(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 December 2015 (03.12.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/184327 A1

(51) International Patent Classification:
C12P 19/18 (2006.01) *C12N 1/26* (2006.01)
C12P 19/12 (2006.01) *C12P 19/00* (2006.01)

(21) International Application Number:
PCT/US2015/033286

(22) International Filing Date:
29 May 2015 (29.05.2015)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/005,603 30 May 2014 (30.05.2014) US

(71) Applicant: **BRASKEM S.A.** [BR/BR]; Rua Eteno 1561, Complexo Petroquímico de Camaçari, 42810-000 Camaçari, BA (BR).

(72) Inventor; and
(71) Applicant *(for US only)*: **SLOVIC, Avram Michael** [US/BR]; c/o Rua Eteno 1561, Complexo, Petroquímico de Camaçari, 42810-000 Camaçari, BA (BR).

(72) Inventor: **KOCH, Daniel Johannes**; c/o Rua Eteno 1561, Complexo, Petroquímico de Camaçari, 42810-000 Camaçari, BA (BR).

(74) Agents: **BETTI, Christopher J.** et al.; K&L GATES LLP, P.O. Box 1135, Chicago, Illinois 60690-1135 (US).

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

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

Declarations under Rule 4.17:

— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:

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

— *with sequence listing part of description (Rule 5.2(a))*

(54) **Title**: MODIFIED MICROORGANISMS COMPRISING AN OPTIMIZED SYSTEM FOR OLIGOSACCHARIDE UTILIZATION AND METHODS OF USING SAME

(57) **Abstract**: The present disclosure generally relates to modified microorganisms comprising an optimized system for oligosaccharide utilization comprising one or more polynucleotides coding for one or more energy independent oligosaccharide transporters for transporting an oligosaccharide into the microorganism, one or more polynucleotides coding for enzymes that catalyze the conversion of the oligosaccharide into at least one phosphorylated saccharide, and one or more polynucleotides coding for enzymes that catalyze the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide that is utilized in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, etc. The present disclosure also generally relates to methods of using the optimized system for oligosaccharide utilization.

WO 2015/184327 A1

TITLE

MODIFIED MICROORGANISMS COMPRISING AN OPTIMIZED SYSTEM FOR OLIGOSACCHARIDE UTILIZATION AND METHODS OF USING SAME

FIELD

[0001] The present disclosure generally relates to modified microorganisms (*e.g.*, non-naturally occurring microorganisms) that comprise one or more polynucleotides coding for proteins and enzymes in a pathway that optimizes the microorganism's utilization of oligosaccharides.

BACKGROUND

[0002] For many years, the chemical industry has been using coal, gas, and oil to produce the vast majority of its industrial products. However, with diminishing supplies of these resources and the looming dangers of excessive carbon dioxide emissions, there is a dire need to develop sustainable and renewable chemicals that can produce the same products in a safe and cost effective way.

[0003] To address the need to develop sustainable and renewal chemical resources, companies have begun using genetically modified microorganisms to convert carbon sources to chemicals, such as terpenes, that can be used to produce industrial products. Typically, this conversion is carried out by fermentation of the carbon source under anaerobic conditions. Use of this process provides a sustainable, renewable source of chemicals for use in industrial applications.

[0004] Oligosaccharides are frequently used as a carbon source or feedstock to fuel the fermentation or other metabolic process used to produce the desired chemicals. However, the efficiency of the chemical producing process is limited by the genetically modified microorganisms' ability to efficiently utilize the carbon source provided.

[0005] All known natural and currently employed heterologous oligosaccharide utilization systems lose energy during transport of the oligosaccharide into the microorganism or during cleavage of the oligosaccharide. This loss of energy reduces the efficiency of the microorganisms' production of chemicals, which in turn increases the cost and time of production.

[0006] Given the demand for sustainable, renewable sources of chemicals and the resulting increased use of genetically modified microorganisms to produce industrially useful chemicals, more efficient microorganisms are needed. In particular, methods for optimizing

microorganisms' utilization of oligosaccharides to reduce the time and expense of producing chemicals is desired, particularly in anaerobic environments.

SUMMARY

[0007] The present disclosure generally relates to microorganisms (e.g., non-naturally occurring microorganisms) having an optimized system (e.g., a genetically modified pathway for increasing microorganisms' adenosine triphosphate (ATP) production from utilization (e.g. metabolism) of oligosaccharides when compared to microorganisms lacking this genetically modified pathway) for oligosaccharide utilization that comprise one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting an oligosaccharide into the microorganism; one or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the oligosaccharide into at least one phosphorylated saccharide; and one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism. Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0008] The present disclosure also provides a method of optimizing utilization of an oligosaccharide in a microorganism that comprises providing an oligosaccharide source; expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide;

expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism; and contacting the oligosaccharide source with the microorganism. Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out. In some embodiments of each or any of the above or below mentioned embodiments, the oligosaccharide source is sugar cane juice. In some embodiments of each or any of the above or below mentioned embodiments the oligosaccharide source is sucrose. In some embodiments of each or any of the above or below mentioned embodiments the oligosaccharide source is maltose. In some embodiments of each or any of the above or below mentioned embodiments the oligosaccharide source is cellobiose. In some embodiments of each or any of the above or below mentioned embodiments, the oligosaccharide source is selected from the group consisting of: lactose, lactulose, isomaltose, melibiose, and trehalose.

[0009] In some embodiments of each or any of the above or below mentioned embodiments, the microorganism is a bacteria selected from the genera consisting essentially of: Propionibacterium, Propionispira, Clostridium, Bacillus, Escherichia, Pelobacter, or Lactobacillus.

[0010] In some embodiments of each or any of the above or below mentioned embodiments, the microorganism is a eukaryote selected from the group consisting essentially of a yeast, filamentous fungi, protozoa, or algae.

[0011] In some embodiments of each or any of the above or below mentioned embodiments, the microorganism is from a genus selected from the group consisting of: Saccharomyces, Yarrowia, Hansenula, Pichia, Ashbya, and Candida.

[0012] In some embodiments of each or any of the above or below mentioned embodiments, the energy independent oligosaccharide transporter is from a class selected from the group consisting of: sugars will eventually be exported transporter (SWEET) proteins, sucrose binding proteins (SBP), sucrose uptake facilitators (SUF), cellodextrin facilitators and aquaporins.

[0013] In some embodiments of each or any of the above or below mentioned embodiments, the polynucleotides encoding the phosphorylase are genes selected from the group consisting of: sucrose phosphorylase genes, such as spl of *Bifidobacterium adolescent* and sucP (742sp) of *Leuconostoc mesenteroides*; and maltose phosphorylase genes, such as LVIS_0358 of *Lactobacillus brevis* and *mapA* of *Lactobacillus sanfranciscensis*; cellobiose phosphorylase genes, such as cbp from *Clostridium thermocellum.*.

[0014] In some embodiments of each or any of the above or below mentioned embodiments, the phosphoglucomutase is selected from the group consisting of: α-phosphoglucomutase and β-phosphoglucomutase. In some embodiments of each or any of the above or below mentioned embodiments, the phosphoglucomutase is selected from the group consisting of: *pgm1* and *pgm2* from *Saccharomyces cerevisiae*, *pgmA* from *Lactobacillus sanfrancisco*, and *pgmB* from *Lactococcus lactis*.

[0015] In some embodiments of each or any of the above or below mentioned embodiments, the isomer is converted to pyruvate via glycolysis.

[0016] In some embodiments of each or any of the above or below mentioned embodiments, the isomer is utilized in an anaerobic metabolic pathway.

[0017] In some embodiments of each or any of the above or below mentioned embodiments, the utilization of the isomer yields an increased amount of adenosine triphosphate (ATP).

[0018] In some embodiments of each or any of the above or below mentioned embodiments, the isomer of the phosphorylated saccharide is utilized in a pathway for the production of an organic molecule.

[0019] In some embodiments of each or any of the above or below mentioned embodiments, the utilization being optimized is the fermentation of di-saccharides. In some embodiments of each or any of the above or below mentioned embodiments, the ATP production resulting from di-saccharide fermentation is increased by at least 25% at 100% efficiency of the optimized oligo-saccharide uptake system. In some embodiments of each or any of the above or

below mentioned embodiments, the increased yield of ATP results in an increased yield of organic molecules produced as a result of the microorganism's metabolism.

[0020] In some embodiments of each or any of the above or below mentioned embodiments, the oligosaccharide source is contacted with the microorganism prior to expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide; and expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism.

[0021] In some embodiments of each or any of the above or below mentioned embodiments, the isomer of the phosphorylated saccharide is utilized in a pathway for the production of an organic molecule.

[0022] The present disclosure also provides a microorganism having an optimized system for oligosaccharide utilization comprising one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting an oligosaccharide into the microorganism; one or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the oligosaccharide into at least one phosphorylated saccharide; and one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0023] The present disclosure also provides a method of optimizing utilization of sucrose in a microorganism comprising: providing sucrose; expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the sucrose into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the sucrose into glucose-1-phosphate and fructose; expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism; and contacting the sucrose with the microorganism. Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0024] The present disclosure also provides a microorganism having an optimized system for sucrose utilization comprising one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting sucrose into the microorganism; one or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the sucrose into glucose-1-phosphate and fructose; and one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be

knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0025] The present disclosure also provides a method of optimizing utilization of maltose in a microorganism comprising: providing maltose; expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the maltose into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the maltose into glucose-1-phosphate and glucose; expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism; and contacting the maltose with the microorganism. Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0026] The present disclosure also provides a microorganism having an optimized system for maltose utilization comprising one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting maltose into the microorganism; one or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the maltose into glucose-1-phosphate and glucose; and one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism. Optionally, the microorganism may naturally lack or may be obtained that lacks

one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0027] The present disclosure also provides a method optimizing utilization of cellobiose in a microorganism comprising: providing cellobiose; expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the cellobiose into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the cellobiose into glucose-1-phosphate and glucose; expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism; and contacting the cellobiose with the microorganism. Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0028] The present disclosure also provides a microorganism having an optimized system for cellobiose utilization comprising one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting cellobiose into the microorganism; one

or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the cellobiose into glucose-1-phosphate and glucose; and one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0029] The present disclosure also provides a method of optimizing utilization of an oligosaccharide in a microorganism to produce an organic molecule comprising: providing an oligosaccharide source; expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide; expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into a an isomer of the phosphorylated saccharide; expressing one or more polynucleotides in the microorganism for catalyzing a conversion of the isomer into an organic molecule; and contacting the oligosaccharide source with the microorganism. Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example,

for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0030] In some embodiments of each or any of the above or below mentioned embodiments, the organic molecule is selected from the group consisting of: acid, alcohol, alkane, alkene, amide, amine, amino acid, aromatic, carbohydrate, diacid, dialcohol, diene, ester (incl. waxes), ether, fat (incl. oils), fatty acid, fatty alcohol, ketone, lactam, peptide, protein, steroid, terpene, vitamin.

[0031] In some embodiments of each or any of the above or below mentioned embodiments, the organic molecule is selected from the group consisting of: acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, methacrylic acid; ethanol, isopropanol, 1-propanol, 2-propanol, n-butanol, isobutanol; hexene, propene; hexamethylenediamine; adipic acid, glucaric acid, itaconic acid, malonic acid, succinic acid; 1,2-ethandiol (ethylene glycol), butanediol, 1,4-butanediol, 1,2-propanediol (monopropylen glycol), 1,3-propanediol; butadiene; methyl methacrylate; caprolactam; isoprene, farnesene.

[0032] The present disclosure also provides a method of increasing the yield of an organic molecule produced by a microorganism under anaerobic conditions comprising: providing an oligosaccharide source for use by the microorganism; expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phsophorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide; expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into an isomer of the phosphorylated sacchardie; expressing one or more polynucleotides in the microorganism for catalyzing a conversion of the isomer into an organic molecule; and contacting the oligosaccharide source with the microorganism.

BRIEF DESCRIPTION OF THE DRAWINGS

[0033] The foregoing summary, as well as the following detailed description of the disclosure, will be better understood when read in conjunction with the appended figures. For the purpose of illustrating the disclosure, shown in the figures are embodiments that are presently preferred. It should be understood, however, that the disclosure is not limited to the precise arrangements, examples and instrumentalities shown.

[0034] Figure 1 depicts an exemplary oligosaccharide utilization system wherein the oligosaccharide being utilized is sucrose.

[0035] Figure 2. Growth profile of strains BRKY30, BRKY427 and BRKY448 with sucrose as sole carbon source.

[0036] Figure 3. Growth profile of strains BRKY474 and BRKY475 with sucrose as sole carbon source.

[0037] Figure 4. Growth kinetics for six uniporter candidates in anaerobic flasks (2% sucrose).

DETAILED DESCRIPTION

[0038] The present disclosure generally relates to microorganisms (*e.g.*, non-naturally occurring microorganisms) that comprise a genetically modified pathway for optimizing the microorganisms' utilization of oligosaccharides (e.g., an oligosaccharide utilization system) (*see*, Figure 1). Such microorganisms can comprise one or more polynucleotides coding for one or more energy independent oligosaccharide transporters for transporting an oligosaccharide into the microorganism. Optionally, the microorganism can be further modified to comprise one or more polynucleotides coding for enzymes (e.g., phosphorylase) that catalyze the conversion (e.g., phosphorolysis) of the oligosaccharide into at least one phosphorylated saccharide, and one or more polynucleotides coding for enzymes (e.g., phosphoglucomutase) that catalyze the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized (e.g., metabolized) in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as a acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, etc. The microorganism can be genetically modified to include polynucleotides coding for one or more energy independent oligosaccharide transporters, either in the presence of or in the absence of naturally occurring energy independent oligosaccharide transporters. Similarly, the microorganism can also be genetically modified to include polynucleotides coding for phosphorylases and/or phosphoglucomutases in addition to an oligosaccharide transporter, either in the presence or absence of naturally occuring phosphorylases and phosphoglucomutases.

[0039] Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the

microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0040] In an embodiment, the microorganism comprises one or more polynucleotides coding for one or more energy independent oligosaccharide transporters for transporting an oligosaccharide into the microorganism, one or more polynucleotides coding for enzymes that catalyze the conversion of the oligosaccharide into at least one phosphorylated saccharide, and one or more polynucleotides coding for enzymes that catalyze the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, etc.

