCT GGGGGAATGTTTTTGcatatgaatatacctccttag
<i>pgm</i> via D&W	TGAGAAGGTTTTCGGAACATCTAAAAC GTTGCAGACAAAGGACAAAGCAgtgtag gctggagctgcttc	CATACGTAAAAAAGGGCGATCTTGCGACCGCCCTT TTTTTATTAAATGTGTcatatgaatatacctcctta g
<i>pgm::kan</i>	TGAGAAGGTTTTCGGAACATCTAAAAC GTTGCAGACAAAGGACAAAGCAACGAAA GGCTCAGTCGAAAG	CATACGTAAAAAAGGGCGATCTTGCGACCGCCCTT TTTTTATTAAATGTGTAGAACTCCAGCATGAGATC C
<i>pgm::GFP</i>	TGAGAAGGTTTTCGGAACATCTAAAAC GTTGCAGACAAAGGACAAAGCAgtgtag gctggagctgcttc	CATACGTAAAAAAGGGCGATCTTGCGACCGCCCTT TTTTTATTAAATGTGTATCCGTCAGGATGGCCTT C
<i>ptsG</i>	gccacgcgtgagaacgtaaaaaagcac ccatactcaggagcactctcaattgtgt aggctggagctgcttc	Cacctgtaaaaaaggcagccatctggctgccttag tctccccaacgtcttacggacatatgaatatacctc cttag
<i>glk</i>	CGAGAAGGCCCGATTGTCATGGACGAT GAGATACACCGGAATATCATGGgtgtag	CCAGGTATTTACAGTGTGAGAAAGAATTATTTTGA CTTTAGCGGAGCAGTTGAAGAcatatgaatatacct

	gctggagctgcttc	ccttag
<i>malPQ</i>	ATATCCAGCCAGTCTTCCGGCTGTAGTC CTAACAGAGCACTGTTACTGTCagcatt acacgtccttgagcg	GCTTTAAGTGGTTGAGATCACATTTCTTGCTCAT CCCCGCAACTCCTCCcatatgaatatectccttag
MP KI	ATATCCAGCCAGTCTTCCGGCTGTAGTC CTAACAGAGCACTGTTACTGTC <i>GTAAAACGACGGCCAGTG</i>	CAACGGCCATTTTTTGCCTTAGATACAGATTTTC TGCGCTGTATTGCATTGCCGGGATCCGATGCATAT <i>GG</i>
<i>ycjU</i>	TTTTATTTTGGCCCTTCAATGGGACCGCT ACCAAACATCAGGAGGATGAATGAAACa gcattacacgtccttgagcg	TTCCGTTGAAGGCAACAGTAATTGCGCCCCGGTTA AGCCCGCGCCGATCCcatatgaatatectccttag
CA via D&W	GTAGCATTGTTCCCTAAGTATGACTCCAT TTTTCCAGGAATGGTCGCAAATCgtgta ggctggagctgcttc	TTACGCCGCATCCGGCAAGCAAACCAGCTCATAA GCCGGGAGAACAACCcatatgaatatectccttag
CA via sacB	TTACGCCGCATCCGGCAAGCAAACCAG CTCATAAGCCGGGAGAACAACCccgctt acagacaagctgtg	GTAGCATTGTTCCCTAAGTATGACTCCATTTTCCA GGAATGGTCGCAAATCagccatgaccgggaatta c
<i>wcaJ</i> via <i>sacB</i>	ATCGCCGACCACTTCGCGCCGCTGATGG TTTTTTCACGTAAGCTCATATCccgctt acagacaagctgtg	GGATCTTCCCTTACCCCACTGCGGGTAAGGGGCTA ATAACAGGAACAACGagccatgaccgggaattac
<i>wcaJ</i> via <i>sacB</i> and fusion PCR	GGGGGCCCCCGGGGTATGAGCTTACGT GAAAAAACCATCAG	GGGCCCCGGGCCGGGCGTTGTTCTGTATTAGCC CCTTACCC
<i>wcaJ</i> via D&W	ATCGCCGACCACTTCGCGCCGCTGATGG TTTTTTCACGTAAGCTCATATCgtgtag gctggagctgcttc	GGATCTTCCCTTACCCCACTGCGGGTAAGGGGCTA ATAACAGGAACAACGcatatgaatatectccttag
<i>wcaJ</i> via D&W_2	TTTGATATCGAACCAGACGCTCCATTC GCGGATGTACTCAAGGTCGAACgtgtag gctggagctgcttc	TCTATGGTGCAACGCTTTTCAGATATCACCATCAT GTTTGCCGGACTATGcatatgaatatectccttag
<i>galET-H1- P22-RBS</i>	TAGCCAAATGCGTTGGCAAACAGAGATT GTGTTTTTTCTTTCAGACTCATCTTTGT TTCCTCCGAATTCG	CGGTTTCGACGCATGCAGGCATGAAACCGCGTCTTT TTTCAGATAAAAAGCcatatgaatatectccttag

<i>galET</i> extended homology	ACCAATCAAATTCACGCGGCCAGGCGCC TGAATGGTGTGAGTGGCAGGGTAGCCAA ATGCGTTGGCAAAC	GTCGGTAGTGCTGACCTTGCCGGAGGCGGCCTTAG CACCTCTCCGGCCAACGGTTCGACGCATGCAGGC
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Example 2. Engineering and usage of Base strain 1 (carbon source: sucrose; converted into glucose-1-phosphate and fructose by sucrose phosphorylase) - Screening of different sucrose phosphorylases

An important requirement for the success of 'base strain 1' is the existence of a potent sucrose phosphorylase. However, though *E. coli* has a putative sucrose phosphorylase (SP), it does not grow on a minimal medium with sucrose as sole carbon source. Therefore, 6 sucrose phosphorylases from a variety of microbial sources were screened (Table 2)

To this end, 6 transformants of the wild type *E. coli* were constructed each carrying a plasmid [pCX-promoter-SP] encoding for one of the sucrose phosphorylase (SP) listed in Table 2. The performance of these strains was evaluated in shake flasks, using sucrose as sole carbon source. The wild type strain (WT) was incorporated in the experimental design as a control ($\mu_{WT} = 0$).

Table 2: Screened sucrose phosphorylases

Source sucrose phosphorylase	Abbreviation
<i>Bifidobacterium adolescentis</i>	BA
<i>Lactobacillus acidophilus</i>	LA
<i>Streptococcus mutans</i>	SM
<i>Leuconostoc mesenteroides</i> B742	LM B742
<i>Leuconostoc mesenteroides</i> B1149	LM B1149
<i>Leuconostoc mesenteroides</i> B1355	LM B1355

In this screening experiment, the growth rate of the various transformants was monitored and linked to the performance of the sucrose phosphorylases. According to this reasoning the best growing strain does possess the best performing sucrose phosphorylase.

The growth rate of the various transformants is depicted in Figure 2, the principle applied on for the production of a specialty carbohydrate is depicted in Figure 1: (a) A normal equilibrium reaction, which occurs in the current production technologies (b) pull/push principle: the equilibrium is shifted towards the saccharide and activated saccharide. The main goal of a cell is to grow, hence it pulls, in this figure at the saccharide to form biomass. Due to this pulling effect, the activated saccharide will accumulate in the cell and pushes the production pathway.

Example 3. Characterization of the sucrose phosphorylase of *Bifidobacterium adolescentis*

Various artificial constitutive promoters have been inserted to evaluate the influence of the promoter strength on the growth rate. To this end, a weak, medium strength, and strong promoter from a promoter library available at The Centre of Expertise - Industrial Biotechnology and Biocatalysis (Ghent University) were introduced. The medium strength promoter, which yielded the highest growth rate, was finally retained.

The affinity constant and the maximal growth rate of the *E. coli* strain carrying a plasmid encoding for the sucrose phosphorylase of *Bifidobacterium adolescentis* were determined. To this end, batch and chemostat experiments were run. The influence of the phosphate concentration on these parameters was checked as well (Table 3).

The kinetic properties of the engineered strain are listed in Table 3. It is clear that the kinetic properties of the engineered strain are adequate in view of future industrial applications.

Table 3: Growth characteristics of an *E. coli* carrying the *Bifidobacterium adolescentis* sucrose phosphorylase. High PO_4^{3-} = 64 mM; low PO_4^{3-} = 8,5 mM.

	High PO_4^{3-}	Low PO_4^{3-}
$\mu_{\max}$ (h^{-1})	0.5	0.46
K_S (mg/L)	<10	+/- 10

Example 4. Engineering strategy for an increased supply of α glucose-1-phosphate

To validate the rational of the engineering strategy it is important to demonstrate an increased pool of α glucose-1-phosphate in the mutant strain (Figure 3), compared to the α glucose-1-phosphate pool in the wild type (Figure 4). In 'Base strain 1' the microbial metabolism is split into two disconnected parts because the main reactions able to convert α glucose-1-phosphate to biomass production were eliminated. One part of the metabolism converts the fructose moiety into biomass and numerous bio-catalytic enzymes (classic central metabolism). The other part converts the α glucose-1-phosphate moiety of sucrose.

The α glucose-1-phosphate concentration was determined both for the wild type and some engineered strains. To this end, batch experiments were performed, using sucrose as sole carbon source.