[0041] In a preferred embodiment, the microorganism comprises one or more polynucleotides coding for a plant energy independent oligosaccharide transporter for transporting an oligosaccharide into the microorganism, and one or more polynucleotides coding for a bacterial phosphorylase for catalyzing the conversion of the oligosaccharide into at least one phosphorylated saccharide, wherein the phosphorylated saccharide is utilized in one or more naturally occurring or genetically modified enzymatic pathways in the microorganism for production of an organic molecule.

[0042] In some embodiments, the microorganism is genetically modified to comprise one or more polynucleotides coding for one or more energy independent oligosaccharide transporters, one or more polynucleotides coding for enzymes that catalyze the conversion of the oligosaccharide into at least one phosphorylated saccharide, and one or more polynucleotides coding for enzymes that catalyze the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, etc. In other embodiments, one or more polynucleotides coding for one or more energy independent oligosaccharide transporters, enzymes that catalyze the conversion of the oligosaccharide into at least one phosphorylated saccharide, and enzymes that catalyze the conversion of the

phosphorylated saccharide into an isomer can be naturally occurring in the microorganism. In some embodiments, the microorganism can contain or be genetically modified to include and also naturally contain any combination of the above-mentioned polynucleotides. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0043] In another embodiment, the microorganism comprises one or more polynucleotides coding for one or more energy independent oligosaccharide transporters for transporting sucrose into the microorganism, one or more polynucleotides coding for a phosphorylase that catalyzes the conversion of the sucrose into glucose-1-phosphate and fructose, one or more polynucleotides coding for a phosphoglucomutase that catalyzes the conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, etc. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0044] In another embodiment, the microorganism comprises one or more polynucleotides coding for one or more energy independent oligosaccharide transporters for transporting maltose into the microorganism, one or more polynucleotides coding for a phosphorylase that catalyzes the conversion of the maltose into glucose-1-phosphate and glucose,

one or more polynucleotides coding for a phosphoglucomutase that catalyzes the conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, etc. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0045] In another embodiment, the microorganism comprises one or more polynucleotides coding for one or more energy independent oligosaccharide transporters for transporting cellobiose into the microorganism, one or more polynucleotides coding for a phosphorylase that catalyzes the conversion of the cellobiose into glucose-1-phosphate and glucose, one or more polynucleotides coding for a phosphoglucomutase that catalyzes the conversion of the glucose-1-phosphate into glucose-6-phosphate, wherein the glucose-6-phosphate is utilized in one or more enzymatic pathways in the microorganism for the production of an organic molecule such as acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid etc. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0046] This disclosure provides, in part, the discovery of a novel system for optimized oligosaccharide utilization that does not require the use of energy during oligosaccharide uptake. Such a system has not previously been used to optimize oligosaccharide utilization.

[0047] The use of energy independent, gradient driven oligosaccharide transporters, also referred to as uniporters, in the oligosaccharide utilization system conserves energy during oligosaccharide uptake by the microorganism by not requiring the use of adenosine triphosphate (ATP). Most known oligosaccharide transporters, such as ATP-binding cassette (ABC) type transporters, phosphotransferase (PTS) based importers (PEP based importers), or proton symporters, require the use of ATP for oligosaccharide uptake.

[0048] No currently known energy independent oligosaccharide transporters of microbial origin have been identified, with the exception of the eukaryotic (yeast) cellodextrin facilitators, and none have been used to optimize oligosaccharide utilization in microorganisms. However, in plants oligosaccharide transporters have been identified that are non-PTS, do not require the use of ATP or guanosine triphosphate (GTP), are pH independent, and are not inhibited by protonophores (e.g. proton gradient uncoupling chemicals, such as dinitrophenol (DNP)). Classes of these known oligosaccharide uniporters include sugars will eventually be exported transporter (SWEET) proteins, sucrose binding proteins (SBP), sucrose uptake facilitators (SUF). Additionally, aquaporins are suspected as being potential oligosaccharide uniporters.

[0049] Although the above-mentioned proteins have been identified as uniporters or potential uniporters, none have been used in an oligosaccharide utilization system with the intent of optimizing energy conversation during oligosaccharide utilization.

[0050] Optionally, a phosphorylase can be used as part of the disclosed oligosaccharide utilization system. The phosphorylase catalyzes a conversion of the oligosaccharide (e.g., phosphorolysis) that cleaves the oligosaccharide, and during said cleavage transfers an orthophosphate ion to one of the resulting mono-saccharides. The energy in the oligosaccharide's glycolytic bond is used to create the mono-saccharide-phosphate, which conserves energy (e.g., ATP). For example, the use of a phosphorylase on a di-saccharide yields one phosphorylated mono-saccharide and one non-phosphorylated mono-saccharide, saving one ATP per di-saccharide. The phosphorylated saccharide is then utilized in one or more enzymatic pathways in the microorganism, such as glycolysis or the pentose-phosphate pathway.

[0051] The majority of known microorganisms use hydrolases to break down oligosaccharides, not phosphorylases. However, some Lactobacilli reportedly use a phosphorylase to utilize the energy potential in the oligosaccharide's glycolytic bond. Yet, the known Lactobacillus sucrose transporters all require the use of ATP, including an ATP driven ABC type importer (MsmEFGK) and a PTS energy dependent transporter (Pts1BCA). Thus, the use of a phosphorylase in the disclosed oligosaccharide utilization system was not previously known.

[0052] Optionally, a phosphoglucomutase can be used as part of the disclosed oligosaccharide utilization system. The phosphoglucomutase catalyzes a conversion of the mono-saccharide-phosphate (e.g. inverts) produced by the phosphorylase described above into a different isomer that is utilized in one or more enzymatic pathways in the microorganism (e.g., glycolysis). The type of oligosaccharide used impacts the isoform of the mono-saccharide-phosphate produced during phosphorolysis, which could require different phosphoglucomutases.

[0053] For example, phosphorylation of sucrose or maltose yields D-glucose and α-D-glucose-1-phosphate or β-D-glucose-1-phosphate, respectively. In this case either an α-phosphoglucomutase or a β-phosphoglucomutase is required. In another example, phosphorylation of cellobiose yields α-D-glucose-1-phosphate and D-glucose. In this case an α-phosphoglucomutase is required.

[0054] As mentioned above, the compound produced by the phosphoglucomutase is utilized in one or more enzymatic pathways in the microorganism, such as glycolysis. Additionally, the compound can be used in any other genetically modified pathway to produce industrially useful chemicals, such as a terpene. Thus, the use of the oligosaccharide utilization system allows for the more efficient production of any chemical the microorganism has been genetically modified to produce.

[0055] In an embodiment, the disclosed oligosaccharide utilization system is used under anaerobic conditions. In another embodiment, the compound produced by either the phosphorylase or the phosphoglucomutase is utilized in an anaerobic enzymatic pathway to produce an organic molecule.

[0056] As used herein, optimizing utilization of an oligosaccharide refers to establishing energy independent oligo-saccharide transport in a genetically modified microorganism, coupled with energy conserving phosphorolysis and isomerization of the transported sugar, optionally coupled with deletion of intrinsic competing/energy dependent oligo-saccharide transport and/or energy wasting oligo-saccharide hydrolysis. Such optimization of oligosaccharide utilization in a microorganism may result in at least a 25%, 50%, 75%, 100%, or greater increase in adenosine triphosphate (ATP) production from fermentation of the oligosaccharide as compared to a microorganism in which utilization of the oligosaccharide has not been optimized.

[0057] As used herein, a system for optimized oligosaccharide utilization refers to a non-naturally occurring system comprising an energy independent oligo-saccharide transporter, an oligo-saccharide phosphorylase, and a phosphoglucomutase. Such optimized oligosaccharide utilization in a microorganism may result in at least a 25%, 50%, 75%, 100%, or greater increase in adenosine triphosphate (ATP) production from fermentation of the oligosaccharide as

compared to a microorganism in which utilization of the oligosaccharide has not been optimized.

[0058] As used herein, the term "acceptor" includes but is not limited to NAD+ or NADP+ or quinone, or oxidized cytochrome c. Additionally, as used herein the term "reduced acceptor" includes but is not limited to NADH or NADPH or quinol or reduced cytochrome c.

[0059] As used herein, the term "biological activity" or "functional activity," when referring to a protein, polypeptide or peptide, may mean that the protein, polypeptide or peptide exhibits a functionality or property that is useful as relating to some biological process, pathway or reaction. Biological or functional activity can refer to, for example, an ability to interact or associate with (*e.g.*, bind to) another polypeptide or molecule, or it can refer to an ability to catalyze or regulate the interaction of other proteins or molecules (*e.g.*, enzymatic reactions).

[0060] As used herein, the term "culturing" may refer to growing a population of cells, *e.g.*, microbial cells, under suitable conditions for growth, in a liquid or on solid medium.

[0061] As used herein, the term "derived from" may encompass the terms originated from, obtained from, obtainable from, isolated from, and created from, and generally indicates that one specified material finds its origin in another specified material or has features that can be described with reference to the another specified material.

[0062] As used herein, "exogenous polynucleotide" refers to any deoxyribonucleic acid that originates outside of the microorganism.

[0063] As used herein, the term "expression vector" may refer to a DNA construct containing a polynucleotide or nucleic acid sequence encoding a polypeptide or protein, such as a DNA coding sequence (*e.g.*, gene sequence) that is operably linked to one or more suitable control sequence(s) capable of affecting expression of the coding sequence in a host. Such control sequences include a promoter to affect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome binding sites, and sequences which control termination of transcription and translation. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome (*e.g.*, independent vector or plasmid), or may, in some instances, integrate into the genome itself (*e.g.*, integrated vector). The plasmid is the most commonly used form of expression vector. However, the disclosure is intended to include such other forms of expression vectors that serve equivalent functions and which are, or become, known in the art.

[0064] As used herein, the term "expression" may refer to the process by which a polypeptide is produced based on a nucleic acid sequence encoding the polypeptides (*e.g.*, a gene). The process includes both transcription and translation.

[0065] As used herein, the term “gene” may refer to a DNA segment that is involved in producing a polypeptide or protein (*e.g.*, fusion protein) and includes regions preceding and following the coding regions as well as intervening sequences (introns) between individual coding segments (exons).

[0066] As used herein, the term “heterologous,” with reference to a nucleic acid, polynucleotide, protein or peptide, may refer to a nucleic acid, polynucleotide, protein or peptide that does not naturally occur in a specified cell, *e.g.*, a host cell. It is intended that the term encompass proteins that are encoded by naturally occurring genes, mutated genes, and/or synthetic genes. In contrast, the term homologous, with reference to a nucleic acid, polynucleotide, protein or peptide, refers to a nucleic acid, polynucleotide, protein or peptide that occurs naturally in the cell.

[0067] As used herein, the term a “host cell” may refer to a cell or cell line, including a cell such as a microorganism which a recombinant expression vector may be transfected for expression of a polypeptide or protein (*e.g.*, fusion protein). Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total genomic DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. A host cell may include cells transfected or transformed in vivo with an expression vector.

[0068] As used herein, the term “introduced,” in the context of inserting a nucleic acid sequence or a polynucleotide sequence into a cell, may include transfection, transformation, or transduction and refers to the incorporation of a nucleic acid sequence or polynucleotide sequence into a eukaryotic or prokaryotic cell wherein the nucleic acid sequence or polynucleotide sequence may be incorporated into the genome of the cell (*e.g.*, chromosome, plasmid, plastid, or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed.

[0069] As used herein, the term "non-naturally occurring" when used in reference to a microbial organism or microorganism of the invention is intended to mean that the microbial organism has at least one genetic alteration not normally found in a naturally occurring strain of the referenced species, including wild-type strains of the referenced species. Genetic alterations include, for example, modifications introducing expressible nucleic acids encoding metabolic polypeptides, other nucleic acid additions, nucleic acid deletions and/or other functional disruption of the microbial organism's genetic material. Such modifications include, for example, coding regions and functional fragments thereof, for heterologous, homologous or both heterologous and homologous polypeptides for the referenced species. Additional modifications include, for example, non-coding regulatory regions in which the modifications alter expression

of a gene or operon. A non-naturally occurring microbial organism of the disclosure can contain stable genetic alterations, which refers to microorganisms that can be cultured for greater than five generations without loss of the alteration. Generally, stable genetic alterations include modifications that persist greater than 10 generations, particularly stable modifications will persist more than about 25 generations, and more particularly, stable genetic modifications will be greater than 50 generations, including indefinitely. Those skilled in the art will understand that the genetic alterations, including metabolic modifications exemplified herein, are described with reference to a suitable host organism such as *E. coli* and their corresponding metabolic reactions or a suitable source organism for desired genetic material such as genes for a desired metabolic pathway. However, given the complete genome sequencing of a wide variety of organisms and the high level of skill in the area of genomics, those skilled in the art will readily be able to apply the teachings and guidance provided herein to essentially all other organisms. For example, the *E. coli* metabolic alterations exemplified herein can readily be applied to other species by incorporating the same or analogous encoding nucleic acid from species other than the referenced species. Such genetic alterations include, for example, genetic alterations of species homologs, in general, and in particular, orthologs, paralogs or nonorthologous gene displacements.

[0070] As used herein, the term "operably linked" may refer to a juxtaposition or arrangement of specified elements that allows them to perform in concert to bring about an effect. For example, a promoter may be operably linked to a coding sequence if it controls the transcription of the coding sequence.

[0071] As used herein, the term "a promoter" may refer to a regulatory sequence that is involved in binding RNA polymerase to initiate transcription of a gene. A promoter may be an inducible promoter or a constitutive promoter. An inducible promoter is a promoter that is active under environmental or developmental regulatory conditions.

[0072] As used herein, the term "a polynucleotide" or "nucleic acid sequence" may refer to a polymeric form of nucleotides of any length and any three-dimensional structure and single- or multi-stranded (*e.g.*, single-stranded, double-stranded, triple-helical, etc.), which contain deoxyribonucleotides, ribonucleotides, and/or analogs or modified forms of deoxyribonucleotides or ribonucleotides, including modified nucleotides or bases or their analogs. Such polynucleiotides or nucleic acid sequences may encode amino acids (*e.g.*, polypeptides or proteins such as fusion proteins). Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and the present disclosure encompasses polynucleotides which encode a particular amino acid sequence. Any type of modified nucleotide or nucleotide analog may be used, so long as the polynucleotide retains the desired functionality under conditions of use, including modifications that increase nuclease resistance (*e.g.*, deoxy, 2'-

O-Me, phosphorothioates, etc.). Labels may also be incorporated for purposes of detection or capture, for example, radioactive or nonradioactive labels or anchors, *e.g.*, biotin. The term polynucleotide also includes peptide nucleic acids (PNA). Polynucleotides may be naturally occurring or non-naturally occurring. The terms polynucleotide, nucleic acid, and oligonucleotide are used herein interchangeably. Polynucleotides may contain RNA, DNA, or both, and/or modified forms and/or analogs thereof. A sequence of nucleotides may be interrupted by non-nucleotide components. One or more phosphodiester linkages may be replaced by alternative linking groups. These alternative linking groups include, but are not limited to, embodiments wherein phosphate is replaced by P(O)S (thioate), P(S)S (dithioate), (O)NR_2 (amidate), P(O)R, P(O)OR', $COCH_2$ (formacetal), in which each R or R' is independently H or substituted or unsubstituted alkyl (1-20 C) optionally containing an ether (–O–) linkage, aryl, alkenyl, cycloalkyl, cycloalkenyl or araldyl. Not all linkages in a polynucleotide need be identical. Polynucleotides may be linear or circular or comprise a combination of linear and circular portions.

[0073] As used herein, the term a "protein" or "polypeptide" may refer to a composition comprised of amino acids and recognized as a protein by those of skill in the art. The conventional one-letter or three-letter code for amino acid residues is used herein. The terms protein and polypeptide are used interchangeably herein to refer to polymers of amino acids of any length, including those comprising linked (*e.g.*, fused) peptides/polypeptides (*e.g.*, fusion proteins). The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, *etc.*), as well as other modifications known in the art.

[0074] As used herein, related proteins, polypeptides or peptides may encompass variant proteins, polypeptides or peptides. Variant proteins, polypeptides or peptides differ from a parent protein, polypeptide or peptide and/or from one another by a small number of amino acid residues. In some embodiments, the number of different amino acid residues is any of about 1, 2, 3, 4, 5, 10, 20, 25, 30, 35, 40, 45, or 50. In some embodiments, variants differ by about 1 to about 10 amino acids. Alternatively or additionally, variants may have a specified degree of sequence identity with a reference protein or nucleic acid, *e.g.*, as determined using a sequence alignment tool, such as BLAST, ALIGN, and CLUSTAL (see, infra). For example, variant proteins or nucleic acid may have at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%,

80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 99.5% amino acid sequence identity with a reference sequence.

[0075] As used herein, the term "recovered," "isolated," "purified," and "separated" may refer to a material (*e.g.*, a protein, peptide, nucleic acid, polynucleotide or cell) that is removed from at least one component with which it is naturally associated. For example, these terms may refer to a material which is substantially or essentially free from components which normally accompany it as found in its native state, such as, for example, an intact biological system.

[0076] As used herein, the term "recombinant" may refer to nucleic acid sequences or polynucleotides, polypeptides or proteins, and cells based thereon, that have been manipulated by man such that they are not the same as nucleic acids, polypeptides, and cells as found in nature. Recombinant may also refer to genetic material (*e.g.*, nucleic acid sequences or polynucleotides, the polypeptides or proteins they encode, and vectors and cells comprising such nucleic acid sequences or polynucleotides) that has been modified to alter its sequence or expression characteristics, such as by mutating the coding sequence to produce an altered polypeptide, fusing the coding sequence to that of another coding sequence or gene, placing a gene under the control of a different promoter, expressing a gene in a heterologous organism, expressing a gene at decreased or elevated levels, expressing a gene conditionally or constitutively in manners different from its natural expression profile, and the like.

[0077] As used herein, the term "selective marker" or "selectable marker" may refer to a gene capable of expression in a host cell that allows for ease of selection of those hosts containing an introduced nucleic acid sequence, polynucleotide or vector. Examples of selectable markers include but are not limited to antimicrobial substances (*e.g.*, hygromycin, bleomycin, or chloramphenicol) and/or genes that confer a metabolic advantage, such as a nutritional advantage, on the host cell.

[0078] As used herein, the term "substantially similar" and "substantially identical" in the context of at least two nucleic acids, polynucleotides, proteins or polypeptides may mean that a nucleic acid, polynucleotide, protein or polypeptide comprises a sequence that has at least about 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or even 99.5% sequence identity, in comparison with a reference (*e.g.*, wild-type) nucleic acid, polynucleotide, protein or polypeptide. Sequence identity may be determined using known programs such as BLAST, ALIGN, and CLUSTAL using standard parameters. (See, *e.g.*, Altshul*et al.* (1990) J. Mol. Biol. 215:403-410; Henikoff *et al.* (1989) Proc. Natl. Acad. Sci. 89:10915; Karin *et al.* (1993) Proc. Natl. Acad. Sci. 90:5873; and Higgins *et al.* (1988) Gene 73:237). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology

Information. Also, databases may be searched using FASTA (Person *et al.* (1988) Proc. Natl. Acad. Sci. 85:2444-2448.) In some embodiments, substantially identical polypeptides differ only by one or more conservative amino acid substitutions. In some embodiments, substantially identical polypeptides are immunologically cross-reactive. In some embodiments, substantially identical nucleic acid molecules hybridize to each other under stringent conditions (*e.g.*, within a range of medium to high stringency).

[0079] As used herein, the term "terpene" refers to a product having the formula $(C_5H_8)_n$. where n is 1 (i.e., isoprene), 2, 3, 4, 5, 6, 7, 8, 9, 10, or more. Terpenes may be classified by the number of isoprene units in the molecule; a prefix in the name indicates the number of terpene units needed to assemble the molecule.

- Hemiterpenes consist of *a single isoprene* unit. Isoprene itself is considered the only hemiterpene, but oxygen-containing derivatives such as prenol and isovalericacid arehemiterpenoids.
- Monoterpenes consist of *two isoprene* units and have the molecular formula $C_{10}H_{16}$. Examples of monoterpenes are: geraniol, limonene and terpeneol.
- Sesquiterpenes consist of *three isoprene* units and have the molecular formula $C_{15}H_{24}$. Examples of sesquiterpenes are: humulene, farnesenes, farnesol.
- Diterpenes are composed of *four isoprene* units and have the molecular formula $C_{20}H_{32}$. They derive from geranylgeranyl pyrophosphate. Examples of diterpenes are cafestol, kahweol, cembrene and taxadiene (precursor of taxol). Diterpenes also form the basis for biologically important compounds such as retinol, retinal, and phytol. They are known to be antimicrobial and antiinflammatory.
- Sesterterpenes, terpenes having 25 carbons and *five isoprene* units, are rare relative to the other sizes. (The *sester-* prefix means half to three, i.e. two and a half.) An example of a sesterterpene is geranylfarnesol.
- Triterpenes consist of *six isoprene* units and have the molecular formula $C_{30}H_{48}$. The linear triterpenesqualene, the major constituent of shark liver oil, is derived from the reductive coupling of two molecules of farnesyl pyrophosphate. Squalene is then processed biosynthetically to generate either lanosterol or cycloartenol, the structural precursors to all the steroids.
- Sesquarterpenes are composed of *seven isoprene* units and have the molecular formula $C_{35}H_{56}$. Sesquarterpenes are typically microbial in their origin. Examples of sesquarterpenes are ferrugicadiol and tetraprenylcurcumene.

- Tetraterpenes contain *eight isoprene* units and have the molecular formula $C_{40}H_{64}$. Biologically important tetraterpenes include the acyclic lycopene, the monocyclic gamma-carotene, and the bicyclic alpha- and beta-carotenes.
- Polyterpenes consist of long chains of *many isoprene* units. Natural rubber consists of polyisoprene in which the double bonds are cis. Some plants produce a polyisoprene with trans double bonds, known as gutta-percha.
- Norisoprenoids, such as the C_{13}-norisoprenoids 3-oxo-α-ionol present in Muscat of Alexandria leaves and 7,8-dihydroionone derivatives, such as megastigmane-3,9-diol and 3-oxo-7,8-dihydro-α-ionol found in Shiraz leaves (both grapes in the species *Vitisvinifera*) or wine (responsible for some of the spice notes in Chardonnay), can be produced by fungal peroxydase or glycosidases.

[0080] As used herein, the term "transfection" or "transformation" may refer to the insertion of an exogenous nucleic acid or polynucleotide into a host cell. The exogenous nucleic acid or polynucleotide may be maintained as a non-integrated vector, for example, a plasmid, or alternatively, may be integrated into the host cell genome. The term transfecting or transfection is intended to encompass all conventional techniques for introducing nucleic acid or polynucleotide into host cells. Examples of transfection techniques include, but are not limited to, calcium phosphate precipitation, DEAE-dextran-mediated transfection, lipofection, electroporation, and microinjection.

[0081] As used herein, the term "transformed," "stably transformed," and "transgenic" may refer to a cell that has a non-native (*e.g.*, heterologous) nucleic acid sequence or polynucleotide sequence integrated into its genome or as an episomal plasmid that is maintained through multiple generations.

[0082] As used herein, the term "vector" may refer to a polynucleotide sequence designed to introduce nucleic acids into one or more cell types. Vectors include cloning vectors, expression vectors, shuttle vectors, plasmids, phage particles, single and double stranded cassettes and the like.

[0083] As used herein, the term "wild-type," "native," or "naturally-occurring" proteins may refer to those proteins found in nature. The terms wild-type sequence refers to an amino acid or nucleic acid sequence that is found in nature or naturally occurring. In some embodiments, a wild-type sequence is the starting point of a protein engineering project, for example, production of variant proteins.

[0084] Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this disclosure belongs. Singleton, *et al.*, Dictionary of Microbiology and Molecular Biology, second

ed., John Wiley and Sons, New York (1994), and Hale & Markham, The Harper Collins Dictionary of Biology, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this disclosure.

[0085] Numeric ranges provided herein are inclusive of the numbers defining the range.

[0086] Unless otherwise indicated, nucleic acids sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively.

[0087] While the present disclosure is capable of being embodied in various forms, the description below of several embodiments is made with the understanding that the present disclosure is to be considered as an exemplification of the disclosure, and is not intended to limit the disclosure to the specific embodiments illustrated. Headings are provided for convenience only and are not to be construed to limit the disclosure in any manner. Embodiments illustrated under any heading may be combined with embodiments illustrated under any other heading.

[0088] The use of numerical values in the various quantitative values specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word "about." Also, the disclosure of ranges is intended as a continuous range including every value between the minimum and maximum values recited as well as any ranges that can be formed by such values. Also disclosed herein are any and all ratios (and ranges of any such ratios) that can be formed by dividing a disclosed numeric value into any other disclosed numeric value. Accordingly, the skilled person will appreciate that many such ratios, ranges, and ranges of ratios can be unambiguously derived from the numerical values presented herein and in all instances such ratios, ranges, and ranges of ratios represent various embodiments of the present disclosure.

Modification of Microorganism

[0089] A microorganism can be modified (*e.g.*, genetically engineered) by any method known in the art to comprise and/or express one or more polynucleotides coding for proteins in a system to optimize oligosaccharide utilization. Such proteins may include any of those proteins as are set forth in Tables 1-4. For example, the microorganism may be modified to comprise one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting oligosaccharides into the microorganism. Optionally, the microorganism may be further modified to comprise one or more polynucleotides coding for a phosphorylase and/or a phosphoglucomutase.

[0090] In an embodiment, a microorganism can be modified to comprise one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporter

oligosaccharides into the microorganism, one or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the oligosaccharide into at least one phosphorylated saccharide, and one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide that is utilized in one or more enzymatic pathways in the microorganism. Optionally, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[0091] Exemplary protein candidates for the disclosed optimized oligosaccharide utilization system and enzyme reaction products are presented in Table 1 below. Exemplary coding genes for some of the proteins are also presented in Table 1. The enzyme reference identifier listed in Table 1 correlates with the enzyme numbering used in Figure 1, which schematically represents the transport of sucrose into a microorganism and the subsequent enzymatic conversion of sucrose into glucose-6-phosphate, which is then utilized in one or more enzymatic pathways in the microorganism.

Table 1. Examples of protein candidates for optimized oligosaccharide utilization.

Ref.	Protein Family/Enzyme Name	E.C. number	Mediated Conversion	Examples	Citations
A	aquaporin	-	Potential sucrose uniport	*Arabidopsis thaliana* STP1	Chaudhuri *et al.* 2008
A	sucrose uptake facilitator (SUF)	-	sucrose uniport	*Pisum sativum* PsSUF1, PsSUF4	Zhou *et al.* 2007
A	sucrose binding protein (SBP)	-	sucrose/ maltose uniport	Soybean *Glycine max*(L) Merrill SBP1, SBP2	Overvoorde *et al.* 1996, Pirovani *et al.* 2002

Ref.	Protein Family/Enzyme Name	E.C. number	Mediated Conversion	Examples	Citations
A	sugars will eventually be exported transporter (SWEET)	-	sucrose uniport	*A. thaliana* AtSWEET10-15; *Oryza sativa* OzSWEET11+14; *Bradyrhizobium japonicum* SemiSWEET1	Chen *et al.* 2010, Chen *et al.* 2012, Xuan *et al.* 2013
B	sucrose phosphorylase	E.C. 2.4.1.7	sucrose →α-D-glucose-1-phosphate + D-fructose	*Bifidobacterium adolescentis* Spl; *Leuconostoc mesenteroides* SucP	Goedl *et al.* 2007
C	phosphoglucomutase	E.C. 5.4.2.2	α-D-glucose-1-phosphate → α-D-glucose-6-phosphate	*S. cerevisiae* PGM1, PGM2	Boles *et al.* 1994

[0092] In some embodiments, the disclosure contemplates the modification (*e.g.*, engineering) of the uniporters provided herein. Such modification may be performed to enhance or alter one or more of the uniporter's properties, such as lowering the substance-transporter binding constant (Km), improving the expression of the protein, increasing the transport speed, or altering the substrate specificity of the protein.

[0093] The one or more proteins can be expressed in a microorganism selected from an archea, bacteria, or eukaryote. In some embodiments, the bacteria is a *Propionibacterium*, *Propionispira*, *Clostridium*, *Bacillus*, *Escherichia*, *Pelobacter,* or *Lactobacillus* including, for example, *Escherichia coli*, *Pelobacter propionicus*, *Clostridium propionicum*, *Clostridium acetobutylicum*, *Lactobacillus*, *Propionibacterium acidipropionici* or *Propionibacterium freudenreichii*. In some embodiments, the eukaryote is a yeast, filamentous fungi, protozoa, or algae. In some embodiments, the yeast is *Saccharomyces cerevisiae* or *Pichia pastoris*.