The α glucose-1-phosphate pool: comparing the wild type and the plug strain

To evaluate the potential of the envisaged metabolic engineering strategy, the α glucose-1-phosphate pool was determined in:

- the wild type *E. coli* MG1655 grown on glucose,
- *E. coli* MG1655 *p22BaSP* grown on sucrose,
- *E. coli* MG1655 Δ *glgC* Δ *pgm* Δ *lacZ* *p22BaSP* grown on sucrose

The size of this pool is of major importance because the metabolically engineered pathways of the various specialty carbohydrates to be produced all use α glucose-1-phosphate as prime precursor. Hence, the larger the α glucose-1-phosphate pool, the more precursors that is available for the production of the various specialty carbohydrates.

The shake flasks results are depicted in Figure 5. The intracellular glucose-1-phosphate concentration is $4.03 \cdot 10^{-3}$ mmol/gcDW, 0.26 mmol/gcDW, and 1.27 mmol/gcDW, respectively. A >20000% increase in the G1P pool is thus already achieved. This increased pool enables the efficient production of a variety of specialty carbohydrates.

In the wild type *E. coli* MG1655 strain glucose-1-phosphate is a precursor of cell wall related components, glycogen, etc. A limited flow of carbon, typically coming from α glucose-6-phosphate, suffices to supply the cell with sufficient α glucose-1-phosphate to produce these minor biomass fractions. Hence, the α glucose-1-phosphate pool is of limited size ($4.03 \cdot 10^{-3}$ mmol/gcDW).

This is in contrast with the proposed strategy to use sucrose as carbon source. Compared to the wild type *E. coli* MG1655 strain an increased glucose-1-phosphate pool has been shown in the mutant strains that contain a potent sucrose phosphorylase that efficiently splits the inexpensive sugar sucrose into fructose and α glucose-1-phosphate.

The results obtained in a 1.5 L batch reactor are depicted in Figure 6. The intracellular α glucose-1-phosphate concentration is $4.03 \cdot 10^{-3}$ mmol/gcDW, 0.65 mmol/gcDW, and 0.89 mmol/gcDW, respectively.

Production of α glucose-1-phosphate by Δ *pgm* Δ *lacZ* Δ *glgC* (3KO) P22-BaSP on buffered LB medium at reactor scale

The ability of $\Delta pgm \Delta lacZ \Delta glgC$ P22-BaSP to produce α glucose-1-phosphate was verified. To this end, a culture with buffered LB medium with about 15 g/L sucrose was run. At about 15h a concentrated sucrose solution, containing phosphate was added. In Figure 7 and Figure 6 the concentration of the most important (by)products are depicted. The maximal growth rate during the batch phase is about 0,552 h⁻¹.

During the batch phase per mole of sucrose that is degraded 0.74 mole of glucose-1-phosphate is generated. Ideally, 1 mole of α glucose-1-phosphate can be generated. However, the 3KO studied still contains genes whose products are able to convert α glucose-1-phosphate, e.g., *agp*.

From the moment all sucrose is consumed, the concentration of glucose-1-phosphate decreases and the concentration of glucose increases which is due to the activity of Agp.

Subsequently at about 15 h additional sucrose is added, which is again converted to glucose-1-phosphate and which accumulates in the medium. Fructose accumulates as well in the medium, which indicates that the cell has limited means to further metabolize this compound (0.64 mole of fructose per mole of sucrose).

In a subsequent experiment, sucrose and phosphate were added on regular time intervals during the course of the fermentation. A maximum α glucose-1-phosphate concentration of about 57 g/L was achieved.

Example 5. Inactivation of the gene coding for phosphoglucomutase

To split the metabolism according to example 1-4 the gene coding for phosphoglucomutase has to be knocked out. Via the classical methodology described by Datsenko and Wanner (19) a knock out results into a chromosomal scar of approximately 84 base pairs. The strains in which this gene was deleted in this manner seem to grow on a complex medium but, to our surprise, did not grow on a minimal medium as described in the materials and methods section. However, the strain did grow on a minimal medium when the kanamycine cassette was left behind. Apparently the removal of the original sequence at this chromosomal location seemed to interfere with growth on a minimal medium but the replacement of this specific sequence (*pgm* gene), coding for phosphoglucomutase, by a sequence with a similar length did not. This fact was validated by replacing the *pgm* gene with a part of the GFP gene which has exactly the same size as the *pgm* gene. This resulted also in a mutant strain that could grow on a minimal medium. The sequences

of these strains at the chromosomal location of *pgm* are shown in Figure 11, Figure 12, Figure 13, and Figure 14.

Example 6. Cellobiose production in E. coli

Cellobiose producing strains have been constructed starting from 'Base strain 1' (Figure 8). To this end a plasmid containing both a sucrose phosphorylase (*Bifidobacterium adolescentis*) and cellobiose phosphorylase (*Cellulomonas uda*) have been inserted in the wild type, in *E. coli* MG1655 $\Delta \Delta glgC \Delta pgm \Delta lacZ$ (3KO), and in *E. coli* MG1655 $\Delta agp \Delta glgC \Delta pgm \Delta lacZ$ (4KO) (Table 4). Additional genes to be knocked out are *glk* and *ptsG*, since both convert glucose into glucose-6-phosphate.

Table 4 Cellobiose producing strain

Knock –out	Reaction
<i>lacZ</i>	Glu +Gal $\leftrightarrow$ Lactose
<i>pgm</i>	G1P $\leftrightarrow$ G6P
<i>glgC</i>	G1P + ATP + H $\leftrightarrow$ ADP-glucose + PPi
<i>agp</i>	G1P + H ₂ O $\rightarrow$ Glu + Pi
<i>ycjM</i>	Suc $\rightarrow$ G1P + Fruc
<i>ptsG</i>	Glu + PEP $\rightarrow$ G6P + Pyr
<i>glk</i>	Glu + ATP $\rightarrow$ G6P + ADP
Knock-in	Reaction
Sucrose phosphorylase	Sucrose + Pi $\rightarrow$ G1P + Fruc
Cellobiose phosphorylase	G1P + Glu $\rightarrow$ Cellobiose + Pi

Comparing the wild type and the plug strain

To evaluate the potential of the envisaged metabolic engineering strategy to produce specialty carbohydrates the production of cellobiose was investigated in various engineered strains:

- *E. coli* MG1655 (WT) P22-BaSP P22-CuCP
- *E. coli* MG1655 $\Delta glgC \Delta pgm \Delta lacZ$ (3KO) P2- BaSP P22-CuCP
- *E. coli* MG1655 $\Delta glgC \Delta pgm \Delta lacZ \Delta agp$ (4KO) P22-BaSP P22-CuCP

To this end shake flask experiments were performed. The medium contained buffered LB medium and sucrose and glucose were added in equal amounts to the shake flasks, so that a final concentration of 1.978 g cellobiose /L was achieved in the shake flask (Table 5). The shake flasks results are depicted in Figure 5. The (extracellular) concentration of the desired product cellobiose increases with the number of mutations that has been introduced.

Table 5 Cellobiose production of various engineered strains

Strain	Abbreviation	Cellobiose (g/L)
<i>E. coli</i> MG1655 P22-BaSP P22-CuCP	WT P22-BaSP P22-CuCP	0
<i>E. coli</i> MG1655 $\Delta glgC$ Δpgm $\Delta lacZ$ P22-BaSP P22-CuCP	3KO P22-BaSP P22-CuCP	0.539
<i>E. coli</i> MG1655 $\Delta glgC$ Δpgm $\Delta lacZ$ Δagp P22-BaSP P22-CuCP	4KO P22-BaSP P22-CuCP	1.978

Production of cellobiose by $\Delta pgm \Delta lacZ \Delta glgC \Delta agp$ (4KO) P22-BaSP P22-CuCP on buffered LB medium at reactor scale.

The ability of $\Delta pgm \Delta lacZ \Delta glgC \Delta agp$ P22-BaSP P22-CuCP to produce cellobiose was verified on reactor scale in a preliminary experiment. To this end, a culture with buffered LB medium was run. At about 9h and on specific time points a solution containing 500 g/L sucrose and 250 g/L glucose was added to the culture.

A conversion efficiency of about 30 % (mol cellobiose produced/ mol sucrose consumed) was achieved and about 40 % of the glucose moiety of sucrose ended up in cellobiose or in glucose-1-phosphate. A titer of about 15 g/L of cellobiose was achieved at the end of the culture.

Secondly, the production of cellobiose was verified in a batch culture starting from 70 g/l sucrose with a high concentration of phosphate and low concentration of phosphate. High phosphate indicates a phosphate concentration of 0.2 M phosphate, low phosphate indicates a concentration of 13 mM phosphate. This affected the production significantly. The high concentration of phosphate resulted in a final titer of approximately 20 g/l and a yield of 0.33 g/g, while a low phosphate concentration resulted in a final titer of 42 g/l and a yield on consumed sucrose of 0.84 g/g (Figure 9).

Example 7. Engineering Base strain 2 (Sucrose- sucrose synthase) and its uses

By metabolically engineering *E. coli* a base strain is constructed that is an efficient producer of specialty carbohydrates and their derivatives whose pathway starts from UDP-glucose.