[0094] In some embodiments, sequence alignment and comparative modeling of proteins may be used to alter one or more of the proteins disclosed herein. Homology modeling or comparative modeling refers to building an atomic-resolution model of the desired protein from its primary amino acid sequence and an experimental three-dimensional structure of a similar

protein. This model may allow for the protein substrate binding site to be defined, and the identification of specific amino acid positions that may be replaced to other natural amino acid in order to redesign its substrate specificity.

[0095] Variants or sequences having substantial identity or homology with the polynucleotides encoding proteins as disclosed herein may be utilized in the practice of the disclosure. Such sequences can be referred to as variants or modified sequences. That is, a polynucleotide sequence may be modified yet still retain the ability to encode a polypeptide exhibiting the desired activity. Such variants or modified sequences are thus equivalents. Generally, the variant or modified sequence may comprise at least about 30%-60%, preferably about 60%-80%, more preferably about 80%-90%, and even more preferably about 90%-95% sequence identity with the native sequence.

[0096] The microorganism may be modified by genetic engineering techniques (*i.e.*, recombinant technology), classical microbiological techniques, or a combination of such techniques and can also include naturally occurring genetic variants to produce a genetically modified microorganism. Some of such techniques are generally disclosed, for example, in Sambrook *et al.*, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Labs Press.

[0097] A genetically modified microorganism may include a microorganism in which a polynucleotide has been inserted, deleted or modified (*i.e.*, mutated; *e.g.*, by insertion, deletion, substitution, and/or inversion of nucleotides), in such a manner that such modifications provide the desired effect of expression (*e.g.*, over-expression) of one or more proteins as provided herein within the microorganism. Genetic modifications which result in an increase in gene expression or function can be referred to as amplification, overproduction, overexpression, activation, enhancement, addition, or up-regulation of a gene. Addition of cloned genes to increase gene expression can include maintaining the cloned gene(s) on replicating plasmids or integrating the cloned gene(s) into the genome of the production organism. Furthermore, increasing the expression of desired cloned genes can include operatively linking the cloned gene(s) to native or heterologous transcriptional control elements.

[0098] Where desired, the expression of one or more of the proteins provided herein is under the control of a regulatory sequence that controls directly or indirectly the protein expression in a time-dependent fashion during the fermentation.

[0099] In some embodiments, a microorganism is transformed or transfected with a genetic vehicle, such as an expression vector comprising an exogenous polynucleotide sequence coding for the proteins provided herein.

[00100] Polynucleotide constructs prepared for introduction into a prokaryotic or eukaryotic host may typically, but not always, comprise a replication system (*i.e.* vector) recognized by the host, including the intended polynucleotide fragment encoding the desired polypeptide, and may preferably, but not necessarily, also include transcription and translational initiation regulatory sequences operably linked to the polypeptide-encoding segment. Expression systems (expression vectors) may include, for example, an origin of replication or autonomously replicating sequence (ARS) and expression control sequences, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, mRNA stabilizing sequences, nucleotide sequences homologous to host chromosomal DNA, and/or a multiple cloning site. Signal peptides may also be included where appropriate, preferably from secreted polypeptides of the same or related species, which allow the protein to cross and/or lodge in cell membranes or be secreted from the cell.

[00101] The vectors can be constructed using standard methods (see, *e.g.*,Sambrook *et al.*, Molecular Biology: A Laboratory Manual, Cold Spring Harbor, N.Y. 1989; and Ausubel, *et al.*, Current Protocols in Molecular Biology, Greene Publishing, Co. N.Y, 1995).

[00102] The manipulation of polynucleotides that encode the proteins disclosed herein is typically carried out in recombinant vectors. Numerous vectors are publicly available, including bacterial plasmids, bacteriophage, artificial chromosomes, episomal vectors and gene expression vectors, which can all be employed. A vector may be selected to accommodate a polynucleotide encoding a protein of a desired size. Following recombinant modification of a selected vector, a suitable host cell is transfected or transformed with the vector. Host cells may be prokaryotic, such as any of a number of bacterial strains, or may be eukaryotic, such as yeast or other fungal cells, insect or amphibian cells, or mammalian cells including, for example, rodent, simian or human cells. Each vector contains various functional components, which generally include a cloning site, an origin of replication and at least one selectable marker gene. A vector may additionally possess one or more of the following elements: an enhancer, promoter, and transcription termination and/or other signal sequences. Such sequence elements may be optimized for the selected host species (*e.g.* humanized). Such sequence elements may be positioned in the vicinity of the cloning site, such that they are operatively linked to the gene encoding a preselected protein.

[00103] Vectors, including cloning and expression vectors, may contain nucleic acid sequences that enable the vector to replicate in one or more selected host cells. For example, the sequence may be one that enables the vector to replicate independently of the host chromosomal DNA and may include origins of replication or autonomously replicating

sequences. Such sequences are well known for a variety of bacteria, yeast and viruses. For example, the origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2 micron plasmid origin is suitable for yeast, and various viral origins (*e.g.*SV 40, adenovirus) are useful for cloning vectors in mammalian cells. Generally, the origin of replication is not needed for mammalian expression vectors unless these are used in mammalian cells able to replicate high levels of DNA, such as COS cells.

[00104] A cloning or expression vector may contain a selection gene (also referred to as a selectable marker). This gene encodes a protein necessary for the survival or growth of transformed host cells in a selective culture medium. Host cells not transformed with the vector containing the selection gene will therefore not survive in the culture medium. Typical selection genes encode proteins that confer resistance to antibiotics and other toxins, *e.g.* ampicillin, neomycin, methotrexate, hygromycin, thiostrepton, apramycin or tetracycline, complement auxotrophic deficiencies, or supply critical nutrients not available in the growth media.

[00105] The replication of vectors may be performed in E. coli (*e.g.,* strain TB1 or TG1, DH5α, DH10β, JM110). An *E. coli*-selectable marker, for example, the β-lactamase gene that confers resistance to the antibiotic ampicillin, may be of use. These selectable markers can be obtained from *E. coli* plasmids, such as pBR322 or a pUC plasmid such as pUC18 or pUC19, or pUC119.

[00106] Expression vectors may contain a promoter that is recognized by the host organism. The promoter may be operably linked to a coding sequence of interest. Such a promoter may be inducible or constitutive. Polynucleotides are operably linked when the polynucleotides are in a relationship permitting them to function in their intended manner.

[00107] Promoters suitable for use with prokaryotic hosts may include, for example, the α-lactamase and lactose promoter systems, alkaline phosphatase, the tryptophan (trp) promoter system, the erythromycin promoter, apramycin promoter, hygromycin promoter, methylenomycin promoter and hybrid promoters such as the tac promoter. Moreover, host constitutive or inducible promoters may be used. Promoters for use in bacterial systems will also generally contain a Shine-Dalgarno sequence operably linked to the coding sequence.

[00108] Viral promoters obtained from the genomes of viruses include promoters from polyoma virus, fowlpox virus, adenovirus (*e.g.,* Adenovirus 2 or 5), herpes simplex virus (thymidine kinase promoter), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, retrovirus (*e.g.,*MoMLV, or RSV LTR), Hepatitis-B virus, Myeloproliferative sarcoma virus promoter (MPSV), VISNA, and Simian Virus 40 (SV40). Heterologous mammalian promoters include, *e.g.,* the actin promoter, immunoglobulin promoter, heat-shock protein promoters.

[00109] The early and late promoters of the SV40 virus are conveniently obtained as a restriction fragment that also contains the SV40 viral origin of replication (see, *e.g.*,Fiers*et al.*, Nature, 273:113 (1978); Mulligan and Berg, Science, 209:1422-1427 (1980); and Pavlakis*et al.*, Proc. Natl. Acad. Sci. USA, 78:7398-7402 (1981)). The immediate early promoter of the human cytomegalovirus (CMV) is conveniently obtained as a Hind III E restriction fragment (see, *e.g.*, Greenaway *et al.*, Gene, 18:355-360 (1982)). A broad host range promoter, such as the SV40 early promoter or the Rous sarcoma virus LTR, is suitable for use in the present expression vectors.

[00110] Generally, a strong promoter may be employed to provide for high level transcription and expression of the desired product. Among the eukaryotic promoters that have been identified as strong promoters for high-level expression are the SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Rous sarcoma virus long terminal repeat, and human cytomegalovirus immediate early promoter (CMV or CMV IE). In an embodiment, the promoter is a SV40 or a CMV early promoter.

[00111] The promoters employed may be constitutive or regulatable, *e.g.*, inducible. Exemplary inducible promoters include jun, fos and metallothionein and heat shock promoters. One or both promoters of the transcription units can be an inducible promoter. In an embodiment, the GFP is expressed from a constitutive promoter while an inducible promoter drives transcription of the gene coding for one or more proteins as disclosed herein and/or the amplifiable selectable marker.

[00112] The transcriptional regulatory region in higher eukaryotes may comprise an enhancer sequence. Many enhancer sequences from mammalian genes are known *e.g.*, from globin, elastase, albumin, α-fetoprotein and insulin genes. A suitable enhancer is an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the enhancer of the cytomegalovirus immediate early promoter (Boshart*et al.* Cell 41:521 (1985)), the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers (see also, *e.g.*,Yaniv, Nature, 297:17-18 (1982) on enhancing elements for activation of eukaryotic promoters). The enhancer sequences may be introduced into the vector at a position 5′ or 3′ to the gene of interest, but is preferably located at a site 5′ to the promoter.

[00113] Yeast and mammalian expression vectors may contain prokaryotic sequences that facilitate the propagation of the vector in bacteria. Therefore, the vector may have other components such as an origin of replication (*e.g.*, a nucleic acid sequence that enables the vector to replicate in one or more selected host cells), antibiotic resistance genes for selection in bacteria, and/or an amber stop codon which can permit translation to read through the codon.

Additional eukaryotic selectable gene(s) may be incorporated. Generally, in cloning vectors the origin of replication is one that enables the vector to replicate independently of the host chromosomal DNA, and includes origins of replication or autonomously replicating sequences. Such sequences are well known, *e.g.,* the ColE1 origin of replication in bacteria. Various viral origins (*e.g.,* SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. Generally, a eukaryotic replicon is not needed for expression in mammalian cells unless extrachromosomal (episomal) replication is intended (*e.g.,* the SV40 origin may typically be used only because it contains the early promoter).

[00114] To facilitate insertion and expression of different genes coding for the proteins as disclosed herein from the constructs and expression vectors, the constructs may be designed with at least one cloning site for insertion of any gene coding for any protein disclosed herein. The cloning site may be a multiple cloning site, *e.g.,* containing multiple restriction sites.

[00115] The plasmids may be propagated in bacterial host cells to prepare DNA stocks for subcloning steps or for introduction into eukaryotic host cells. Transfection of eukaryotic host cells can be any performed by any method well known in the art. Transfection methods include lipofection, electroporation, calcium phosphate co-precipitation, rubidium chloride or polycation mediated transfection, protoplast fusion and microinjection. Preferably, the transfection is a stable transfection. The transfection method that provides optimal transfection frequency and expression of the construct in the particular host cell line and type is favored. Suitable methods can be determined by routine procedures. For stable transfectants, the constructs are integrated so as to be stably maintained within the host chromosome.

[00116] Vectors may be introduced to selected host cells by any of a number of suitable methods known to those skilled in the art. For example, vector constructs may be introduced to appropriate cells by any of a number of transformation methods for plasmid vectors. For example, standard calcium-chloride-mediated bacterial transformation is still commonly used to introduce naked DNA to bacteria (see, *e.g.,* Sambrook *et al.*, 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), but electroporation and conjugation may also be used (see, *e.g.,* Ausubel *et al.*, 1988, Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y.).

[00117] For the introduction of vector constructs to yeast or other fungal cells, chemical transformation methods may be used (*e.g.,* Rose *et al.*, 1990, Methods in Yeast Genetics, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.). Transformed cells may be isolated on selective media appropriate to the selectable marker used. Alternatively, or in addition, plates or filters lifted from plates may be scanned for GFP fluorescence to identify transformed clones.

[00118] For the introduction of vectors comprising differentially expressed sequences to mammalian cells, the method used may depend upon the form of the vector. Plasmid vectors may be introduced by any of a number of transfection methods, including, for example, lipid-mediated transfection ("lipofection"), DEAE-dextran-mediated transfection, electroporation or calcium phosphate precipitation (see, *e.g.,* Ausubel *et al.*, 1988, Current Protocols in Molecular Biology, John Wiley & Sons, Inc., NY, N.Y.).

[00119] Lipofection reagents and methods suitable for transient transfection of a wide variety of transformed and non-transformed or primary cells are widely available, making lipofection an attractive method of introducing constructs to eukaryotic, and particularly mammalian cells in culture. For example, LipofectAMINE™ (Life Technologies) or LipoTaxi™ (Stratagene) kits are available. Other companies offering reagents and methods for lipofection include Bio-Rad Laboratories, CLONTECH, Glen Research, InVitrogen, JBL Scientific, MBIFermentas, PanVera, Promega, Quantum Biotechnologies, Sigma-Aldrich, and Wako Chemicals USA.

[00120] The host cell may be capable of expressing the construct encoding the desired protein, processing the protein and transporting a secreted protein to the cell surface for secretion. Processing includes co- and post-translational modification such as leader peptide cleavage, GPI attachment, glycosylation, ubiquitination, and disulfide bond formation. Immortalized host cell cultures amenable to transfection and in vitro cell culture and of the kind typically employed in genetic engineering are preferred. Examples of useful mammalian host cell lines are monkey kidney CV1 line transformed by SV40 (COS-7, ATCCCRL 1651); human embryonic kidney line (293 or 293 derivatives adapted for growth in suspension culture, Graham *et al.*, J. Gen Virol., 36:59 (1977); baby hamster kidney cells (BHK, ATCC CCL 10); DHFR−Chinese hamster ovary cells (ATCC CRL-9096); dp12.CHO cells, a derivative of CHO/DHFR-(EP 307,247 published 15 Mar. 1989); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCCCRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y. Acad. Sci., 383:44-68 (1982)); PEER human acute lymphoblastic cell line (Ravid*et al.* Int. J. Cancer 25:705-710 (1980)); MRC 5 cells; FS4 cells; human hepatoma cell line (Hep G2), human HT1080 cells, KB cells, JW-2 cells, Detroit 6 cells, NIH-3T3 cells, hybridoma and myeloma cells. Embryonic cells used for generating transgenic animals are also suitable (*e.g.,* zygotes and embryonic stem cells).