By introducing sucrose synthase (e.g., coming from *Solanum tuberosum*), sucrose is split into fructose and UDP-glucose. By additionally knocking-out genes coding for UDP-glucose 4 epimerase (*galE*), UDP-glucose galactose-1-P uridyltransferase (*galT*), glucose-1-P uridyltransferase (*galU*, *galF*), 5'-nucleotidase / UDP-sugar hydrolase (*ushA*), UDP-glucose 6-dehydrogenase (*ugd*), belonging to the colanic acid operon (*ca*) a mutant is constructed which accumulates UDP-glucose (Table 6).

10 Table 6 Base strain UDP-Glucose

Knock –out	Reaction
<i>ca</i>	→ colanic acid
<i>galU</i>	G1P +UTP <-->UDP-Glc + PPi
<i>galF</i>	G1P +UTP <-->UDP-Glc + PPi
<i>galE</i>	UDP-Glc <--> UDP-Gal
<i>galT</i>	UDP-Glc + Gal1P <--> UDP-Gal + G1P
<i>ushA</i>	UDP-sugar + H ₂ O <--> uridine-5'-phosphate + 2H ⁺ + an aldose-1-phosphate
<i>ugd</i>	2 NAD +UDP-sugar + H ₂ O <--> 2 NADH + UDP-glucuronate + 3H ⁺
Knock-in	Reaction
Sucrose synthase	Suc + UDP→ UDP Glu + Fruc

Example 8. Expression of sucrose synthase in E. coli

The activity of sucrose synthase was determined using an in vitro assay. A sucrose synthase from *Solanum tuberosum* was heterologously expressed in *E. coli* BL21. From this culture an enzyme extract was prepared, which was incubated with 500mM sucrose and 2mM UDP. The evolution of the amount UDP-Glucose produced is given in Table 7.

Table 7 Sucrose synthase enzym assay demonstrating the activity of cleavage reaction of sucrose synthase (Mixture contained 500mM sucrose and 2mM UDP)

Sampling time	UDP-Glucose formed
0h 10 min	5mg/L

1h 40 min	64mg/L
24h	300mg/L

Example 9. Sophorose production

Starting from base strain 2 (UDP-Glucose), a strain is constructed which produces large quantities of sophorose, as a polymer of sophorose units. This is achieved by additionally introducing the gene *tts* from *Streptococcus pneumoniae* (50). Sophorose units can be generated out of the sophorose polymer in two ways, i.e., via acid hydrolysis or via enzymatic conversion. Such an enzyme can be (over)expressed in the producer strain as well.

To evaluate the potential of the metabolic engineering strategy the sophorose polymer was determined for *E. coli* MG1655 P22-BaSP P22-*tts*, and a 6KO P22-BaSP P22-*tts* strain (*E. coli* MG1655 Δ *glgC* Δ *pgm* Δ *lacZ* Δ *agp* Δ *ptsG* Δ *glk*) by growing these strains on a minimal medium containing lactose as sole carbon source. The results are depicted in Figure 15. These results indicate that the mutant strains produces significantly more sophorose polymer in comparison to the wild type strain.

Example 10. Engineering Base strain 3 (Lactose- lactose phosphorylase) and its uses

By introducing lactose phosphorylase (20), lactose is split into glucose and galactose-1-P. By additionally knocking-out genes coding for *agp*, *galE*, *galT*, *lacZ*, and the metabolism is split into two disconnected parts. However, all possible combinations of these genes result in increased accumulation of galactose-1-phosphate. Instead of knocking-out these genes, this goal can also be achieved by rendering them defective or by reducing their expression (Table 8).

Table 8 Base strain 3 Galactose-1-phosphate (Lactose- lactose phosphorylase)

Knock –out	Reaction
<i>lacZ</i>	Glu +Gal $\leftrightarrow$ Lactose
<i>galE</i>	UDP-Glc $\leftrightarrow$ UDP-Gal

agp G1P + H₂O → Glu + Pi
galT UDP-Glc + Gal1P ↔ UDP-Gal + G1P

Knock-in	Reaction
Lactose phosphorylase	Lactose + Pi → Gal1P + Glucose

Example 11. Galactose(β1-4)L-Rhamnose production:

Starting from base strain 3, a Gal(β1-4)L-Rha producer is constructed by additionally introducing a gene coding for an (Ga)lacto-N-biose D-galactosyl-(β1-4)-L-rhamnose phosphorylase, which convert Galactose-1-phosphate and rhamnose into Gal(β1-4)L-Rha and phosphate.

A fermentation is performed using lactose as main carbon source yielding quantities of Gal(β1-4)L-Rha. L-rhamnose is added to the medium. Undesired degradation of rhamnose is prevented by knocking out genes involved in the degradation of rhamnose (*rhaA*, *rhaB*, *rhaC*, *rhaD*).

Example 12. Engineering Base strain 4 (Lactose – lactose synthase) and its uses

By introducing lactose synthase (71, 72) lactose is split into glucose and UDP-galactose. By additionally knocking-out genes coding for beta-galactosidase (*lacZ*), UDP-glucose, galactose-1-P uridyltransferase (*galT*) UDP-glucose 4 epimerase (*galE*), 5'-nucleotidase / UDP-sugar hydrolase (*ushA*), UDP-glucose 6-dehydrogenase (*ugd*), belonging to the colanic acid operon (*ca*) a mutant is constructed which accumulates UDP-Galactose (Table 9).

Table 9: Base strain 4 UDP-Galactose (Lactose synthase)

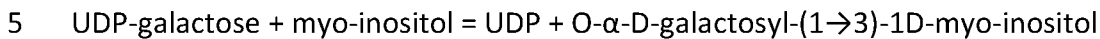
Knock –out	Reaction
<i>lacZ</i>	Glu + Gal ↔ Lactose
<i>galE</i>	UDP-Glc ↔ UDP-Gal
<i>galT</i>	UDP-Glc + Gal1P ↔ UDP-Gal + G1P

<i>ca</i>	→ colanic acid
<i>ushA</i>	UDP-sugar + H ₂ O <--> uridine-5'-phosphate + 2H ⁺ + an aldose-1-phosphate
<i>ugd</i>	2 NAD + UDP-sugar + H ₂ O <--> 2 NADH + UDP-glucuronate + 3H ⁺

Knock-in	Reaction
Lactose synthase	Lactose → UDP-Gal + Glu

Example 13. Galactinol production:

Starting from base strain 4, a galactinol producer is constructed by additionally introducing a gene coding for an Inositol 3-alpha-galactosyltransferase which catalyzes the conversion:



A fermentation is performed using lactose as main carbon source yielding quantities of galactinol in which myo-inositol is added to the medium.

Example 14. Globotriose production:

10 Starting from base strain 4, a globotriose producer is constructed by additionally introducing the gene *lgtC* from *Neisseria meningitidis* (3) encoding for a α-1,4-Gal transferase, which catalyzes the conversion UDP-Gal + Lactose → UDP + Globotriose. A fermentation is performed using lactose as main carbon source yielding quantities of globotriose.

Example 15. Engineering Base strain 5 and producing fucosylated sugars

15 Starting from base strain 5, which accumulates fructose-6-phosphate, (described in Example 20 and Example 28) fucosylated sugar derivatives such as fucosyllactose and more specifically 1,2-fucosyllactose can be produced. To this end, the strain is modified so that the cell is forced to produce fructose-6-phosphate which is a precursor of GDP-fucose. Glucose or glucose-1-phosphate (if the starting enzyme is either a sucrase or a sucrose phosphorylase) is then fed to the central carbon metabolism via the pentose phosphate pathway. Figure 10 shows the route
20 towards product and biomass and the needed knock outs to achieve this. To avoid loss of fructose-6-phosphate via glycolysis, *pfkA*, *pfkB* and *pgi* are knocked out. To avoid accumulation of pyruvate, the Entner Doudoroff route is knocked out (*edd* and *eda*).

Because GDP-fucose is an intermediate of the colanic acid biosynthesis pathway, this pathway has to be modified. Genes from the colanic acid operon that can potentially reduce product yield are
25 knocked out. These genes are *gmm*, *wcaA*, *wcaBi*, *wcaC*, *wcaD*, *wcaE*, *wcaF*, *wcaI*, *wcaJ*, *wcaK*,

wcaL and/or, *wcaM*. The genes *manA*, *cpsG*, *cpsB*, *gmd* and, *fcl* (coding for Mannose-6-phosphate isomerase, phosphomannomutase, mannose-1-phosphate guanylyltransferase, GDP-mannose 4,6-dehydratase and GDP-fucose synthase, respectively) are enhanced in expression to increase the flux towards GDP-fucose. Either the colanic acid operon is knocked out completely, with the reintroduction of the needed genes that are overexpressed with an artificial promoter from a promoter library, or the operon is modified as such that the genes described above are knocked out one by one and the remaining genes in the operon are enhanced in expression by changing the promoter of the operon with an artificial constitutive promoter (23). Finally GDP-fucose is linked to lactose into α -1,2-fucosyllactose by a α -1,2-fucosyltransferase. The fucosyltransferases tested originate from *Helicobacter pylori*, *Bacteroides sp.*, *Homo sapiens*, *Mus musculus*, *Bos taurus* and, *Dictyostelium discoideum*.

Example 16. Engineering E. coli Base strain 3 (Galactose-1-P) and its uses

By knocking-out genes coding for (a) phosphatase(s) (*agp*), UDP-glucose, galactose-1-P uridilyltransferase (*galT*), UDP-glucose-4-epimerase (*galE*) a mutant is constructed which accumulates galactose-1-P. By additionally overexpressing genes coding for galactokinase (*galK*) and/or galactose-1-epimerase (*galM*) the formation of galactose-1-P is enhanced (Table 10).

Table 10 Base strain galactose-1-phosphate

Knock –out	Reaction
<i>galT</i>	Gal1P + UDP-Glucose $\leftrightarrow$ UDP-Galactose + G1P
<i>galE</i>	UDP-Glucose $\leftrightarrow$ UDP-Galactose
<i>agp</i>	Glucose-1-phosphate + H ₂ O $\rightarrow$ Pi + glucose
Knock-in	Reaction
<i>galK</i>	α -galactose + ATP $\rightarrow$ Gal1P + ADP
<i>galM</i>	β -galactose $\leftrightarrow$ α -galactose

Example 17. Engineering Base strain 3 (Galactose-1P) and its uses

By knocking-out genes coding for (a) phosphatase(s) (*agp*), UDP-glucose, galactose-1-P uridilyltransferase (*galT*), UDP-glucose-4-epimerase (*galE*) and by additionally overexpressing genes coding for galactokinase (*galK*) a mutant is constructed which accumulates galactose-1-P (Table 11).

Table 11 *E. coli* base strain galactose-1-phosphate

Knock –out	Reaction
<i>galT</i>	Gal1P + UDP-Glucose $\leftrightarrow$ UDP-Galactose + G1P
<i>galE</i>	UDP-Glucose $\leftrightarrow$ UDP-Galactose
<i>galK</i>	α -galactose + ATP $\rightarrow$ Gal1P + ADP
<i>galM</i>	β -galactose $\leftrightarrow$ α -galactose
<i>agp</i>	glucose-1-phosphate + H ₂ O $\rightarrow$ Pi + glucose
Knock-in	Reaction
<i>galK</i>	α -galactose + ATP $\rightarrow$ Gal1P + ADP

To evaluate the potential of the metabolic engineering strategy, the galactose-1-phosphate concentration was determined for the wild type, *E. coli* MG1655 $\Delta galET$ P22 *galK*, *E. coli* MG1655 $\Delta galETKM$ Δagp P22 *galK*, *E. coli* MG1655 $\Delta galET$ P22 *galK*, and *E. coli* MG1655 $\Delta galETKM$ Δagp P22 *galK* + orotate (2 g/L) by growing these strains on a minimal medium containing lactose (15 g/L) as main carbon source. The results are depicted in Table 12.

10

Table 12 Galactose-1-P concentration of various *E. coli* mutants

strain	Galactose-1-P (mg/L)
<i>E. coli</i> MG1655	Not detectable
<i>E. coli</i> MG1655 $\Delta galET$ P22 <i>galK</i>	15,73
<i>E. coli</i> MG1655 $\Delta galETKM$ Δagp P22 <i>galK</i>	69,08

<i>E. coli</i> MG1655 $\Delta galET$ P22 <i>galK</i>	12,48
<i>E. coli</i> MG1655 $\Delta galETKM \Delta agp$ P22 <i>galK</i> + orotate (2 g/L)	64,90

Example 18. Production of Glucose-6-phosphate using sucrose phosphorylase in E. coli

By introducing sucrose phosphorylase sucrose is split into glucose-1-P and fructose. By additionally knocking-out genes coding for (a) phosphatase(s) (*agp*), glucose 6-phosphate-1-dehydrogenase (*zwf*), phosphoglucose isomerase (*pgi*), glucose-1-phosphate adenylyltransferase (*glgC*) a mutant is constructed which accumulates glucose-6-P.

The KO mutants are chosen in such a way that the metabolism is split into two disconnected parts. However, all possible combinations of these genes result in increased supply. Instead of knocking-out these genes, this goal is also achieved by rendering them defective or by reducing their expression.

By metabolically engineering *E. coli* a base strain is constructed that is an efficient producer of specialty carbohydrates and their derivatives whose pathway starts from Glucose-6-P (Table 13).

Table 13 Base strain glucose-6-phosphate using a sucrose phosphorylase

Knock –out	Reaction
<i>zwf</i>	$G6P + NADP \rightarrow 6PG + NADPH$
<i>agp</i>	$G1P + H_2O \rightarrow Glc + Pi$
<i>glgC</i>	$G1P + ATP + H \leftrightarrow ADP\text{-}glucose + PPi$
<i>pgi</i>	$G6P \leftrightarrow F6P$

Knock-in	Reaction
Sucrose phosphorylase	$\text{Suc} + \text{Pi} \rightarrow \text{G1P} + \text{Fru}$
<i>pgm</i>	$\text{G6P} \leftrightarrow \text{G1P}$

Example 19. Production of Glucose-6-phosphate using invertase in E. coli

By introducing sucrose hydrolase/invertase sucrose is split into glucose and fructose. By additionally knocking-out genes coding for (a) phosphatase(s) (*agp*), glucose 6-phosphate-1-dehydrogenase (*zwf*), phosphoglucose isomerase (*pgi*), glucose-1-phosphate adenylyltransferase (*glgC*), phosphoglucomutase (*pgm*) a mutant is constructed which accumulates Glucose-6-P (Table 14).

Table 14: Base strain glucose-6-phosphate using invertase in *E. coli*

Knock –out	Reaction
<i>zwf</i>	$\text{G6P} + \text{NADP} \rightarrow \text{6PG} + \text{NADPH}$
<i>agp</i>	$\text{G1P} + \text{H}_2\text{O} \rightarrow \text{Glu} + \text{Pi}$
<i>pgi</i>	$\text{G6P} \leftrightarrow \text{F6P}$
<i>pgm</i>	$\text{G6P} \leftrightarrow \text{G1P}$
Knock-in	Reaction
invertase	$\text{Suc} + \text{H}_2\text{O} \rightarrow \text{Glc} + \text{Fru}$

Example 20. Production of fructose-6-phosphate using invertase in E. coli

By introducing sucrose hydrolase/invertase sucrose is split into glucose and fructose. By additionally knocking-out genes coding for (a) phosphatase(s) (*agp*), phosphofructokinase (*pfkA* and *pfkB*), phosphoglucose isomerase (*pgi*), glucose-1-phosphate adenylyltransferase (*glgC*), phosphoglucomutase (*pgm*) a mutant is constructed which accumulates fructose-6-phosphate (Table 15).

Table 15 Base strain fructose-6-phosphate

Knock –out	Reaction
<i>pfkA</i>	$\text{F6P} + \text{ATP} \rightarrow \text{FBP} + \text{ADP}$
<i>pfkB</i>	$\text{F6P} + \text{ATP} \rightarrow \text{FBP} + \text{ADP}$
<i>agp</i>	$\text{G1P} + \text{H}_2\text{O} \rightarrow \text{Glc} + \text{Pi}$

<i>pgi</i>	G6P $\leftrightarrow$ F6P
<i>pgm</i>	G6P $\leftrightarrow$ G1P
Knock-in	Reaction
invertase	Suc + H ₂ O $\rightarrow$ Glc + Fru

Example 21. Production of β -Glucose-1-phosphate using maltose phosphorylase in *E. coli*

By introducing maltose phosphorylase, maltose is split into β -D-glucose 1-phosphate and glucose. By additionally knocking-out genes coding for (a) phosphatase(s) (*agp*, *yfbT*), phosphoglucomutase (*ycjU*), maltose hydrolase (*malPQ*) a mutant is constructed which accumulates β -Glucose -1-P. The KO mutants are chosen in such a way that the metabolism is split into two disconnected parts. However, all possible combinations of these genes result in increased supply. Instead of knocking-out these genes, this goal is also achieved by rendering them defective or by reducing their expression (Table 16).

10

Table 16: Base strain β -D-Glucose-1-phosphate using maltose phosphorylase in *E. coli*

Knock –out	Reaction
<i>malPQ</i>	Maltose $\rightarrow$ glucose
<i>yfbT</i>	β -D-glucose 1-phosphate + H ₂ O $\rightarrow$ glucose + Pi
<i>agp</i>	Glucose-1-phosphate + H ₂ O $\rightarrow$ glucose + Pi
<i>ycjU</i>	β -D-glucose 1-phosphate $\rightleftharpoons$ β -D-glucose-6-phosphate
Knock-in	Reaction
Maltose phosphorylase (MP)	Maltose + Pi $\rightarrow$ Glucose-1P + Glucose

Example 22. Production of β -Glucose-1-phosphate using trehalose phosphorylase in *E. coli*

By introducing trehalose phosphorylase trehalose is split into β -D-glucose 1-phosphate and glucose. By additionally knocking-out genes coding for (a) phosphatase(s) (*agp*, *yfbT*), phosphoglucomutase (*ycjU*), undesired native trehalose degrading enzymes (*treABC*, *treC*, *treE*, *treF*) a mutant is constructed which accumulates β -Glucose -1-phosphate (Table 17).

15

The KO mutants are chosen in such a way that the metabolism is split into two disconnected parts. However, all possible combinations of these genes result in increased productivity. Instead of knocking-out these genes, this goal is achieved by rendering them defective or by reducing their expression.