[00121] Suitable host cells for cloning or expressing polynucleotides (*e.g.,* DNA) in vectors may include, for example, prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes for this purpose include eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *Escherichia*, *e.g.,E. coli*, *Enterobacter*, *Erwinia*, *Klebsiella*, *Proteus*, *Salmonella*, *e.g.,Salmonella typhimurium*, *Serratia*, *e.g.,Serratiamarcescans*, and *Shigella*, as well as *Bacilli* such as *B. subtilis* and *B. licheniformis* (*e.g.,B. licheniformis* 41 P disclosed in DD 266,710 published Apr. 12, 1989), *Pseudomonas* such as *P. aeruginosa*, and *Streptomyces*. One preferred *E. coli* cloning host is *E. coli* 294 (ATCC 31,446), although other strains such as *E. coli* B, E. coli X1776 (ATCC 31,537), *E. coli* JM110 (ATCC 47,013) and *E. coli* W3110 (ATCC 27,325) are suitable.

[00122] In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast may be suitable cloning or expression hosts for vectors comprising polynucleotides coding for one or more enzymes. *Saccharomyces cerevisiae*, or common baker's yeast, is the most commonly used among lower eukaryotic host microorganisms. However, a number of other genera, species, and strains are commonly available and useful herein, such as *Schizosaccharomycespombe*; *Kluyveromyces* hosts such as, *e.g.,K. lactis, K. fragilis* (ATCC 12,424), *K. bulgaricus*(ATCC 16,045), *K. wickeramii*(ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906), *K. thermotolerans*, and *K. marxianus*; *Yarrowia* (EP 402,226); *Pichia pastors* (EP 183,070); *Candida*; *Trichodermareesia* (EP 244,234); *Neurosporacrassa*; *Schwanniomyces*such as *Schwanniomycesoccidentalis*; and filamentous fungi such as, *e.g.,Neurospora*, *Penicillium*, *Tolypocladium*, and *Aspergillus* hosts such as *A. nidulans* and *A. niger*.

[00123] When the protein is glycosylated, suitable host cells for expression may be derived from multicellular organisms. Examples of invertebrate cells include plant and insect cells. Numerous baculoviral strains and variants and corresponding permissive insect host cells from hosts such as *Spodopterafrugiperda* (caterpillar), *Aedesaegypti* (mosquito), *Aedesalbopictus* (mosquito), *Drosophila melanogaster* (fruitfly), and *Bombyxmori* (silk moth) have been identified. A variety of viral strains for transfection are publicly available, *e.g.,* the L-1 variant of AutographacalifornicaNPV and the Bm-5 strain of BombyxmoriNPV, and such viruses may be used as the virus herein according to the present disclosure, particularly for transfection of *Spodopterafrugiperda* cells.

[00124] Plant cell cultures of cotton, corn, potato, soybean, petunia, tomato, tobacco, lemna, and other plant cells can also be utilized as host cells.

[00125] Examples of useful mammalian host cells are Chinese hamster ovary cells, including CHOK1 cells (ATCC CCL61), DXB-11, DG-44, and Chinese hamster ovary cells/-

DHFR (CHO, Urlaub *et al.*, Proc. Natl. Acad. Sci. USA 77: 4216 (1980)); monkey kidney CV1 line transformed by SV40 (COS-7, ATCCCRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, (Graham *et al.*, J. Gen Virol. 36: 59, 1977); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, (Biol. Reprod. 23: 243-251, 1980); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCCCRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary tumor (MMT 060562, ATCC CCL51); TRI cells (Mather *et al.*, Annals N.Y Acad. Sci. 383: 44-68 (1982)); MRC 5 cells; FS4 cells; and a human hepatoma line (Hep G2).

[00126] Host cells are transformed or transfected with the above-described expression or cloning vectors for production of one or more proteins as disclosed herein or with polynucleotides coding for one or more proteins as disclosed herein and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

[00127] Host cells containing desired nucleic acid sequences coding for the disclosed enzymes may be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM), Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham *et al.*, Meth. Enz. 58: 44, (1979); Barnes *et al.*, Anal. Biochem. 102: 255 (1980); U.S. Patent Nos. 4,767,704; 4,657,866; 4,927,762; 4,560,655; or 5,122,469; WO90103430; WO 87/00195; or U.S. Patent Re. No. 30,985 may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleotides (such as adenosine and thymidine), antibiotics (such as GENTAMYCIN™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

Polynucleotides and Encoded Proteins

[00128] Any known polynucleotide (*e.g.*, gene) that codes for a protein or variant thereof that can be used in a system for optimizing oligosaccharide utilization including, for example, a protein as set forth in Table 1 or Figure 1, is contemplated for use by the present disclosure. Additional exemplary protein candidates for the disclosed optimized oligosaccharide utilization system and enzyme reaction products are presented in Tables 2-4 below. Such polynucleotides can be modified (*e.g.*, genetically engineered) to modulate (*e.g.*, increase or decrease) the substrate specificity of an encoded protein, or the polynucleotides may be modified to change the substrate specificity of the encoded protein (*e.g.*, a polynucleotide that codes for a protein with specificity for a substrate may be modified such that the protein has specificity for an alternative substrate). For an energy independent saccharide transporter protein, such polynucleotides can be also be modified to lower the substance-transporter binding constant (Km), improve the expression of the protein, or increase the transport speed. Preferred microorganisms may comprise polynucleotides coding for one or more of the proteins as set forth in Tables 1-4 and Figure 1.

Table 2. Exemplary uniporter proteins and candidate proteins to be evolved into uniporter proteins for optimized oligosaccharide utilization.

SEQ ID NO:	Gene	GenBank or Uniprot ID	Organism
1	PsSUF1	A3DSX2	*Pisum sativum*
2	PsSUF1	DQ221698.2	*Pisum sativum*
3	PsSUF4	A3DSX1	*Pisum sativum*
4	PsSUF4	DQ221697.2	*Pisum sativum*
5	PvSUF1	A3DSX4	*Phaseolus vulgaris*
6	PvSUF1	DQ221700.1	*Phaseolus vulgaris*
7	LjSUT4	Q84RQ3	*Lotus japonicus*
8	LjSUT4	AJ538041.1	*Lotus japonicus*
9	SIP1-1	Q9M8W5	*Arabidopsis thaliana*
10	SIP1-1	AK226432.1	*Arabidopsis thaliana*
11	SIP1-2	Q9FK43	*Arabidopsis thaliana*
12	SIP1-2	BT005263.1	*Arabidopsis thaliana*
13	SBP1	Q04672	*Glycine max*
14	SBP1	L06038.1	*Glycine max*

SEQ ID NO:	Gene	GenBank or Uniprot ID	Organism
15	SBP2	Q84V19	*Glycine max*
16	SBP2	AY234869.1	*Glycine max*
17	AtSWEET10	Q9LUE3	*Arabidopsis thaliana*
18	AtSWEET10	AY064674.1	*Arabidopsis thaliana*
19	AtSWEET11	Q9SMM5	*Arabidopsis thaliana*
20	AtSWEET11	AF361825.1	*Arabidopsis thaliana*
21	AtSWEET12	O82587	*Arabidopsis thaliana*
22	AtSWEET12	AY059108.1	*Arabidopsis thaliana*
23	AtSWEET13	Q9FGQ2	*Arabidopsis thaliana*
24	AtSWEET13	AY087516	*Arabidopsis thaliana*
25	AtSWEET14	Q9SW25	*Arabidopsis thaliana*
26	AtSWEET14	CP002687.1	*Arabidopsis thaliana*
27	AtSWEET15	Q9FY94	*Arabidopsis thaliana*
28	AtSWEET15	AY045949.1	*Arabidopsis thaliana*
29	OzSWEET11	Q6YZF3	*Oryza sativa japonica*
30	OzSWEET11	AK106127.1	*Oryza sativa japonica*
31	OzSWEET14	Q2R3P9	*Oryza sativa japonica*
32	OzSWEET14	AK101913.1	*Oryza sativa japonica*
33	glpF	P0AER0	*Escherichia coli*
34	glpF	X15054.1	*Escherichia coli*
35	Suc	B0RRG6	*Xanthomonas campestris pv.campestris*
36	Suc	Accession No.: NC_010688, 1961593	*Xanthomonas campestris pv.campestris*
37	Cdt-1	Q7SCU1	*Neurospora crassa*
38	Cdt-1	NCBI Ref. Seq. NW 001849825.1	*Neurospora crassa*
39	Cdt-2	Q7SD12	*Neurospora crassa*
40	Cdt-2	NCBI Ref. Seq. NW 001849743.1	*Neurospora crassa*

Table 3. Exemplary phosphorylase proteins for optimized oligosaccharide utilization.

SEQ ID NO:	Gene	GenBank or Uniprot ID	Organism
41	LVIS_0358	Q03TE9	*Lactobacillus brevis*
42	LVIS_0358	NC_008497, 382638	*Lactobacillus brevis*
43	mapA	O87772	*Lactobacillus sanfranciscensis*
44	mapA	AJ224340.2	*Lactobacillus sanfranciscensis*
45	Spl	A0ZZH6	*Bifidobacterium adolescentis*
46	Spl	NC_008618, 104792	*Bifidobacterium adolescentis*
47	SucP	Q59495	*Leuconostoc mesenteroides*
48	SucP	D90314.1	*Leuconostoc mesenteroides*
49	Cbp	Q8VP44	*Clostridium thermocellum*
50	Cbp	AY072794.1	*Clostridium thermocellum*

Table 4. Exemplary phosphoglucomutase proteins for optimized oligosaccharide utilization.

SEQ ID NO:	Gene	GenBank or Uniprot ID	Organism
51	PGM1	P33401	*Saccharomyces cerevisiae*
52	PGM1	X72016.1	*Saccharomyces cerevisiae*
53	PGM2	P37012	*Saccharomyces cerevisiae*
54	PGM2	X74823.1	*Saccharomyces cerevisiae*
55	pgmA	O87773	*Lactobacillus sanfranciscensis*
56	pgmA	AJ224340.2	*Lactobacillus sanfranciscensis*
57	pgmB	P71447	*Lactococcus lactis lactis*
58	pgmB	Z70730.1	*Lactococcus lactis lactis*

Methods of Optimizing Oligosaccharide Utilization

[00129] A microorganism's utilization of one or more oligosaccharides can be optimized by contacting any of the disclosed genetically modified microorganisms with an oligosaccharide source (e.g., a fermentable carbon source). Such methods can comprise contacting an oligosaccharide source with a microorganism comprising one or more polynucleotides coding for energy independent oligosaccharide transporter proteins (e.g. uniporters), such as those set forth in Table 1 and Table 2. Preferred nucleotide sequences for energy independent oligosaccharide

transporter proteins and candidate proteins for evolution into energy independent oligosaccharide transporter proteins are provided by SEQ ID Nos.: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 and 40. Preferred protein sequences for energy independent oligosaccharide transporter proteins or candidate proteins for evolution into energy independent oligosaccharide transporter proteins are provided by SEQ ID Nos.: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37 and 39. Such methods can also comprise contacting an oligosaccharide source with a microorganism comprising one or more polynucleotides coding for a uniporter, one or more polynucleotides coding for a phosphorylase, and one or more polynucleotides coding for a phosphoglucomutase. Preferred nucleotide sequences for phosphorylases are provided by SEQ ID Nos.: 42, 44, 46, 48 and 50. Preferred protein sequences for phosphorylases are provided by SEQ ID Nos.: 41, 43, 45, 47 and 49. Preferred nucleotide sequences for phosphoglucomutases are provided by SEQ ID Nos.: 52, 54, 56 and 58. Preferred protein sequences for phosphoglucomutases are provided by SEQ ID Nos.: 51, 53, 55 and 57.

[00130] Optionally, the methods may further comprise knocking-out (*i.e.*, deleting) one or more naturally competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. Alternatively, the microorganism may naturally lack or may be obtained that lacks one or more competing transporter systems (*e.g.*, one or more nucleotides coding for one or more proteins involved in the transporter system) for transporting the oligosaccharide into the microorganism. For example, for sucrose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out. Additonally, for example, for maltose utilization in *S. cerevisiae* one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out. Further, for example, for maltose utilization in *E. coli* at least one genes of the group malE, malF, malK, malG, or lamb may be knocked-out. In other embodiments, for example, for cellobiose utilization in *S. cerevisiae*, for sucrose utilization in *E. coli*, or for cellobiose utilization in *E. coli*, :the microorganism does not have any naturally competing transporter systems knocked-out.

[00131] The compound or molecule resulting from the optimized oligosaccharide utilization method is then utilized in one or more enzymatic pathways in the microorganism that have been engineered to produce industrially important compounds.

[00132] The metabolic pathways that lead to the production of industrially important compounds involve oxidation-reduction (redox) reactions. For example, during fermentation, glucose is oxidized in a series of enzymatic reactions into smaller molecules with the concomitant release of energy. The electrons released are transferred from one reaction to another through universal electron carriers, such Nicotinamide Adenine Dinucleotide (NAD) and

Nicotinamide Adenine Dinucleotide Phosphate (NAD(P)), which act as cofactors for oxidoreductase enzymes. In microbial catabolism, glucose is oxidized by enzymes using the oxidized form of the cofactors (NAD(P)+ and/or NAD+) as cofactor thus generating reducing equivalents in the form of the reduced cofactor (NAD(P)H and NADH). In order for fermentation to continue, redox-balanced metabolism is required, *i.e.*, the cofactors must be regenerated by the reduction of microbial cell metabolic compounds.

[00133] Microorganism-catalyzed fermentation for the production of natural products is a widely known application of biocatalysis. Industrial microorganisms can affect multistep conversions of renewable feedstocks to high value chemical products in a single reactor. Products of microorganism-catalyzed fermentation processes range from chemicals such as ethanol, lactic acid, amino acids and vitamins, to high value small molecule pharmaceuticals, protein pharmaceuticals, and industrial enzymes. In many of these processes, the biocatalysts are whole-cell microorganisms, including microorganisms that have been genetically modified to express heterologous genes.