5 Table 17 Base strain β -D-Glucose-1-phosphate using trehalose phosphorylase in *E. coli*

Knock –out	Reaction
<i>treA</i>	trehalose + H ₂ O $\rightarrow$ 2 β -D-glucose
<i>treC</i>	trehalose 6-phosphate + H ₂ O $\rightarrow$ β -D-glucose-6-phosphate + β -D-glucose
<i>treE</i>	trehalose 6-phosphate + H ₂ O $\leftrightarrow$ β -D-glucose-6-phosphate + β -D-glucose
<i>treF</i>	trehalose + H ₂ O $\leftrightarrow$ 2 β -D-glucose
<i>yfbT</i>	β -D-glucose 1-phosphate + H ₂ O $\rightarrow$ Glucose + Pi
<i>agp</i>	glucose 1-phosphate + H ₂ O $\rightarrow$ Glucose + Pi
<i>ycjU</i>	β -D-glucose 1-phosphate $\leftrightarrow$ β -D-glucose-6-phosphate
Knock-in	Reaction
Trehalose Phosphorylase (TP)	Trehalose + Pi $\rightarrow$ β -D-Glucose-1P + Glucose
<i>otsB</i>	Trehalose-6-phosphate + H ₂ O $\rightarrow$ trehalose + Pi

Example 23. Production of kojibiose

By additionally introducing kojibiose phosphorylase in a strain accumulating β -D-glucose 1-phosphate and additionally knocking-out genes coding for glucokinase (*glk*) and, phosphotransferase system (*ptsG*) a mutant is constructed which produces kojibiose.

A fermentation is performed with an *E. coli* mutant strain ($\Delta lacZ \Delta glgC \Delta agp \Delta ptsG \Delta malPQ \Delta ycjU$ pCXp22MPp22KP) using maltose and glucose as main carbon sources yielding kojibiose. (Figure 16).

Example 24. Production of UDP glucose from sucrose via a sucrose phosphorylase

By introducing sucrose phosphorylase (e.g., originating from *Bifidobacterium adolescentis*) sucrose is split into fructose and glucose-1-P. Starting from a glucose-1-phosphate accumulating strain (see examples above) and by additionally knocking-out genes coding UDP-glucose 4 epimerase (*galE*), UDP-glucose galactose-1-P uridyltransferase (*galT*), 5'-nucleotidase / UDP-sugar hydrolase (*ushA*),
 5 UDP-glucose 6-dehydrogenase (*ugd*) a mutant is constructed which accumulates UDP-glucose by additionally overexpressing genes coding for UDP-glucose pyrophosphorylase (e.g. coming from *Bifidobacterium bifidum*) (Table 18).

Table 18 Base strain UDP-Glucose from sucrose via a sucrose phosphorylase in *E. coli*

Knock –out	Reaction
<i>lacZ</i>	Glu +Gal $\leftrightarrow$ Lactose
<i>galE</i>	UDP-Glucose $\leftrightarrow$ UDP-Galactose
<i>galT</i>	Gal1P + UDP-Glucose $\leftrightarrow$ UDP-Galactose + G1P
<i>ca</i>	$\rightarrow$ colanic acid
<i>ushA</i>	a UDP-sugar + H ₂ O $\leftrightarrow$ uridine-5'-phosphate + an α -D-aldose-1-phosphate + 2 H ⁺
<i>ugd</i>	2 NAD ⁺ + UDP-D-glucose + H ₂ O $\leftrightarrow$ 2 NADH + UDP-D-glucuronate + 3 H ⁺
<i>pgm</i>	G1P $\leftrightarrow$ G6P
<i>glgC</i>	G1P + ATP + H $\leftrightarrow$ ADP-glucose + PPi
<i>agp</i>	G1P + H ₂ O $\rightarrow$ Glu + Pi
<i>ptsG</i>	Glc + PEP $\rightarrow$ G6P + Pyr
<i>glk</i>	Glc + ATP $\rightarrow$ G6P + ADP
Knock-in	Reaction
<i>galU/F</i>	G1P +UTP $\rightarrow$ UDP-Glucose + PPi
Sucrose phosphorylase	Suc $\rightarrow$ G1P + Fru

Example 25. Production of UDP-glucose in *E. coli* with a sucrose-6-phosphate synthase combined with sucrose PTS

By introducing Sucrose PTS (94) and Sucrose-6-phosphate synthase (69), sucrose is converted into fructose-6-phosphate and UDP-glucose. Starting from the strain described in example 4, without a

sucrose phosphorylase, and by additionally knocking-out genes coding for (a) phosphatase(s) (*agp*), UDP-glucose 4 epimerase (*galE*), UDP-glucose galactose-1-P uridyltransferase (*galT*), 5'-nucleotidase / UDP-sugar hydrolase (*ushA*), UDP-glucose 6-dehydrogenase (*ugd*) a mutant is constructed which accumulates UDP-glucose By additionally overexpressing genes coding for

5 UDP-glucose pyrophosphorylase (*Bifidobacterium bifidum*) (Table 19).

Table 19 Base strain UDP-Glucose with sucrose-6-phosphate synthase combined with sucrose PTS

Knock –out	Reaction
<i>galE</i>	UDP-Glucose $\leftrightarrow$ UDP-Galactose
<i>galU/galF</i>	UTP + G1P $\leftrightarrow$ UDP-Glucose + PPi
<i>galT</i>	Gal1P + UDP-Glucose $\leftrightarrow$ UDP-Galactose + G1P
<i>ca</i>	$\rightarrow$ colanic acid
<i>ushA</i>	a UDP-sugar + H ₂ O $\leftrightarrow$ uridine-5'-phosphate + an α -D-aldose-1-phosphate + 2 H ⁺
<i>ugd</i>	2 NAD ⁺ + UDP-D-glucose + H ₂ O $\leftrightarrow$ 2 NADH + UDP-D-glucuronate + 3 H ⁺
Knock-in	Reaction
Sucrose 6P synthase	UDP-glucose + D-fructose 6-phosphate $\rightarrow$ UDP + sucrose 6-phosphate
Sucrose PTS	Sucrose + PEP $\rightarrow$ sucrose-6-phosphate + Pyruvate

Example 26. Production of UDP galactose via galactokinase

By overexpressing genes coding for galactokinase (*galK*) and Galactose-1-phosphate uridylyltransferase (for example originating from *Bifidobacterium bifidum*) the formation of UDP-galactose is enhanced by additionally knocking-out genes coding for (a) phosphatase(s) (*agp*),
 5 UDP-glucose, galactose-1-P uridylyltransferase (*galT*), UDP-glucose-4-epimerase (*galE*) a mutant is constructed which accumulates galactose-1-P (Table 20).

Table 20 Base strain UDP-Galactose via galactokinase

Knock –out	Reaction
<i>galT</i>	Gal1P + UDP-Glucose $\leftrightarrow$ UDP-Galactose + G1P
<i>galE</i>	UDP-Glucose $\leftrightarrow$ UDP-Galactose
<i>galK</i>	α -galactose + ATP $\rightarrow$ Gal1P + ADP
<i>galM</i>	β -galactose $\leftrightarrow$ α -galactose
<i>agp</i>	glucose 1-phosphate + H ₂ O $\rightarrow$ Pi + glucose
Knock-in	Reaction
<i>galK</i>	α -galactose + ATP $\rightarrow$ Gal1P + ADP
Galactose-1-phosphate uridylyltransferase	Gal1P + UTP $\rightarrow$ UDP-Galactose + PPi

Example 27. Production of UDP galactose via lactose phosphorylase

By introducing lactose phosphorylase (20), lactose is split into glucose and galactose-1-P. By knocking-out genes coding for (a) phosphatase(s) (*agp*), UDP-glucose, galactose-1-P uridylyltransferase (*galT*), UDP-glucose-4-epimerase (*galE*) a mutant is constructed which
 15 accumulates galactose-1-P. By additionally overexpressing genes coding for Galactose-1-phosphate uridylyltransferase (for example coming from *Bifidobacterium bifidum*) the formation of UDP-galactose is enhanced (Table 21).

The KO mutants are chosen in such a way that the metabolism is split into two disconnected parts. However, all possible combinations of these genes result in increased productivity. Instead of
 20 knocking-out these genes, this goal is achieved by rendering them defective or by reducing their expression.

Table 21 Base strain UDP-Galactose via lactose phosphorylase

Knock-out	Reaction
<i>lacZ</i>	$\text{Glu} + \text{Gal} \leftrightarrow \text{Lactose}$
<i>galE</i>	$\text{UDP-Glc} \leftrightarrow \text{UDP-Gal}$
<i>agp</i>	$\text{G1P} + \text{H}_2\text{O} \rightarrow \text{Glu} + \text{Pi}$
<i>galT</i>	$\text{UDP-Glc} + \text{Gal1P} \leftrightarrow \text{UDP-Gal} + \text{G1P}$
Knock-in	Reaction
Lactose phosphorylase	$\text{Lactose} + \text{Pi} \rightarrow \text{Gal1P} + \text{Glucose}$
Galactose-1-phosphate uridylyltransferase	$\text{Gal1P} + \text{UTP} \rightarrow \text{UDP-Galactose} + \text{PPi}$

Example 28. Fructose-6-phosphate accumulation in E. coli

- 5 The metabolism is split in order to accumulate fructose-6-phosphate. This is achieved by knocking out the genes coding for phosphoglucose isomerase and phosphofructokinase activity. In *E. coli* these activities are coded by the genes *pgi*, *pfkA* and *pfkB*. The growth rate of strains devoid of these activities is described in Table 22 for growth on glucose and sucrose. The growth rate of the wild type strain is somewhat affected when grown on sucrose after introduction of a sucrose phosphorylase in comparison with growth on glucose, however the introduction of *pgi* knock outs and *pfkA* and *pfkB* double mutations lead to significant reduction of growth rate, with the latter being extremely low (0.02 h⁻¹) on glucose. Surprisingly the mutant strain $\Delta pgi \Delta pfkA \Delta pfkB$ has a similar growth rate to that of the Δpgi single mutant.

15 Table 22: Specific growth rates of the glycolysis knock out strains on a minimal medium with glucose and sucrose. In the strains grown on sucrose a plasmid coding for sucrose phosphorylase was introduced.

Strain	Growth rate on glucose (h ⁻¹)	Growth rate on sucrose (h ⁻¹)
Wild type	0.64	0.41
Δpgi	0.18	0.23
$\Delta pfkA \Delta pfkB$	0.02	n.d.
$\Delta pgi \Delta pfkA \Delta pfkB$	0.23	0.24

Only the $\Delta pgi\Delta pfkA\Delta pfkB$ mutant strain accumulated fructose-6-phosphate in the medium when grown on sucrose, the other strains did not indicate any F6P accumulation. The growth profile and F6P accumulation by this strain is shown Figure 17.

*Example 29. α Glucose-1-phosphate accumulation in *Saccharomyces cerevisiae**

5 Because *Saccharomyces cerevisiae* splits sucrose by nature, all alternative sucrose degrading reactions (invertases), which are coded by the genes SUC2, MAL32, MAL12, YJL216C, YGR287c, are knocked out. To avoid the assimilation of α -glucose-1-phosphate, the enzymes that convert this activated carbohydrate are rendered less functional or non-functional. These enzymes are phosphoglucomutase, coded by PGM1 and PGM2, and glucose-1-phosphatase, coded by INM1
10 and INM2. By introducing a sucrose phosphorylase (e.g., originating from *Bifidobacterium adolescentis*), similar to the split metabolism of *E. coli* (see example above). The *Saccharomyces cerevisiae* metabolism is split into two parts resulting in the accumulation of α -glucose-1-phosphate.

*Example 30. Cellobiose production with *Saccharomyces cerevisiae**

15 Because *Saccharomyces cerevisiae* splits sucrose by nature, all alternative sucrose degrading reactions (invertases), which are coded by the genes SUC2, MAL32, MAL12, YJL216C, YGR287c, are knocked out. To avoid the assimilation of α -glucose-1-phosphate, the enzymes that convert this activated carbohydrate are rendered less functional or non-functional. These enzymes are phosphoglucomutase, coded by PGM1 and PGM2, and glucose-1-phosphatase, coded by INM1
20 and INM2. By introducing a sucrose phosphorylase (e.g., originating from *Bifidobacterium adolescentis*), similar to the split metabolism of *E. coli* (see example above). The *Saccharomyces cerevisiae* metabolism is split into two parts resulting in the accumulation of α -glucose-1-phosphate. By introducing a cellobiose phosphorylase gene originating from *Cellulomonas uda*, *Saccharomyces cerevisiae* is able to produce cellobiose.

25 To avoid degradation of glucose, glucokinase activity, coded by GLK1 is knocked out as well. Because hexokinases in *Saccharomyces cerevisiae* are not specific, these are replaced by a specific heterologous substrate specific hexokinase. Fructokinases originating from *E. coli* or *Bifidobacterium adolescentis* show lower activity for glucose and can replace the genes coding for the native hexokinases coded by HXK1 and HXK2.

Example 31. Fructose-6-phosphate accumulation in Saccharomyces cerevisiae by introducing a sucrose phosphorylase

Because *Saccharomyces cerevisiae* splits sucrose by nature, all alternative sucrose degrading reactions (invertases), which are coded by the genes SUC2, MAL32, MAL12, YJL216C, YGR287c, are knocked out. By introducing sucrose phosphorylase from *Bifidobacterium adolescentis* sucrose is split into fructose and glucose-1-phosphate. To avoid the conversion of fructose-6-phosphate into biomass, the activity of the enzymes phosphoglucose isomerase and phosphofructokinase is reduced or eliminated by rendering the genes PGM1, PFK1 and PFK2 less functional or non-functional, respectively.

10 *Example 32. Galactose-1-phosphate accumulation in Saccharomyces cerevisiae*

Galactose-1-phosphate is derived from the disaccharide lactose. Because *Saccharomyces cerevisiae* does not split lactose by nature, a heterologous β -galactosidase (e.g. from *E. coli*) is introduced. This leads to the degradation of lactose to galactose and glucose. The goal is to increase the supply of galactose-1-phosphate to a biosynthetic pathway of a specialty carbohydrate. Therefore, galactose-1-phosphate may not be converted anymore into biomass, which is catalysed by UDP-glucose-hexose-1-phosphate uridylyltransferase, the aldose reductase. This is achieved by knocking out the genes coding for UDP-glucose-hexose-1-phosphate uridylyltransferase and aldose reductase, GAL7 and GRE3 respectively. To avoid degradation of said galactose-1-phosphate, the genes encoding for galactose-1-phosphatase are be knocked out. These are INM1 and INM2. A galactokinase is overexpressed in order to enhance the formation of galactose-1-phosphate from galactose.

Example 33. Glucose-6-phosphate accumulation in Saccharomyces cerevisiae via its native invertase

To split the *Saccharomyces cerevisiae* metabolism into two parts (to enhance the supply of glucose-6-phosphate so that it can be used as a building block for a specialty carbohydrate) glucose-6-phosphate dehydrogenase, phosphoglucomutase and phosphoglucose isomerase, which are coded by the genes ZWF1, PGM1 and PGM2, and PGI1, respectively, are knocked out. In such a strain sucrose is split in fructose and glucose by the native invertases and phosphorylated into fructose-6-phosphate and glucose-6-phosphate by the native hexokinases. Glucose-6-phosphate is then supplied to the specialty carbohydrate biosynthesis pathway and fructose-6-phosphate is converted into biomass.

Example 34. Glucose-6-phosphate accumulation in Saccharomyces cerevisiae via sucrose phosphorylase

Saccharomyces cerevisiae is modified to produce glucose-6-phosphate from sucrose with a sucrose phosphorylase originating from *Bifidobacterium adolescentis*. Because sucrose phosphorylase competes for sucrose with invertase, the genes coding for invertase activity are knocked out. These genes are SUC2, MAL32, MAL12, YJL216C, and YGR287c. Glucose-1-phosphate is then further converted into glucose-6-phosphate with a phosphoglucomutase coded by PGM1 and PGM2. To avoid degradation of glucose-1-phosphate into glucose, glucose-1-phosphatase encoding genes are knocked out, coded by INM1 and INM2. The assimilation of this activated saccharide is further reduced by eliminating the UTP glucose-1-phosphate uridylyltransferase activity in the cell, by rendering the genes YHL012W and UGP1 less functional or non-functional. The fructose moiety is phosphorylated by the native hexokinases coded by HXK1 and HXK2 and converted into biomass.

Example 35. Enhanced UDP-glucose formation in Saccharomyces cerevisiae via sucrose synthase

Because *Saccharomyces cerevisiae* splits sucrose by nature, all alternative sucrose degrading reactions (invertases), which are coded by the genes SUC2, MAL32, MAL12, YJL216C, YGR287c, are knocked out. A sucrose synthase, e.g. originating from *Solanum tuberosum*, is introduced so that sucrose is split into UDP-Glucose and fructose. To avoid UDP-glucose conversion into biomass, the activity of the enzymes UDP-glucose diphosphorylase, UDP-glucose 4-epimerase, UDP-glucose-hexose-1-phosphate uridylyltransferase, UDP-glucose-glycogen glucosyltransferase, UDP-glucose-1,3-beta-D-glucan glucosyltransferase, UDP-glucose-glucosephosphate glucosyltransferase are rendered less functional or non-functional, these enzymes are coded by the genes UGP1 and YHL012W, GAL10, GAL7, GSY1 and GSY2, FEN1 and FKS1 and GSC2, and TPS1, respectively. The fructose moiety is phosphorylated by the native hexokinases coded by HXK1 and HXK2 and converted into biomass.

Example 36. Enhanced UDP-glucose formation in Saccharomyces cerevisiae via sucrose sucrose phosphorylase

In order to enhance UDP-glucose supply in *Saccharomyces cerevisiae*, a strain as described in Example 29 is further modified by overexpressing the gene GAL7 which codes for a UTP-glucose-1-phosphate uridylyltransferase that catalyzes the conversion of α -glucose-1-phosphate into UDP-glucose.

Example 37. Enhanced UDP-galactose formation in *Saccharomyces cerevisiae* via β -galactosidase

In order to enhance UDP-galactose supply in *Saccharomyces cerevisiae*, a strain as described in Example 32 is further modified by overexpressing a gene which codes for a UTP-galactose-1-phosphate uridylyltransferase (e.g. coming from *Bifidobacterium bifidum*) that catalyzes the conversion of α -galactose-1-phosphate into UDP-galactose.

Example 38. *Dictyostellium discoideum* α 1,2-fucosyltransferase activity
Introduction

The *Dictyostellium discoideum* α 1,2-fucosyltransferase (FT) is part of a rather new glycosyltransferase class. All known FT's belong to class GT11, while *D. discoideum* FT belongs to class GT74. Such an FT has, up to now, only been found in two other organisms namely *Desulfococcus oleovorans* and *Desulfotomaculum reducens*. The third class is GT37, which only contains plant FT's (Figure 18). A clustalW and Tcoffee alignment indicated that the identity with *H. pylori* is only 9.7% and 15.5%, respectively. The conserved motives of GT11 are shown in Figure 19 and these do not occur at all in the *Dictyostellium* protein (67). These domains differentiate for the fucosylation activity of the transferases. The first two motives are the same for α -1,2-fucosyltransferase and α -6-fucosyltransferase, while the third differentiates the enzymes from each other. α -1,3-fucosyltransferase contains two completely different conserved motives.

The *Dictyostellium discoideum* FT was however described to be only active on lacto-N-biose and not on galactose phenyl- β -galactoside, Gal β 1-6-GlcNac, lactose, Gal β 1-6Gal, Xyl, Glc, GlcNac and GalNac (92).

In Figure 20 a LC MSMS analysis of the *Dictyostellium discoideum* fucosyltransferase assay is shown. This assay showed surprisingly that this heterologous expressed fucosyltransferase is active with lactose as acceptor and GDP-fucose as fucose donor. The activity of this enzyme was 0.19 ± 0.01 U/mg protein and was determined following the method of Persson and Palcic (68).

Because only half of the enzyme of *Dictyostellium discoideum* is responsible for its α 1,2-fucosyltransferase activity, this part was cloned in a similar way to the complete enzyme, however with an additional start codon (coded by ATG) in front of the nucleotide sequence. This new enzyme was produced in a $\Delta lacZ \Delta glgC \Delta manA \Delta CA$ mutant strain and assayed for α 1,2-fucosyltransferase activity with an LC MSMS. Figure 21 shows clearly that this partial enzyme is still able to form 2-fucosyllactose from GDP-fucose and lactose.

Example 39. *Myo-inositol production in E. coli*

Starting from a base strain that accumulates glucose-6-phosphate, a myo-inositol producer is constructed by additionally introducing a gene coding for myo-inositol-1-phosphate synthase (INO1, originating from *Saccharomyces cerevisiae*) and myo-inositol-1(or 4)-monophosphatase (INM1 and INM2, originating from *Saccharomyces cerevisiae*).

Example 40. *Lacto-N-biose production in E. coli*

Starting from a base strain that accumulates galactose-1-phosphate, a Lacto-N-biose producer is constructed by additionally introducing a gene coding for lacto-N-biose phosphorylase (Inbp, *Bifidobacterium longum*). A fermentation is performed using lactose and N-acetylglucoamine as carbon sources. Degradation of N-acetylglucosamine is inhibited in the producer strain by eliminating any N-acetyl-D-glucosamine kinase activity (*nagK*) and N-acetylglucosamine PTS activity (*nagE*, *ptsH*, *ptsI*, *manXYZ*).

List of abbreviations used in the text

3KO	a mutant strain in which the genes <i>pgm</i> , <i>lacZ</i> and <i>glgC</i> are knocked out
4KO	a mutant strain in which the genes <i>pgm</i> , <i>lacZ</i> , <i>glgC</i> and <i>agp</i> are knocked out
6PG	6-phosphogluconate
BA	<i>Bifidobacterium adolescentis</i>
BaSP	a sucrose phosphorylase originating from <i>Bifidobacterium adolescentis</i>
CA	Colanic acid operon defined by Stevenson <i>et al.</i> (86)
CDW	Cell dry weight
CuCP	<i>Cellulomonas uda</i> cellobiose phosphorylase
F6P	Fructose-6-phosphate
FBP	Fructose-1,6-bisphosphate
Fru	Fructose
FT	α 1,2-Fucosyltransferase
G1P	Glucose-1-phosphate

	Gal1P	Galactose-1-phosphate
	Glc	Glucose
	Glu	Glucose
	KI	Knock in
5	KO	Knock out
	KP	Kojibiose phosphorylase
	LA	<i>Lactobacillus acidophilus</i>
	LB	Luria Bertani Broth
	LM	<i>Leuconostoc mesenteroides</i>
10	MP	Maltose phosphorylase
	P22	promoter 22 of the promoter library of De Mey <i>et al</i> (2007)
	pCXp22	a plasmide that contains the P22 promoter according to Aerts <i>et al</i> (1)
	PEP	Phosphoenolpyruvate
	Pi	Inorganic phosphate
15	PPi	Pyrophosphate
	Pyr	Pyruvate
	rpm	rotations per minute
	SM	<i>Streptococcus mutans</i>
	SP	Sucrose phosphorylase
20	Suc	Sucrose
	UDP-gal	UDP-galactose
	UDP-glc	UDP-glucose

WT

Wild type strain

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Claims

1. A metabolically engineered organism for the production of at least one specialty product chosen from the group consisting of a saccharide, an activated saccharide, a nucleoside, a glycoside, a glycolipid and a glycoprotein, characterized in that:
 - 5 a) said organism is genetically modified with the introduction of at least: i) a gene encoding for a carbohydrate hydrolase in combination with a gene encoding for a carbohydrate kinase, ii) a gene encoding for a carbohydrate synthase, or, iii) a gene encoding for a carbohydrate phosphorylase, so that said organism is capable to split a disaccharide, oligosaccharide, polysaccharide or a mixture thereof into an activated saccharide and a saccharide, and
 - 10 b) said organism is further genetically modified so that at least one other gene than any of the introduced genes of step a) of said organism is rendered less- functional or non-functional and wherein said other gene encodes for an enzyme which converts said activated saccharide into biomass and/or bio-catalytic enzymes.
2. A metabolically engineered organism according to claim 1 wherein said organism is further
15 genetically modified with the introduction of at least one other gene which converts said activated saccharide into a specialty product, or, wherein at least one other endogenous gene of said organism which converts said activated saccharide into a specialty product is over-expressed.
3. A metabolically engineered organism according to claims 1 to 2 wherein said organism is
20 capable to grow on a disaccharide, oligosaccharide, polysaccharide or a mixture thereof as the main carbon source.
4. A metabolically engineered organism according to claims 1 to 3 wherein the genetic modification of step a) is optional, or, is replaced by over-expressing at least: i) an endogenous gene encoding for a carbohydrate hydrolase in combination with a gene encoding for
25 carbohydrate kinase, ii) an endogenous gene encoding for a carbohydrate synthase, or, iii) an endogenous gene encoding for a carbohydrate phosphorylase, and wherein said organism is capable to split a disaccharide, oligosaccharide, polysaccharide or a mixture thereof into an activated saccharide and a saccharide.

5. A metabolically engineered organism according to claims 1-4 wherein said activated saccharide in step b) is replaced by said saccharide, and, wherein said activated saccharide in said further genetic modification according to claims 2-4 is replaced by said saccharide.
6. A metabolically engineered organism according to claims 1-4 wherein said gene in step a) splits a disaccharide, oligosaccharide, or polysaccharide in two activated saccharides or in two saccharides.
7. A metabolically engineered organism according to claims 1-6, wherein said organism is a microorganism chosen from the list consisting of a bacterium, a yeast, a fungus cell, or is a plant cell or an animal cell.
8. A metabolically engineered organism according to claim 7, wherein said bacterium is *Escherichia coli* or wherein said yeast is *Saccharomyces cerevisiae*.
9. A metabolically engineered organism according to claims 1-8, wherein said activated saccharide is selected from the group consisting of alpha-glucose-1-phosphate, alpha-galactose-1-phosphate, beta-glucose-1-phosphate, beta-galactose-1-phosphate, UDP-glucose, glucose-6-phosphate, fructose-6-phosphate and UDP-galactose and wherein said saccharide is selected from the group consisting of fructose, glucose, and galactose.
10. A metabolically engineered organism according to claims 1-9, wherein said carbohydrate hydrolase is a lactase, invertase, sucrase, maltase, trehalase, sucrose-6-phosphate hydrolase and/or amylase, and, wherein said carbohydrate kinase is a galactokinase, a fructokinase, a glucokinase and/or mannokinase.
11. A metabolically engineered organism according to claims 1-3, 6-10 wherein:
 - said gene in step a) is encoding for a sucrose phosphorylase, and/or
 - the following genes in step b) are rendered non-functional: a gene encoding for a beta-galactosidase, and/or, a gene encoding for a phosphoglucomutase, and/or, a gene encoding for a glucose-1-phosphate adenylyltransferase, and/or, a gene encoding for a phosphatase and/or, a gene encoding for a glucose-1-phosphate uridylyltransferase, and/or, a gene encoding for a UDP-glucose-4-epimerase, and/or, a gene encoding for UDP-glucose:galactose-1-phosphate uridylyltransferase, and/or, a gene encoding for UDP-

galactopyranose mutase, and/or, a gene encoding for UDP-galactose: (glucosyl)lipopolysaccharide-1,6-galactosyltransferase, and/or, a gene encoding for a UDP-galactosyltransferase, and/or, a gene encoding for a UDP-glucosyltransferase, and/or, a gene encoding for an UDP-glucuronate transferase, and/or, a gene encoding for an UDP-glucose lipid carrier transferase, and/or, a gene encoding for an UDP-sugar hydrolase, and/or, a gene encoding for an invertase, and/or, a gene encoding for a maltase, and/or, a gene encoding for a trehalase, and/or, a gene encoding for a sugar transporting phosphotransferase, and/or, a gene encoding for a hexokinase.

12. A metabolically engineered organism according to claim 11 wherein:

- said gene in step a) is encoding for a sucrose phosphorylase

- said genes in step b) are : the gene *lacZ*, the gene *pgm*, the gene *ycjU*, the gene *glgC*, the gene *agp*, the gene *ptsG*, the gene *glk*, the gene *glf*, the gene *waaB*, the gene *ushA*, the gene *wcaA*, the gene *wcaC*, the gene *wcaE*, the gene *wcaI*, the gene *wcaL*, the gene *wcaJ*, the gene *galU*, the gene *galF*, the gene *galE*, the gene *galT*, the gene *malP*, and/or, the gene *malQ*.

13. A metabolically engineered organism according to claim 11 wherein:

-said gene in step a) is encoding for a sucrose phosphorylase

-said genes in step b) are: the gene PGM1, the gene PGM2, the gene GLG1, the gene GLG2, the gene INM1, the gene INM2, the gene GLK1, the gene HXK1, the gene HXK2, the gene GAL10, the gene GAL7, the gene YHL012W, the gene UGP1, the gene GSY1, the gene GSY2, the gene DIE2, the gene ALG8, the gene ATG26, the gene SUC2, the gene MAL32, the gene MAL12, the gene YJL216C, and/or, the gene YGR287C, and/or, FEN1, and/or, FKS1, and/or, GSC2, and/or, TPS1.

14. A metabolically engineered organism according to claim 12-13 wherein said sucrose phosphorylase originates from a Lactic acid bacterium and wherein said Lactic acid bacterium is *Bifidobacterium adolescentis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, or *Streptococcus mutans*.

15. A metabolically engineered organism according to claims 11-14 wherein said sucrose phosphorylase of step a) is replaced by a sucrose synthase, a maltose phosphorylase or a maltose synthase.

16. A metabolically engineered organism according to claims 11-14 wherein said sucrose phosphorylase of step a) is replaced by a lactose phosphorylase or a lactose synthase.

17. A metabolically engineered organism according to claim 5 wherein:

-said gene in step a) is encoding for a sucrose phosphorylase, and/or

-the following genes in step b) are rendered non-functional: a gene encoding for a beta-galactosidase, and/or, a gene encoding for a glucose-6-phosphate isomerase, and/or a gene encoding for a glucose-1-phosphate adenylyltransferase, and/or, a gene encoding for a glucose-1-phosphatase, and/or, a gene encoding for a phosphofructokinase A, and/or, a gene encoding for a phosphofructokinase B, and/or, a gene encoding for phosphogluconate dehydratase, and/or, a gene encoding for 2-keto-3-deoxygluconate-6-phosphate aldolase, and/or, a gene encoding for a glucose-1-phosphate uridyltransferase, and/or, a gene encoding for an UDP-glucose-4-epimerase, and/or, a gene encoding for an UDP-glucose:galactose-1-phosphate uridyltransferase, and/or, a gene encoding for an UDP-galactopyranose mutase, and/or, a gene encoding for an UDP-galactose:(glucosyl)lipopolysaccharide-1,6-galactosyltransferase, and/or, a gene encoding for an UDP-galactosyltransferase, and/or, a gene encoding for an UDP-glucosyltransferase, and/or, a gene encoding for an UDP-glucuronate transferase, and/or, a gene encoding for an UDP-glucose lipid carrier transferase, and/or, a gene encoding for a GDP-mannose hydrolase, and/or, a gene encoding for an UDP-sugar hydrolase, and/or, a gene encoding for a mannose-6-phosphate isomerase, and/or, a gene encoding for an UDP-N-acetylglucosamine enoylpyruvoyl transferase, and/or, a gene encoding for an UDP-N-acetylglucosamine acetyltransferase, and/or, a gene encoding for an UDP-N-acetylglucosamine-2-epimerase, and/or, a gene encoding for an undecaprenyl-phosphate- α -N-acetylglucosaminyl transferase, and/or, a gene encoding for a glucose-6-phosphate-1-dehydrogenase, and/or, a gene encoding for a L-glutamine:D-fructose-6-phosphate aminotransferase, and/or, a gene encoding for a mannose-6-phosphate isomerase, and/or a gene encoding for a sorbitol-6-phosphate dehydrogenase, and/or, a gene encoding for a

mannitol-1-phosphate 5-dehydrogenase, and/or a gene encoding for a allulose-6-phosphate 3-epimerase, and/or, a gene encoding for an invertase, and/or, a gene in coding for a maltase, and/or, and/or a gene encoding for a trehalase, and/or, a gene encoding for a sugar transporting phosphotransferase, and/or, a gene encoding for a hexokinase.

5 18. A metabolically engineered organism according to claim 17 wherein:

- said gene in step a) is a gene encoding for a sucrose phosphorylase

- said genes in step b) are: the gene *lacZ*, the gene *pgi*, the gene *glgC*, the gene *agp*, the gene *pfkA*, the gene *pfkB*, the gene *waaB*, the gene *ushA*, the gene *eda*, the gene *edd*, the gene *wcaA*, the gene *wcaC*, the gene *wcaE*, the gene *wcaI*, the gene *wcaL*, the gene *wcaJ*,
10 the gene *wcaB*, the gene *wcaF*, the gene *wcaK*, the gene *wcaD*, the gene *galU*, the gene *galF*, the gene *galE*, the gene *gmm*, the gene *galT*, the gene *manA*, the gene *murA*, the gene *lpxA*, the gene *rffE*, the gene *rfe*, the gene *glmS*, the gene *srlD*, the gene *mtlD*, the gene *alsE* and/or, *zwf*.

19. A metabolically engineered organism according to claim 18 wherein:

15 -said gene in step a) is a gene encoding for a sucrose phosphorylase

-said genes in step b) are: the gene *pgi1*, the gene *PFK1*, the gene *PFK2*, the gene *PFK26*, the gene *PFK26*, the gene *PFK27*, the gene *GND1*, the gene *GND2*, the gene *PMI40*, the gene *ZWF1*, the gene *GFA1*, the gene *GLG1*, the gene *GLG2*, the gene *INM1*, the gene *INM2*, the gene *GLK1*, the gene *HXK1*, the gene *HXK2*, the gene *GAL10*, the gene *GAL7*, the
20 gene *YHL012W*, the gene *UGP1*, the gene *GSY1*, the gene *GSY2*, the gene *DIE2*, the gene *ALG8*, the gene *ATG26*, the gene *SUC2*, the gene *MAL32*, the gene *MAL12*, the gene *YJL216C*, and/or, the gene *YGR287C*, and/or, *FEN1*, and/or, *FKS1*, and/or, *GSC2*, and/or, *TPS1*.

20. A metabolically engineered organism according to claim 18-19 wherein said sucrose
25 phosphorylase originates from a Lactic acid bacterium and wherein said Lactic acid bacterium is *Bifidobacterium adolescentis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*, or *Streptococcus mutants*.

21. A metabolically engineered organism according to claims 2-20 wherein the gene which converts said activated saccharide into a specialty product codes for an epimerase, a transferase, a reductase, a (pyro)phosphorylase, a (de)carboxylase, a dehydratase, a permease, a synthase, a dehydrogenase, an oxidase or an isomerase.
- 5 22. A metabolically engineered organism according to claim 21 wherein said epimerase is UDP-galactose-4-epimerase or UDP-N-acetylglucosamine epimerase, and/or, wherein said transferase is a glycosyltransferase, a fucosyltransferase, a sialyltransferase or a sulfotransferase.
- 10 23. A metabolically engineered organism according to claims 1-22 wherein said specialty product is a monosaccharide, a disaccharide, a trisaccharide, a tetrasaccharide, a pentasaccharide, a polysaccharide, a nucleoside, an O-glycoside, an S-glycoside, an N-glycoside, a C-glycoside, a glycoprotein, a glycolipid or an activated carbohydrate.
24. A method to produce a specialty product according to any of claims 1-23 comprising:
- i) metabolically engineering a microorganism according to in any of claims 1-23, and
- 15 ii) cultivating said genetically engineered microorganism, and
- iii) extracting and purifying said specialty product.
25. Use of a 2-fucosyltransferase originating from *Dictyostellium discoideum* having an amino acid sequence given by SEQ ID N° 1, or, a fragment thereof having 2-fucosyltransferase activity, or, a variant thereof having a sequence identity of at least 75 % and having 2-fucosyltransferase activity
- 20 to produce fucosyllactose.
26. Use according to claim 25 wherein said fragment having 2-fucosyltransferase activity has an amino acid sequence as given by SEQ ID N° 4.
27. Use of a nucleic acid encoding for a 2-fucosyltransferase according to any of claim 25 and 26 to produce fucosyllactose.
- 25 28. Use according to claim 27 wherein said nucleic acids have a nucleic acid sequence as given by SEQ ID N° 2, SEQ ID N° 3, SEQ ID N° 5 or SEQ ID N° 6.