[00134] Some key parameters for efficient microorganism-catalyzed fermentation processes include the ability to grow microorganisms to a greater cell density, increased yield of desired products, increased amount of volumetric productivity, removal of unwanted co-metabolites, improved utilization of inexpensive carbon and nitrogen sources, adaptation to varying fermenter conditions, increased production of a primary metabolite, increased production of a secondary metabolite, increased tolerance to acidic conditions, increased tolerance to basic conditions, increased tolerance to organic solvents, increased tolerance to high salt conditions and increased tolerance to high or low temperatures. Inefficiencies in any of these parameters can result in high manufacturing costs, inability to capture or maintain market share, and/or failure to bring fermented end-products to market.

[00135] The methods and compositions of the present disclosure can be adapted to conventional fermentation bioreactors (*e.g.*, batch, fed-batch, cell recycle, and continuous fermentation).

[00136] In some embodiments, a microorganism (*e.g.*, a genetically modified microorganism) as provided herein is cultivated in liquid fermentation media (*i.e.*, a submerged culture) which leads to excretion of the fermented product(s) into the fermentation media. In one embodiment, the fermented end product(s) can be isolated from the fermentation media using any suitable method known in the art.

[00137] In some embodiments, formation of the fermented product occurs during an initial, fast growth period of the microorganism. In one embodiment, formation of the fermented product occurs during a second period in which the culture is maintained in a slow-growing or

quiescent state. In one embodiment, formation of the fermented product occurs during more than one growth period of the microorganism. In such embodiments, the amount of fermented product formed per unit of time is generally a function of the metabolic activity of the microorganism, the physiological culture conditions (*e.g.*, pH, temperature, medium composition), and the amount of microorganisms present in the fermentation process.

[00138] In some embodiments, the fermentation product is recovered from the periplasm or culture medium as a secreted metabolite. In one embodiment, the fermentation product is extracted from the microorganism, for example when the microorganism lacks a secretory signal corresponding to the fermentation product. In one embodiment, the microorganisms are ruptured and the culture medium or lysate is centrifuged to remove particulate cell debris. The membrane and soluble protein fractions may then be separated if necessary. The fermentation product of interest may then be purified from the remaining supernatant solution or suspension by, for example, distillation, fractionation, chromatography, precipitation, filtration, and the like. In one embodiment, the microorganism cells (or portions thereof) may be used as biocatalysts or for other functions in a subsequent process without substantial purification.

Methods of Modifying Transporter Proteins

[00139] An energy independent transporter (e.g., uniporter) for a given saccharide can be selected or identified from a set of unspecified transporters, evolved (e.g. modified) from a known energy dependent transporter, or improved from an existing uniporter wildtype. All three of these can be accomplished by selecting the desired uniport activity through growth selection.

[00140] Libraries of mutated candidate genes can be used to select candidate genes. The selected candidate genes are then expressed in a microorganism, such as yeast, that is deficient in the uptake or transport of the oligosaccharide of interest, but otherwise capable of metabolizing saccharides. In an embodiment, the *S. cerevisiae* INV8 or SUSY7 strain for sucrose transport is used to express candidate genes. (Riesmeier *et al.* 1992.)

[00141] The microorganism strains expressing the candidate genes are then grown on or in minimal media with the saccharide of interest as the only carbon source. For example, the strains expressing candidate genes can be grown in a chemostat or turbidostat. Growth of a strain on this minimal media indicates that the candidate gene encodes a saccharide transporter. The strains expressing candidate genes for the most efficient saccharide transporters will have the highest growth rate. A strain that has a candidate gene encoding an improved saccharide transporter will outcompete other clones in the same culture and, eventually, will dominate the culture.

[00142] This approach is also known as evolutionary fermentation. Through the above-described process, saccharide transporters can be identified from non-transporters. Additionally, this process can be used to continuously improve a known uniporter, such as by lowering the substance-transporter binding constant (Km), improving the expression of the protein, increasing the transport speed, or altering the substrate specificity of the protein, for a substrate of choice and in a strain of choice. For example, the well known *S. cerevisiae* hexose uniporters of the Hxt family could be mutated and selected from sucrose acceptance. In another example, the sucrose uniporters mentioned in this application can be optimized for maltose or cellbiose acceptance, particularly since SBP1 already demonstrates maltose transport activity. In yet another example, the *E. coli* glycerol uniporter can be evolved to accept maltose, sucrose or cellobiose.

[00143] The above-described process can also be used to evolve an energy dependent transporter into an energy independent transporter. Instead of assessing the growth rate of the cultured strains, the biomass formation of the strains should be measured. Strains that express an energy independent transporter would have more ATP and, therefore, higher biomass formation (e.g., an increase in biomass formation of, for example, 25% or 33%) from growth on limited media than strains expressing an energy dependent transporter. In an embodiment, the strains expressing candidate genes are cultured individually, such as in 96 well plates, to avoid competition by growth speed instead of total biomass formation. The biomass of each strain is then measured using simple, high throughput optical density methods, such as by using an OD600 plate reader. For example, the bacterial maltose/ sucrose H+ symporter Suc from *Xanthomonas campestris* can be evolved to function as an uniporter.

[00144] Symporters could potentially be evolved into uniporters using the above-described process. This is based, in part, on the fact that some SUF sucrose uniporters are closely related to sucrose H+ (SUT) symporters in clade I (PvSUT3 and PvSUF1) and clade II (LjSUT4 and PsSUF4) of the sucrose transporter family (Zhou *et al.* 2007). The close relationship of SUF uniporters and SUT symporters suggests that symporters could be successfully evolved into uniporters.

[00145] Additionally, transporters utilizing energy in the form of ATP or GTP could potentially be evolved into energy independent uniporters. This is based on the fact that sucrose binding protein 2 (SBP2) binds GTP, but mutant versions of SBP2 unable to bind GTP still function as sucrose transporters in yeast (Pirovani *et al.* 2002). While SBP2 can bind GTP and presumably use its energy, it obviously does not need GTP to function as importer. Thus, it seems possible that an energy independent uniporter could be evolved from some transporters that usually utilize PEP, ATP, or GTP.

EXAMPLES

Example 1: Modification of microorganism to optimize oligosaccharide utilization.

[00146] A microorganism such as yeast or bacteria is genetically modified to optimize its utilization of oligosaccharides.

[00147] In an exemplary method, a microorganism, such as the bacterium *E. coli*, is genetically engineered by any methods known in the art to comprise a maltose uniporter, a maltose phosphorylase, and a β-phosphoglucomutase for the transport, phosphorolysis and inversion of maltose, for instance by expressing a glycerol uniporter GlpF mutant from *E. coli* evolved to accept sucrose or a maltose/ sucrose H+ symporter Suc mutant from *Xanthomonas campestris* evolved to function as uniporter, LVIS_0358 from *Lactobacillus brevis* and PgmB from *Lactococcus lactis*.

[00148] In another exemplary method, a microorganism, such as the yeast *S. cerevisiae*, is genetically engineered by any methods known in the art to comprise a maltose uniporter, a maltose phosphorylase, and a β-phosphoglucomutase for the transport, phosphorolysis and inversion of maltose, for instance by expressing SBP1 from *Glycine max*, LVIS_0358 from *Lactobacillus brevis* and PgmB from *Lactococcus lactis*.

[00149] In an exemplary method, a microorganism, such as the bacterium *E. coli*, is genetically engineered by any methods known in the art to comprise a cellobiose uniporter, a cellobiose phosphorylase, and an α-phosphoglucomutase for the transport, phosphorolysis and inversion of cellobiose, for instance by expressing a glycerol uniporter GlpF mutant from *E. coli* evolved to accept cellobiose, Cbp from *Clostridium thermocellum* and PGM1 from *S. cerevisiae*.

[00150] In another exemplary method, a microorganism, such as the yeast *S. cerevisiae*, is genetically engineered by any methods known in the art to comprise a cellobiose uniporter, a cellobiose phosphorylase, and an α-phosphoglucomutase for the transport, phosphorolysis and inversion of cellobiose, for instance by expressing CDT2 from *Neurospora crassa*, Cbp from *Clostridium thermocellum* and PGM1 from *S. cerevisiae*.

[00151] In another exemplary method, a microorganism, such as the bacterium *E. coli*, is genetically engineered by any methods known in the art to comprise a sucrose uniporter, a sucrose phosphorylase, and an α-phosphoglucomutase for the transport, phosphorolysis and inversion of sucrose, for instance by expressing a glycerol uniporter GlpF mutant from *E. coli* evolved to accept sucrose or a maltose/ sucrose H+ symporter Suc mutant from *Xanthomonas campestris* evolved to function as uniporter, SucP from *Leuconostoc mesenteroides* and PGM1 from *S. cerevisiae*.

[00152] In another exemplary method, a microorganism, such as the yeast *S. cerevisiae*, is genetically engineered by any methods known in the art to comprise a sucrose uniporter, a

sucrose phosphorylase, and an α-phosphoglucomutase for the transport, phosphorolysis and inversion of sucrose, for instance by expressing SUF1 from *Pisum sativum*, SucP from *Leuconostoc mesenteroides* and PGM1 from *S. cerevisiae*.

[00153] The sucrose uniporter passively imports the sucrose to the microorganism without using any energy. The phosphorylase utilizes the energy content of the glycoside bond to yield a phosphorylated glucose and a non-phosphorylated fructose. The α-phosphoglucomutase then converts the phosphorylated glucose to an isomer that is utilized by the microorganism. The phosphorylated glucose is then degraded into pyruvate and ATP by glycolysis. The steps involved and the resulting ATP production from the use of the optimized oligosaccharide utilization system are set forth below:

Import: sucrose →(uniporter)→ sucrose

Phosphorylation: sucrose+phosphate →(phosphorylase)→ glucose-1-phosphate+fructose

Conversion: glucose-1-phosphate+fructose →(phosphoglucomutase)
→glucose-6-phosphate+fructose

[Glycolysis]

Summary: sucrose+5ADP+5phosphate → 4 pyruvate+5ATP

[00154] A naturally occurring oligosaccharide utilization system does not produce as much ATP as the optimized oligosaccharide utilization system described above. For example, *S. cerevisiae* naturally uses an excreted invertase (EC 3.2.1.26) to hydrolyze sucrose to glucose and fructose. This results in the loss of the energy present in the glycolytic bond. The glucose and fructose are then passively imported into the yeast and phosphorylated to glucose-6-phosphate and fructose-6-phosphate, using one ATP for each hexose (Barnett 1976, Lagunas 1993). The steps involved in this process and the resulting ATP production are set forth below:

Hydrolysis: sucrose →(invertase) →glucose+fructose

Import: glucose+fructose →glucose+fructose

Phosphorylation: glucose+ATP →glucose-6-phosphate+ADP →fructose-6-phosphate+ADP

fructose+ATP →fructose-6-phosphate+ADP

Glycolysis: 2 fructose-6-phosphate+6 ADP+6 phosphate→4 pyruvate+6 ATP

Summary: sucrose+4ADP+4 phosphate→4 pyruvate + 4 ATP

[00155] Likewise, other sucrose utilization systems, including heterologous systems for microbial use, also do not produce as much ATP as the optimized oligosaccharide utilization system. Heterologous systems for microbial use mainly utilize sucrose/proton (H^+) symporters where sucrose is imported in an energy dependent manner. This manner of importing sucrose is

energy dependent because the imported proton must then be exported by an ATPase, which requires the use of one ATP per proton (Serrano 1984). In the majority of systems, once imported the sucrose is then hydrolyzed to glucose and fructose, which, as mentioned above, causes the loss of the energy present in the glycolytic bond. The steps involved in this process and the resulting ATP production are set forth below:

Import: sucrose+H^+→(symporter) →sucrose+H^+

Export: H^++ATP→H^++ADP+P

Hydrolysis: sucrose→(hydrolase) →glucose+fructose

[Glycolysis]

Summary: sucrose+3 ADP+3 phosphate→4 pyruvate+3 ATP

[00156] The examples above show differences in ATP yield from 3 to 5 ATP per sucrose. Use of the disclosed optimized oligosaccharide utilization system on larger saccharides will lead to a higher ATP gain per released hexose. Large saccharides up to tetra-saccharides can be imported by the disclosed transporters, although at low efficiency. Table 5 below shows the effect of the optimized oligosaccharide utilization system on ATP yield per hexose. The values provided are shown for degradation of the saccharide to pyruvate, which is a common intermediate for aerobic and anaerobic conditions. During anaerobic fermentation, pyruvate would be further reduced to ethanol to re-oxidize the NADH produced during glycolysis.

Table 5. Effect of optimized oligosaccharide utilization on ATP yield per hexose.

	ATP per hexose (degradation to pyruvate using glycolysis)			
saccharide	active transport, hydrolysis	hydrolysis, passive transport	active transport, phosphorolysis	passive transport, phosphorolysis (the optimized oligosaccharide utilization system)
mono-	1	2	1	2
di-	1.5	2	2	2.5
tri-	1.67	2	2.33	2.67
tetra-	1.75	2	2.5	2.75

[00157] As shown in Table 5, above, the impact of the optimized oligosaccharide utilization system is particularly high under anaerobic conditions, where di-saccharide fermentation to ethanol usually yields 2 ATP per hexose. The use of the disclosed optimized oligosaccharide utilization system increases the ATP yield to 2.5 ATP per hexose for di-saccharides, an increase of 25%. A 25% increase in ATP production can translate into 25% more of the resulting product. In industrial applications, where the microorganism has also been

genetically modified to produce a desired chemical, this can mean a significant increase in chemical production.

[00158] The use of higher order oligosaccharides can result in a greater increase in the ATP yield per hexose. The use of higher order oligosaccharides, such as tri-saccharides, even with the use of phosphorolysis with active transport, can significantly increase the ATP yield per hexose. However, the uptake or import of these higher order oligosaccharides is very inefficient (Zhou *et al.* 2007) and they are not commonly used as feedstocks.

Example 2: Impact of optimization of sucrose utilization in a microorganism anaerobically producing the organic molecules isoprene and ethanol.

[00159] As an example, the potential impact of optimized oligosaccharide utilization on the anaerobic production of isoprene in *S. cerevisiae* is calculated. In U.S. Provisional Patent Application No. 62/003,919, anaerobic isoprene production via mevalonate pathway is claimed, utilizing partial non-oxydative glycolysis via PK/PTA pathway to achieve redox neutrality and therefore retaining maximum carbon in the product. Optimized for maximum isoprene production, the theoretic maximum yields are 17.45 wt% isoprene and 23.60 wt% ethanol, which is a necessary co-product to deliver ATP (+2 ATP per molecule of ethanol produced), as calculated by a metabolic model. This maximum yield is calculated for redox and ATP neutral conditions (0 NADH, 0 ATP). This scenario applies to fermentations based on glucose as well as sucrose, where a *S. cerevisiae* strain naturally utilizes secreted sucrose invertase and passive hexose uptake.

[00160] Using a strain with implemented optimized oligo-saccharide utilization for sucrose, assuming 100% efficiency, the maximum theoretical yield would be 21.81 wt% isoprene and 16.72 wt% ethanol on sucrose. The increase from 17.45 wt% to 21.81 wt% equals +25%. This +25% increase stems from the fact that the optimized system delivers +25% ATP per hexose, thus 25% less saccharide is needed to form ethanol and can be used for synthesis of the main product isoprene instead. This increase will have a dramatic effect on the economic viability of the anaerobic fermentative production of this bulk chemical in a highly competitive market with low margins, where feedstock costs are usually by far the main factor in production costs (~50% of total costs). It also shows the advantage of using a method that combines an engineered strain with implemented optimized oligo-saccharide utilization and the feedstock sugar cane juice, in contrast to using natural sucrose utilization or in contrast to using glucose as feedstock.

Example 3: Establishing *S. cerevisiae* S288c with an optimized sucrose uptake system

[00161] In order to test and demonstrate functionality of the individual components and the entire optimized oligo-saccharide optimization system for sucrose in *S. cerevisiae* intrinsic, competing utilization pathways, like energy dependent transport, in the utilized strain were knocked-out (*i.e.*, deleted). These deletions also ensure that the full benefit of the optimized oligo-sacharose utilization system can obtained and that no energy is lost. In addition to the secreted sucrose invertase SUC2 (GeneID 854644), parts of the maltose utilization system, the maltose transporters MAL11 (Gene ID 853207) and MAL31 (Gene ID 852601) and maltases MAL12 (Gene ID: 853209) and MAL32 (Gene ID: 852602), are known to be involved in the transport of sucrose (Mwesigye *et al.* 1994, Stambuk *et al.* 1999). Thus, these five genes may be deleted to obtain a strain without a natural sucrose utilization system, regarding transport as well as hydrolysis. Briefly, the KanMX4 marker gene was amplified from the plasmid pUG6 (GenBank: AM701829.1) with primers BKO1008 (SEQ ID NO: 76) and BKO1009 (SEQ ID NO: 77). These primers contain 40 bp sequence identity to the regions upstream and downstream of the MAL11-MAL12 genes, which are located at adjacent loci in the S288c genome.

[00162] The resulting linear DNA cassette BKO1008- BKO1009 (SEQ ID NO. 68) was transformed into wild-type S288c (ATCC 204508, with auxotrophies his3 leu2 ura3 and trp1), creating the deletion mutant S288c (mal11 mal12). G418 (Gentamycin) resistant candidates, demonstrating successful replacement of the targeted MAL11-MAL12 gene region with the KanMX4 marker gene, were confirmed by colony PCR and sequencing. Likewise, the SUC2 gene was deleted: The HIS3 marker gene (GeneID 854377) was amplified from chromosomal DNA isolated from *S. cerevisiae* S288c strain (ATCC 204508) using the primers BKO1016 (SEQ ID NO: 78) and BKO1017 (SEQ ID NO: 79) with 40 bp sequence identity to the regions upstream and downstream of the SUC2 gene. The resulting PCR product BKO1016-BKO1017 (SEQ ID NO. 69) was transformed into S288c (mal11 mal12) to generate BRKY427 (mal11 mal12 suc2), selected through complementation of the histidine auxotrophy, and verified by colony-PCR and sequencing. MAL31 and MAL32, also co-located in the genome, were deleted through markerless deletion from position 802631 to 807105 on chromosome 2, and verified by colony-PCR and sequencing. The constructed strain should be unable to utilize sucrose. In order to confirm the suc^- phenotype of the constructed strain, growth tests were conducted in shake flasks in triplicate with strains BRKY30 (S288c wildtype), BRKY427 (mal11 mal12 suc2) and BRKY448 (mal11 mal12 mal31 mal32 suc2) (Fig. 2). The strains were pre-grown in YNB media with 2% glucose overnight, washed and inoculated in YNB with 2% sucrose as sole carbon source to an OD of 0.2. To complement the remaining auxotrophies of the parent strain, all cultures required addition of leucine, tryptophan and uracil in a minimal media. The flasks were incubated at 30°C and 250 rpm.

[00163] As expected, the wildtype (WT) strain consumed all the sucrose within 20 hours. Both BRKY427 and BRKY448 were unable to grow with sucrose as sole carbon source even after 160 hours of cultivation, proving that the suc^- mal^- phenotype was established and is stable over a long period of time under the assayed conditions. Two sucrose phosphorylase candidates, SucP from *Leuconostoc mesenteroides* (LmSUCP) and SPL from *Bifidobacterium adolescent* (BaSPL), were tested in BRKY448 overexpressing its natural, energy dependent sucrose/ maltose H+ symporter AGT1 (MAL11) (see Fig. 3). The genes were cloned as transcription units with the promotor and terminator as described in table 6 by standard means and methods known to a person skilled in the art into and expressed episomally from the plasmid pRS415 (GenBank: U03449.1) (SEQ ID NO. 70). Expression and growth tests were performed as described above. Both strains showed strong growth, albeit slower than the wildtype. A phosphoglucomutase gene was not overexpressed in this test, suggesting that there is sufficient induction and activity of the intrinsic PGM2. As LmSUCP demonstrated faster growth complementation, it was selected to establish the full optimized sucrose utilization system.

[00164] Plant based uniporter candidate genes were cloned, as a transcription unit with a strong promotor and terminator (SEQ IDs 71-75), on the plasmid pRS415 and combined with the transcription units pTDH3-LmSUCP-tTDH3 and pTDH3-PGM2-tADH1, to establish the complete optimized sucrose utilization system (see, Table 6). The given promotor sequences were cloned by standard methods directly upstream of the gene start codon and terminator sequences directly downstram of the stop codon of the given gene. Growth complementation tests of BRKY448 harboring those plasmids were performed as described previously, using glass tubes with liquid media. Each experiment was stopped with an end-point OD-measurement after 8 days, or earlier if significant growth occurred before (Table 6). For strains not showing growth at 2% sucrose, the experiment was repeated with 4% sucrose, and AtSWEET14 as positive control. Transcription units for LmSUCP and PGM2 were episomally expressed in BRKY448 without a transporter gene as a negative control, the S288C wildtype without further genetic modifications served as positive control. While growing slower than the wildtype, 9 out of 13 tested uniporter candidates showed growth complementation in YNB with 2% or 4% succrose. Thus, for all uniporter candidate families tested, at least one member each was able to complement growth of BRKY448 if co-expressed with a functional sucrose phosphorylase and phosphoglucomutase (Table 6). None of the uniporter candidates could complement growth of BRKY448 with sucrose as sole carbon source if expressed without a functional phosphorylase (data not shown).

Table 6. Aerobic growth complementation of BRKY448 from sucrose uniporter candidate genes, cloned together with pTDH3-LmSPase-tTDH3 and pTDH3-PGM2-tADH1.

Tested transcription unit (promoter/gene/terminator)	**Function/Protein Family**	**Growth with 2% sucrose (days)***	**Growth with 4% sucrose (days)***
pTDH3-PsSUF1-tTDH3	sucrose uptake facilitator (SUF)	-	5.9 (5 d)
pTDH3-PsSUF4-tTDH3	sucrose uptake facilitator (SUF)	-	-
pTDH3-PvSUF1-tTDH3	sucrose uptake facilitator (SUF)	-	5.0 (6 d)
pTDH3-GmSBP1-tTDH3	sucrose binding protein (SBP)	5.8 (8 d)	ND
pTDH3-GmSBP2-tTDH3	sucrose binding protein (SBP)	-	5.1 (3 d)
pTDH3-AtSWEET10-tTDH3	SWEET transporter	4.0 (7 d)	ND
pTDH3-AtSWEET11-tTDH3	SWEET transporter	4.2 (7 d)	ND
pTDH3-AtSWEET12-tTDH3	SWEET transporter	-	-
pTDH3-AtSWEET13-tTDH3	SWEET transporter	-	ND
pTDH3-AtSWEET14-tTDH3	SWEET transporter	3.4 (8 d)	5.1 (6 d)
pTDH3-AtSWEET15-tTDH3	SWEET transporter	3.2 (8 d)	ND
pTDH3-OzSWEET11-tTDH3	SWEET transporter	-	-
pTDH3-OzSWEET12-tTDH3	SWEET transporter	-	6.1 (3 d)
No transporter	Negative control	-	-
Wildtype	Positive control	6.0 (2 d)	ND

*ND: not determined/ assayed. "-": no detectable growth observed within 8 days.

[00165] For a further characterization, some uniporter candidates were tested in an anaerobic growth complementation assay, in duplicate (Fig. 4). The conditions were as described previously (2% sucrose), but utilizing an anaerobic, closed flask system with separately operable gas and sample lines for the main culture. The main-culture was sparged with nitrogen before and for 2h after inoculation, closed and shaken at 30°C/ 250 rpm. The wildtype strain was capable of reaching the stationary phase after around 20 hours. Several BRKY448 strains were modifed for an optimized uptake system which showed growth complementation after a lag phase of more than 100 h (Fig. 4), in contrast to the negative control lacking a transporter.

[00166] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present disclosure. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[00167] Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the disclosure are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[00168] The terms "a," "an," "the" and similar referents used in the context of describing the disclosure (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (*e.g.,* "such as") provided herein is intended merely to better illuminate the disclosure and does not pose a limitation on the scope of the disclosure otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the disclosure.

[00169] Groupings of alternative elements or embodiments of the disclosure disclosed herein are not to be construed as limitations. Each group member can be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group can be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[00170] Certain embodiments of this disclosure are described herein, including the best mode known to the inventors for carrying out the disclosure. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the disclosure to be practiced otherwise than specifically described herein. Accordingly, this disclosure includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the disclosure unless otherwise indicated herein or otherwise clearly contradicted by context.

[00171] Specific embodiments disclosed herein can be further limited in the claims using consisting of or and consisting essentially of language. When used in the claims, whether as filed or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the disclosure so claimed are inherently or expressly described and enabled herein.

[00172] It is to be understood that the embodiments of the disclosure disclosed herein are illustrative of the principles of the present disclosure. Other modifications that can be employed are within the scope of the disclosure. Thus, by way of example, but not of limitation, alternative configurations of the present disclosure can be utilized in accordance with the teachings herein. Accordingly, the present disclosure is not limited to that precisely as shown and described.

[00173] While the present disclosure has been described and illustrated herein by references to various specific materials, procedures and examples, it is understood that the disclosure is not restricted to the particular combinations of materials and procedures selected for that purpose. Numerous variations of such details can be implied as will be appreciated by those skilled in the art. It is intended that the specification and examples be considered as exemplary, only, with the true scope and spirit of the disclosure being indicated by the following claims. All

references, patents, and patent applications referred to in this application are herein incorporated by reference in their entirety.

What is claimed is:

1. A method of optimizing utilization of an oligosaccharide in a microorganism comprising:

a) providing an oligosaccharide source;

b) expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism;

c) expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide;

d) expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism; and

e) contacting the oligosaccharide source with the microorganism.

2. The method of claim 1, wherein the oligosaccharide source is sucrose, maltose, or cellobiose.

3. The method of claim 1, wherein the oligosaccharide source is selected from the group consisting of: lactose, lactulose, isomaltose, melibiose, and trehalose.

4. The method of claim 1, wherein the microorganism is a bacteria selected from the genera consisting essentially of: Propionibacterium, Propionispira, Clostridium, Bacillus, Escherichia, Pelobacter, or Lactobacillus.

5. The method of claim 1, wherein the microorganism is a eukaryote selected from the group consisting essentially of a yeast, filamentous fungi, protozoa, or algae.

6. The method of claim 1, wherein the microorganism is from a genus selected from the group consisting of: Saccharomyces, Yarrowia, Hansenula, Pichia, Ashbya, and Candida.

7. The method of claim 1, wherein the energy independent oligosaccharide transporter is from a class selected from the group consisting of: sugars will eventually be exported transporter (SWEET) proteins, sucrose binding proteins (SBP), sucrose uptake facilitators (SUF), cellodextrin facilitators and aquaporins.

8. The method of claim 1, wherein the polynucleotides encoding the phosphorylase are genes selected from the group consisting of: *spl* of *Bifidobacterium adolescentis,* sucP of *Leuconostoc mesenteroides*, LVIS_0358 of *Lactobacillus brevis*, mapA of *Lactobacillus sanfranciscensis* and cbp from *Clostridium thermocellum.*

9. The method of claim 1, wherein the phosphoglucomutase is selected from the group consisting of: α-phosphoglucomutase and β-phosphoglucomutase.

10. The method of claim 1, wherein the phosphoglucomutase is selected from the group consisting of: *pgm1* and *pgm2* from *Saccharomyces cerevisiae*, *pgmA* from *Lactobacillus sanfrancisco*, and *pgmB* from *Lactococcus lactis.*

11. The method of claim 1, wherein the oligosaccharide source is contacted with the microorganism prior to expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism; expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide; and expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism.

12. The method of claim 1, wherein the isomer of the phosphorylated saccharide is utilized in a pathway for the production of an organic molecule.

13. The method of claim 1 further comprising knocking-out one or more naturally competing transporter systems for transporting the oligosaccharide into the microorganism.

14. The method of claim 2, wherein the microorganism is *S. cerevisiae* and one or more of suc2, mal11, mal12, mal31, or mal32 may be knocked-out, or one or more of suc2, mal11, mal12, mal31, mal32, mph2, or mph3 may be knocked-out.

15. A microorganism having an optimized system for oligosaccharide utilization comprising:

a) one or more polynucleotides coding for an energy independent oligosaccharide transporter for transporting an oligosaccharide into the microorganism;

b) one or more polynucleotides coding for a phosphorylase for catalyzing the conversion of the oligosaccharide into at least one phosphorylated saccharide; and

c) one or more polynucleotides coding for a phosphoglucomutase for catalyzing the conversion of the phosphorylated saccharide into an isomer of the phosphorylated saccharide, wherein the isomer of the phosphorylated saccharide is utilized in one or more enzymatic pathways in the microorganism.

16. The method of claim 15, wherein the microorganism is a bacteria selected from the genera consisting essentially of: Propionibacterium, Propionispira, Clostridium, Bacillus, Escherichia, Pelobacter, or Lactobacillus.

17. The method of claim 15, wherein the microorganism is a eukaryote selected from the group consisting essentially of a yeast, filamentous fungi, protozoa, or algae.

18. The method of claim 15, wherein the microorganism is from a genus selected from the group consisting of: Saccharomyces, Yarrowia, Hansenula, Pichia, Ashbya, and Candida.

19. A method of optimizing utilization of an oligosaccharide in a microorganism to produce an organic molecule comprising:

providing an oligosaccharide source;

expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism;

expressing one or more polynucleotides in the microorganism that encode a phosphorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide;

expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into a an isomer of the phosphorylated saccharide;

expressing one or more polynucleotides in the microorganism for catalyzing a conversion of the isomer into an organic molecule; and

contacting the oligosaccharide source with the microorganism.

20. The method of claim 19, wherein the organic molecule is selected from the group consisting of: acid, alcohol, alkane, alkene, amide, amine, amino acid, aromatic, carbohydrate,

diacid, dialcohol, diene, ester (incl. waxes), ether, fat (incl. oils), fatty acid, fatty alcohol, ketone, lactam, peptide, protein, steroid, terpene, vitamin.

21. The method of claim 19, wherein the organic molecule is selected from the group consisting of: acetic acid, acrylic acid, 3-hydroxypropionic acid, lactic acid, methacrylic acid; ethanol, isopropanol, 1-propanol, 2-propanol, n-butanol, isobutanol; hexene, propene; hexamethylenediamine; adipic acid, glucaric acid, itaconic acid, malonic acid, succinic acid; 1,2-ethandiol (ethylene glycol), butanediol, 1,4-butanediol, 1,2-propanediol (monopropylen glycol), 1,3-propanediol; butadiene; methyl methacrylate; caprolactam; isoprene, farnesene.

22. A method of increasing the yield of an organic molecule produced by a microorganism under anaerobic conditions comprising:

a) providing an oligosaccharide source for use by the microorganism;

b) expressing one or more polynucleotides in the microorganism that encode an energy independent oligosaccharide transporter for transporting the oligosaccharide into the microorganism;

c) expressing one or more polynucleotides in the microorganism that encode a phsophorylase for catalyzing a conversion of the oligosaccharide into at least one phosphorylated saccharide;

d) expressing one or more polynucleotides in the microorganism that encode a phosphoglucomutase for catalyzing a conversion of the phosphorylated saccharide into an isomer of the phosphorylated sacchardie;

e) expressing one or more polynucleotides in the microorganism for catalyzing a conversion of the isomer into an organic molecule; and

f) contacting the oligosaccharide source with the microorganism.

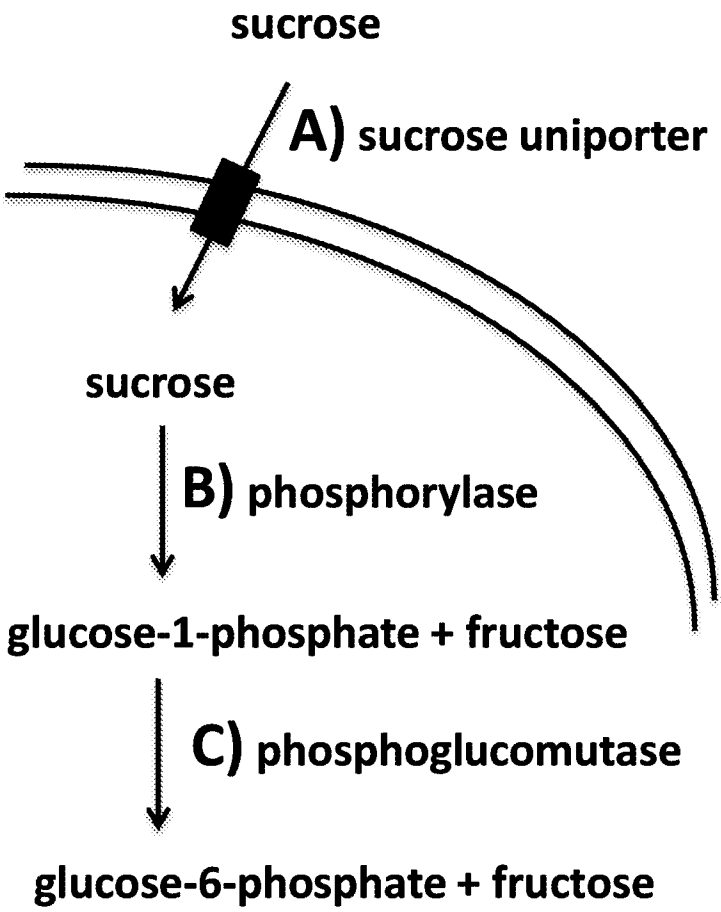
sucrose
A) sucrose uniporter
sucrose
B) phosphorylase
glucose-1-phosphate + fructose
C) phosphoglucomutase
glucose-6-phosphate + fructose

Fig. 1

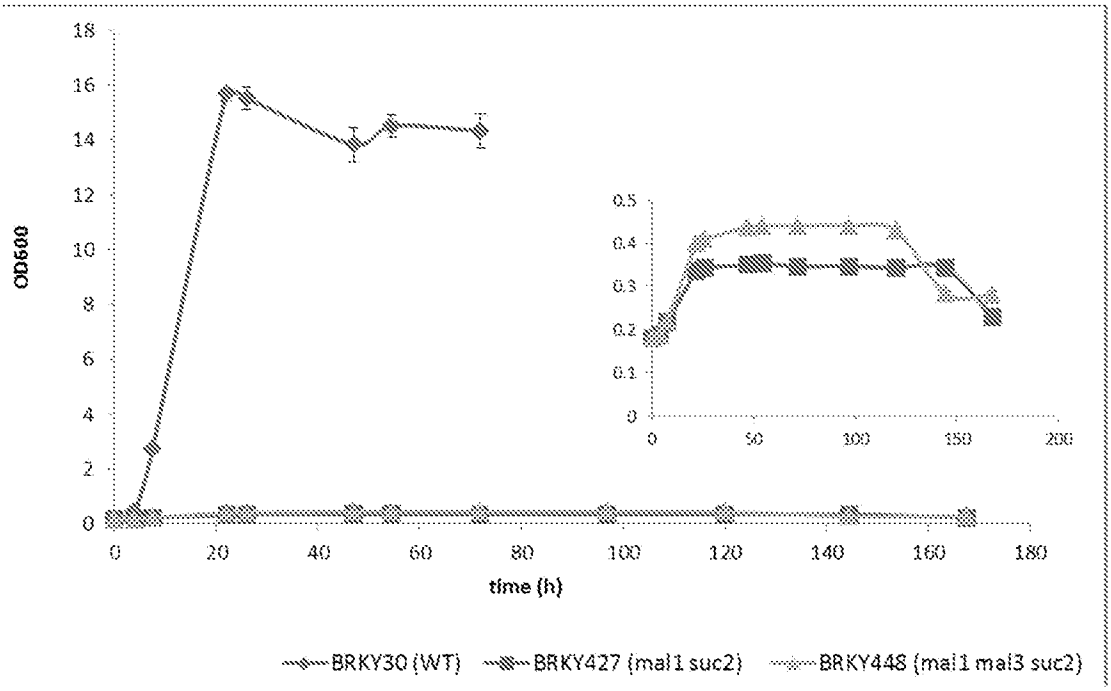
OD600
time (h)
BRKY30 (WT)
BRKY427 (mal1 suc2)
BRKY448 (mal1 mal3 suc2)

Fig. 2

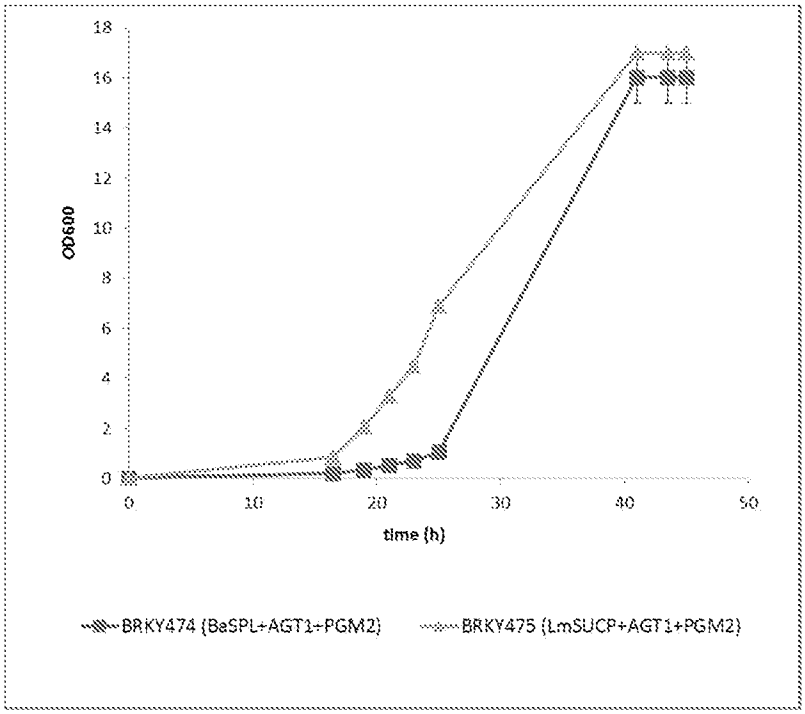
18
16
14
12
10
8
6
4
2
0
OD600
0
10
20
30
40
50
time (h)
BRKY474 (BaSPL+AGT1+PGM2)
BRKY475 (LmSUCP+AGT1+PGM2)

Fig. 3

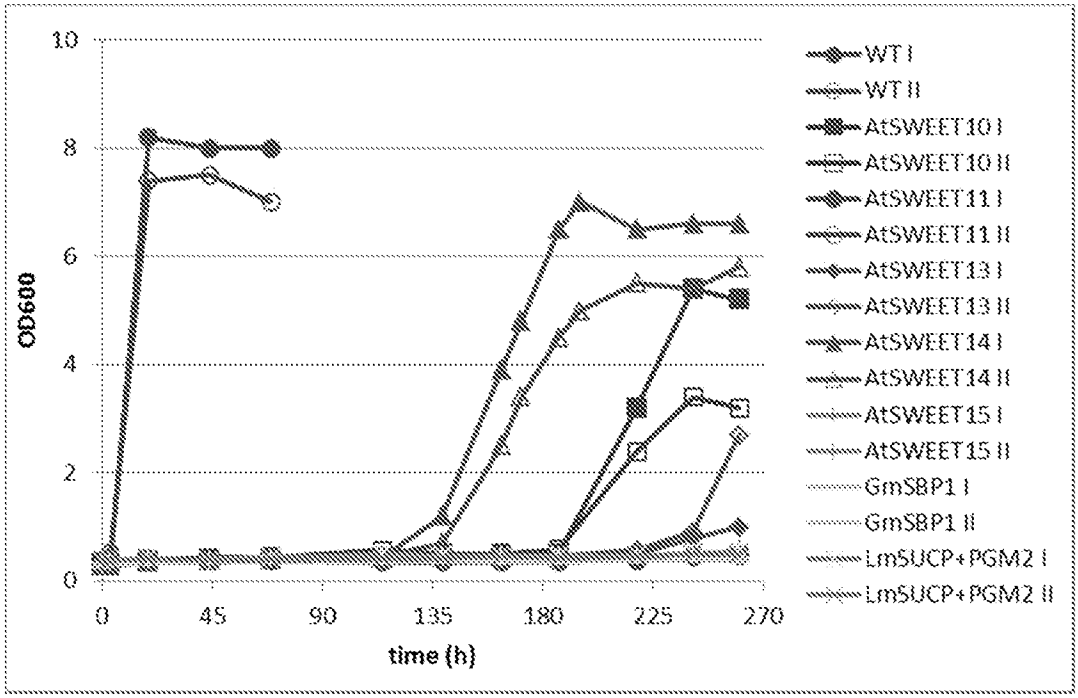
10
8
6
4
2
0
OD600
0
45
90
135
180
225
270
time (h)
WT I
WT II
AtSWEET10 I
AtSWEET10 II
AtSWEET11 I
AtSWEET11 II
AtSWEET13 I
AtSWEET13 II
AtSWEET14 I
AtSWEET14 II
AtSWEET15 I
AtSWEET15 II
GmSBP1 I
GmSBP1 II
LmSUCP+PGM2 I
LmSUCP+PGM2 II

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/33286

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 19/18, C12P 19/12, C12N 1/26, C12P 19/00 (2015.01)
CPC - C12P 19/18, C12P 19/12, C12N 1/26, C12P 19/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (8): C12P 19/18, C12P 19/12, C12N 1/26, C12P 19/00 (2015.01)
CPC: C12P 19/18, C12P 19/12, C12N 1/26, C12P 19/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/97; 435/248, 435/253.6, 435/254.11, 435/100, 435/72, 435/243

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Google patents, Google scholar, Google web, PatBase, Proquest Dialog
microorganism/microbe/bacteria/yeast/fungi/algae/Saccharomyces; oligosaccharide/saccharide/sugar/sucrose; oligosaccharide transporter; phosphorylase/spl/sucP/mapA/cbp; phosphoglucomutase/ pgm1/pgm2/ pgmA/pgmB; transgenic/recombinant/engineered

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/109274 A1 (DOUDNA et al.) 16 August 2012 (16.08.2012) para [0325]; para [0010]; para [0011]; claim 265; para [0009]; para [0026]; para [0077]; para [0217]; para [0098]; para [0309]; para [0268]; para [0161]; para [0048]-para [0325]; para [0203]; para [0204]; para [0242]; claim 145; para [0327]; para [0050]	1-22
Y	ZHOU et al. A suite of sucrose transporters expressed in coats of developing legume seeds includes novel pH-independent facilitators. Plant J. February 2007. Vol. 49. No. 4. pp 750-764 especially, Abstract	1-22
Y	US 2006/0275319 A1 (MULLER et al.) 07 December 2006 (07.12.2006) Abstract; para [0068]	13-14

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 August 2015 (05.08.2015)	25 AUG 2015
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/33286

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments: