(11) Application No. AU 2009204313 A2

(19) AUSTRALIAN PATENT OFFICE

(54) Title
Transgenic photosynthetic microorganisms and photobioreactor

(51)[6] International Patent Classification(s)
C12M 1/00 (2006.01) 6BHUS C12N 1/21 20060101ALI2009071
C12N 1/13 (2006.01)
C12N 1/21 (2006.01)
C12N 15/00 (2006.01) 6BHUS C12N 15/00 20060101ALI2009071
C12P 19/00 (2006.01) 6BHUS C12P 19/00 20060101ALI2009071
C12M 1/00 20060101AFI2009071 6BHUS C12N 1/13 20060101ALI2009071 6BHUS PCT/US2009/030162

(21) Application No: 2009204313 (22) Application Date: 2009.01.05

(87) WIPO No: WO09/089185

(30) Priority Data

(31) Number	(32) Date	(33) Country
61/018,798	2008.01.03	US
61/085,797	2008.08.01	US

(43) Publication Date : 2009.07.16

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 July 2009 (16.07.2009)

PCT

(10) International Publication Number
WO 2009/089185 A1

(51) International Patent Classification:
C12M 1/00 (2006.01) *C12N 15/00* (2006.01)
C12N 1/21 (2006.01) *C12P 19/00* (2006.01)
C12N 1/13 (2006.01)

(21) International Application Number:
PCT/US2009/030162

(22) International Filing Date: 5 January 2009 (05.01.2009)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/018,798 3 January 2008 (03.01.2008) US
61/085,797 1 August 2008 (01.08.2008) US

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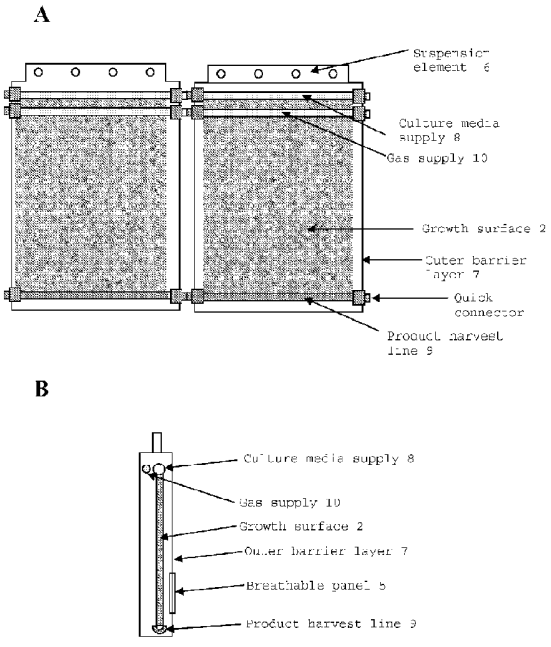
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM,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

[Continued on next page]

(54) Title: TRANSGENIC PHOTOSYNTHETIC MICROORGANISMS AND PHOTOBIOREACTOR

FIG. 12



(57) Abstract: Provided herein is a transgenic bacteria engineered to accumulate carbohydrates, for example disaccharides. Also provided is a photobioreactor for cultivating photosynthetic microorganisms comprising a non-gelatinous, solid cultivation support suitable for providing nutrients and moisture to photosynthetic microorganisms and a physical barrier covering at least a portion of the surface of the cultivation support. Devices for the large scale and continuous cultivation of photosynthetic microorganisms incorporating photobioreactors and methods of use are disclosed. Also disclosed are methods of producing fermentable sugar from photosynthetic microorganisms using a photobioreactor of the invention.

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European (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, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

TITLE OF THE INVENTION

TRANSGENIC PHOTOSYNTHETIC MICROORGANISMS AND PHOTOBIOREACTOR

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Prov. App. Ser. No. 61/085,797 (filed 01 August 2008) and U.S. Prov. App. Ser. No. 61/018,798 (filed 03 January 2008), each of which are incorporated herein by reference in their entirety.

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN COMPUTER READABLE FORM

[0002] The Sequence Listing, which is a part of the present disclosure, includes a computer readable form and a written sequence listing comprising nucleotide and/or amino acid sequences of the present invention. The sequence listing information recorded in computer readable form is identical to the written sequence listing. The subject matter of the Sequence Listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention generally relates to transgenic microorganisms and methods and devices for their cultivation.

BACKGROUND

[0004] To address the world's increasing energy requirements, efficient and environmentally sound alternatives to the use of fossil fuels are sought after. Alternative fuels, such as ethanol or biodiesel, can be produced from plant biomass. For example, the key ingredient used to produce ethanol from current processes is termed fermentable sugar. Most often, fermentable sugar is in the form of sucrose, glucose, or high-fructose corn syrup. Plants currently grown to produce such biomass include corn, sugarcane, soybeans, canola, jatropha, and so forth. But much of the plant biomass used to produce fermentable sugar requires extensive energy-intensive pre-processing. Further, use of such plant biomass can lead to soil depletion, erosion, and diversion of the food supply.

[0005] It is known that some cyanobacteria produce sucrose through the action of sucrose phosphate synthase and sucrose phosphate phosphatase, where it has been studied exclusively as an osmoprotectant. With respect to salt tolerance, cyanobacteria can be divided into three groups. Strains having low tolerance (less than 700 mM) synthesize either sucrose, as is the case with Synechococcus elongatus PCC 7942, or another dissaccharide known as trehalose [Blumwald et al., Proc Natl Acd Sci USA (1983) 80:2599-2602 and Reed et al., FEMS Microbiol Rev (1986) 39:51-56]. Glucosylglycerol is produced by strains having moderate halotolerance (0.7-1.8 mM), such as Synechocystis sp. PCC 6803. High salt tolerance (up to 2.5 M) results from the accumulation of either glycine betaine or glutamate betaine. Miao et al. [FEMS Microbiol Lett (2003) 218:71-77] determined that when glucosylglycerol biosynthesis is blocked by deletion of the agp gene, however, Synechocystis sp. PCC 6803 produces sucrose as its osmoprotectant. Desiccation tolerant cyanobacteria also produce sucrose and trehalose in response to matric water stress [Hershkovitz et al., Appl Environ Microbiol (1991) 57:645-648].

[0006] *Synechocystis* spp. PCC 6803 (ATCC 27184) and *Synechococcus elongatus* PCC 7942 (ATCC 33912) are relatively well-studied, have genetic tools available and the sequences of their genomes are known (*see e.g.*, Koksharova, O. A. and·Wolk, C. P. 2002. Appl Microbiol Biotechnol 58, 123–137; Ikeuchi1, M. and Satoshi Tabata, S. 2001. Photosynthesis Research 70, 73–83; Golden, S. S., Brusslan, J. and Haselkorn, R. 1987. Methods in Enzymology 153, 215-231; Friedberg, D. 1988. Methods in Enzymology 167, 736-747; Kaneko, T. *et al.* 1996. DNA Research 3, 109-136).

[0007] The commercial cultivation of photosynthetic microorganisms such as Spirulina maximum, Spirulina platensis, Dunaliella salina, Botrycoccus braunii, Chlorella vulgaris, Chlorella pyrenoidosa, Serenastrum capricomutum, Scenedesmus auadricauda, Porphyridium cruentum, Scenedesmus acutus, Dunaliella sp., Scenedesmus obliquus, Anabaenopsis, Aulosira, Cylindrospermum, Scenecoccus sp., Scenecosystis sp., and Tolypothrix is desirable for numerous applications including the production of fine chemicals, pharmaceuticals, cosmetic pigments, fatty acids, antioxidants, proteins with prophylactic action, growth factors, antibiotics, vitamins and polysaccharides. The algic biomass can also be useful, in a low dose, to replace or decrease the level of antibiotics in animal food or be useful as a source of proteins. Furthermore, the algic biomass provided in a wet form, as opposed to a dried

form, can be fermented or liquefied by thermal processes to produce fuel. Thus, there is great interest in the ability to increase the efficiency of cultivating such organisms.

[0008] In general, current photosynthetic bioreactors rely on the cultivation of microorganisms in a liquid phase system to produce biomass. These systems are usually open-air pond-type reactors or enclosed tank-type reactors. Enclosed bioreactors, however, typically are considered to be an improvement over pond type reactors in many respects. Importantly, enclosed systems provide a barrier against environmental contamination. In addition, these systems allow for greater control of temperature and gas content of the liquid media.

[0009] Still, the uses of enclosed photobioreactors tend to be limited by photosynthetic microorganisms' requirement for light (*i.e.*, actinic radiation provides the energy required by photosynthetic microorganisms to fix carbon dioxide into organic molecules). Thus, sufficient illumination of the photosynthetic microorganisms is an unyielding requirement. Nevertheless, as the cell density in a liquid phase photobioreactor increases, the ability of light to penetrate into the media decreases, which typically limits the cell density that may be achieved. Additionally, some type of agitation of the liquid media is generally required to prevent unwanted sedimentation of the organisms, a process that requires the input of energy.

[0010] Numerous attempts have been made to devise a method of bringing light to the organisms in liquid phase systems. For example, some systems involve circulating the liquid culture media through transparent tubes. Other attempts involve placing a light source within the media or introducing reflecting particles into the culture media to adjust the radiation absorbance of the culture. Despite these efforts, a significant increase in the ability to culture organisms in liquid phase systems at higher cell densities has not yet been achieved.

[0011] In addition to the aforementioned light requirement, the use of liquid phase photobioreactors has been burdened with providing the photosynthetic microorganisms enough carbon dioxide for photosynthesis. Typically, these systems generally incorporate some type of additional aeration system to increase the concentration of carbon dioxide dissolved in the media. Eliminating the need for aeration would greatly simplify the system thus reducing operating costs.

[0012] Liquid phase photobioreactors also tend not to be well suited for conventional methods of continuous production. In general, the transportation of large volumes of liquid is complex and burdensome. Further, because liquid phase systems usually require mechanisms for circulation, agitation, aeration, and the like, it is generally simpler and more cost effective to operate only one or a few large cultivation devices rather than numerous smaller ones. Therefore, currently practiced methods involve processing relatively large batches (*i.e.*, a batch of photosynthetic microorganisms is cultivated and the entire resulting biomass is then harvested).

[0013] Thus, there is a great need in the art for advancement in photosynthetic bioreactor design. Providing a new type of photosynthetic bioreactor capable of efficiently cultivating and harvesting relatively high densities of photosynthetic microorganisms without large volumes of water or other liquid media, without the aforementioned extraordinary measures for supplying adequate light and carbon dioxide, and at a reasonable cost would represent a substantial advance in the art, and benefit industry and consumers alike.

SUMMARY OF THE INVENTION

[0014] Provided herein is a transgenic bacteria engineered to accumulate carbohydrates, for example disaccharides. Also provided is a photobioreactor for cultivating photosynthetic microorganisms comprising a non-gelatinous, solid cultivation support suitable for providing nutrients and moisture to photosynthetic microorganisms and a physical barrier covering at least a portion of the surface of the cultivation support. Devices for the large scale and continuous cultivation of photosynthetic microorganisms incorporating photobioreactors and methods of use are disclosed. Also disclosed are methods of producing fermentable sugar from photosynthetic microorganisms using a photobioreactor of the invention.

[0015] One aspect provides a photobioreactor for cultivating photosynthetic microorganisms. The photobioreactor comprises a non-gelatinous, solid cultivation support suitable for providing nutrients and moisture to photosynthetic microorganisms on at least a portion of a surface thereof, wherein said portion of the surface has a topography that allows photosynthetic microorganisms to adhere thereto when said portion of the surface is oriented non-horizontally; and a physical barrier covering at least said portion of the surface of the

cultivation support, wherein the physical barrier is configured so as to allow inoculation of said portion of the surface of the cultivation support, formation and maintenance of an environment suitable for the cultivation of such photosynthetic microorganisms, and harvesting of such cultivated photosynthetic microorganisms.

[0016] In some embodiments, the photobioreactor comprises photosynthetic microorganisms on said portion of the surface of the cultivation support. In some embodiments, the photobioreactor further comprises a cell engineered to accumulate a disaccharide, as described further below, wherein the cell is adhered to the solid cultivation support. In some embodiments, said portion of the surface of the cultivation support is capable of cultivating photosynthetic microorganisms at a density of at least about 50 grams of dry biomass per liter equivalent.

[0017] In some embodiments, the cultivation support is flexible. In some embodiments, the cultivation support comprises one or more rigid materials. In some embodiments, the cultivation support of the photobioreactor comprises at least two layers, a first layer adjacent to a second layer, wherein material of the at least two layers is the same material or different materials. In some embodiments, the first layer comprises a high surface area growth material and the second layer a permeable type material. In some embodiments, the cultivation support of the photobioreactor comprises flexibly connected rigid portions, wherein the rigid portions are comprised of the one or more rigid materials. In some embodiments, the photobioreactor comprises a single cultivation support. In some embodiments, the photobioreactor comprises a plurality of cultivation supports.

[0018] In some embodiments, the cultivation support comprises a fabric. In some embodiments, the fabric is comprised of fibers that are natural, modified natural, synthetic, or a combination thereof. In some embodiments, the fabric is a woven fabric, a knitted fabric, a felt, a mesh of cross-linked fiber polymers, or a combination thereof. In some embodiments, the natural fibers are selected from the group consisting of cotton, wool, hemp, tree fiber, other cellulosic fibers, and combinations thereof. In some embodiments, the modified natural fibers are selected from the group consisting of nitrocellulose, cellulose acetate, cellulose sulfonate, crosslinked starches, and combinations thereof. In some embodiments, the synthetic fibers are selected from the group consisting of polyester, polyacrylate, polyamine, polyamide, polysulfone, and combinations thereof.

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[0019] In some embodiments, the cultivation support is coated with a moisture absorbent polymer. In some embodiments, the fabric, the fiber of the fabric, or both, are coated with a moisture absorbent polymer. In some embodiments, the moisture absorbent polymer is selected from the group consisting of agar, polyacrylate, polyamide, polyamine, polyethylene glycol, modified starches, and combinations thereof.

[0020] In some embodiments, the physical barrier of the photobioreactor is at least substantially impermeable to solid particulate and liquid but does not prevent the transport of gas or vapor to and from the space proximate to said portion of the surface of the cultivation support nor actinic irradiation of said portion of the surface of the cultivation support. In some embodiments, the physical barrier is sufficiently impermeable to water vapor so that the cultivation support upon being moistened will retain enough of the moisture so the photosynthetic microorganisms remain adequately hydrated during cultivation. In some embodiments, the barrier is configured to enclose the cultivation support and any photosynthetic microorganisms thereon, and to be releasably sealed during at least a portion of the cultivation of the photosynthetic microorganisms. In some embodiments, the physical barrier is flexible. In some embodiments, the physical barrier further comprises a first portion that is at least substantially impermeable to solid particulate, liquid, gas, and vapor, and a second portion that is permeable to gas and vapor but at least substantially impermeable to solid particulate and liquid. In some embodiments, the second portion of the barrier has a gas or vapor exchange rate that is from at least about 5 Gurley seconds to no greater than about 10,000 Gurley seconds. In some embodiments, the second portion of the barrier comprises a selective membrane comprising olefin fiber or polyethylene fiber material, polytetrafluoroethylene filtration media, cellulosic filter material, fiberglass filter material, polyester filter material, polyacrylate filter material, polysulfone membranes, or nylon membranes. In some embodiments, the first portion is at least substantially transparent to actinic radiation and the second portion is not at least substantially transparent to actinic radiation, and the configuration of the first and second portions relative to each other and at least said portion of the surface of the cultivation support is such that there a sufficient amount of actinic radiation and gas exchange to support photosynthesis by photosynthetic microorganisms.

[0021] In some embodiments, the photobioreactor further comprises a source of actinic radiation situated between the cultivation support and the physical barrier. In some embodiments, the physical barrier is between the cultivation support and a source of actinic radiation and is sufficiently transparent to such actinic radiation and sufficiently gas permeable to allow for photosynthesis by the photosynthetic microorganisms during cultivation.

[0022] In some embodiments, the photobioreactor further comprises water, nutrients, or a combination thereof on, within, or on and within, the cultivation support. In some embodiments, the photobioreactor further comprises one or more attachment points for attaching the photobioreactor to a structure. In some embodiments, the solid cultivation support further comprises one or more attachment points for attaching the cultivation support. In some embodiments, the photobioreactor further comprises at least one of a fluid supply system, a nutrient supply system, a gas supply system, and a microorgansim supply system.

[0023] Another aspect provides a device for cultivating photosynthetic microorganisms. Such device comprises at least one photobioreactor as described above, and a structure to which the at least one photobioreactor is attached that orientates at least one cultivation support of the at least one photobioreactor non-horizontally. In some embodiments, the at least one photobioreactor is suspended from the structure. In some embodiments, the structure is substantially covered by the physical barrier. In some embodiments, the structure comprises a conveyor system or a component thereof such that the at least one cultivation support is capable of being conveyed along the path of the conveyor system. In some embodiments, the device further comprises one, two, or three of the following: an inoculation station such that each cultivation support as it is conveyed along the path of the conveyor system may be inoculated with photosynthetic microorganisms; a cultivating station such that the photosynthetic microorganisms on each inoculated cultivation support are cultivated as each cultivation support is conveyed along the path of the conveyor system; and a harvesting station to which the cultivation support is conveyed so that at least a portion of the cultivated photosynthetic microorganisms may be harvested from each cultivation support. In some embodiments, the inoculation station and the harvesting station are substantially adjacent to each other or are substantially coextensive. In some embodiments, the device further comprises an inducing station for inducing the synthesis of fermentable sugar by photosynthetic

microorganisms on each cultivation support. In some embodiments, the device futher comprises at least one of a fluid supply system, a nutrient supply system, a gas supply system, or a microorgansim supply system. In some embodiments, the device further comprises a photosynthetic microorganisms adhered on the solid cultivation support. In some embodiments, the device further comprises a cell engineered to accumulate a disacharide, as described further below, wherein the cell is adhered to the solid cultivation support.

[0024] Another aspect provides a transgenic photosynthetic microorganism cell engineered to accumulate a disaccharide. The transgenic photosynthetic microorganism cell comprises, as operably associated components in the 5' to 3' direction of transcription: a promoter functional in the photosynthetic microorganism cell; a polynucleotide comprising a nucleotide sequence encoding a polypeptide having a disaccharide biosynthetic activity selected from the group consisting of a disaccharide phosphate synthase and a disaccharide phosphate phosphatase; and a transcriptional termination sequence; wherein the transgenic photosynthetic microorganism cell accumulates increased levels of the disaccharide compared to a photosynthetic microorganism cell not comprising the DNA construct.

[0025] In some embodiments, the transgenic photosynthetic microorganism cell comprises a polynucleotide comprising a first nucleotide sequence encoding a polypeptide having disaccharide phosphate synthase activity and a second nucleotide sequence encoding a polypeptide having disaccharide phosphate phosphatase activity. In some embodiments, the comprises a polynucleotide comprising a nucleotide sequence encoding a polypeptide having disaccharide phosphate synthase activity and disaccharide phosphate phosphatase activity. In some embodiments, the comprises a a first nucleotide sequence encoding a polypeptide having disaccharide phosphate synthase activity; a second nucleotide sequence encoding a polypeptide having disaccharide phosphate phosphatase activity; and a third nucleotide sequence encoding a polypeptide having disaccharide phosphate synthase activity and disaccharide phosphate phosphatase activity.

[0026] In some embodiments, the polynucleotide of the transgenic photosynthetic microorganism cell is selected from the group consisting of: (a) a polynucleotide comprising a nucleotide sequence encoding a polypeptide selected from the group consisting of: SEQ ID NO: 2 or a sequence 95% identical thereto having sucrose phosphate synthase and sucrose phosphate

phosphatase (ASF) activity; SEQ ID NO: 4 or a sequence 95% identical thereto having sucrose phosphate synthase (SPS) activity; SEQ ID NO: 6 or a sequence 95% identical thereto having a sucrose phosphate phosphatase (SPP) activity; SEQ ID NO: 77 or a sequence 95% identical thereto having trehalose phosphate synthase (TPS) activity; SEQ ID NO: 79 or a sequence 95% identical thereto having trehalose phosphate phosphatase (TPP) activity; SEQ ID NO: 81 or a sequence 95% identical thereto having glucosylglycerol phosphate synthase (GPS) acitivity; SEQ ID NO: 83 or a sequence 95% identical thereto having glucosylglycerol phosphate phosphatase (GPP) activity; SEQ ID NO: 85 or a sequence 95% identical thereto having mannosylfructose phosphate synthase (MPS) activity; and SEQ ID NO: 87 or a sequence 95% identical thereto having mannosylfructose phosphate phosphatase (MPP) activity; (b) an isolated polynucleotide comprising SEQ ID NO: 1 or a sequence 95% identical thereto encoding sucrose phosphate synthase / sucrose phosphate phosphatase (ASF) activity; SEQ ID NO: 3 or a sequence 95% identical thereto encoding sucrose phosphate synthase (SPS) activity; SEQ ID NO: 5 or a sequence 95% identical thereto encoding sucrose phosphate phosphatase (SPP) activity; SEQ ID NO: 76 or a sequence 95% identical thereto encoding trehalose phosphate synthase (TPS) activity; SEQ ID NO: 78 or a sequence 95% identical thereto encoding trehalose phosphate phosphatase (TPP) activity; SEQ ID NO: 80 or a sequence 95% identical thereto encoding glucosylglycerol phosphate synthase (GPS) acitivity; SEQ ID NO: 82 or a sequence 95% identical thereto encoding glucosylglycerol phosphate phosphatase (GPP) activity; SEQ ID NO: 84 or a sequence 95% identical thereto encoding mannosylfructose phosphate synthase (MPS) activity; and SEQ ID NO: 86 or a sequence 95% identical thereto encoding mannosylfructose phosphate phosphatase (MPP) activity; (c) an isolated polynucleotide that hybridizes under stringent conditions to a nucleic acid sequence selected from the group consisting of: SEQ ID NO: 1, wherein the isolated polynucleotide encodes a polypeptide having ASF activity; SEQ ID NO: 3, wherein the isolated polynucleotide encodes a polypeptide having SPS activity; SEQ ID NO: 5, wherein the isolated polynucleotide encodes a polypeptide having SPP activity; SEQ ID NO: 76, wherein the isolated polynucleotide encodes a polypeptide having TPS activity; SEQ ID NO: 78, wherein the isolated polynucleotide encodes a polypeptide having TPP activity; SEQ ID NO: 80, wherein the isolated polynucleotide encodes a polypeptide having GPS activity; SEQ ID NO: 82, wherein the isolated polynucleotide encodes a polypeptide having

GPP activity; SEQ ID NO: 84, wherein the isolated polynucleotide encodes a polypeptide having MPS activity; SEQ ID NO: 86, wherein the isolated polynucleotide encodes a polypeptide having MPP activity; wherein said stringent conditions comprise incubation at 65°C in a solution comprising 6X SSC (0.9 M sodium chloride and 0.09 M sodium citrate); and (d) an isolated polynucleotide complementary to the polynucleotide sequence of (a), (b), or (c).

[0027] In some embodiments, monomers of the accumulated disaccharide are endogenous to the cell. In some embodiments, a monomer(s) of the accumulated disaccharide are exogenous to the cell and expression of such monomer(s) is engineered into the cell.

[0028] In some embodiments, the cell is a cyanobacterium cell, a photosynthetic bacteria; or a green algae. In some embodiments, the cell is a cyanobacterium cell. In some embodiments, the cell is a cyanobacterium selected from the group consisting of Synechococcus and Synechocystis.

[0029] In some embodiments, the promoter is an inducible promoter. In some embodiments, the promoter is iducible by an agent selected from the group consisting of temperature, pH, a metabolite, light, an osmotic agent, a heavy metal, and an antibiotic. In some embodiments, the promoter is selected from the group consisting of *carB*, *nirA*, *psbAII*, *dnaK*, *kaiA*, and λ_{PR}.

[0030] In some embodiments, the DNA construct of the cell comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 19 (pLybAL11 encoding *asf*); SEQ ID NO: 20 (pLybAL12 encoding *asf*); SEQ ID NO: 44 (pLybAL15 encoding *asf*); SEQ ID NO: 45 (pLybAL16 encoding *asf*); SEQ ID NO: 46 (pLybAL17 encoding *asf*); SEQ ID NO: 47 (pLybAL18 encoding *asf*); SEQ ID NO: 48 (pLybAL19 encoding *asf*); SEQ ID NO: 49 (pLybAL21 encoding *asf*); SEQ ID NO: 50 (pLybAL22 encoding *asf*); SEQ ID NO: 51 (pLybAL13f encoding *asf*); SEQ ID NO: 52 (pLyAL13r encoding *asf*); SEQ ID NO: 53 (pLybAL14f encoding *asf*); SEQ ID NO: 54 (pLybAL14r encoding *asf*); SEQ ID NO: 65 (pLybAL7f encoding *asf*); SEQ ID NO: 69 (pLybAL8f encoding *asf*); SEQ ID NO: 118 (pLybAL23 encoding *tps* and *tpp*); SEQ ID NO: 121 (pLybAL28 encoding *tps* and *tpp*); SEQ ID NO: 122 (pLybAL29 encoding *tps* and *tpp*); SEQ ID NO: 123 (pLybAL30 encoding *tps* and *tpp*); SEQ ID NO: 124 (pLybAL31 encoding *tps* and *tpp*); SEQ ID NO: 125 (pLybAL36

encoding *tps* and *tpp*); SEQ ID NO: 126 (pLybAL37 encoding *tps* and *tpp*); SEQ ID NO: 130 (pLybAL24 encoding *tps* and *tpp*); and SEQ ID NO: 133 (pLybAL33 encoding *tps* and *tpp*).

[0031] In some embodiments, the cell accumulates at least about 0.1 micrograms of the disaccharide per minute per gram dry biomass. In some embodiments, the cell accumulates at least about 0.1 micrograms of the disaccharide per minute per gram dry biomass up to about 10 micrograms of the disaccharide per minute per gram dry biomass.

[0032] In some embodiments, the cell does not comprise a nucleotide sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, and SEQ ID NO: 74, or a nucleotide variant thereof having at least 95% identity thereto and invertase activity or sucraseferridoxin activity. In some embodiments, the cell does not express a polypeptide sequence selected from the group consisting of SEQ ID NO: 71, SEQ ID NO: 73, and SEQ ID NO: 75, or a polypeptide variant thereof having at least 95% identity thereto and invertase activity or sucraseferridoxin activity. In some embodiments, the cell expresses a small interfering RNA specific a nucleotide sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, and SEQ ID NO: 74, or a nucleotide variant thereof having at least 95% identity thereto and invertase activity or sucraseferridoxin activity.

[0033] In some embodiments, the cell further comprises an isolated polynucleotide comprising SEQ ID NO: 94 or a sequence 95% identical thereto encoding an active porin polypeptide; an isolated polynucleotide encoding a polypeptide comprising SEQ ID NO: 95 or a sequence 95% identical thereto and having porin activity; or an isolated polynucleotide comprising SEQ ID NO: 91 (pLybAL32 encoding a porin); wherein the accumulated disaccacharide is sucrose, the cell expresses porin, and the expressed porin secretes the accumulated sucrose from the cell.

[0034] Another aspect provides an artificial DNA construct. In some embodiments, the artificial DNA construct comprises at least one sequence selected from the group consisting of SEQ ID NO: 19 (pLybAL11 encoding *asf*); SEQ ID NO: 20 (pLybAL12 encoding *asf*); SEQ ID NO: 44 (pLybAL15 encoding *asf*); SEQ ID NO: 45 (pLybAL16 encoding *asf*); SEQ ID NO: 46 (pLybAL17 encoding *asf*); SEQ ID NO: 47 (pLybAL18 encoding *asf*); SEQ ID NO: 48 (pLybAL19 encoding *asf*); SEQ ID NO: 49 (pLybAL21 encoding *asf*); SEQ ID NO: 50

(pLybAL22 encoding *asf*); SEQ ID NO: 51 (pLybAL13f encoding *asf*); SEQ ID NO: 52 (pLyAL13r encoding *asf*); SEQ ID NO: 53 (pLybAL14f encoding *asf*); SEQ ID NO: 54 (pLybAL14r encoding *asf*); SEQ ID NO: 65 (pLybAL7f encoding *asf*); SEQ ID NO: 69 (pLybAL8f encoding *asf*); SEQ ID NO: 118 (pLybAL23 encoding *tps* and *tpp*); SEQ ID NO: 121 (pLybAL28 encoding *tps* and *tpp*); SEQ ID NO: 122 (pLybAL29 encoding *tps* and *tpp*); SEQ ID NO: 123 (pLybAL30 encoding *tps* and *tpp*); SEQ ID NO: 124 (pLybAL31 encoding *tps* and *tpp*); SEQ ID NO: 125 (pLybAL36 encoding *tps* and *tpp*); SEQ ID NO: 126 (pLybAL37 encoding *tps* and *tpp*); SEQ ID NO: 130 (pLybAL24 encoding *tps* and *tpp*); SEQ ID NO: 133 (pLybAL33 encoding *tps* and *tpp*); SEQ ID NO: 91 (pLybAL32 encoding a porin); SEQ ID NO: 102 (pLybAL3f encoding SS-UPP); SEQ ID NO: 103 (pLybAL5f encoding SE-UPP); SEQ ID NO: 106 (pLybAL4f encoding SE-UPP); SEQ ID NO: 107 (pLybAL9f encoding SE-UPP); SEQ ID NO: 109 (pLybAL6fb encoding SE-UPP); SEQ ID NO: 110 (pLybAL10fb encoding SE-UPP); and SEQ ID NO: 91 (pLybAL32 encoding a porin).

[0035] Another aspect provides a method of cultivating a photosynthetic microorganism. The method of cultivating a photosynthetic microorganism can use any of photobioreactor or device described above. The method comprises inoculating a cultivation support with photosynthetic microorganisms; cultivating the photosynthetic microorganisms on the inoculated cultivation support; and harvesting at least a portion of the cultivated photosynthetic microorganisms from the cultivation support. In some embodiments, the method further comprises sealing the physical barrier of the photobioreactor after the inoculation of the cultivation support such that all or a substantial portion of the cultivation of the photosynthetic microorganisms occurs while the physical barrier is sealed. In some embodiments, the physical barrier is releasably sealed. In some embodiments, the method further comprises conveying each cultivation support to an inoculation station, a cultivation station, and a harvesting station. In some embodiments, the method further comprises at least one of: supplying fluid to the cultivation support; supplying nutrients to the cultivation support; or supplying gas to the cultivation support. In some embodiments, the photosynthetic microorganisms are cultivated to a density of at least about 50 grams of dry biomass per liter equivalent. In some embodiments, the photosynthetic microorganisms comprise a transgenic photosynthetic microorganism engineered to accumulate a disaccharide, as described above.

[0036] Another aspect provides a method of producing a fermentable sugar. The method producing a fermentable sugar can use any of photobioreactor or device described above. The method of producing a fermentable sugar comprises inoculating a cultivation support with photosynthetic microorganisms capable of accumulating a fermentable sugar; cultivating the photosynthetic microorganisms on the inoculated cultivation support; isolating accumulated fermentable sugar. In some embodiments, the fermentable sugar accumulates within the photosynthetic microorganisms. In some embodiments, isolating the accumulated fermentable sugar comprises: harvesting at least a portion of the cultivated photosynthetic microorganisms from cultivation support; and recovering the fermentable sugars from the harvest. In some embodiments, the accumulated fermentable sugar is secreted from the photosynthetic microorganisms and isolated from a cultivation media. In some embodiments, isolating the accumulated fermentable sugar comprises isolating the accumulated fermentable sugar from a cultivation media. In some embodiments, the method further comprises releasably sealing the physical barrier of the photobioreactor after the inoculation of the cultivation support such that all or a substantial portion of the cultivation of the photosynthetic microorganisms occurs while the physical barrier is sealed. In some embodiments, the method further comprises at least one of: supplying fluid to the cultivation support; supplying nutrients to the cultivation support; or supplying gas to the cultivation support. In some embodiments, the method further comprises conveying the cultivation support to at least one of an inoculation station, a cultivation station, and a harvesting station.

[0037] In some embodiments, the method further comprises inducing synthesis of the fermentable sugar by the photosynthetic microorganisms. In some embodiments, inducing synthesis of the fermentable sugar comprises exposing the photosynthetic microorganism to an inducing agent selected from the group consisting of temperature, pH, a metabolite, light, an osmotic agent, a heavy metal, and an antibiotic. In some embodiments, inducing synthesis of the fermentable sugar comprises treating the photosynthetic microorganisms with a salt compound. In some embodiments, the salt compound is sodium chloride. In some embodiments, the salt compound is added at a concentration of between about 0.01 mM and 1.5 M or between about 0.2 and 0.9 M. In some embodiments, the inducing agent is applied to the growth surface by aerosol spray. In some embodiments, the photosynthetic microorganisms are cultivated to a

density of at least about 50 grams of dry biomass per liter equivalent. In some embodiments, the fermentable sugar comprises at least one sugar selected from the group consisting of glucose, fructose, sucrose, trehalose, glucosylglyerol, and mannosylfructose. In some embodiments, the fermentable sugar comprises at least one sugar selected from the group consisting of sucrose and trehalose.

[0038] In some embodiments, the photosynthetic microorganisms comprise naturally occurring photosynthetic microorganisms. In some embodiments, the photosynthetic microorganisms comprise genetically modified photosynthetic microorganisms. In some embodiments, the photosynthetic microorganisms comprise cyanobacteria. In some embodiments, the photosynthetic microorganisms comprise cyanobacteria selected from the group consisting of Synechococcus or Synechocystis. In some embodiments, the photosynthetic microorganisms comprise a transgenic photosynthetic microorganism engineered to accumulate a disaccharide, as described above.

[0039] Other objects and features will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

[0040] Those of skill in the art will understand that the drawings, described below, are for illustrative purposes only. The drawings are not intended to limit the scope of the present teachings in any way.

[0041] FIG. 1 illustrates a front view of the photobioreactor of the invention including a solid cultivation support, an outer protective transparent barrier layer, a selective panel, resealable closures, and support elements for suspending the device.

[0042] FIG. 2 illustrates a side view of the photobioreactor of the invention including a solid cultivation support, an outer protective transparent barrier layer, a selective panel, resealable closures, and support elements for suspending the device.

[0043] FIG. 3 illustrates an arrangement of multiple photobioreactors or cultivation supports of the invention along multiple closed loop conveyor systems radiating out from common inoculation and harvesting centers to comprise a photobioreactor farm.

[0044] FIG. 4 is a cartoon depicting photosynthetic production of sucrose in cyanobacteria.

[0045] FIG. 5 is a polypeptide sequence alignment of the *Synechocystis* spp. PCC 6803 (Ssp6803) sucrose phosphate synthase (SPS) and sucrose phosphate phosphatase (SPP) proteins with the *Synechococcus elongatus* PCC 7942 (Selo7942) active SPS/SPP fusion (ASF). Ssp6803 contains separate genes encoding SPS and SPP activities. The SPS protein from *Synechocystis* spp. PCC 6803 bears a presumably inactive SPP domain, as many of the active site residues are not conserved. The canonical HAD hydrolase active site residues are shown above the alignment with <u>*conserved*</u> amino acids shown underlined and non-conserved residues double underlined. An eight amino acid insertion within the inactive SPP domain of *Synechocystis* spp. PCC 6803 SPS is italicized. Further details regarding methodology are provided in Example 4.

[0046] FIG. 6 is schematic depiction of pLybAL11. pLybAL11 allows construction of libraries of cyanobacterial DNA and selection for promoter sequences. The promoterless *asf* gene is behind bidirectional terminators, separated by a multiple cloning site (MCS). *oriV* allows for plasmid replication in most Gram-negative organisms. *oriT* allows for conjugal transfer of the plasmid from *E. coli* to a chosen cyanobacterium (or other organism) with the assistance of the pRK2013 helper plasmid. The β–lactamase gene (*bla*) is present for selection in *E. coli*. DNA libraries can be constructed in *E. coli* by cloning cyanobacterial genomic DNA into the MCS. The plasmid library can then be transferred to cyanobacteria by conjugation or direct transformation. Active promoters can then be isolated by selection for resistance to chloramphenicol through expression of the chloramphenicol acetyltransferase gene (*cat*). The strength of the promoters can be assessed by both assay for chloramphenicol acetyltransferase activity and direct examination of sucrose production. Further details regarding methodology are provided in Example 5.

[0047] FIG. 7 is schematic depiction of pLybAL12. pLybAL12 allows analysis of the capacity of preselected promoters to drive *asf* expression. The only difference between pLybAL12 and pLybAL11 is the presence of an active promoter in front of the chloramphenicol acetyltransferase gene (*cat*). Specific DNA sequences isolated from cyanobacterial chromosomal DNA amplified by PCR can be cloned into the MCS. Both chloramphenicol and

ampicillin can be used for selection in *E. coli*. The plasmid library can then be transferred to cyanobacteria by conjugation or direct transformation. Plasmid bearing cyanobacteria can then be isolated by selection for resistance to chloramphenicol through expression of the chloramphenicol acetyltransferase gene (*cat*). The strength of the promoters can be assessed by both assay for chloramphenicol acetyltransferase activity and direct examination of sucrose production. Further details regarding methodology are provided in Example 5.

[0048] FIG. 8 is a cartoon depicting construction of a cyanobacterial promoter library. Further details regarding methodology are provided in Example 8.

[0049] FIG. 9 is a schematic diagram depicting pSMART-LCKan. Further details regarding methodology are provided in Example 8.

[0050] FIG. 10 is a sequence listing showing a possible promoter within *Synechococcus elongatus* PCC 7942 *asf*. Shown is the amplified PCR product containing the *asf* gene from *Synechococcus elongatus* PCC 7942 that was cloned upstream of the chloramphenicol resistance marker. The regions of *asf* encoding the sucrose phosphate synthase and sucrose phosphate phosphatase polypeptide activities are single underlined and double underlined, respectively. All DNA sequence elements are italicized and labeled above. *Start* and *Stop* represent the start and stop codons, respectively. *SD* represents the Shine-Delgarno sequence. The -35 and -10 regions of the putative promoters are highlighted in gray. Further details regarding methodology are provided in Example 8.

[0051] FIG. 11 is a schematic diagram depicting a two-step protocol for markerless deletion of genes in the cyanobacterial genome. This strategy assumes that the cyanobacterial strain being used has had its *upp* gene deleted. The *upp* gene will have been deleted during the sucrose biosynthetic insertions. The gene of interest that has been targeted for deletion must be identified. The starting strain is resistant to 5-fluorouracil, but sensitive to kanamycin. The gene is either completely or partially deleted by the insertion of a cassette containing a kanamycin resistance marker and an active *upp*, making the strain resistant to kanamycin, but sensitive to 5-fluorouracil. The upp and kanamycin resistance markers can then be removed, making the strain once again resistant to 5-fluorouracil, but sensitive to kanamycin. Further details regarding methodology are provided in Example 12.

[0052] FIG. 12 is a schematic diagram of a photobioreactor embodiment. FIG. 12A provides a front view while FIG. 12B provides a side view. The photobioreactor includes suspension element (6); culture media supply (8); gas supply (10); growth surface (2); outer barrier layer (7); quick connector; and product harvest line (9).

[0053] FIG. 13 is a schematic diagram of a growth surface in a single material format (FIG. 13A) and a hybrid material format (FIG. 13B).

DETAILED DESCRIPTION OF THE INVENTION

[0054] The present application relates to fermentable sugar accumulating photosynthetic microorganisms, solid-phase photoreactor devices, and methods of using each.

[0055] In the fermentable sugar accumulating photosynthetic microorganisms, it may be preferable to produce a dissaccharide sugar not generally utilized by the photosynthetic microorganisms, which therefore can accumulate within the cultivated biomass (e.g., sucrose, trehalose). In some embodiments, photosynthetic microorganisms are genetically engineered to synthesize a dissaccharide sugar normally produced according to osmotic stress pathways (e.g., sucrose or trehalose) such that the sugar is produced in the absence of, or at reduced levels of, osmotic stress. Because of the greater efficiency and lower environmental impact of growing photosynthetic microorganisms compared to higher plants, the method represents important improvements in sustainability over current biofuel production practices. Advantageously, the foregoing method of synthesizing a dissaccharide sugar has been adapted to occur within the photobioreactor(s) of the present invention.

[0056] The photobioreactor described herein utilizes a solid cultivation support. Advantageously, the difficulty of providing adequate light exposures is alleviated, at least in part. Utilizing the aforementioned solid cultivation support in a photobioreactor can allow for cultivation and growth of photosynthetic microorganisms at cell densities greater than those of commercial-scale liquid phase bioreactors (*e.g.*, cell densities in excess of 200 grams of dry biomass per liter equivalent). In addition, various embodiments of the photobioreactor described herein can be operated using less energy and more simply than conventional commercial-scale liquid phase photobioreactors.

[0057] Embodiments of the photobioreactor described herein provide additional benefits over conventional liquid phase photobioreactors. For example, liquid systems typically require special equipment to deliver adequate concentrations/amount of carbon dioxide to the photosynthetic microorganisms to support their growth and photosynthesis. In contrast, by growing the microorganisms on a solid cultivation support, carbon dioxide can be provided in a relatively simple, less costly manner, such as exposure to surrounding air. If additional carbon dioxide is desired, it can easily be delivered by, for example, adding it to the atmosphere (*e.g.*, air) surrounding or in contact with the cultivation support. Another benefit is ease of transport. Liquid phase photobioreactors can be a pond (completely immobile) or bulky tanks or collections of tubing. In contrast, in various embodiments, the photobioreactor is flat and flexible, which allows for it or a multiplicity of them to be stacked, rolled up, folded, and/or configured in a similar manner for relatively easy transport. In various embodiments, the photobioreactor can be configured in a manner such that it is suspended from a system that allows for easy conveyance of one or more photobioreactors from one location to another. This portability may be utilized on a commercial scale to allow for efficient methods of handling and processing large numbers of photobioreactors in a continuous-type manner.

[0058] One aspect of the application is directed to a method of fermentable sugar feedstock production by photosynthetic microorganisms. Preferably, the fermentable sugar is a fermentable disaccharide sugar. Examples of fermentable disaccharide sugars include, but are not limited to sucrose and trehalose. The fermentable sugar can be a disaccharide not generally utilized by photosynthetic microorganisms. For example, trehalose is not generally utilized by cyanobacteria and therefore can accumulate within the cultivated biomass without substantial degradation by endogenous metabolic pathways. The fermentable sugar can be a disaccharide that is generally utilized by photosynthetic microorganisms. For a disaccharide not used as a primary energy source, the disaccharide can often be accumulated to sufficient levels even in the presence of endogenous metabolic pathways. Where endogenous degradation pathways specific for the target fermentable sugar, the photosynthetic microorganism can be engineered to reduce or eliminate such activity. For example, a cyanobacterium engineered to accumulate sucrose can be further engineered to reduce or eliminate sucrose invertase activity. In various embodiments, strains of photosynthetic microorganisms that synthesize fermentable disaccharide sugar in

response to osmotic or matric water stress can be used. In other embodiments transgenic strains of photosynthetic microorganisms engineered to accumulate fermentable disaccharide sugar in the absence of, or reduced levels of, osmotic stress. Advantageously, the foregoing methods of synthesizing fermentable disaccharide sugar can be adapted to occur within photobioreactors described herein.

[0059] Because of the greater efficiency and lower environmental impact of growing photosynthetic microorganisms compared to higher plants, compositions, devices, and methods described herein represent important improvements in sustainability over current biofuel production practices.

[0060] Photosynthetic Microorganism

[0061] Provided herein is a photosynthetic microorganism genetically engineered to accumulate a dissaccharide sugar. The photosynthetic microorganism can be, for example, a naturally photosynthetic microorganism, such as a cyanobacterium, or an engineered photosynthetic microorganism, such as an artificially photosynthetic bacterium. Examples of the accumulated dissaccharide sugar include, but are not limited to sucrose, trehalose, gluocosylglycerol, and mannosylfructose. In various embodiments, one or more genes encoding the protein(s) responsible for producing the desired dissaccharide from corresponding phosphorylated monomers is engineered in a host photosynthetic microorganism (e.g., cyanobacterium) so as to result in the accumulation of the desired dissaccharide. In some embodiments, an endogenous pathway of the host photosynthetic microorganism is engineered so as to accumulate a dissaccharide sugar. For example, the osmotic sucrose pathway in cyanobacteria can be engineered to accumulate sucrose in the absence of osmotic stress. In some embodiments, an exogenous dissaccharide pathway is engineered in cyanobacteria so as to accumulate a dissaccharide sugar. For example, the osmotic trehalose pathway from E. coli can be engineered to accumulate trehalose in cyanobacteria.

[0062] Synthase and Phosphotase

[0063] A photosynthetic microorganism can be transformed so as to have a synthase activity and a phosphotase activity for the desired dissaccharide. For example, a cyanobacterium can be engineered to have sucrose phosphate synthase activity and sucrose phosphate

phosphatase activity. As another example, a cyanobacterium can be engineered to have trehalose phosphate synthase activity and trehalose phosphate phosphatase activity. As another example, a cyanobacterium can be engineered to have gluocosylglycerol phosphate synthase activity and gluocosylglycerol phosphate phosphatase activity. As another example, a cyanobacterium can be engineered to have mannosylfructose phosphate synthase activity and mannosylfructose phosphate phosphatase activity. It is contemplated these activities can likewise be engineered in other photosynthetic microorganisms.

[0064] Synthase activity and phosphotase activity can be engineered into a photosynthetic microorganism by way of the individual genes, one encoding a polypeptide having synthase activity and the other encoding a polypeptide having phosphatase activity; or by one gene encoding both synthase activity and phosphatase activity. For example, synthase activity and phosphatase activity can be present in a fusion polypeptide.

[0065] The monomeric sugars of the desired dissaccharide can be endogenous or exogenous to the photosynthetic microorganism. Where monomeric sugars of the desired dissaccharide are endogenous, the photosynthetic microorganism can be engineered to produce increased levels of such monomers. Where monomeric sugars of the desired dissaccharide are exogenous, the photosynthetic microorganism can be engineered to produce such exogenous monomers.

[0066] The photosynthetic microorganism can be engineered to synthesize and accumulate the desired dissaccharide continuously, after some developmental state, or upon being induced to do so. Induction of dissaccharide synthesis can be according to the actions of an inducible promoter associated with the encoded synthase or phosphotase and an inducing agent, as discussed in further detail herein.

[0067] In some embodiments, transformed cyanobacteria, as described herein, can accumulate at least about 0.1 micrograms of a dissaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass. In some embodiments, transformed cyanobacteria can accumulate at least about 0.1 up to about 10 micrograms of a dissaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass. For example, transformed cyanobacteria can accumulate at

least about 0.2, at least about 0.3, at least about 0.4, at least about 0.5, at least about 0.6, at least about 0.7, at least about 0.8, or at least about 0.9 micrograms of a dissaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass. In other embodiments, various transformed photosynthetic microorganisms accumulate similar amounts of a dissaccharide.

[0068] It is contemplated that that various embodiments will accumulate a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) at defined ranges of the values above. For example, some transformed cyanobacteria can accumulate at least about 0.1 up to about 0.9 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.1 up to about 0.8 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.1 up to about 0.7 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; etc. Similarly, some transformed cyanobacteria can accumulate at least about 0.2 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.3 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.4 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.5 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.6 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.7 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; at least about 0.8 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass; or at least about 0.9 up to about 1.0 micrograms of a disaccharide (e.g., sucrose, trehalose, glucosylglycerol, or mannosylfructose) per minute per gram dry biomass. Methods for assaying sugar accumulation is host cells are well-known to those of skill in the art (*see e.g.*, Example 10).

[0069] Host

[0070] The host genetically engineered to accumulate a dissaccharide sugar can be any photosynthetic microorganism. The photosynthetic microorganism can be, for example, a naturally photosynthetic microorganism, such as a cyanobacterium, or an engineered photosynthetic microorganism, such as an artificially photosynthetic bacterium. Exemplary microorgansims that are either naturally photosynthetic or can be engineered to be photosynthetic include, but are not limited to, bacteria; fungi; archaea; protists; microscopic plants, such as a green algae; and animals such as plankton, planarian, and amoeba. Examples of naturally occurring photosynthetic microorganisms include, but are not limited to, Spirulina maximum, Spirulina platensis, Dunaliella salina, Botrycoccus braunii, Chlorella vulgaris, Chlorella pyrenoidosa, Serenastrum capricomutum, Scenedesmus auadricauda, Porphyridium cruentum, Scenedesmus acutus, Dunaliella sp., Scenedesmus obliquus, Anabaenopsis, Aulosira, Cylindrospermum, Synechoccus sp., Synechocystis sp., and/or Tolypothrix.

[0071] Preferably, the host photosynthetic microorganism is a cyanobacterium. Cyanobacteria, also known as blue-green algae, are a broad range of oxygengenic photoautotophs. The host cyanobacterium can be any photosynthetic microorganism from the phylum Cyanophyta. The host cyanobacterium can have a unicellular or colonial (*e.g.*, filaments, sheets, or balls) morphology. Preferably, the host cyanobacterium is a unicellular cyanobacterium. Examples of cyanobacteria that can be engineered to accumulate a disaccharide sugar include, but are not limited to, the genus Synechocystis, Synechococcus, Thermosynechococcus, Nostoc, Prochlorococcu, Microcystis, Anabaena, Spirulina, and Gloeobacter. Preferably the host cyanobacterium is a Synechocystis spp. or Synechococcus spp. More preferably, the host cyanobacterium is *Synechococcus elongatus* PCC 7942 (ATCC 33912) and/or *Synechocystis* spp. PCC 6803 (ATCC 27184).

[0072] Sucrose

[0073] Biosynthesis of sucrose in a photosynthetic microorganism, such as cyanobacteria, can be accomplished through the catalytic action of two enzyme activities, sucrose phosphate synthase (*sps*) and sucrose phosphate phosphatase (*spp*), functioning in sequence (*see e.g.*, FIG. 4). Such activities are present in some cyanobacteria for acclimation to

osmotic and matric water stress (*see e.g.*, Lunn, J. E. 2002. Plant Physiol 128, 1490-1500). Either or both of these activities can be engineered in a cyanobacterium so as to result in accumulation of sucrose.

[0074] A gene of particular interest for engineering a photosynthetic microorganism to accumulate sucrose is the active *sps/spp* fusion (*asf*) gene from *Synechococcus elongatus* PCC 7942. *Asf* has both *sps* and *spp* biosynthetic functions (*see e.g.*, Example 4). In some embodiments, an ASF-encoding nucleotide sequence is cloned from its native source (e.g., *Synechococcus elongatus* PCC 7942) and inserted into a host cyanobacterium (*see e.g.*, Examples 4-9). In some embodiments, a transformed host photosynthetic microorganism comprises an *asf* polynucleotide of SEQ ID NO: 1. In some embodiments, a photosynthetic microorganism is transformed with a nucleotide sequence encoding ASF polypeptide of SEQ ID NO: 2. In further embodiments, a transformed host photosynthetic microorganism comprises a nucleotide sequence having at least about 80% sequence identity to SEQ ID NO: 1 or a nucleotide sequence encoding a polypeptide having *sps* and *spp* activity and at least about 80% sequence identity to SEQ ID NO: 2. As an example, a transformed host photosynthetic microorganism, such as a cyanobacterium, can comprise a nucleotide sequence having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 1, wherein the transformed host exhibits ASF, SPS, and/or SPP activity and/or accumulation of sucrose. As an example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 2, wherein the transformed host exhibits ASF, SPS, and/or SPP activity and/or accumulation of sucrose. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence that hybridizes under stringent conditions to SEQ ID NO: 1 over the entire length of SEQ ID NO: 1, and which encodes an active SPS/SPP fusion (ASF) polypeptide. As a further example, a transformed host photosynthetic microorganism can comprise the complement to any of the above sequences.

[0075] In some embodiments, a sucrose phosphate synthase (*sps*) (see e.g., SEQ ID NO: 3 encoding *sps* gene and SEQ ID NO: 4 encoding SPS polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism.

For example, a photosynthetic microorganism can be transformed with a nucleotide having a sequence of SEQ ID NO: 3 so as to express sucrose phosphate synthase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 3 encoding a polypeptide having sucrose phosphate synthase. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 4, wherein the transformed host exhibits SPS activity and/or accumulation of sucrose.

[0076] In some embodiments, sucrose phosphate phosphatase (*spp*) (see e.g., SEQ ID NO: 5 encoding *spp* gene and SEQ ID NO: 6 encoding SPP polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism. For example, a photosynthetic microorganism, such as a cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 5 so as to express sucrose phosphate phosphatase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 5 encoding a polypeptide having sucrose phosphate phosphatase activity. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 6, wherein the transformed host exhibits SPP activity and/or accumulation of sucrose.

[0077] In some embodiments, a photosynthetic microorganism is engineered to express one or more of ASF, SPS, and/or SPP. For example, a photosynthetic microorganism, such as a cyanobacterium, can be engineered to express ASF and SPS; ASF and SPP; SPS and SPP; or ASF, SPS, and SPP.

[0078] Trehalose

[0079] Biosynthesis of trehalose can be accomplished through the catalytic action of two enzyme activities, trehalose phosphate synthase (*tps*) and trehalose phosphate phosphatase (*tpp*), functioning in sequence. Either or both of these activities can be engineered in a

photosynthetic microorganism so as to result in accumulation of trehalose. Biosynthesis of trehalose does not naturally occur in some photosynthetic microorganisms, such as cyanobacteria.

[0080] In some embodiments, a trehalose phosphate synthase (*tps*) (see e.g., SEQ ID NO: 76 encoding *tps* gene and SEQ ID NO: 77 encoding TPS polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism. For example, a photosynthetic microorganism, such as cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 76 so as to express trehalose phosphate synthase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 76 encoding a polypeptide having trehalose phosphate synthase. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 77, wherein the transformed host exhibits TPS activity and/or accumulation of trehalose.

[0081] In some embodiments, trehalose phosphate phosphatase (*tpp*) (see e.g., SEQ ID NO: 78 encoding *tpp* gene and SEQ ID NO: 79 encoding TPP polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism. For example, a photosynthetic microorganism, such as a cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 78 so as to express trehalose phosphate phosphatase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 78 encoding a polypeptide having trehalose phosphate phosphatase activity. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 79, wherein the transformed host exhibits TPP activity and/or accumulation of trehalose.

[0082] Glucosylglycerol

[0083] In some embodiments, a glucosylglycerolphosphate synthase (*gps*) (see e.g., SEQ ID NO: 80 encoding *gps* gene and SEQ ID NO: 81 encoding GPS polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism. For example, a photosynthetic microorganism, such as a cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 80 so as to express glucosylglycerolphosphate synthase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 80 encoding a polypeptide having glucosylglycerolphosphate synthase. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 81, wherein the transformed host exhibits GPS activity and/or accumulation of glucosylgycerol.

[0084] In some embodiments, glucosylglycerolphosphate phosphatase (*gpp*) (see e.g., SEQ ID NO: 82 encoding *gpp* gene and SEQ ID NO: 83 encoding GPP polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism. For example, a photosynthetic microorganism, such as a cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 82 so as to express glucosylglycerolphosphate phosphatase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 82 encoding a polypeptide having glucosylglycerolphosphate phosphatase activity. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 83, wherein the transformed host exhibits GPP activity and/or accumulation of glucosylgycerol.

[0085] Mannosylfructose

[0086] In some embodiments, a mannosylfructose phosphate synthase (*mps*) (see e.g., SEQ ID NO: 84 encoding *mps* gene and SEQ ID NO: 85 encoding MPS polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed

photosynthetic microorganism. For example, a photosynthetic microorganism, such as a cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 84 so as to express mannosylfructose phosphate synthase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 84 encoding a polypeptide having mannosylfructose phosphate synthase. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 85, wherein the transformed host exhibits MPS activity and/or accumulation of mannosylfructose.

[0087] In some embodiments, mannosylfructose phosphate phosphatase (*mpp*) (see e.g., SEQ ID NO: 86 encoding *mpp* gene and SEQ ID NO: 87 encoding MPP polypeptide), or homologue thereof, is engineered to be expressed or overexpressed in a transformed photosynthetic microorganism. For example, a photosynthetic microorganism, such as a cyanobacterium, can be transformed with a nucleotide having a sequence of SEQ ID NO: 86 so as to express mannosylfructose phosphate phosphatase. As another example, a photosynthetic microorganism can be transformed with a nucleotide having at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 99% percent identity to SEQ ID NO: 86 encoding a polypeptide having mannosylfructose phosphate phosphatase activity. As another example, a transformed host photosynthetic microorganism can comprise a nucleotide sequence encoding a polypeptide having at least about 85%, at least about 90%, at least about 95%, or at least about 99% sequence identity to SEQ ID NO: 87, wherein the transformed host exhibits MPP activity and/or accumulation of mannosylfructose.

[0088] Molecular Engineering

[0089] Design, generation, and testing of the variant nucleotides, and their encoded polypeptides, having the above required percent identities to an *asf* sequence and retaining a required activity of the expressed protein and/or sugar accumulation phenotype is within the skill of the art. For example, directed evolution and rapid isolation of mutants can be according to methods described in references including, but not limited to, Link et al. (2007) Nature Reviews 5(9), 680-688; Sanger et al. (1991) Gene 97(1), 119-123; Ghadessy et al. (2001) Proc Natl Acad

Sci USA 98(8) 4552-4557. Thus, one skilled in the art could generate a large number of nucleotide (e.g., *asf*, *sps*, *spp*, *tps*, *tpp*, *gps*, *gpp*, *mps*, or *mpp*) and/or polypeptide (e.g., ASF, SPS, SPP, TPS, TPP, GPS, GPP, MPS, or MPP) variants having, for example, at least 95-99% identity to the reference sequence described herein and screen such for phenotypes including disaccharide accumulation according to methods routine in the art. Generally, conservative substitutions can be made at any position so long as the required activity is retained.

[0090] Nucleotide and/or amino acid sequence identity percent (%) is understood as the percentage of nucleotide or amino acid residues that are identical with nucleotide or amino acid residues in a candidate sequence in comparison to a reference sequence when the two sequences are aligned. To determine percent identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum percent sequence identity. Sequence alignment procedures to determine percent identity are well known to those of skill in the art. Often publicly available computer software such as BLAST, BLAST2, ALIGN2 or Megalign (DNASTAR) software is used to align sequences. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full-length of the sequences being compared. When sequences are aligned, the percent sequence identity of a given sequence A to, with, or against a given sequence B (which can alternatively be phrased as a given sequence A that has or comprises a certain percent sequence identity to, with, or against a given sequence B) can be calculated as: percent sequence identity = X/Y100, where X is the number of residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B and Y is the total number of residues in B. If the length of sequence A is not equal to the length of sequence B, the percent sequence identity of A to B will not equal the percent sequence identity of B to A.

[0091] "Highly stringent hybridization conditions" are defined as hybridization at 65 °C in a 6 X SSC buffer (*i.e.,* 0.9 M sodium chloride and 0.09 M sodium citrate). Given these conditions, a determination can be made as to whether a given set of sequences will hybridize by calculating the melting temperature (T_m) of a DNA duplex between the two sequences. If a particular duplex has a melting temperature lower than 65°C in the salt conditions of a 6 X SSC, then the two sequences will not hybridize. On the other hand, if the melting temperature is above 65 °C in the same salt conditions, then the sequences will hybridize. In general, the

melting temperature for any hybridized DNA:DNA sequence can be determined using the following formula: $T_m = 81.5\ °C + 16.6(\log_{10}[Na^+]) + 0.41(\text{fraction G/C content}) - 0.63(\%\ \text{formamide}) - (600/l)$. Furthermore, the T_m of a DNA:DNA hybrid is decreased by 1-1.5°C for every 1% decrease in nucleotide identity (see e.g., Sambrook and Russel, 2006).

[0092] Host cells can be transformed using a variety of standard techniques known to the art (*see, e.g.*, Sambrook and Russel (2006) Condensed Protocols from Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, ISBN-10: 0879697717; Ausubel et al. (2002) Short Protocols in Molecular Biology, 5th ed., Current Protocols, ISBN-10: 0471250929; Sambrook and Russel (2001) Molecular Cloning: A Laboratory Manual, 3d ed., Cold Spring Harbor Laboratory Press, ISBN-10: 0879695773; Elhai, J. and Wolk, C. P. 1988. Methods in Enzymology 167, 747-754). Such techniques include, but are not limited to, viral infection, calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, receptor-mediated uptake, cell fusion, electroporation, and the like. The transfected cells can be selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome.

[0093] Promoter

[0094] One or more of the nucleotide sequences discussed above (e.g., *asf*, *sps*, *spp*, *tps*, *tpp*, *mps*, *mpp*, *gps*, *gpp*) can be operably linked to a promoter that can function in the host photosynthetic microorganism. Where the host is cyanobacteria, preferably, the promoter can function efficiently in both cyanobacteria and a bacteria, such as E. coli. Promoter selection can allow expression of a desired gene product under a variety of conditions.

[0095] Promoters can be selected for optimal function in a photosynthetic microorganism host cell, such as a cyanobacterium, into which the vector construct will be inserted. Promoters can also be selected on the basis of their regulatory features. Examples of such features include enhancement of transcriptional activity and inducibility.

[0096] The promoter can be an inducible promoter. For example, the promoter can be induced according to temperature, pH, a hormone, a metabolite (e.g., lactose, mannitol, an amino acid), light (e.g., wavelength specific), osmotic potential (e.g., salt induced), a heavy

metal, or an antibiotic. Numerous standard inducible promoters will be known to one of skill in the art.

[0097] In some embodiments, the promoter is a temperature inducible promoter. For example, the Lambda promoter is a temperature inducible promoter that can function in cyanobacteria. Surprisingly, the Lambda promoter functions at a temperature different than when utilized in E. coli. In E. coli, the Lambda promoter is most active at 42°C, a temperature above the normal viability range for cyanobacteria. Generally, in E. coli, the Lambda promoter has about a 5% to 10% increased expression from about 30°C to 35°C and at about 37°C has about a 20% increased expression; but from about 37°C to 42°C provides about 100% increased expression. In cyanobacteria, the Lambda promoter is most active at around 30°C to 35°C, an ideal growth temperature range for cyanobacteria and a range much lower than optimal expression of the Lambda promoter in E. coli. So, the Lambda promoter provides for effective expression of disaccharide biotsynthetic activity in cyanabcteria.

[0098] Examples of promoters that can be inserted into the plasmid include, but are not limited to, *carB*, *nirA*, *psbAII*, *dnaK*, *kaiA*, and λ_{PR} (*see e.g.*, Example 6). In some embodiments, the promoter can function efficiently in both cyanobacteria and *E. coli*. In some embodiments, the *asf* coding region comprises a promoter with said coding region (*see e.g.*, Example 8). For example, the *asf* coding region can comprise a promoter in front of the SPP domain of *asf* (*see e.g.*, FIG. 10). Such an internal promoter can occur with or without a promoter at the start of the *asf* coding region.

[0099] The term "chimeric" is understood to refer to the product of the fusion of portions of two or more different polynucleotide molecules. "Chimeric promoter" is understood to refer to a promoter produced through the manipulation of known promoters or other polynucleotide molecules. Such chimeric promoters can combine enhancer domains that can confer or modulate gene expression from one or more promoters or regulatory elements, for example, by fusing a heterologous enhancer domain from a first promoter to a second promoter with its own partial or complete regulatory elements. Thus, the design, construction, and use of chimeric promoters according to the methods disclosed herein for modulating the expression of operably linked polynucleotide sequences are encompassed by the present invention.

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[0100] Novel chimeric promoters can be designed or engineered by a number of methods. For example, a chimeric promoter may be produced by fusing an enhancer domain from a first promoter to a second promoter. The resultant chimeric promoter may have novel expression properties relative to the first or second promoters. Novel chimeric promoters can be constructed such that the enhancer domain from a first promoter is fused at the 5' end, at the 3' end, or at any position internal to the second promoter.

[0101] Constructs

[0102] Any of the transcribable polynucleotide molecule sequences described above can be provided in a construct. Constructs of the present invention generally include a promoter functional in the host photosynthetic microorganism, such as cyanobacteria, operably linked to a transcribable polynucleotide molecule for disaccharide biosynthesis (e.g., *asf*, *sps*, *spp*, *tps*, *tpp*, *mps*, *mpp*, *gps*, *gpp*), such as provided in SEQ ID NO: 1, 3, 5, 76, 78, 80, 82, 84, and 86, and variants thereof as discussed above.

[0103] Exemplary promoters are discussed above. One or more additional promoters may also be provided in the recombinant construct. These promoters can be operably linked to any of the transcribable polynucleotide molecule sequences described above.

[0104] The term "construct" is understood to refer to any recombinant polynucleotide molecule such as a plasmid, cosmid, virus, autonomously replicating polynucleotide molecule, phage, or linear or circular single-stranded or double-stranded DNA or RNA polynucleotide molecule, derived from any source, capable of genomic integration or autonomous replication, comprising a polynucleotide molecule where one or more polynucleotide molecule has been linked in a functionally operative manner, i.e. operably linked. The term "vector" or "vector construct" is understood to refer to any recombinant polynucleotide construct that may be used for the purpose of transformation, *i.e.*, the introduction of heterologous DNA into a host photosynthetic microorganism, such as a cyanobacterium.

[0105] In addition, constructs may include, but are not limited to, additional polynucleotide molecules from an untranslated region of the gene of interest. These additional polynucleotide molecules can be derived from a source that is native or heterologous with respect to the other elements present in the construct.

[0106] Plasmid

[0107] In some embodiments, a host photosynthetic microorgansim, such as a cyanobacterium, is transformed with a plasmid-based expression system (*see e.g.*, Example 5). Preferably the plasmid encoding the gene of interest comprises a promoter, such as one or more of those discussed above. For plasmid based transformation, preferred is a broad host range plasmid that enables function in both *E. coli* and cyanobacteria, which provides the advantage of working in a convenient fast growing well understood system (*E. coli*) that can be efficiently transferred to the final host (cyanobacteria). In some embodiments, plasmid based transformation and chromosomal integration are used in conjunction, where the plasmid protocol is used for design and testing of gene variants followed by chromosomal integration of identified variants.

[0108] Host strains developed according to the approaches described herein can be evaluated by a number of means known in the art (*see e.g.*, Studier (2005) Protein Expr Purif. 41(1), 207–234; Gellissen, ed. (2005) Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems, Wiley-VCH, ISBN-10: 3527310363; Baneyx (2004) Protein Expression Technologies, Taylor & Francis, ISBN-10: 0954523253).

[0109] Provided herein are nucleotide sequences for plasmid constructs encoding *sps*, *spp*, and/or *asf*. Examples of plasmid constructs encoding *sps*, *spp*, and/or *asf* include, but are not limited to, pLybAL11 (SEQ ID NO: 19) (*see e.g.*, FIG. 6) and pLybAL12 (SEQ ID NO: 20) (*see e.g.*, FIG. 7). Also provided herein are nucleotide sequences for plasmid constructs encoding *tps* and *tpp*. Examples of plasmid constructs encoding *tps* and *tpp* include, but are not limited to, pLybAL23 (SEQ ID NO: 118). A skilled artisan will understand that similar contructs can be generated for biosynthetic genes necessary for accumulation of other disaccharides, such as glucosylglycerol and mannosylfructose.

[0110] In some embodiments, the transformed host photosynthetic microorganism comprises pLybAL11 (SEQ ID NO: 19) or pLybAL12 (SEQ ID NO: 20). In some embodiments, the transformed host photosynthetic microorganism comprises pLybAL23 (SEQ ID NO: 118). For example, a transformed cyanobacterium can comprise pLybAL11 (SEQ ID NO: 19), pLybAL12 (SEQ ID NO: 20), or pLybAL23 (SEQ ID NO: 118).

[0111] A plasmid construct comprising a disaccharide biosynthetic gene(s) can also include a promoter. Examples of plasmid constructs comprising *sps*, *spp*, and/or *asf* and a promoter include, but are not limited to, pLybAL7f (SEQ ID NO: 65); pLybAL8f, including kanamycin resistance (SEQ ID NO: 69); pLybAL13f (SEQ ID NO: 51), pLyAL13r (SEQ ID NO: 52), pLybAL14f (SEQ ID NO: 53), pLybAL14r (SEQ ID NO: 54), pLybAL15 (SEQ ID NO: 44), pLybAL16 (SEQ ID NO: 45), pLybAL17 (SEQ ID NO: 46), pLybAL18 (SEQ ID NO: 47), pLybAL19 (SEQ ID NO: 48), pLybAL21 (SEQ ID NO: 49), and pLybAL22 (SEQ ID NO: 50). Examples of plasmid constructs comprising *tps* and *tpp* and a promoter include, but are not limited to, pLybAL23 (SEQ ID NO: 118), pLybAL28 (SEQ ID NO: 121), pLybAL29 (SEQ ID NO: 122), and pLybAL30 (SEQ ID NO: 123). A skilled artisan will understand that similar promoter containing contructs can be generated for biosynthetic genes necessary for accumulation of other disaccharides, such as glucosylglycerol and mannosylfructose.

[0112] In some embodiments, the transformed host cyanobacterium comprises pLybAL7f (SEQ ID NO: 65); pLybAL8f (SEQ ID NO: 69); pLybAL13f (SEQ ID NO: 51), pLyAL13r (SEQ ID NO: 52), pLybAL14f (SEQ ID NO: 53), pLybAL14r (SEQ ID NO: 54), pLybAL15 (SEQ ID NO: 44), pLybAL16 (SEQ ID NO: 45), pLybAL17 (SEQ ID NO: 46), pLybAL18 (SEQ ID NO: 47), pLybAL19 (SEQ ID NO: 48), pLybAL21 (SEQ ID NO: 49), and pLybAL22 (SEQ ID NO: 50). In some embodiments, the transformed host cyanobacterium comprises pLybAL28 (SEQ ID NO: 121), pLybAL29 (SEQ ID NO: 122), pLybAL30 (SEQ ID NO: 123), and pLybAL23 (SEQ ID NO: 118).

[0113] Sugar Secretion

[0114] In various embodiments, a transformed disaccharide-accumulating photosynthetic microorganism can secrete the accumulated disaccharide from within the cell into its growth environment. Secretion of the disaccharide can be an inherent effect of transforming the photosynthetic microorganism to accumulate a disaccharide or the photosynthetic microorganism can be further engineered to secrete the disaccharide. For example, some cyanobacteria transformed to accumulate trehalose inherently secrete trehalose from the cell (see e.g., Examples 19-20). As another example, a cyanobacterium transformed to accumulate sucrose can be further engineered to secrete sucrose from the cell (see e.g., Example 16).

[0115] A host photosynthetic microorganism, such as a cyanobacterium, can be further engineered to secrete a disaccharide. In some embodiment, a transformed host photosynthetic microorganism is engineered to express a porin specific for the accumulated disaccharide. For example, a cyanobacterium engineered to accumulate sucrose can be further engineered to express a sucrose porin (see e.g., Example 16). In one embodiment, the transformed disaccharide-accumulating cyanobacterium comprises an *scrY* nucleic acid, such as SEQ ID NO: 94. In one embodiment, the transformed disaccharide-accumulating cyanobacterium comprises a nucleic acid encoding a *scrY* polypeptide, such as SEQ ID NO: 95. In one embodiment, the transformed disaccharide-accumulating cyanobacterium comprises a plasmid containing *scrY*, such as pLybAL32 (SEQ ID NO: 91). It is contemplated that a similar approach can be applied to other photosynthetic microorganisms or other target disaccharides.

[0116] Modulation of Sugar Degradation

[0117] In some embodiments, a host photosynthetic microorganism, such as a cyanobacterium, is further engineered to improve disaccharide production by modulation of degradation activity (*see e.g.*, Example 14). In some embodiments, an invertase homologue can be down-regulated or eliminated in a transformed photosynthetic microorgansim. For example an invertase homologue from *Synechocystis* spp. PCC 6803 (nucleotide sequence SEQ ID NO: 70; polypeptide sequence SEQ ID NO: 71) can be down-regulated or eliminated in a transformed cyanobacterium. As another example, an invertase homologue from *Synechococcus elongatus* PCC 7942 (nucleotide sequence SEQ ID NO: 72; polypeptide sequence SEQ ID NO: 73) can be down-regulated or eliminated in a transformed cyanobacterium. In some embodiments, a sucraseferredoxin-like protein is down-regulated or eliminated in a transformed cyanobacteriuma. For example, a sucraseferredoxin-like protein from *Synechocystis* spp. PCC 6803 (nucleotide sequence SEQ ID NO: 74; polypeptide sequence SEQ ID NO: 75) (Machray G.C. *et al.* 1994. FEBS Lett 354, 123-127) can be down-regulated or eliminated in a transformed cyanobacterium. These genes can be deleted using the markerless deletion protocol described in, for example, FIG. 11 (see e.g., Examples 12-13) A similar approach can be taken for other disaccharides engineered to be accumulated in a cyanobacterium.

[0118] Other methods of down-regulation or silencing the above genes are known in the art. For example, disaccharide degradative activity can be down-regulated or eliminated

using antisense oligonucleotides, protein aptamers, nucleotide aptamers, and RNA interference (RNAi) (e.g., small interfering RNAs (siRNA), short hairpin RNA (shRNA), and micro RNAs (miRNA) (see e.g., Fanning and Symonds (2006) Handb Exp Pharmacol. 173, 289-303G, describing hammerhead ribozymes and small hairpin RNA; Helene, C., et al. (1992) Ann. N.Y. Acad. Sci. 660, 27-36; Maher (1992) Bioassays 14(12): 807-15, describing targeting deoxyribonucleotide sequences; Lee et al. (2006) Curr Opin Chem Biol. 10, 1-8, describing aptamers; Reynolds et al. (2004) Nature Biotechnology 22(3), 326 – 330, describing RNAi; Pushparaj and Melendez (2006) Clinical and Experimental Pharmacology and Physiology 33(5-6), 504-510, describing RNAi; Dillon et al. (2005) Annual Review of Physiology 67, 147-173, describing RNAi; Dykxhoorn and Lieberman (2005) Annual Review of Medicine 56, 401-423, describing RNAi). RNAi molecules are commercially available from a variety of sources (*e.g.*, Ambion, TX; Sigma Aldrich, MO; Invitrogen). Several siRNA molecule design programs using a variety of algorithms are known to the art (*see e.g.*, Cenix algorithm, Ambion; BLOCK-iT™ RNAi Designer, Invitrogen; siRNA Whitehead Institute Design Tools, Bioinofrmatics & Research Computing). Traits influential in defining optimal siRNA sequences include G/C content at the termini of the siRNAs, Tm of specific internal domains of the siRNA, siRNA length, position of the target sequence within the CDS (coding region), and nucleotide content of the 3' overhangs.

[0119] In some embodiments, a host photosynthetic microorganism can be further engineered to promote disaccharide secretion from the cells. For example, a cyanobacterium can be further engineered to promote sucrose secretion from the cells (*see e.g.*, Example 15-16). When in a low osmotic environment, the sucrose can be automatically expunged from the cells, as done with osomoprotectants by some organisms when transitioning from high to low salt environments (Schleyer, M., Schmidt, R. and Bakker, E. P. 1993. Arch Microbiol 160, 424-43; Koo, S. P., Higgins, C. F. and Booth, I. R. 1991. J Gen Microbiol 137, 2617-2625; Lamark, T., Styrvold, O. B. and Strgim, A. R. 1992. FEMS Microbiol. Lett 96, 149-154). Sucrose porins can be engineered to be expressed in a transformed cyanobacterium (see e.g., Example 16). These genes can be cloned and transformed into cyanobacteria according to techniques described above. Such approaches can be adapted to other photosynthetic microorganisms.

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[0120] In some embodiments, a host photosynthetic microorganism is transformed by stable integration into a chromosome of the host. For example, a host cyanobacterium can be transformed by stable integration into a chromosome of the host (*see e.g.*, Examples 11-13). Chromosomal integration can insure that the target gene(s) is installed into the organism without risk of expulsion as sometimes occurs with plasmid-based gene expression. Chromosomal integration can also reduce or eliminate the need for antibiotics to maintain target genes.

[0121] Preferably, the strategy for chromosomal integration targets gene insertion into what is termed the *upp* locus on the chromosome (*see e.g.*, Example 11-13). This site codes for the enzyme uracil phosphoribosyltransferase (UPRTase) which is a scavenger enzyme in pyrimidine biosynthesis. Using this strategy allows candidate selection by 5-fluorouracil (5-FU), which can eliminate non-integrated organisms. Segregation methods are generally used in cyanobacterial systems because these organisms contain multiple copies of their chromosomes (*e.g.*, up to 12 for *Synechocystis* spp. PCC 6803 and 16 for *Synechococcus elongatus* PCC 7942). This strategy is particularly attractive for cyanobacteria, because this approach can avoid the use of traditional segregation techniques that rely on selective pressure and statistical integration for successful segregation. Using 5-FU as a screening agent can be more efficient because it can prevent growth for any organism that contains even a single active *upp* gene. In this manner, fully integrated candidates can be selected rapidly over fewer generation cycles compared to the processes required of traditional techniques.

[0122] Solid Phase Photosynthetic Bioreactor

[0123] Provided herein is a photobioreactor for culturing photosynthetic microorganisms comprising a solid phase cultivation support for the growth of photosynthetic microorganisms. A solid phase cultivation support, or solid cultivation support, or solid support, or the like, is generally understood to mean a cultivation support that is neither a liquid nor a gas. Although the support itself is a solid, the support structure may be selected so that it absorbs a liquid (*e.g.*, growth media), a gas, or both. In certain preferred embodiments, as described more fully below, the solid support can absorb moisture for use by the microorganisms during cultivation.

[0124] Various embodiments of the photobioreactor(s) described herein can support the growth a photosynthetic microorganism. The photosynthetic microorganism grown in the photobioreactor can be, for example, a naturally photosynthetic microorganism, such as a cyanobacterium, or an engineered photosynthetic microorganism, such as an artificially photosynthetic bacterium. Exemplary microorganisms that are either naturally photosynthetic or can be engineered to be photosynthetic include, but are not limited to, bacteria; fungi; archaea; protists; microscopic plants, such as a green algae; and animals such as plankton, planarian, and amoeba. Examples of naturally occurring photosynthetic microorganisms include, but are not limited to, Spirulina maximum, Spirulina platensis, Dunaliella salina, Botrycoccus braunii, Chlorella vulgaris, Chlorella pyrenoidosa, Serenastrum capricomutum, Scenedesmus auadricauda, Porphyridium cruentum, Scenedesmus acutus, Dunaliella sp., Scenedesmus obliquus, Anabaenopsis, Aulosira, Cylindrospermum, Synechoccus sp., Synechocystis sp., and/or Tolypothrix.

[0125] Preferably, the bioreactor is configured to support innoculation, growth, and/or harvesting of cyanobacteria transformed to accumulate a disaccharide, as described above.

[0126] The photobioreactor can be an open or a closed system, as described more fully below. In various embodiments, the photobioreactor includes a solid phase cultivation support, a protective barrier layer, and a suspension element. Some embodiments of the photobioreactor can contain a system for delivery and/or removal of gas, fluids, nutrients, and/or photosynthetic microorganisms. Delivery systems can be, for example, standard plumbing fixtures. Any of the various lines can include quick-connect plumbing fixtures. The photobioreactor can have a gas delivery line, which can deliver, for example, delivering carbon dioxide or normal atmospheric air. The photobioreactor can have a fluid delivery line. Preferably, the fluid delivery line connects to a trickle or drip system which conveys a fluid (e.g., water) to the solid phase cultivation support. The photobioreactor can have a nutrient delivery line. Formulation of a nutrient composition for the growth and maintenance of a photosynthetic microorganism is within the ordinary skill of the art. In some embodiments, the nutrient and fluid delivery lines can be combined, for example to supply a fluid-based nutrient mixture. In some embodiments, the fluid delivery line or the nutrient delivery line can be a spray device for

distributing a liquid medium over the growth surface. In such spray devices, the photobioreactor is large enough to accommodate, for example, a spray device between an outer layer, such as a barrier layer, and the solid phase cultivation support. Usually, nutrients are supplied in a water-based composition. It can be advantageous to provide for different water delivery line(s) and nutrient delivery line(s) so as to provide for independent control of moisture and nutrient levels. The photobioreactor can have a product harvest line so as to provide for collection of photosynthetic microorganisms and/or liquid suspended/soluble products. The photobioreactor can have an inoculation line so as to provide for inoculation of photosynthetic microorganisms. In some embodiments, the fluid, nutrient, and/or inoculation lines can be combined.

[0127] One embodiment of a solid-phase photobioreactor is depicted in FIG 1 (front view) and FIG 2 (side view). In these embodiments, a solid phase cultivation support 2 is enclosed by protective barrier 7. FIG 2 shows that the solid cultivation support is between protective barrier layers 3 that comprise the protective barrier 7. The solid cultivation support 2 provides the surface upon which photosynthetic microorganisms are cultivated. The protective barrier layers 3 that make up the protective barrier 7 are transparent to allow actinic radiation to reach the surface of the solid cultivation support 2 to support the growth of photosynthetic microorganisms. Resealable closures 4 allow for a protective barrier 7 that is releasably sealed. Exchange of gases and vapor occurs through a selective panel 5 of material that is incorporated into the protective barrier 7. The photobioreactor 1 can be suspended by support elements 6 to allow for a vertical or non-horizontal orientation.

[0128] Another embodiment of a solid-phase photobioreactor is depicted in FIG. 12A (front view) and FIG. 12B (side view). The reactor 1 can be designed in a segmented format, which can aid in servicing and minimizes potential contamination of the surface and/or plumbing. Each segment can be connected to the reactor through plumbing (e.g., quick connect type plumbing) of the various supply and product harvest lines. The reactor can be supported by a suspension element 6 from, for example, rails, which allows the reactor 1 to hang in space and aid in rapid servicing of each segment. The outer protective barrier 7 can be a transparent material that enables light penetration facilitating photosynthesis on the growth surface 2, while preventing environmental contamination and moisture loss from evaporation. The growth surface 2 can be composed of a material that retains moisture, supplies nutrients, removes

products, and/or enables high density growth of photosynthetic microorganisms. The growth surface 2 can be serviced by plumbing that provides continuous feeding/product harvest from the surface by liquid culture media. The media tubing 8 can be a porous hose that seeps liquid to the surface 2, which can percolate through the growth surface 2 by gravity. The liquid can be harvested at the bottom of the reactor by a harvesting tube 9, which collects products and excess liquid media for transport from the reactor 1. Gases, such as carbon dioxide and air, can be supplied to the reactor by a gas dispersion tube 10. The gas supply tube 10 can provide a positive pressure environment and is expected to supply gases necessary for growth in a controlled, efficient manner. The gas supply line 10 can also assist in minimizing moisture loss by humidifying incoming gas streams. Excess gas from the reactor can be vented by a breathable panel 5 (on the reverse side, not shown) that is a porous material that allows for gas passage but minimizes or eliminates environmental contamination. Contamination is expected to be minimized by the positive pressure configuration of the reactor 1 through filtration of the incoming gas delivered by the supply line 10. Positive pressure can also prevent contamination from the environment by providing an inside out pathway for gas flow.

[0129] In the embodiment depicted in FIG. 12B, features of the reactor 1 are depicted in an orientation relative to the growth surface. The breathable panel 5 allowing for excess gas to escape the reactor 1 can be located toward the bottom of the device to provide a path for gas to migrate across the growth surface 2. Location of the breathable panel 5 on the bottom of the barrier surface 7 also minimizes or prevents the possibility of carbon dioxide segregation and build up resulting from its higher density relative to air. The dimensions of the breathable panel 5 can be determined based on gas flow rate requirements for optimal growth on the cultivation surface 2.

[0130] Solid Phase Cultivation Support

[0131] The solid phase cultivation support of a photobioreactor as described herein provides a surface on and/or in which a photosynthetic microorganism can grow. Preferably, the solid phase cultivation support comprises a material that provides or facilitates the provision and/or retention of moisture and/or nutrients to the organisms, so as to promote and sustain growth. Embodiments of the invention are not limited to the type or strain of photosynthetic microorganisms that can be cultivated. One of ordinary skill in the art will recognize that the

amount of moisture and the amount and composition of nutrients desirable for cell growth will vary with the type or strain of photosynthetic microorganism and the application for which it is to be grown. Materials (or the substances contained within or on those materials) that may have a deleterious effect on the growth of photosynthetic microorganisms are generally avoided.

[0132] A single photobioreactor can be used to cultivate a single type or multiple types or strains of photosynthetic microorganisms. Further, the solid cultivation support can comprise material(s) such that it is suitable for a single cultivation cycle or multiple cycles of cultivation, with or without sterilization between cultivation cycles. Still further, a photobioreactor can be configured to cultivate a single type or strain of microorganism or multiple types or strains of microorganisms on a single or multiple solid supports. In some embodiments, instead of an axenic culture, a community of different photosynthetic microorganisms, or a community of photosynthetic and non-photosynthetic microorganisms, can be grown together simultaneously on one cultivation support. A single photobioreactor can also comprise multiple cultivation supports. Thus in another embodiment, multiple cultivation supports within a single protective barrier can cultivate one or more types or strains of photosynthetic microorganisms simultaneously.

[0133] The solid cultivation support preferably comprises a relatively porous material. A relatively porous material generally has increased surface area and can retain and/or absorb more moisture than a relatively non-porous material. Also preferred is a solid cultivation support that has a textured or topographical surface(s). A textured or topographical surface can enhance cell density compared to a relatively non-textured or smooth surface. Although the choice of support material and surface topography are typically selected to enhance the adhesion of microorganisms to the support, it generally is desirable that the organisms not so tightly adhere so as to impede their removal or harvest. In some embodiments, the solid cultivation support comprises a material suitable for adhesion and growth of microorganisms. In some embodiments, the solid cultivation support comprises a material that reduces or eliminates biofilm formation.

[0134] The solid-phase supports of the photobioreactors described herein are believed to be different from solid supports that have been utilized in the art (*e.g.*, the most commonly used solid phase support for the growth of microorganisms is agar). Agar is generally cast into

rigid forms, such as a petri dish, and used while therein to maintain its physical integrity because agar tends to break or tear when subjected to minimal levels of stress, strain, or both. In contrast, various embodiments of the cultivation support is sufficiently strong and durable that it can be used in a photobioreactor while maintaining its physical integrity without the need of a stronger, more durable "frame". Or stated another way, the prior art involved a sufficient portion of the weak agar support in contact with a substantially stronger, more durable material (*e.g.*, a petri dish) such that a composite is formed. Thus, the solid-phase supports of various embodiments of the photobioreactor are suitable in themselves for the cultivation of microorganisms and are sufficiently strong and durable.

[0135] Other desirable physical characteristics and/or operation parameters of the solid-phase support are described below. For example, the support can be relatively flat and rigid (like a plate) or it may consist of a multiplicity of flat and rigid sections flexibly connected by, *e.g.*, hinges, springs, wires, threads, etc. Suitable rigid materials include, but are not limited to, various metals, polymers, ceramics, and composites thereof. The rigid materials preferably have surface topographies that enhance the adherence of the photosynthetic microorganisms thereto. Further, the rigid materials may be formed with a desired level of porosity to enhance the ability to deliver moisture and/or nutrients to the photosynthetic microorganisms. Still further, the rigid materials may be coated with absorbent or super absorbent polymer formulations (see below). Alternatively, the support may consist essentially of flexible material, such as a fabric. Fabrics for use in a solid-phase support include, but are not limited to, cotton, polyester, and/or cotton polyester blends, optionally coated with absorbent or super absorbent polymer formulations. Flexibility of the cultivation support can be greatly advantageous because it allows for the cultivation support to be folded, twisted, draped, or rolled for storage, transport, or handling.

[0136] In addition, the solid-phase cultivation support is preferably structurally stable at elevated temperatures (*e.g.*, about 120°C and above), such as would be typically encountered during autoclave sterilization, and will not melt like agar. Thus, in one embodiment, the cultivation support may be sterilized by autoclaving and then placed within the protective barrier of the invention. In another embodiment, the cultivation support can be placed within the protective barrier, and the entire photobioreactor may then be autoclaved. Although autoclaving

is one method for sterilization, one of skill in the art will recognize that any other appropriate method of sterilization may be utilized.

[0137] The solid cultivation support of the present invention can comprise or be made of any material appropriate for supporting the growth of photosynthetic microorganisms. For example, the support may be composed of natural materials, modified natural materials, synthetic materials, or any combination thereof. Natural materials can include, but are not limited to cotton, wool, processed woven plant fibers, and natural polysaccharides (e.g., agar, starches, cellulosics). Modified natural materials can include, but are not limited to, chemically modified plant fibers such as nitrocellulose or cellulose esters, in addition to natural fibers co-woven or blended with polyester or polyamide fibers. Synthetic materials can include, but are not limited to, fibers composed of nylon, fiberglass, polysiloxanes, polyester, polyolefins, polyamide, copolyester polyethylene, polyacrylates, or polysulfonates. Further examples of solid cultivation support materials include wire mesh, polyurethane foams, polyethylene foams, vitreous carbon foams, polyester/polyethylene foams, polyimide foams, polyisocyanate foams, polystyrene foams, and polyether foams, or combinations thereof.

[0138] In various embodiments, the solid cultivation support is a fabric. The fabric can be formed by methods such as, but not limited to, weaving, knitting, felting, and the bonding or cross-linking of fibers or polymers together. The construction of the fabric can be loose or open. Alternatively, the fabric can be tightly constructed. That said, fabrics that have a significant texture, surface area, topographical variability, and/or roughness may provide more mechanical bonding or adherence of the photosynthetic microorganisms to the cultivation support and thus may be preferable, especially in embodiments wherein the photobioreactor is handled, transported, or otherwise moved during the process for inoculating the support with, and/or growing and/or harvesting the organisms. Preferably, in most applications the adherence of the organisms to the substrate should not be so great as to unduly hinder their removal during a harvesting operation. Still further, the ability of a fabric to retain moisture and/or nutrients for use by the organisms can be controlled by selecting fibers that are generally hydrophobic, hydrophilic, or a mixture of such fibers. These properties allow for moisture and/or nutrients dissolved therein to be retained and/or transported by the solid support so that they are available to the microorganisms growing on the surface.

[0139] The properties of the cultivation support, especially moisture and/or nutrient retention, can be enhanced by coating the support with a material selected to enhance photosynthetic microorganism growth. For example, the cultivation support can be coated with agar or a super absorbent polymer such as modified cellulose ester, acrylate or acrylate/polyamine copolymer blends. These coating materials are typically able to absorb and retain greater than 10 to 100 times their dry weight in water. In some embodiments, these materials are formulated such that they would retain their superabsorbent properties in the presence of ionic culture media components. The coating material can coat the surface of the cultivation support, or the fibers of a fabric if used, or both. In one embodiment, a swatch of terrycloth serving as the cultivation support is coated in agar. When a solid cultivation support is coated as such, the "surface" of the cultivation support includes the surface of the coating if photosynthetic microorganisms attach to such. To keep the cultivation support thin, pliable, and light, the coating is preferably thin, for example, no greater than about 100 microns. However, thicker coatings can also be used depending on the application desired, or on the combination of solid cultivation support and coating material selected.

[0140] The solid-phase cultivation support can be a composite, layered structure. The solid-phase cultivation support can comprise at least two layers arranged so as to be adjacent. Multiple layers of the solid-phase cultivation support can be coupled, such as by bonding, stitching, adhesive, compression, or any other suitable means. The various layers can each independently be selected from among the several materials discussed above. For example, the solid-phase cultivation support can comprise a first material layer of fabric bonded to a second material layer of synthetic foam. An another example, the solid-phase cultivation support can comprise a first material layer of synthetic foam bonded to a second material layer of synthetic foam of the same or different density. Preferably, the solid-phase cultivation support is a composite, layered structure comprising at least a first layer, which is composed of a high surface area growth material, and a second layer, which is composed of a permeable type material.

[0141] In addition to supplying moisture, nutrients, and a surface for attachment, the cultivation support can provide a surface for capturing actinic radiation. Thus, in some embodiments, the dimensions of the solid cultivation support are sheet-like. That is, the depth of

the support is small relative to the length and width of the support. In one embodiment, the cultivation support is a sheet-like layer between film-like layers of a protective barrier. Such a flat bioreactor can be suspended like a flat panel. In another embodiment, just the cultivation support is suspended like a curtain enclosed by the outer barrier of the photobioreactor. A thin sheet of a traditional solid phase support such as agar would easily rip apart, and would likely not be able to be suspended as such. Therefore, it is preferable that the solid cultivation support alone be able to maintain its integrity when suspended, even when saturated with liquid.

[0142] As shown herein, a fabric with a terrycloth-type weave can provide a suitable solid support (*see e.g.*, Example 1). One of skill in the art will understand that other natural, modified-natural, and synthetic materials may also be acceptable. Terrycloth provides many of the attributes believed to be desirable in a solid support of the present invention. For example, it is flexible, and not prone to tearing, ripping, breaking, or cracking when handled in accordance with non-destructive techniques (*e.g.*, bending, folding, twisting, or rolling) under conventional conditions (*e.g.*, temperature). Likewise, terrycloth is typically not prone to tearing, ripping, or breaking when modestly stretched (even when saturated with liquid). Additionally, terrycloth tends to be highly textured because it is composed of the many loops of fibers. This provides a large amount of surface area for the attachment of microorganisms thereby increasing the amount of microorganisms that can be grown on a support of any given size. Further, a cotton terrycloth typically absorbs at least about three times its own weight, which allows for moisture and any nutrients dissolved therein to be retained by the fabric support so that they are available to the microorganisms growing on the surface of the support. Thus, various embodiments provide for a solid cultivation support that is thin or sheet-like in dimension, able to support its own wet weight while suspended, flexible, pliable, absorbent, highly textured, or any combination thereof.

[0143] The above-described supports can be, and in many applications preferably are, used repeatedly and more preferably for so long as they are structurally sound and provide a surface adequate to support the growth of the microorganisms disposed of after a single use thereby reducing operational costs and waste. That said, there can be certain applications in which single-use supports would be desirable, such as cultivation of recombinant photosynthetic microorganisms useful in producing pharmaceutical products such as small organic molecules or

therapeutic proteins and peptides. To reduce the costs of such single-use supports and in view of the fact that that they will not be reused, such supports need not be as durable and therefore can be made or constructed using methods and/or materials that are less costly and less durable. For example, supports comprised of paper fibers similar to that of paper towels may be appropriate.

[0144] Several embodiments of a solid phase cultivation support are depicted in FIG. 13. The solid phase cultivation support material depicted in FIG. 13A is a single material that can provide sustainable surface for organism growth, access to moisture and nutrients, point of organism attachment, and/or removal of cultivation products. The material can allow for liquid percolation and equilibrium diffusion to exchange nutrients, moisture, and products between the surface and organisms. The rendering of the structure configuration is an example of a high surface area material, which can be optimized for dimension and shape. The solid phase cultivation support material depicted in FIG. 13B is a hybrid material that is composed of multiple layers of materials, each having specific functions for the growth surface. The base layer can be a porous material that efficiently allows for supply of nutrients and moisture as well as removal of products that are percolated through the material. The base material can also provide physical support for the growth surface. The outer layer(s) is expected to be attached to the base layer and can be optimized to provide point of attachment for the organisms. The surface layer can achieve more control of the surface growth environment in terms of surface area and compatibility with the cultivated organism.

[0145] Protective Barrier

[0146] A photobioreactor as described herein can comprise a barrier that protects the solid cultivation support and growth surface from contamination and/or moisture loss. At the same time, the photobioreactor provides for actinic radiation, either sunlight or artificial light, and carbon dioxide reaching the photosynthetic microorganisms. In various embodiments, the photobioreactor comprises at least one solid support and a protective barrier for the cultivation of photosynthetic microorganisms.

[0147] Protection from Physical Handling and/or Contamination

[0148] To prevent contamination, a protective physical barrier can at least partially cover the solid cultivation support. In certain embodiments, the physical barrier can enclose the

cultivation support. The protective barrier can also control, at least in part, the loss of the moisture from the support and/or the atmosphere within the photobioreactor to the atmosphere outside the photobioreactor. One of skill in the art will recognize that the protective barrier can be constructed from any of numerous types of materials depending on the embodiment of the invention desired.

[0149] The protective barrier can completely enclose the cultivation support. If the protective barrier is permanently sealed, the barrier must be breached, cut, torn, or the like to access the cultivation support within. Thus, in some embodiments, access is provided through the protective barrier to the cultivation support and the surface on which the microorganisms are grown.

[0150] In preferred embodiments, the protective barrier is releasably sealed. The releasable seal can be any of a number of closure types including, but not limited to zipper-type closures such as found in Ziploc® storage bags (SC Johnson Company), hook-and-loop type fasteners (*e.g.*, Velcro USA, Inc.), twist ties, zipties, snaps, clips, pressure sensitive adhesive backed surfaces, and all art recognized equivalents thereto. A complete seal, however, is not necessarily required; and it may be more efficient not to completely seal the outer barrier to allow for easier access to the cultivation support.

[0151] The photobioreactor can comprise a single cultivation support or multiple cultivation supports within a protective barrier. In some embodiments, a single cultivation support is enclosed within a single protective barrier. For example, a plastic bag may form a protective barrier within which a single solid cultivation support is enclosed (*see e.g.*, FIG. 1). In other embodiments, a single protective barrier may enclose multiple solid cultivation supports. For example, a greenhouse-type structure may form a protective barrier within which multiple solid cultivation supports are enclosed.

[0152] Transmission of Actinic Radiation

[0153] The photobioreactor can provide for transmission of actinic radiation, either sunlight or artificial light, to the photosynthetic microorganisms. But the protective barrier of the invention need not necessarily be transparent to light. Some embodiments can comprise a cultivation support enclosed within a non-transparent protective barrier if a sufficient light source

for the growth of photosynthetic microorganisms is provided within. It may be desirable, simpler, more economical, and the like to provide a transparent barrier to utilize sunlight, for instance, as a light source.

[0154] Preferred embodiments provide for a transparent barrier comprising a material such as, but not limited, glass or any type of transparent or generally visible light transmitting polymer such as polyethylene, acrylic polymers, polyethylene terephthalate, polystyrene, polytetrafluoroethylene, or co-polymers thereof, or combinations thereof. The transparent barrier can be selected from materials that are durable and not prone to ripping, tearing, cracking, fraying, shredding, or other such physical damage. The transparent barrier material can be selected for its ability to withstand autoclave sterilization or other exposure to temperature extremes. Further, the transparent barrier materials can be selected to withstand prolonged exposure to sunlight or other radiation without discoloring or deteriorating. One of skill in the art will recognize that certain coatings or formulations that resist photooxidation can be particularly useful. In addition, infrared reflecting or absorbing coatings can be selected to reduce and/or otherwise regulate the buildup of temperature within the photobioreactor of the invention.

[0155] One of skill in the art will recognize that the thickness of the transparent barrier material will vary depending on mechanical properties of scale. For example, the transparent barrier material may be of an industrial/marine type plastic about 10 mil thick or it may be of the type used in a household plastic bag, *i.e.*, around 2 mil thick. In one embodiment, the transparent barrier material is thin and flexible. For example, the transparent barrier material can be less than about 10 mil.

[0156] In some embodiments, the barrier forms a protective layer or film covering the two sides of a thin, flexible, solid cultivation support. The assembled photobioreactor of this embodiment would be flexible, and could be bent, rolled, folded, twisted, or the like for storage, transport, conveying, or handling. In another embodiment, the transparent barrier material is rigid. For example, the barrier can be a glass greenhouse. Most likely, the thickness of the greenhouse glass would preferably be consistent with building practices but it is possible that it could be altered. The photobioreactor of such an embodiment would be for practical purposes

immovable, but multiple solid supports could be handled, transported, conveyed and the like within the confines of one protective, transparent barrier.

[0157] Although a protective barrier can be selected to provide sufficient light for the growth of photosynthetic microorganisms, it is not necessary that the entire barrier be transparent. Thus, in some embodiments, portions of the barrier, such as one or more edges, are made from a non-transparent material. The non-transparent material can be composed of materials including, but not limited to polyethylene fiber material (Tyvek®), polytetrafluoroethylene filtration media, cellulosic filter material, fiberglass filter material, polyester filter material and polyacrylate filter material, and combinations thereof. The non-transparent material can be selected for durability. In such an embodiment, a transparent portion of the barrier would be further protected from tearing, ripping, fraying, shredding, and the like by a durable, non-transparent portion. In one embodiment, a non-transparent portion provides or comprises an attachment structure and/or reinforcement for suspending the photobioreactor by further comprising mounting or attachment points (*e.g.*, holes, loops, hooks, grommets, or other art equivalent device, opening or, recess) and/or or a mechanism for securing the photobioreactor to a structure. Although it is not required that any such mounting points, etc., be located in or on the non-transparent portion, they can be contained within or on a non-transparent portion of the barrier, within or on a transparent portion of the barrier, or within or on a non-transparent and a transparent portion of the barrier. The attaching structure may also be contained within or on, or pass through, the solid cultivation support.

[0158] In some embodiments, the device has a discernable front side and back side. The front side of this device is meant to face a light source, and thus the portion of the barrier on the front side is preferably transparent, while the portion of the protective barrier on the side facing away from the light source is not necessarily transparent.

[0159] Provision of Gas Exchange

[0160] During photosynthesis, photosynthetic microorganisms consume carbon dioxide and release oxygen. A photobioreactor as described herein can provide carbon dioxide sufficient for a desired amount of photosynthesis to occur. One way to supply carbon dioxide to the inside of the photobioreactor is to allow direct gas exchange between the air inside and the

air surrounding the photobioreactor. For example, holes, vents, windows, or other such openings can be provided in the protective barrier so that the system is open to the surrounding atmosphere.

[0161] But such an open configuration may not be desirable when contamination of the photosynthetic microorganisms is a concern. To address this concern, the protective barrier can completely seal off the solid support or supports enclosed within from the outside air. In such an embodiment, the desired concentration of carbon dioxide can be maintained by introducing it into the enclosure. For example, one of skill in the art would recognize that plumbing or tubing from a tank of compressed carbon dioxide would allow for carbon dioxide to be mixed into the air enclosed within the photobioreactor. In addition, it is known that the emissions from factories, industrial plants, power plants, or the like can be harnessed as a source of carbon dioxide for photosynthetic microorganisms, thus reducing carbon emissions. In one embodiment, a gas supply line can provide carbon dioxide to the growth surface local area.

[0162] It may be desirable, simpler, more economical, and the like to provide a selective barrier that is gas permeable to utilize atmospheric carbon dioxide. Thus, some photobioreactor embodiments provide for a selective barrier that allows gas and vapor exchange between the environment enclosed within the protective barrier and the surrounding air, while still providing a sealed physical barrier against contamination. Such barrier can be at least partially gas/vapor permeable (*e.g.*, much less permeable than conventional textile fabrics, higher than that of plastic films, and/or similar to that of coated papers), thus allowing the exchange of gases such as carbon dioxide and oxygen but is additionally at least partially and preferably considered to be impermeable to solids and liquids. In some embodiments, the photobioreactor can contain a semi-permeable barrier layer and a gas supply line to maintain an elevated carbon dioxide concentration in the area around or near the growth surface.

[0163] In some embodiments, a selective barrier can have an average pore size or diameter of no greater than about 10 micrometers and a gas exchange rate that is at least about 5 and no greater than about 10,000 Gurley seconds (a Gurley second or Gurley is a unit describing the number of seconds required for 100 cubic centimeters of gas to pass through 1.0 square inch of a given material at a given pressure differential). Therefore, in addition to allowing gas exchange, the selective barrier can prevent loss of moisture from the enclosed system.

[0164] The selective barrier portion of the protective barrier can be composed of any appropriate polymer-based material, such as spunbonded olefin barriers. Spunbonded olefin barriers (very fine polyethylene fibers) with various properties are readily available from DuPont under the brand name Tyvek®. Such materials are particularly advantageous because of their combination of physical properties, *i.e.*, they tend to resist the transmission of liquids such as water yet they have a sufficiently high degree of gas/vapor permeability; they are relatively strong, absorb little or no moisture, are rip-resistant, have a significant degree of elasticity, and are highly flexible. Spunbonded olefin can exceed 20,000 cycles when tested on an MIT flex tester (TAPPI method T-423). In addition, they are inert to most acids, bases and salts although a prolonged exposure to oxidizing substances, such as concentrated nitric acid or sodium persulfate, will cause some loss of strength. Spunbonded olefin barriers have good dimensional stability in that sheet dimensions tend to change less than 0.01% between 0 and 100% relative humidity at constant temperature. Certain products meet the requirements of Title 21 of the United States Code of Federal Regulations (21 CFR 177.1520) for direct food contact applications. They also have excellent mold and mildew resistance; and are of a neutral pH. Unfortunately, however, their UV resistance is not exceptional. That said, at least one to three months of useful outdoor life can usually be expected. Additionally, their UV resistance can be improved with opaque coatings or by including UV inhibitors in the polymer fibers. Additionally, because the spunbonded oelefins produced to date are opaque, the portion of the protective barrier that would comprise such material is preferably not situated and/or so extensive as to compromise the cultivation of the photosynthetic microorganisms.

[0165] In particular, spunbonded olefin can be produced in "hard" and "soft" structure types. Type 10, a "hard," area-bonded product, is a smooth, stiff non-directional paper-like form. Types 14 and 16 are "soft," point-bonded products with an embossed pattern, providing a fabric-like flexible substrate. Type 14 styles (or the equivalent thereof) can be used, for example, where barrier, durability, and breathability are required. Type 16 styles are pin perforated with 5-20 mil (0.13-0.51 mm) holes, giving them much higher air and moisture permeability, additional softness, and greater flexibility and drape than Type 14 styles, but at the expense of lower tear strength and barrier properties. Thus, the particular properties of the

selective barrier can be customized by selecting one or more types of spunbonded olefin products.

[0166] Other examples of selective polymer barriers include, but are not limited to nylon, polysulfone, polytetrafluoroethylene, cellulosic, fiberglass, polyester and polyacrylate membranes and filter material, and combinations thereof.

[0167] The entirety of the protective barrier need not be gas permeable to provide for a barrier that is sufficiently selective for the growth of photosynthetic microorganisms. Only a portion of the protective barrier sufficient to allow for adequate gas exchange need be gas permeable. In one embodiment, the selective portion is a panel of the protective barrier (*see e.g.*, FIG 1). The size and placement of the selective panel in relation to the area of the support surface can be altered to achieve a desired amount of gas exchange for a particular application without unduly hindering the cultivation of the microorganisms. One of skill in the art will recognize that the percentage of the area of the outer barrier composed of the gas permeable selective material will depend on the gas permeability rate of the material. In fact, because the gas permeable portion will still allow the transport of water vapor across it, in various embodiments, the size of the gas permeable portion of the protective barrier is selected so as to allow for sufficient transport of oxygen and carbon dioxide while minimizing the loss of moisture.

[0168] Suspension and Conveyance System

[0169] Photobioreactors described herein can be configured for large scale production and/or harvesting through, for example, integration into a handling and conveyance system. FIG 3 shows an above view of an exemplary design of a photobioreactor farm for handling large numbers of photobioreactors in a continuous process. The photobioreactors or cultivation panels (not individually shown) are attached to conveyor systems 8. The conveyor systems 8 move the cultivation panels along their paths. Multiple conveyor systems converge at centrally located inoculation and harvesting centers 9. Thus, the cultivation panels are moved into the inoculation and harvesting centers 9 where they can be processed (*e.g.*, harvested and/or inoculated) and then the panels are moved away from the centers following inoculation and during the period of cultivation of the biomass. The panels are then moved back towards the

centers during the latter period of cultivation prior to harvesting, eventually arriving back at the centers with mature biomass for harvest. The cycle is then repeated. Harvested biomass can be transported through a pipeline 10 for further processing. The capacity of the photobioreactor farm can be increased by adding additional conveyor systems or additional inoculation and harvest centers to form large arrays dedicated to biomass production.

[0170] Suspension of PhotoBioreactor

[0171] To supply light to photosynthetic microorganisms, a favored embodiment of the photobioreactor is one in which the cultivation support is thin and sheet-like. When oriented horizontally, the efficient utilization of floor space tends to decrease, therefore in certain embodiments of the invention the cultivation support is oriented non-horizontally, preferably substantially vertically, or more preferably vertically. Nevertheless, the cultivation support may be oriented in essentially any manner so long as a sufficient amount of actinic radiation can reach the microorganisms. Thus, when the photobioreactor is of the type where the protective barrier forms a closely associated film or layer around the solid support, a preferred orientation of the entire photobioreactor is vertical, but any orientation is acceptable. To be clear, the aforementioned orientations (*e.g.*, vertical, horizontal, substantially vertical, non-horizontal, *etc.*) are relative to the floor or ground beneath the cultivation support, assuming that the floor or ground is horizontal.

[0172] Various structures, scaffolding, stands, racks, *etc.* may be used to hold or suspend a cultivation support or an entire photobioreactor in a desired orientation. In particular, the cultivation support and/or the protective barrier can be suspended from, or attached to. a rope, line, hook, cable, track, rail, chain, shelf, pole, tube, scaffold, stand, beam or any other such structure capable of suspending the solid cultivation support and/or photobioreactor. Multiple cultivation supports and/or photobioreactors may be suspended from a common structure, like sheets hanging from a clothes line. The cultivation support(s) and/or photobioreactor(s) may be suspended statically, or in a manner that allows for their movement. The position of the holes, loops, hooks, or the like will preferably distribute the weight of the cultivation support and/or photobioreactor substantially evenly.

[0173] Suspension of the photobioreactor or cultivation support, especially in a vertical orientation, is space efficient and may provide advantages in handling. However, the bioreactor or cultivation support of the invention need not be suspended. For example, in certain embodiments of the present invention, the cultivation support is sufficiently rigid that if oriented non-horizontally, vertically, or substantially vertically (*e.g.*, by securing or placing its base to/on a surface, in an embodiment in which the support is like a rigid plate, panel, grid, etc.) it can support its own weight and will remain so oriented. In another embodiment, the protective barrier is free standing, such as a greenhouse, and multiple cultivation supports are suspended and/or free-standing within.

[0174] Suspension of the photobioreactor and/or cultivation support, especially in a vertical orientation, is space efficient and may provide advantages in handling. However, the bioreactor or cultivation support of the invention need not be suspended. For example, in certain embodiments of the present invention, the cultivation support is sufficiently rigid that if oriented non-horizontally, vertically, or substantially vertically (*e.g.*, by securing or placing its base to/on a surface, in an embodiment in which the support is like a rigid plate, panel, grid, *etc.*) it can support its own weight and will remain so oriented. In another embodiment, the protective barrier is free standing, such as a greenhouse, and multiple cultivation supports are suspended and/or free-standing within.

[0175] Conveyance

[0176] Also described herein is a system for conveying photobioreactors, cultivation supports within the protective barrier of a photobioreactor, or some combination thereof from one location to another. The ability to transport a photobioreactor and/or cultivation support can be advantageous for a variety of reasons. For example, it may allow for optimizing their position(s) for receiving light, and for maintaining a desired temperature or gas content. The transportability can be particularly advantageous when multiple photobioreactors or cultivation supports are to be subject to discrete steps, such as inoculating, cultivating, inducing, and/or harvesting, because it is likely to be more efficient to move the photobioreactors or cultivation supports to several assigned locations in a continuous-type process instead of transporting the necessary materials and equipment to stationary photobioreactors or cultivation supports.

[0177] Thus, the growing surface, whether the cultivation support alone, or the cultivation support enclosed in a protective barrier, can be conveyed, even after inoculation. One of skill in the art will be familiar with numerous types of conveyor systems frequently used in industrial applications. The conveyance system is not limited to any particular type so long as it is capable of moving one or more photobioreactors or cultivation supports. One skilled in the art will recognize that the type of attachment between the photobioreactor or cultivation support and the conveyor system will vary with the type of conveyance system employed and will be selected to work cooperatively with any mounting points that are part of the cultivation support and/or the protective barrier. Although it is envisioned that the cultivation support(s) or photobioreactor(s) will be conveyed in a mechanized manner powered by one or more motors (*e.g.*, through the action of a chain and gears), it is also possible for them to be conveyed with human effort (*e.g.*, by simply pushing suspended bioreactors that are attached to a rail by a bearing mechanism that slides along the rail).

[0178] A conveyor system that suspends photobioreactor(s) and/or cultivation support(s), especially in a vertical orientation, is space efficient and may provide advantages in handling. But the conveyor system need not rely on suspending photobioreactor(s) or cultivation support(s). For example, a photobioreactor may move along on top of the conveyor system, such as by sliding over a roller conveyor. In one embodiment, the conveyor system may move photobioreactors comprising a cultivation support enclosed in a protective barrier. Alternatively, the protective barrier of a photobioreactor may be a large enclosure protecting one or more conveyor systems moving multiple cultivation supports.

[0179] Photobioreactor Farm

[0180] For large scale applications, it may be impractical to construct a single cultivation support of sufficient size. Thus is provided use of two or several or tens or hundreds or thousands or more cultivation supports to cultivate photosynthetic microorganisms in a photobioreactor "farm." These cultivation supports can all reside within a single protective barrier, thus comprising a single photobioreactor, or multiple cultivation supports may be part of multiple photobioreactors. In either case, it can be beneficial to organize the multiple photobioreactors or cultivation supports within a photobioreactor farm for ease and efficiency of handling and processing. It can also be beneficial to organize their arrangement to maximize the

amount of energy captured from a light source such as the sun. Such organization can consist of arranging numerous photobioreactors or cultivation supports in an orderly fashion such as, but not limited to, rows, columns, concentric circles, in grids, radiating outward from a central point, and so forth.

[0181] In various embodiments, the farm comprises multiple photobioreactors or cultivation supports suspended from a common structure such as a track, rail, chain, line, or the like. In further embodiments, the structure is part of a conveyor system and the photobioreactors or cultivation supports move along the path of the conveyor system from one location to another.

[0182] A photobioreactor farm can comprise one or an arrangement of multiple conveyor systems handling numerous photobioreactors or cultivation supports. Such an arrangement could be scaled up to comprise two or several or tens or hundreds or thousands or more conveyor systems together handling two or several or tens or hundreds or thousands or more photobioreactors or cultivation supports. In addition to the conveyor system(s), a photobioreactor farm can include defined areas, stations, or centers for performing steps such as inoculating, cultivating, inducing, and/or harvesting photosynthetic microorganisms. Such centers can be the location of specialized equipment for performing certain steps. The paths of the conveyor systems can bring the photobioreactors or cultivation supports to such centers where a particular step is performed. The photobioreactor or cultivation support can then be moved along to the next area or center in the sequence. Different photobioreactors or cultivation supports along the conveyor system can reside at different centers along the path and thus be subject to different steps simultaneously. In one embodiment, the path of the conveyor system is a loop. Once a photobioreactor or cultivation support completes one round of steps in the cultivation process, it can repeat the process. Allowing for some units to be damaged or otherwise eventually needing replacement, essentially the same set of photobioreactors or solid cultivation supports can be used repeatedly.

[0183] In a further embodiment, cultivation and harvest can occur at the same or nearly the same location. This location is termed an inoculation and harvest center (*see e.g.*, FIG 3). Inoculation of the photobioreactors and/or solid cultivation supports occurs at the inoculation and harvest center. The conveyor system forms a loop that then transports the photobioreactors or cultivation supports away from the inoculation and harvest center. The photobioreactors or

cultivation supports then travel along the path of the conveyor system for an amount of time sufficient for the desired amount of cell growth. The conveyor system then returns the photobioreactors or cultivation supports back to the inoculation and harvest center for harvest. Multiple conveyor systems can share a common inoculation and harvest center from which they radiate out from. If even more capacity is needed, a photobioreactor farm can comprise multiple inoculation and harvest centers handling the photobioreactors or cultivation supports from multiple conveyor systems. Although increased efficiencies may be realized, it is not necessary that the location of inoculation and of harvest be the same or nearly the same location.

[0184] Methods of Using a Photobioreactor

[0185] Cultivation of Photosynthetic Microorganisms

[0186] A solid phase photobioreactor, as described herein, can be used for cultivating photosynthetic microorganisms. Photosynthetic microorganisms that can be grown in the solid phase photobioreactor include, but are not limited to, a naturally photosynthetic microorganism, such as a cyanobacterium, or an engineered photosynthetic microorganism, such as an artificially photosynthetic bacterium. Exemplary microorgansims that are either naturally photosynthetic or can be engineered to be photosynthetic include, but are not limited to, bacteria; fungi; archaea; protists; microscopic plants, such as a green algae; and animals such as plankton, planarian, and amoeba. Examples of naturally occurring photosynthetic microorganisms that can be grown in the bioreactor include, but are not limited to, Spirulina maximum, Spirulina platensis, Dunaliella salina, Botrycoccus braunii, Chlorella vulgaris, Chlorella pyrenoidosa, Serenastrum capricomutum, Scenedesmus auadricauda, Porphyridium cruentum, Scenedesmus acutus, Dunaliella sp., Scenedesmus obliquus, Anabaenopsis, Aulosira, Cylindrospermum, Synechoccus sp., Synechocystis sp., and/or Tolypothrix.

[0187] Preferably, the photosynthetic microorganisms grown in the solid phase photobioreactor comprise cyanobacteria. The cyanobacterium grown in the bioreactor can be any photosynthetic microorganism from the phylum Cyanophyta. The cyanobacterium grown in the bioreactor can have a unicellular or colonial (*e.g.*, filaments, sheets, or balls) morphology. Preferably, the cyanobacterium grown in the bioreactor is a unicellular cyanobacterium. Examples of cyanobacteria that can be grown in the bioreactor include, but are not limited to, the

genus Synechocystis, Synechococcus, Thermosynechococcus, Nostoc, Prochlorococcu, Microcystis, Anabaena, Spirulina, and Glocobacter. Preferably the cyanobacterium grown in the bioreactor is a Synechocystis spp. or Synechococcus spp. (e.g., *Synechococcus elongatus* PCC 7942 (ATCC 33912) and/or *Synechocystis* spp. PCC 6803 (ATCC 27184)). More preferably, the photosynthetic microorganism grown in the bioreactor is a transgenic photosynthetic microorganism engineered to accumulate a disaccharide, as disclosed herein.

[0188] A solid cultivation support of a photobioreactor can be inoculated with a photosynthetic microorganism, along with addition of moisture and other components including, but not limited to, nutrients, salts, buffers, metals, nitrogen, phosphate, sulfur, etc. The photobioreactor can then be releasably sealed with the cultivation support within the protective barrier. The sealed photobioreactor can be placed, for example by suspending it, in a location and manner to allow for control of illumination and temperature. The placement can be static, or the photobioreactor can be moved, such as to ensure maximum exposure to the sun's radiation over the course of a day. The photosynthetic microorganisms can be cultivated for a desired amount of time. One of skill in the art will recognize that the length of time will vary according to the type of microorganism and the density of cell growth desired. For example, for certain strains of cyanobacteria, a cultivation period that is within the range of about four to about seven days can provide a yield of cells that is within the range of about 50 to about 250 grams of dry biomass per liter equivalent. Following a period for cultivation, the releasable seal can be opened and the photosynthetic microorganisms can be harvested.

[0189] As used herein, "grams of dry biomass per liter equivalent" is a unit determined by calculating the average depth of the biomass layer (*e.g.*, about 150 microns) growing on the cultivation surface and multiplying that value by the length and the width of the cultivation surface. This calculation provides a volume. The weight of the collected biomass from the cultivation surface can then be correlated to the volume and expressed as "grams of dry biomass per liter equivalent."

[0190] Method of Continuous Cultivation

[0191] Greater efficiencies can be realized if the process of cultivating photosynthetic microorganisms were to be made continuous, for example, like an assembly line. Instead of

requiring the equipment and capacity to handle a large amount of biomass all at once that then sits idle in between batches, a continuous system would require less total capacity, but would utilize that capacity more efficiently through continuous operation. By dividing cultivation into smaller but more numerous components, the components can be organized in a spatially continuous arrangement. Different discrete steps of the overall production process can then occur simultaneously. After a cultivation component is subjected to a process step, the component moves forward in the process while another component replaces it in that step. Therefore, production of the end product would not be limited to the maturation of a large batch, but can occur regularly as individual components complete the assembly line-like process. Further, following the completion of one round of the process, the components can immediately start the process over and do so repeatedly.

[0192] More specifically, continuous cultivation relates to methods of using conveyable photobioreactors or cultivation supports for cultivating photosynthetic microorganisms in a continuous manner. Continuous or continuous process is understood as the spatial relationship that can allow the photobioreactors or solid cultivation supports to progress from one step of the cultivation process to another. Alternatively, it is possible for a single large structural support to be utilized in a continuous process. Specifically, the support can be a loop of material (*e.g.*, terry cloth fabric) that is made to travel along a circuit (*e.g.*, like a conveyor belt that is arranged preferably vertically). The end result is that biomass production can be achieved regularly as multiple photobioreactors or solid cultivation supports finish the process sequentially and repeatedly. This type of process presents opportunities in large scale applications for increased efficiencies over producing biomass in large, but infrequent batches.

[0193] In a preferred embodiment, the continuous spatial relationship is along the path of a conveyor system. The manner of operation is analogous to an assembly line. Such a conveyor system can operate in a number of ways. For example, the conveyor system can operate without interruption while moving the photobioreactors or cultivation supports from one location to another. In such an embodiment, inoculation, harvesting, and the like occur while the photobioreactors or cultivation supports are in motion. Alternatively, the conveyor system can stop to allow for steps to be performed, and then resume to move the photobioreactors or cultivation supports to the location of the next step. Further, the conveyor system can operate

without interruption, and the photobioreactors or cultivation supports can be detached from the movement of the conveyor system for processing, and then reattached to re-enter into the stream of conveyance. One skilled in the art will realize that other permutations of this general theme are also possible.

[0194] In one embodiment of a method of continuous cultivation, multiple photobioreactors are inoculated at one location along the conveyor system. The conveyor system then moves the photobioreactors to an area where cultivation of the photosynthetic microorganisms occurs. During this portion of conveyance, the photobioreactors can be positioned to allow for optimal illumination to promote growth and photosynthesis. Next, the photobioreactors would arrive at a location where the photosynthetic microorganisms can be harvested. The photobioreactors can then return along the path of the conveyor system to the point of inoculation to begin the process again. To improve efficiency, the time between when the photobioreactors leave the location of inoculation and arrive at the location of harvest can be made to coincide with the time it takes for the desired amount of growth of the photosynthetic microorganisms to occur. The steps of the process are not limited to inoculation, cultivation, and harvest; additional steps can include inducement of the cells to synthesize a desired product or sterilization. Although the above embodiment describes a system of conveyable photobioreactors, it will be appreciated that the same type of continuous cultivation can be practiced within a single protective barrier to convey and process multiple solid cultivation supports.

[0195] Method of Producing Fermentable Sugars

[0196] One technology that can benefit from the ability to more efficiently grow photosynthetic microorganisms is the production of biomass for alternative fuels such as ethanol or biodiesel. Relative to plants currently grown to produce biomass such as corn, sugarcane, soybeans, canola, jatropha, and so forth, photosynthetic microorganisms, such as cyanobacteria, produce biomass at a much faster rate, which may lead to much greater productivity. In addition, direct production of disaccharides by microorganisms avoids much of the extensive energy-intensive pre-processing of using plant biomass to produce fermentable sugar. Further, the use of phototrophic microorganisms instead of plants can lead to higher yields of fermentable sugars without soil depletion, erosion, and diversion of the food supply. Relative to other

microorganisms, preference is given to phototrophic microorganisms because their sources of carbon (CO_2) and energy (light) can be supplied from the environment, making them far less expensive to cultivate. In addition, phototrophic microorganisms can be utilized to consume carbon emissions from industrial processes, thus providing further benefits to the environment.

[0197] One obstacle to producing high quantities of fermentable sugars from photosynthetic microorganisms is that they generally consume produced carbohydrates rather than accumulating them. While some sugars, such as sucrose or trehalose, are not utilized as a primary carbon source by photosynthetic microorganisms, there are mechanisms for slow assimilation. In spite of reprocessing mechanisms, such material can accumulate without being metabolized. If the organism is engineered appropriately, the assimilation mechanism can be inactivated, which enables high yields of sugars to be produced.

[0198] Provided herein is a method for producing fermentable sugars, especially disaccharide sugars, by photosynthetic microorganisms. Examples of fermentable sugars include, but are not limited to, sucrose, trehalose, glucosylglycerol, and mannosylfructose. Preferably, the fermentable sugar is sucrose or trehalose. The method can be adapted to occur in a continuous manner to improve the cost effectiveness of production.

[0199] Various embodiments of this method can be practiced using a photosynthetic microorganism capable of synthesizing fermentable sugars. Some embodiments harness and control the natural phenomena of osmo- and matric water protection for the generation of fermentation feedstocks. In one embodiment, synthesis of fermentable sugars is inducible. In another embodiment, synthesis of fermentable sugars can be modified by genetic manipulation to be produced constitutively.

[0200] Fermentable sugar-producing photosynthetic microorganisms are preferably cyanobacteria. In some embodiments, a cyanobacterium accumulates a disaccharide according to inducible endogenous pathways. In some embodiments, a transgenic cyanobacterium accumulates a disaccharide according to engineered exogenous pathways. Both endogenous and exogenous pathways are discussed in further detail above.

[0201] Preferably, the transgenic photosynthetic microorganisms are one or more of those discussed above.

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[0202] Two non-limiting examples of strains of cyanobacteria capable of accumulating a disaccharide are Synechococcus elongatus PCC 7942 and Synechocystis sp. PCC 6803. Naturally occurring Synechococcus elongatus PCC 7942 synthesizes sucrose upon exposure to salt concentrations of up to about 700 mM, its tolerance limit. When glucosylglycerol biosynthesis is blocked by deletion of the agp gene, Synechocystis sp. PCC 6803 produces sucrose as its osmoprotectant upon exposure to salt concentrations up to its tolerance limit which may approach 900 mM. In some embodiments, salt induction can be accomplished by introducing aerosolized saline solution applied directly to the cultivation surface. One advantage of this process is application can be controllably introduced along the growing surface depending on growth time of the cultivar thereby balancing accumulation of biomass and production of a disaccharide such as sucrose.

[0203] For producing fermentable sugars, the photosynthetic microorganisms can be cultured and grown on a solid medium or in a liquid or gel medium. Culture and growth of photosynthetic microorganisms are well known in the art. Except as otherwise noted herein, therefore, culture and growth of photosynthetic microorganisms can be carried out in accordance with such known processes. For example, a transgenic cyanobacteria engineered to accumulate a disaccharide can be cultured and grown in a liquid medium. The accumulated sugar can be isolated from such liquid medium if excreted from the cell. The accumulated sugar can be isolated from photosynthetic microorganisms harvested from the liquid medium. In one embodiment, a transgenic cyanobacteria engineered to accumulate trehalose, as discussed above, is cultured and grown in a liquid medium. Trehalose secreted from the transgenic cyanobacteria can be isolated directly from the liquid medium. In one embodiment, a transgenic cyanobacteria engineered to accumulate sucrose, as discussed above, is cultured and grown in a liquid medium. Sucrose can be isolated directly from engineered cyanobactria harvested from the liquid medium. In one embodiment, a transgenic cyanobacteria engineered to accumulate and secrete sucrose, as discussed above, is cultured and grown in a liquid medium. Sucrose secreted from the transgenic cyanobacteria can be isolated directly from the liquid medium.

[0204] Preferably, photosynthetic microorganisms are cultivated to a relatively high cell density of at least about 50 grams of dry biomass per liter equivalent prior to induction. Such relatively high cell densities can be achieved using a solid phase photobioreactor, as

described herein. Disaccharide (e.g., sucrose) production can then be initiated/induced by treating the accumulated biomass with defined concentrations of suitable salt compounds effective at altering the activity of water in the culture media as measured by solution conductivity. In a further preferred embodiment, sodium chloride is the salt used. Following an appropriate response time period (*e.g.*, at least about 1 hour to no greater than about 48 hours), the sucrose laden cells can be harvested and processed to isolate and recover the sucrose produced. Typically, an appropriate response period is within the range of at least about 5 hours to no greater than about 24 hours. More typically, the appropriate response period is within the range of at least about 10 hours to no greater than about 20 hours.

[0205] In one embodiment, the majority of disaccharide (e.g., sucrose, trehalose, glucosylglycerol, mannosylfructose) synthesized accumulates within the cells. In another embodiment, the disaccharide is secreted by the cells which can then be recovered from the photobioreactor. Regardless of whether the disaccharide is within the cells or secreted, the disaccharide can be obtained using any appropriate harvesting process including, but not limited to, an aqueous spray wash applied to the cultivation surface. The wash comprising cells and/or disaccharide can be collected and processed to isolate and recover the disaccharide.

[0206] Having described the invention in detail, it will be apparent that modifications, variations, and equivalent embodiments are possible without departing the scope of the invention defined in the appended claims. Furthermore, it should be appreciated that all examples in the present disclosure are provided as non-limiting examples.

EXAMPLES

[0207] The following non-limiting examples are provided to further illustrate the present invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent approaches the inventors have found function well in the practice of the invention, and thus can be considered to constitute examples of modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1: SOLID PHASE PHOTOBIOREACTOR

[0208] A static prototype device was constructed composed of a 2 mil polyethylene barrier layer with a Ziploc® resealable closure. A 60 sq. cm breathable panel was incorporated into one surface, and a 225 sq. cm woven cotton fabric cultivation support surface was placed inside. The device was sterilized by treatment with 70% volume aqueous ethanol followed by drying of the device at 50°C with a stream of sterile filtered air. 30 ml of sterile BG-11 culture media was absorbed onto the cultivation support followed by inoculation of the growing surface with a pre-culture of Synechococcus elongates PCC 7942. using an aerosol applicator. The preculture was grown in BG-11 media at 26°C for 2 days prior to inoculation. The photobioreactor was placed in an incubation chamber maintained at 33°C and illuminated at 300 microeinsteins with cool white fluorescent lamps. After 2 days, the reactor displayed active growth of organisms and was allowed to continue growth for an additional 2 days whereupon the reactor was removed from the incubator and the growth surface washed with deionized water. The water was removed by evaporation to afford 254 mg dry weight biomass.

EXAMPLE 2: PRODUCTION OF SUCROSE BY PHOTOSYNTHETIC MICROORGANISMS

[0209] The following is a prophetic example to illustrate a method for production of sucrose by photosynthetic microorganism in combination with a photobioreactor. At least one photobioreactor, for example a photobioreactor of the current invention such as described in Example 1 or Example 3, may be run for approximately 4-7 days with either Synechocystis sp. PCC6803. or engineered Synechocystis sp. at a temperature range of between about 15 and 40°C, under illumination of between about 60 and 300 microeinsteins, and carbon dioxide concentration of between about 0.2 and 15 volume%. Following the initial cultivation period the growth surface may be treated with an aqueous salt solution in the concentration range of between about 0.01 and 1.5 M, more preferably between about 0.2 and 0.9 M, using an aerosol spray. The cultivation may be allowed to continue for approximately an additional one to two days to allow sucrose production. The growth surface may then be harvested by washing the surface with deionized water. In a further embodiment the wash water is sterile fresh cultivation media and the washing stringency is such that between about 70 and 90% of the cell mass is collected. The biomass remaining on the cultivation support may then be allowed to continue

growth as a subsequent cycle. It is anticipated that the yield for these cultivations should be between about 200 and 600 mg dry biomass depending on the growth surface material and organism employed.

EXAMPLE 3: SOLID CULTIVATION SUPPORT COATED WITH AN ABSORBENT POLYMER

[0210] The growth surface of a static photobioreactor of the type described in Example 1 was prepared by dip coating the sterile dry surface of the material with a heated solution of sterile 1.5 weight percent agar dispersed in BG-11 culture media. The coated growth surface was allowed to cool and harden upon which the surface was inserted into a sterilized protective barrier to form a photobioreactor device and inoculated with Synechococcus sp. grown in preculture as described in Example 1. Cultivation and harvesting were performed essentially as described in Example 1.

EXAMPLE 4: ASF GENE TARGET

[0211] Biosynthesis of sucrose in cyanobacteria was explored through modulation of sucrose phosphate synthase (sps) and sucrose phosphate phosphatase (spp) activities. Such activities are already present in many cyanobacteria for acclimation to osmotic and matric water stress (*see e.g.*, Lunn, J. E. 2002. Plant Physiol 128, 1490-1500).

[0212] Lunn, J. E. (2002. Plant Physiol 128, 1490-1500) analyzed the genomic organization of the *sps* and *spp* genes of several organisms, including *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942. Lunn proposed that the sucrose phosphate synthase (SPS) of *Synechocystis* spp. PCC 6803 (SEQ ID NO: 3) has an inactive sucrose phosphate phosphatase (SPP-like) domain and a distinct SPP activity. The SPP-like domain has a high level of identity with the spp, but is missing many of the conserved active site residues of the haloacid dehalogenase (HAD) superfamily. While no work has yet been done on *Synechococcus elongatus* PCC 7942, Lunn proposed that both activities are contained within a single enzyme. An alignment of these enzymes is shown in FIG. 5.

[0213] Searches of the *Synechococcus elongatus* PCC 7942 genome did not reveal a distinct *sps* gene elsewhere on the chromosome. The *Synechococcus elongatus* PCC 7942

enzyme (SEQ ID NO: 2) was utilized so as to avoid the necessity of multiple gene expression. While the gene from PCC 7942 has been termed *sps*, because it is a single enzyme fusion bearing both SPS and SPP activities, it was termed *asf* for active SPS/SPP fusion (SEQ ID NO: 1) (see below for further information on the possible expression of a distinct SPP enzyme.)

[0214] There are two approaches to expressing the *Synechococcus elongatus* PCC 7942 *asf* gene product (SEQ ID NO: 2).

[0215] The first approach is a plasmid-based expression system built upon the broad host range vector pMMB67EH (Furste, J. P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M. and Lanka, E. 1986. Gene 48, 119-131). Plasmid pMMB67EH is a derivative of RSF1010, which replicates in most Gram-negative and even some Gram-positive organisms, thus allowing for plasmid-based analysis of sucrose production in *E. coli*, *Synechocystis* spp. PCC 6803, *Synechococcus elongatus* PCC 7942 and a variety of other cyanobacteria (Kreps, S., Ferino, F., Mosrin, C., Gerits, J., Mergeay, M. and Thuriaux, P. 1990. Mol Gen Genet 221, 129-133; Marraccini, P., Bulteau, S., Cassier-Chauvat, C., Mermet-Bouvier, P. and Chauvat, F. 1993. Plant Molecular Biology 23, 905-909; Gormley, E. P. and Davies, J. 1991. J Bacteriology 173, 6705-8).

[0216] The second approach is stable integration into the chromosome of *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 at the *upp* (uracil phosphoribosyltransferase) locus. The *upp* locus was chosen for reasons described below.

EXAMPLE 5: PLASMID-BASED EXPRESSION

[0217] Two plasmids were designed for plasmid-based expression of the *asf* gene product, pLybAL11 (*see e.g.*, FIG. 6; SEQ ID NO: 19) and pLybAL12 (*see e.g.*, FIG. 7; SEQ ID NO: 20). Plasmid pLybAL12 was constructed for expression from predetermined promoters and pLybAL11 was constructed for expression from promoters selected at random.

[0218] Both plasmids were constructed as follows. The *asf* gene from *Synechococcus elongatus* PCC 7942 was amplified by PCR with the oligonucleotides 5′-AGACTACAATTGGGGCGTTTTCTGTGAG-3′ (the *Mfe*I restriction endonuclease site is nucleotide positions 7-12) (SEQ ID NO: 7) and 5′-

CTTACGTGCCGATCAACGTCTCATTCTGAAAAGGTTAAGCGATCGCCTC-3′ (SEQ ID NO: 8) using whole cells as the template, yielding the product of SEQ ID NO: 1.

[0219] The gene encoding for chloramphenicol acetytransferase (*cat*), both with and without the upstream promoter, was amplified from pBeloBAC11 (GenBank Accession U51113).

[0220] The *cat* gene lacking the promoter was amplified from pBeloBAC11 by PCR with the oligonucleotides 5′- TTATCGCGATCGTCAGGAGCTAAGGAAGCTAAAATGGAG-3′ (SEQ ID NO: 9) and 5′-CGACCAATTCACGTGTTTGACAGCTTATC-3′ (SEQ ID NO: 10) (the *Pvu*I and *Pml*I restriction endonuclease sites are at nucleotide positions 4-9 and 10-15, respectively) to yield the product of SEQ ID NO: 11.

[0221] The *cat* gene bearing the promoter was amplified from pBeloBAC11 by PCR with the oligonucleotides 5′-TTTTGGCGATCGTGAGACGTTGATCGGCACGTAAG-3′ (SEQ ID NO: 12) and 5′-CGACCAATTCACGTGTTTGACAGCTTATC-3′ (SEQ ID NO: 13) (the *Pvu*I and *Pml*I restriction endonuclease sites are at nucleotide positions 7-12 and 10-15, respectively) to yield the product of SEQ ID NO: 14.

[0222] The PCR products bearing the *cat* gene were digested with *Pvu*I and the ends blunted with T4 DNA polymerase. They were then individually ligated to the *asf* PCR product. The resultant products were purified by agarose gel electrophoresis, digested with *Mfe*I and *Pml*I and then ligated with T4 DNA ligase to the 6.6 Kbp product of pMMB67EH digested with *Eco*RI and *Hpa*I. The ligation products were transformed into chemically competent NEB5α (New England Biolabs; Ipswich, MA) and selected for at 37°C on LB agar supplemented with 100μg/ml ampicillin. Selected candidates were grown at 37°C in LB supplemented with 100μg/ml ampicillin for miniprep, analyzed by restriction endonuclease digest and then verified by sequence analysis with the oligonucleotides 5′-GCTTCTGCGTTCTGATTTAATCTGTATCAG-3′ (SEQ ID NO: 15), 5′-TATCACTTATTCAGGCGTAGCAACCAG-3′ (SEQ ID NO: 16), 5′-GTCGTTAGTGACATCGACAACACACTG-3′ (SEQ ID NO: 17), and 5′-GATCGCGATACTGATCGAGATAGGTC-3′ (SEQ ID NO: 18). Candidate number 5 of pLybAL11 (pLybAL11-5) (SEQ ID NO: 19) and Candidate number 1 of pLybAL12 (pLybAL12-1) (SEQ ID NO: 20) were chosen for further study.

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[0223] Based upon plasmid yield during minipreps, it appears that the copy number of these plasmids is greatly reduced when propagated in the *E. coli* strain NEB Turbo (New England Biolabs; Ipswich, MA), suggesting the importance in choice of host strain for these plasmids.

EXAMPLE 6: PROMOTER INSERTION

[0224] Six promoters were chosen for insertion into pLybAL12-5. The presumed promoter for *Synechocystis* spp. PCC 6803 *carB* encoding carbamoyl phosphate synthase, which is likely to be immediately upstream of the gene *pyrR* where they would be co-transcribed as an operon, was chosen because it is likely to be strong due to its role in both pyrimidine and arginine biosynthesis. The nitrate reductase (*nirA*) promoters from both *Synechocystis* spp. PCC 6803 (Aichi, M., Takatani, N. and Omata, T. 2001. J Bacteriol. 183, 5840–5847) and *Synechococcus elongatus* PCC 7942 (Maeda, S-I. *et al.* 1998. J Bacteriol 180, 4080-4088) were chosen for their ability to be regulated by the source of nitrogen. The strong light-phase promoter for the photosystem II D1 protein (*psbAII*) from *Synechococcus elongatus* PCC 7942 (Golden, S. S., Brusslan, J. and Haselkorn, R. 1986. EMBO Journal 5, 2789-2798) and two dark-phase promoters from *Synechocystis* spp. PCC 6803 [*dnaK* (Aoki, S., Kondo, T. and Ishiura M. 1995. J Bacteriol 177, 5606-11) and *kaiA* (Kucho, K-I. *et al.* 2005. J Bacteriol 187, 2190–2199)] were also selected as regulated cyanobacterial derived promoters. Lastly, the λ_{PR} temperature-regulated promoter, which has been shown to be active in cyanobacteria, was chosen (Ferino, F. and Chauvat, F. 1989. Gene 84, 257-66; Mermet-Bouvier, P. and Chauvat, F. 1994. Current Microbiology 28, 145-148).

[0225] The following oligonucleotides were used to amplify the promoters by PCR using whole cells as the template, yielding the products shown. The restriction endonuclease sites incorporated for cloning are provided in the sequence.

[0226] *Synechocystis* spp. PCC 6803 *pyrR* (*SphI*/*KpnI*) (SEQ ID NO: 23) was amplified from whole cells by PCR with the oligonucleotides 5′-CGGTGTGCATGCCGTTATTGATGGAATG-3′ (SEQ ID NO: 21) and 5′-

TCACTAGGTACCTAAATTACCTGGGAAGCCAG-3′ (SEQ ID NO: 22), having restriction endonuclease sites at nucleotide positions 7-12 for both.

[0227] *Synechocystis* spp. PCC 6803 *nirA* (SphI/KpnI) (SEQ ID NO: 26) was amplified from whole cells by PCR with the oligonucleotides 5′-CCCAAGGCATGCAGGAAAACAAGCTCAGAATGCTG-3′ (SEQ ID NO: 24) and 5′-TTTATTGGTACCAACGCTTCAAGCCAGATAACAGTAGAGATC-3′ (SEQ ID NO: 25), having restriction endonuclease sites at nucleotide positions 7-12 for both.

[0228] *Synechococcus elongatus* PCC 7942 *psbAII* (*SphI/KpnI*) (SEQ ID NO: 29) was amplified from whole cells by PCR with the oligonucleotides 5′-ATCTTTGCGTTCCGTGACGGCTACTG-3′ (SEQ ID NO: 27) and 5′-GCAGATGGTACCGGTCAGCAGAGTG-3′ (having restriction endonuclease sites at nucleotide positions 7-12) (SEQ ID NO: 28).

[0229] *Synechococcus elongatus* PCC 7942 *nirA* (*SphI/KpnI*) (SEQ ID NO: 32) was amplified from whole cells by PCR with the oligonucleotides 5′-CAGCCAGCATGCATAAATTTCTGTTTTGACCAAACCATCC-3′ (SEQ ID NO: 30) and 5′-GTGGCTGGTACCATGGATTCATCTGCCTACAAAG-3′ (SEQ ID NO: 31), having restriction endonuclease sites at nucleotide positions 7-12 for both.

[0230] λ_{PR} (*XbaI/KpnI*) (SEQ ID NO: 35) was amplified from whole cells by PCR with the oligonucleotides 5′-GTGCATTCTAGATGGCTACGAGGGCAGACAGTAAG-3′ (SEQ ID NO: 33) and 5′-TTCTGTGGTACCATATGGATCCTCCTTCTTAAGATGCAACCATTATCACC-3′ (SEQ ID NO: 34), having restriction endonuclease sites at nucleotide positions 7-12 for both.

[0231] *Synechocystis* spp. PCC 6803 *dnaK* (*SphI/KpnI*) (SEQ ID NO: 38) was amplified from whole cells by PCR with the oligonucleotides 5′-GCCCCAGCATGCACCAGTAAACATAAATCTC-3′ (SEQ ID NO: 36) and 5′-ATTGGTGGTACCGAGGTCAATCCCAACAAC-3′ (SEQ ID NO: 37), having restriction endonuclease sites at nucleotide positions 7-12 for both.

[0232] *Synechocystis* spp. PCC 6803 *kiaA* (*SphI/KpnI*) (SEQ ID NO: 41) was amplified from whole cells by PCR with the oligonucleotides 5′-

GCCAGAGCATGCAAAGCTCACTAACTGG-3′ (SEQ ID NO: 39) and 5′-GGAAAAGGTACCTGAGTCTATGGGCAACGTG-3′ (SEQ ID NO: 40), having restriction endonuclease sites at nucleotide positions 7-12 for both.

[0233] After amplification, the PCR products were digested with the restriction endonucleases shown above, gel purified, and ligated into similarly digested pLybAL12-1 to yield plasmids pLybAL15 (SEQ ID NO: 44), pLybAL16 (SEQ ID NO: 45), pLybAL17 (SEQ ID NO: 46), pLybAL18 (SEQ ID NO: 47), pLybAL19 (SEQ ID NO: 48), pLybAL21 (SEQ ID NO: 49), and pLybAL21 (SEQ ID NO: 50), respectively. The ligation products were transformed into electrocompetent NEB5α (New England Biolabs; Ipswich, MA) and selected for at 30°C on LB agar supplemented with 100µg/ml ampicillin, 34 µg/ml chloramphenicol, and 5% sucrose. Selected candidates were grown at 30°C in LB supplemented with 100µg/ml ampicillin, 34 µg/ml chloramphenicol and 5% sucrose for miniprep, analyzed by restriction endonuclease digest, and then verified by sequence analysis with the oligonucleotides 5′-GCTTCTGCGTTCTGATTTAATCTGTATCAG-3′ (SEQ ID NO: 42) and 5′-ATGGGTCTGAATGTGCAGAATGTAGAG-3′ (SEQ ID NO: 43). Candidates 6 and 7 (pLybAL15-6 and pLybAL15-7), 2 (pLybAL16-2), 4 and 5 (pLybAL17-4 and pLybAL17-5), 1 and 2 (pLybAL18-1 and pLybAL18-2), 1 and 2 (pLybAL19-1 and pLybAL19-2), 3 and 5 (pLybAL21-3 and pLybAL21-5) and 4 and 8 (pLybAL22-4 and pLybAL22-8) were chosen for plasmids pLybAL15 (SEQ ID NO: 44), pLybAL16 (SEQ ID NO: 45), pLybAL17 (SEQ ID NO: 46), pLybAL18 (SEQ ID NO: 47), pLybAL19 (SEQ ID NO: 48), pLybAL21 (SEQ ID NO: 49), and pLybAL21 (SEQ ID NO: 50), respectively.

[0234] Selection and growth of these plasmids on LB supplemented with sucrose and both antibiotics was essential to obtaining clones. Selection was originally conducted on LB supplemented with ampicillin alone, but plasmids containing a promoter could not be isolated. Isolates were either re-ligation of the vector alone or of varying size and lacking the ability to be propagated in the presence chloramphenicol. It is thought that internal sucrose was being produced, creating an osmotic shock for the cells that leads to deletions preventing sucrose production. Subsequent experiments indicated that, once isolated, the plasmids may be stable in the absence of sucrose, possibly through the eventual induction of osmotic stress machinery and/or sucrose consumption enzymes.

EXAMPLE 7: TRANSFORMATION OF SYNECHOCYSTIS AND SYNECHOCOCCUS

[0235] The promoter-containing plasmids, pLybAL15 (SEQ ID NO: 44), pLybAL16 (SEQ ID NO: 45), pLybAL17 (SEQ ID NO: 46), pLybAL18 (SEQ ID NO: 47), pLybAL19 (SEQ ID NO: 48), pLybAL21 (SEQ ID NO: 49), and pLybAL21 (SEQ ID NO: 50), as well as the promoterless pLybAL12-1 vector (SEQ ID NO: 20) (see Examples 5-6), were placed into both *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 by triparental conjugation, performed consistent with Elhai, J. and Wolk, C. P. 1988. Methods in Enzymology 167, 747-754, unless indicated otherwise.

[0236] Overnight cultures of the cargo strains (NEB5α bearing the plasmids to be transferred), as well as an overnight culture of HB101 bearing the helper plasmid pRK2013 (ATCC 37159) grown at 30°C were pelleted by centrifugation, washed twice with LB and then resuspended in LB in one-tenth the original volume. Each cyanobacterium was grown at 30°C in BG11-A, which is the same as BG11 except the trace elements have been replaced with Nitsch's trace elements (Nitsch, J. P. and Nitsch, C. 1956. American Journal of Botany 43, 839-851) under constant illumination to an OD_{730} of approximately 0.5. The cells were pelleted by centrifugation, washed twice with BG11-A, and resuspended in BG11-A with a 7.5-fold increase in concentration. A series of 10-fold dilutions of the cyanobacteria in BG11-A were prepared down to 10^{-5}. At each dilution, 100 µl of the cyanobacterium was combined with 50 µl each of the cargo and helper strains of *E. coli*. 150 µl of each mixture was then plated onto BG11-A agar (1.5%) plates supplemented with 5% LB. The plates were incubated at 26-28°C under constant illumination for 16 to 24 hours. The agar (app. 30 ml) on each plate was lifted and 300 µl of a 100X chloramphenicol solution was added. The final concentration of chloramphenicol was 25 µg/ml for *Synechocystis* spp. PCC 6803 and 7.5 µg/ml for *Synechococcus elongatus* PCC 7942. Incubation continued for 8-12 days. Individual colonies of transconjugants were purified away from contaminating *E. coli* by restreaking onto BG11-A supplemented with the appropriate amount of chloramphenicol to, again, obtain isolated colonies.

EXAMPLE 8: PROMOTER LIBRARY IN PLYBAL11-5

[0237] The following example describes construction of a library of cyanobacterial DNA for promoter selection using pLybAL11-5 (SEQ ID NO: 19) (see Example 5). A modified, scaled up version of the chromosomal DNA isolation protocol of Wilson, K. (1997. Preparation of Genomic DNA from Bacteria. *In* Current Protocols in Molecular Biology. John Wiley and Sons Vol. 1, pp. 2.4.1-2.4.5) was employed, where the primary differences were much longer incubation times and the replacement of SDS with Sarkosyl. The DNA isolated was of sufficient quality for partial *Sau*3AI digest for insertion into the *Bam*HI site of pLybAL11-5. As shown in FIG. 8, some of the fragments would have promoters and others would not.

[0238] During the process of library construction, a possible promoter within the *asf* gene was discovered. To function as a promoter cloning vector, plasmid pLybAL11-5 (SEQ ID NO: 19) is supposed to only be resistant to chloramphenicol when a promoter has been inserted in front of the *asf* gene, as the marker lacks its normal promoter and the promoter upstream of *asf* was not included. Once constructed, however, the chloramphenicol resistance conferred by this plasmid was examined in *E. coli*. When NEB5α bearing pLybAL11-5 was cultured on LB agar (1.5%) supplemented with 34 μg/ml chloramphenicol at 37°C, growth was observed. When cultured in liquid LB medium supplemented with 34 μg/ml chloramphenicol, however, little-to-no growth was observed. NEB5α bearing pLybAL12-1 (SEQ ID NO: 20) grows in the presence of chloramphenicol on both solid and in liquid LB medium.

[0239] To verify there was no missed promoter upstream of the *asf* gene but downstream of the transcription terminators, the insert placed into pMMB67EH to make pLybAL11 was cloned into Lucigen Corp.'s (Middleton, WI) pSMART-LCKan blunt-end cloning vector using Lucigen's CloneSmart kit with the Lucigen strain of *E. coli* (*E. cloni* 10G) competent cells (*see e.g.*, FIG. 9). Because it was blunt-ended cloning, the inserts could ligate to the plasmid in either direction to create pLybAL13f (SEQ ID NO: 51) and pLyAL13r (SEQ ID NO: 52). This vector is specifically designed to eliminate transcription read through from the vector by surrounding the cloning site with terminators. As a control, the insert used to construct pLybAL12 was also placed into this vector, creating pLybAL14f (SEQ ID NO: 53) and pLybAL14r (SEQ ID NO: 54). The plasmids looked to be the appropriate size on an agarose gel but inserts were not verified by DNA sequencing to confirm the integrity of the clones. Similar

results, however, were seen for *E.cloni* 10G bearing pLybAL13 and pLybAL14 (with the cloned DNA ligated in either direction f or r) as were seen for NEB5α bearing pLybAL11 (SEQ ID NO: 19) and pLybAL12 (SEQ ID NO: 20), respectively. This indicates that the activity of this promoter is weak in *E. coli*.

[0240] Many *E. coli* promoters do not function in cyanobacteria, and vice versa. It is possible that this promoter activity would not be observed in *Synechocystis* spp. PCC 6803 or *Synechococcus elongatus* PCC 7942. To check this, pLybAL11-5 (SEQ ID NO: 19) was inserted into both organisms by conjugation, as described above. On BG11-A agar (1.5%) supplemented with chloramphenicol (25 μg/ml and 7.5 μg/ml for *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942, respectively), growth was observed.

[0241] Growth of these organisms bearing pLybAL11-5 (SEQ ID NO: 19) on liquid BG11-A supplemented with chloramphenicol was examined. It is possible that this activity is very weak and is only observable when present on a multiple-copy plasmid. This may be the case with *E. coli*, but is not likely with the cyanobacteria. RSF1010 is a relatively low-copy plasmid, having only 12 copies in *E. coli* (Frey, J., Bagdasarian, M. M. and Bagdasarian, M. 1992). *Gene* 113,101-106). *E. coli* undergoing rapid division has at most 2 copies of its chromosome, thus at least a 6-fold increase in copy number. A comparable copy number in cyanobacteria for this plasmid is likely. The chromosomal copy numbers of *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 of 10-12 and 16, respectively, are similar (Labarre, J., Chauvat, F. and Thuriaux, P. 1989. J Bacteriol 171, 3449-57). The results above suggest the presence of a promoter within the *asf* gene of cyanobacteria.

[0242] FIG. 10 shows a possible location of a promoter (or promoters) within the *asf* gene. Transcription initiation elements have been described by Curtis, S. E. [1994. The transcription apparatus and the regulation of transcription initiation. *In* The Molecular Biology of Cyanobacteria. Bryant, D. A. (ed). Kluwer Academic Publishers pp. 613-699]. Translation initiation elements have been defined by Sazuka, T. and Ohara, O. (1996. DNA Research 3, 225-232).

[0243] Based upon alignment to known SPS enzymes and the presence of a stop codon only two codons upstream, the translation initiation of the *asf* gene is predicted to start at a

GTG start codon. While ATG start codons are the most common, GTG and TTG are less common, but not rare. A typical *E. coli*-like Shine-Delgarno sequence (GGAG or GAGG) complementary the 3´-end of the 16S rRNA for which the adenine nucleotide is optimally 9-12 bp away from the first nucleotide of the start codon is also present, except with somewhat longer spacing. This sequence is found in about half the genes studied by Sazuka and Ohara. Less optimal spacing is not uncommon, but often leads to reduced levels of expression. There is too little sequence upstream of the Shine-Delgarno sequence but downstream of the *Mfe*I site to incorporate a promoter. It is possible that a partial promoter may be incorporated, but the rest of the promoter would have to produced by the vector sequence of all three plasmids (pLybAL11-5 (SEQ ID NO: 19); pLybAL13f (SEQ ID NO: 51); and pLybAL13r (SEQ ID NO: 52)), which is improbable.

[0244] Thus it likely that the promoter activity is located within the *asf* gene. If the promoter is within the *asf* gene, one potential position is in front of the SPP domain of *asf*. This would give the sucrose biosynthetic enzymes of *Synechococcus elongatus* PCC 7942 a similar quaternary structure to those from *Synechocystis* spp. PCC 6803. Each organism would have two proteins, an SPS domain with a translationally fused SPP or SPP-like domain and a distinct SPP that may (or may not) interact with each other.

[0245] First, it was determined whether the SPP domain of *asf* could even be translated separately. As can be seen in FIG. 10 and Table 1, there is a TTG start codon immediately upstream of the SPP domain that is preceded by a Shine-Delgarno sequence.

Table 1: Nucleotides immediately surrounding the proposed spp start codon. The nucleotides immediately surrounding the proposed spp start codon are compared to the consensus of 72 cyanobacterial genes. Nucleotides matching the consensus are italicized, whereas nucleotides that do not match the consensus are underlined. Nucleotide numbers are relative to the first nucleotide of the start codon.

NT#	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	1 2 3	4	5	6
Consensus	A/G	A/G	A/T	A/T	A/T	A/T	A/T	A/T	C/T	T/C	ATG	A/G	C	C/T
Selo7942 asf	T	*G*	*A*	C	*T*	*A*	G	C	G	*C*	GTG	G	*C*	A
Selo7942 spp	T	C	G	C	*A*	*A*	*A*	C	G	*C*	TTG	A	T	*T*

[0246] The region surrounding the start codon matches the consensus determined by Sazuka and Ohara for 72 cyanobacterial genes almost as well as the native start codon. While determining cyanobacterial promoters based upon rules established for *E. coli* promoters, the typical -35 and -10 elements were searched for since the promoter does appear to be active in *E. coli*. Two possible promoters were identified, as seen in FIG. 10. There remains the possibility of an additional promoter(s) elsewhere in *asf*.

EXAMPLE 9: TRANSFER OF PLASMIDS FROM E. COLI TO CYANOBACTERIA

[0247] Conjugation was used for transfer of the pMMB67EH-based plasmids into cyanobacteria. Protocols exist for the transformation of these organisms (Zang, X., Liu, B., Liu, S., Arunakumara, K. K. I. U. and Zhang, X. 2007. Journal of Microbiology 45, 241-245; Golden, S. S. and Sherman, L. A.1984. Journal of Bacteriology 158, 36-42), but such approaches were unsuccessful for placing these plasmids into *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 using natural transformation.

[0248] The presence of the plasmids in the cyanobacteria was verified. Transconjugants were analyzed for the presence of plasmid by PCR of the *asf/cat* gene combination with the oligonucleotides 5′-AGACTACAATTGGGGCGTTTTCTGTGAG-3′ (SEQ ID NO: 7) and 5′-GGTGGTTGTGTTTGACAGCTTATC-3′ (SEQ ID NO: 55), yielding a 3.1 kb product. In addition, plasmids were isolated and analyzed. Cultures of cells grown in BG11-A supplemented with chloramphenicol (at the concentrations described above) are pelleted by centrifugation, resuspended in TE, heat-treated and miniprepped by the Promega Wizard SV Plus miniprep kit. But with poor yield, direct plasmid analysis is difficult. As such, the isolated DNA is transformed into *E. coli* NEB5α, re-isolated using the Promega Wizard SV Plus miniprep kit, and then subjected to restriction endonuclease analysis.

EXAMPLE 10: SUCROSE PRODUCTION ASSAY AND ANALYSIS

[0249] Synechococcus transformed with pLybAL19 or pLybAL17 (see Example 7) was assayed for sucrose accumulation. Sucrose is measured with BioVision, Inc.'s (Mountain

View, CA) sucrose assay kit. Assays were run following a 4 hour induction period (increased light to 180 microeinsteins from 50 microeinsteins for pLybAL17 (SEQ ID NO: 46) and increased temperature from 26 to 39°C for pLybAL19 (SEQ ID NO: 48)). Data was corrected for background glucose present in the cells.

[0250] Results showed Synechococcus transformed with pLybAL19 (SEQ ID NO: 48) accumulated 0.78 nanomoles of sucrose per mg of dry biomass. Results also showed that Synechococcus transformed with pLybAL17 (SEQ ID NO: 46) accumulated 0.95 nanomoles of sucrose per mg of dry biomass.

[0251] Further analysis for plasmid-based sucrose production in *E. coli*, *Synechocystis* spp. PCC 6803, and *Synechococcus elongatus* PCC 7942 was performed. Because bacteria can consume sucrose, detection may be difficult. As such, cells are grown under suppressing conditions and then assayed shortly after induction. The *pyrR* promoter may be suppressed by growth with uracil and induced by transfer medium lacking uracil. The *nirA* promoters can be suppressed by growth with ammonium ions as the nitrogen source and induced by transfer to medium with nitrate as the nitrogen source. The *psbAII* promoter can be shifted from low light to high light. The dark phase promoters can be shifted from light to dark. And, the λ_{PR} promoter can be shifted from low (25°C) to high (39°C) temperature.

EXAMPLE 11: EXPRESSION THROUGH STABLE CHROMOSOMAL INTEGRATION

[0252] Insertion of sucrose biosynthetic genes can cause a negative impact on cell growth, leading to difficulties in obtaining complete segregation of the 10-16 chromosomes. With normal selection for an antibiotic resistance marker, having additional copies of the marker does not dramatically impact the cells ability to survive in the presence of antibiotic. Therefore, complete chromosomal segregation can be difficult to achieve using antibiotic selection when faced with a negative phenotype.

[0253] Deletion of the *upp* gene (encoding for uracil phosphoribosyltransferase) in most organisms leads to resistance to the otherwise toxic 5-fluorouracil. To obtain complete resistance, all copies of the *upp* gene must be deleted. Thus integrating into the *upp* locus of *Synechocystis* spp. PCC 6803 (SEQ ID NO: 56) and *Synechococcus elongatus* PCC 7942 (SEQ

ID NO: 58) will lead to 5-fluorouracil resistance and allow for positive selection of complete segregation, even in the presence of a negative phenotype.

EXAMPLE 12: THE UPP/KANAMYCIN RESISTANCE CASSETTE

[0254] A general strategy for genomic manipulation using a *upp*/kanamycin resistance cassette is outlined in FIG. 11. Deletion of a gene is depicted, but the strategy can easily be modified at the "replacement" step for insertions and mutations.

[0255] An *upp*/kanamycin resistance cassette was constructed. The cassette was constructed in Epicentre Biotechnologies CopyControl cloning kit with blunt-end cloning vector pCC1 and *E. coli* strain EPI300 according to manufacturer protocols. The *upp* gene from *Bacillus subtilis* 168 was amplified from whole cells using the oligonucleotides 5′-AAGAAGCAAGACAGCGTGTAGCTGCTCTGACTG-3′ (SEQ ID NO: 60) and 5′-TCCCGGGATTTGGTACCTTATTTTGTTCCAAACATGCGGTCACCCGCATC-3′ (having restriction endonuclease sites at nucleotide positions 2-7 and 12-17) (SEQ ID NO: 61), yielding the product of SEQ ID NO: 62.

[0256] The PCR product was cloned into pCC1 and those bearing the insert were selected for on LB supplemented with chloramphenicol as described in Epicentre Biotechnologies' protocol. The forward orientation, relative to *lacZ*, was screened for by restriction endonuclease digest, yielding pLybAL7f (SEQ ID NO: 65). The exact sequence of the insert was verified by DNA sequencing with the oligonucleotides 5′-GTAATACGACTCACTATAGGGC-3′ (SEQ ID NO: 63) and 5′-CACACAGGAAACAGCTATGACCAT-3′(SEQ ID NO: 64) for candidates 3 and 8 (pLybAL7-3 and pLybAL7-8).

[0257] The kanamycin resistance marker from the Lybradyn vector pLybAA1 [originally derived from pACYC177 (Rose, R. E. 1988. Nucleic Acids Res. 16, 356] was amplified with the oligonucleotides 5′-GTCAGTGCACTGCTCTGCCAGTGTTACAACC-3′ (having *Apa*LI restriction endonuclease sites at nucleotide positions 5-10) (SEQ ID NO: 66) and 5′-CTCAGTGGCGCCAAAACTCACGTTAAGGGATTTTGGTC-3′ (SEQ ID NO: 67) (having

*Nar*I restriction endonuclease sites at nucleotide positions 7-12), yielding the product of SEQ ID NO: 68.

[0258] The PCR product was digested with *Apa*LI and *Nar*I and ligated into similarly digested pLybAL7f, creating pLybAL8f (SEQ ID NO: 69). The proper plasmid was selected for on LB supplemented with 50 µg/ml neomycin and examined by restriction endonuclease digestion.

EXAMPLE 13: UPP DELETION

[0259] One strategy to force segregation of chromosomal inserts for the expression of sugars, including sucrose, trehalose, glucosylglycerol, and mannosylfructose, utilizes deletion of *upp* from the chromosome leading to resistance to 5-fluorouracil. While this has been established in many organisms (such as *E. coli* and *B. subtilis*), it has not previously been established for cyanobacteria, such as *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942.

[0260] Testing showed that growth of each of these organisms was completely inhibited by 1 µg/ml, 5-fluorouracil. Growth of *Synechocystis* spp. PCC 6803 is completely inhibited by 0.5 µg/ml, 5-fluorouracil and is sensitive to as little as little as 0.1 µg/ml, 5-fluorouracil.

[0261] The *upp* gene and surrounding sequences of both *Synechocystis* spp. PCC 6803 was amplified with the oligonucleotides Sspupp-F (SEQ ID NO: 96) and Sspupp-R (SEQ ID NO: 97). The *upp* gene and surrounding sequences of *Synechococcus elongatus* PCC 7942 was amplified with the oligonucleotides Seloupp-F (SEQ ID NO: 98) and Seloupp-R (SEQ ID NO: 99). The PCR products (*upp* of *Synechocystis* spp. PCC 6803, SEQ ID NO: 100; *upp* of *Synechococcus elongatus* PCC 7942, SEQ ID NO: 101) were then cloned into the Epicentre Biotechnologies' (Madison, WI) blunt cloning vector pCC1, as per the manufacturer's instructions.

[0262] While the PCR product (SEQ ID NO: 100 or SEQ ID NO: 101) can ligate into pCC1 in either direction, the forward orientation relative to the *lac* promoter was chosen, generating pLybAL3f (SEQ ID NO: 102) (containing *upp* of *Synechocystis* spp. PCC 6803) and

pLybAL5f (SEQ ID NO: 103) (containing *upp* of *Synechococcus elongatus* PCC 7942), respectively. The inserts were sequenced using oligonucleotides T7long (SEQ ID NO: 104) and M13rev (SEQ ID NO: 105). The nucleotide sequence of *upp* of *Synechocystis* spp. PCC 6803 is represented by SEQ ID NO: 111 and the polypeptide sequence by SEQ ID NO: 112. The nucleotide sequence of *upp* of *Synechococcus elongatus* PCC 7942 is represented by SEQ ID NO: 113 and the polypeptide sequence by SEQ ID NO: 114.

[0263] Plasmid pLybAL4f (SEQ ID NO: 106) was created from pLybAL3f (SEQ ID NO: 102) by removal of the *Blp*I and *Apa*LI fragment, blunt ending with T4 DNA polymerase and then recircularizing with T4 DNA ligase. Part of the *Synechocystis* spp. PCC 6803 *upp* gene was then deleted by digesting pLybAL4f with *Avr*II and *Sgf*I, blunt ending with T4 DNA polymerase and then recircularizing with T4 DNA ligase, creating pLybAL9f (SEQ ID NO: 107). The SacI/SphI fragment (SEQ ID NO: 108) bearing the cyanobacterial DNA was excised from pLybAL9f (SEQ ID NO: 107) and ligated into similarly digested pARO180 (sequence not completely known; Parke, D. 1990. Construction of mobilizable vectors derived from plasmids RP4, pUC18 and pUC19. Gene 93:135-137; ATCC 77123), creating pLybAL25. Plasmid pLybAL6fb (SEQ ID NO: 109) was created from pLybAL5f by removal of the *Sap*I and *Apa*LI fragment, blunt ending with T4 DNA polymerase and then recircularizing with T4 DNA ligase. Part of the *Synechococcus elongatus* PCC 7942 *upp* gene was then deleted by digesting pLybAL6fb with BssHII and *Bsa*I, blunt ending with T4 DNA polymerase and then recircularizing with T4 DNA ligase, creating pLybAL10fb (SEQ ID NO: 110). The SacI/SphI fragment (SEQ ID NO: 138) bearing the cyanobacterial DNA was excised from pLybAL10fb and ligated into similarly digested pARO180, creating pLybAL26.

[0264] Plasmids pLybAL25 and pLybAL26 were placed in E. coli S17-1 (ATCC 47055). Plasmids pLybAL25 and pLybAL26 are to be transferred to *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 by biparental conjugation. Since these plasmids do not replicate in cyanobacteria, they should function as suicide vectors and cross over into the chromosome, deleting *upp* on one of the copies of the chromosome. An optimized protocol will enable speeding of segregation without killing the cells by premature exposure to too much 5-fluorouracil.

EXAMPLE 14: MODIFICATION OF SUCROSE DEGRADATION ENZYMES

[0265] Cyanobacteria transformed with *asf* are further engineered to improve sucrose production by modulation of sucrose degradation activity.

[0266] The inventors have identified genes encoding invertase homologues in both *Synechocystis* spp. PCC 6803 (nucleotide sequence SEQ ID NO: 70; polypeptide sequence SEQ ID NO: 71) and *Synechococcus elongatus* PCC 7942 (nucleotide sequence SEQ ID NO: 72; polypeptide sequence SEQ ID NO: 73). *Synechocystis* spp. PCC 6803 also encodes a sucraseferredoxin-like protein (nucleotide sequence SEQ ID NO: 74; polypeptide sequence SEQ ID NO: 75) (Machray G.C. *et al.* 1994. FEBS Lett 354, 123-127).

[0267] These genes are deleted using the markerless deletion protocol described in FIG. 11.

EXAMPLE 15: MODIFICATION OF SUCROSE DEGRADATION ENZYMES

[0268] Cyanobacteria transformed with *asf* are further engineered to promote sucrose secretion from the cells.

[0269] When in a low osmotic environment, the sucrose may be automatically expunged from the cells, as done with osomoprotectants by some organisms when transitioning from high to low salt environments (Schleyer, M., Schmidt, R. and Bakker, E. P. 1993. Arch Microbiol 160, 424-43; Koo, S. P., Higgins, C. F. and Booth, I. R. 1991. J Gen Microbiol 137, 2617-2625; Lamark, T., Styrvold, O. B. and Strgim, A. R. 1992. FEMS Microbiol. Lett 96, 149-154). Engineering of cyanobacteria can promote such a process.

[0270] Cyanobacteria transformed with *asf* are further engineered to express sucrose permease, such as those used by *E. coli* and *Salmonella* or in the transport of sucrose to nitrogen-fixing cysts of certain cyanobacteria (Jahreis K. et al. 2002. J Bacteriol 184, 5307–5316; Cumino, A. C. 2007. Plant Physiol 143, 1385-97). These genes are cloned and transformed into cyanobacteria according to techniques described above.

EXAMPLE 16: SUCROSE SECRETION BY CYANOBACTERIA TRANSFORMED WITH PORIN

[0271] Sucrose secretion from *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 can be facilitated by transformation with sucrose porin.

[0272] The gene encoding sucrose porin (*scrY*) from *Enterobacter sakazakii* ATCC BAA-894 was cloned for expression in *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942. The function of this gene has been inferred from its sequence and those of its neighbors. *Enterobacter sakazakii scrY* was amplified from chromosomal DNA by PCR with the oligonucleotides EsscrYBamHI-F (SEQ ID NO: 88) and EsscrYSacI-R (SEQ ID NO: 89). The PCR product (SEQ ID NO: 90) was digested with *Bam*HI and *Sac*I and ligated into similarly digested pLybAL19 and cloned into NEB5α, creating pLybAL32 (SEQ ID NO: 91). The *scrY* gene (nucleic acid SEQ ID NO: 94; polypeptide sequence, SEQ ID NO: 95) was then sequenced with the oligonucleotides EsscrYmidseq-F (SEQ ID NO: 92) and EsscrYmidseq–R (SEQ ID NO: 93). When introduced into the host, this construct allows for the co-expression of the genes *scrY* and *asf* under the control of the temperature-inducible promoter. This plasmid was transferred by tri-parental conjugation (as described above) into *Synechocystis* spp. PCC 6803. The transformed *Synechocystis* spp. PCC 6803 is tested for efficacy in the secretion of sucrose. Similar transformation and testing of *Synechococcus elongatus* PCC 7942 follows.

EXAMPLE 17: GENERATION OF TREHALOSE ACCUMULATING CYANOBACTERIA

[0273] The trehalose biosynthetic genes encoding trehalose phosphate synthase and trehalose phosphate phosphatase (*otsA* and *otsB*, respectively) from *E. coli* are found in a two gene operon, *otsBA* (SEQ ID NO: 115). The operon was cloned by PCR amplification of E. coli K12 genomic DNA with the oligonucleotides EcotsBA-F (SEQ ID NO: 116) and EcotsBA-R (SEQ ID NO: 117). The PCR product was digested with *Afl*II and *Nhe*I and was cloned into pLybAL19 (SEQ ID NO: 48), replacing most of the *asf* gene. The new plasmid, pLybAL23 (SEQ ID NO: 118), places the trehalose biosynthetic genes under the control of the temperature-inducible λ_{PR} promoter. The genes were sequenced to verify their integrity with the oligonucleotides EcotsBAmidseq-F (SEQ ID NO: 119) and EcotsBAmidseq-R (SEQ ID NO: 120). Expression of the *otsBA* operon was then placed under control of the *pyrR*, *psbAII*, *dnaK* and *kiaA* promoters (as described above) by ligating the *Afl*II (blunt –ended with T4 DNA

polymerase)/*Nhe*I fragment of pLybAL23 bearing the *otsBA* operon, into pLybAL15, pLybAL17, pLybAL21 and pLybAL22 digested with *Sac*I (blunt-ended with T4 DNA polymerase) and *Nhe*I, creating pLybAL28 (SEQ ID NO: 121), pLybAL29 (SEQ ID NO: 122), pLybAL30 (SEQ ID NO: 123), and pLybAL31 (SEQ ID NO: 124), respectively.

[0274] Each of plasmids pLybAL28 (SEQ ID NO: 121), pLybAL29 (SEQ ID NO: 122), pLybAL30 (SEQ ID NO: 123), and pLybAL31 (SEQ ID NO: 124) were moved into *Synechocystis* spp. PCC 6803 by tri-parental conjugation (as described above).

[0275] Expression of the *otsBA* operon from pLybAL23 was placed under the control of the *Synechocystis* spp. PCC 6803 and *Synechococcus elongatus* PCC 7942 *nirA* promoters (as described above) in pLybAL16 and pLybAL18 in the same way as just described for the other promoters, creating pLybAL36 (SEQ ID NO: 125) and pLybAL37 (SEQ ID NO: 126), respectively.

EXAMPLE 18: TREHALOSE ASSAY

[0276] Biomass was separated from the culture broth as necessary by centrifugation and residual biomass was removed from the clarified culture broth by filtration through 0.2 micron filter. The culture broth was concentrated to a residue by evaporation under reduced pressure. The concentrated culture broth was dissolved in 1 ml of de-ionized water and then 10 microliters of solution was sampled in a trehalose assay. The biomass collected by centrifugation was transferred to a weigh dish and heated to 100 °C to remove residual moisture. The dry biomass was weighed and then a 100 mg sample was dissolved in 1 ml of de-ionized water. The mixture was then ground and the solids were removed by centrifugation. A 10 microliter sample of the clarified supernatant was diluted 100 fold with de-ionized water and 10 microliters of the diluted sample were tested for trehalose.

[0277] The assay for trehalose used a modified procedure of a commercially supplied sucrose assay kit available through Biovision, Inc. The modification to the standard protocol was the substitution of trehalase for the kit supplied invertase enzyme solution. The kit involves the hydrolysis of trehalose with trehalase to release glucose. The glucose is oxidized by glucose oxidase to produce hydrogen peroxide which is detected by the action of peroxidase in the

presence of a colored indicator. The colored indicator is quantitatively measured by its characteristic absorbance at 570nm to afford the concentration of glucose originally present in the sample.

[0278] Trehalase (*treA* nucleic acid SEQ ID NO: 134 encoding trehalase polypeptide SEQ ID NO: 135) was prepared from the recombinant E. coli treA gene which has been engineered into a plasmid and transformed into an E. coli host by a similar method as described by Gutierrez C, Ardourel M, Bremer E, Middendorf A, Boos W, Ehmann U.Mol Gen Genet. 1989 Jun;217(2-3):347-54. Periplasmic trehalase was cloned from *E. coli* K12, encoded by *treA*. The *treA* PCR product (SEQ ID NO: 127) was digested with *Afl*II/*Xba*I and then ligated into similarly digested pLybCB6, a proprietary plasmid with a constitutive version of the strong *E. coli trp* promoter, creating pLybAL24 (SEQ ID NO: 130). The integrity of the insert was analyzed by sequencing with the oligonucleotides EctreAmidseq-F and EctreAmidseq-R.

[0279] A C-terminal His_6-tagged version of the trehalase was constructed. The gene was amplified by PCR with the oligonucleotides EctreA-F2 (SEQ ID NO: 131) and EctreA-R2 (SEQ ID NO: 132). The PCR product (SEQ ID NO: 136) was then digested with *Afl*II/*Xba*I and then ligated into similarly digested pLybAL24, creating pLybAL33 (SEQ ID NO: 133).

[0280] Strong constitutive expression of the periplasmic trehalase is detrimental to the cells, causing a strong growth defect at 37°C. This can be significantly alleviated by growing the cells at 30°C.

[0281] The protein was expressed in E. coli BW25113 on a plasmid pLYBAL24 (SEQ ID NO: 130) which was grown in 2xYT media containing kanamycin. The protein was produced constitutively using the Trp promoter and contains a signal peptide which allows the protein to be transported to the periplasm. Following fermentation and harvesting of the biomass, the enzyme was purified by selective periplasmic release by treatment of the washed and resuspended cell pellet with 2 % v/v dichloromethane in 50 mM Tris buffer pH 8. The lysate was separated from cell debris by centrifugation and further processed by concentration using an Amicon ultrafilter fitted with a 10,000 Dalton membrane. The concentrated lysate may be used in assays directly or the enzyme can be further purified by metal ion affinity

chromatography using the engineered 6X poly histidine tag on the C-terminus of the enzyme (SEQ ID NO: 137).

EXAMPLE 19: SOLID PHASE TREHALOSE PRODUCTION

[0282] A solid composite fabric covered hydrophilic foam composed of a substrate foam used as a media/moisture reservoir (Foamex Aquazone) was bound to a fabric layer (DuPont Sontara) used as a growth surface measuring 15 cm by 15 cm. The composite material was sterilized by soaking in 70% ethanol in water and then hung in a vertical bioreactor plumbed to deliver solutions to the top of the composite material. The solutions were allowed to percolate through the growing composite surface by gravity. Residual ethanol was removed from the sterilized growing surface by passage of 1 liter of sterile de-ionized water flowing at 0.2 ml/min. The growing surface was equilibrated with culture media by flowing 0.5 liters of BG11A growth medium containing 10 micrograms/ml chloramphenicol through the composite material at 0.2ml/min.

[0283] The equilibrated, sterile growth surface was inoculated by flooding the surface with 10 ml of a 4 day pre-culture of Synechocystis spp. PCC 6803 transformed by plasmid pLYBAL23. Following 30 minute incubation the reactor was turned to a vertical position and the fermentation was begun. The reactor was illuminated with 80 microeisteins of light from a white LED array. Temperature was maintained at 28 °C, by a resistive heating device attached to the bioreactor. The reactor was continuously purged with 0.2 micron filtered air at 0.2 L/min and fresh culture media was supplied by pump and gravity percolation through the foam layer of the growth composite at a rate of 0.2 ml/min for 30 minutes every 6 hours. The reactor was run continuously for 4-7 days during which the growth surface of the composite was overspread with a dense lawn of cyanobacteria. Following the initial cultivation period the temperature of the bioreactor was increased to 40°C and maintained at this temperature for an additional 24 hours. During the elevated temperature period spent culture broth was collected and processed for trehalose determination. At the completion of the fermentation run the biomass was collected by rinsing the growth surface with de-ionized water which can be processed for trehalose assay.

[0284] The amount of trehalose produced and retained in the biomass grown on the solid surface was up to 2.5 wt % of the total dry weight biomass recovered from the bioreactor following temperature induction. 0.8 wt% of the dry biomass equivalent weight of trehalose was recovered from the culture medium following temperature induction.

EXAMPLE 20: TREHALOSE PRODUCTION LIQUID PHASE

[0285] 1 liter of sterile BG11A media was prepared in a Bioflow reactor to which chloramphenicol was added to a concentration of 10 micrograms/ml. The reactor was then inoculated with a 5% by volume, 4 day pre-culture of Synechocystis spp. PCC 6803 transformed with plasmid pLYBAL23. The reactor was run at 28 °C, 300 RPM, 0.2 L/min 0.2 micron filtered air purge and illuminated at 80 microeinsteins of light using a fluorescent bulb array. The cultivation was maintained for 4-7 days following which a 200ml sample was removed for processing and trehalose assay. The temperature of the fermentation was then elevated to 40 °C for 24 hours. A 200ml sample was then removed from the bioreactor for processing and trehalose assay.

[0286] Following temperature induction the dried biomass produced up to 3 wt% trehalose while the spent culture broth contained 0.3 wt% trehalose equivalent relative to biomass.

[0287] With reference to the use of the word(s) "comprise" or "comprises" or "comprising" in the foregoing description and/or in the following claims, unless the context requires otherwise, those words are used on the basis and clear understanding that they are to be interpreted inclusively, rather than exclusively, and that each of those words is to be so interpreted in construing the foregoing description and/or the following claims.

REFERENCES

[0288] All publications, patents, patent applications, and other references cited in this application are incorporated herein by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application or other reference was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. Citation of a reference herein shall not be construed as an admission that such is prior art to the present invention.

The claims defining the invention are as follows:

Claim 1. A transgenic photosynthetic microorganism cell comprising an artificial DNA construct comprising, as operably associated components in the 5' to 3' direction of transcription:

a promoter functional in the photosynthetic microorganism cell;

a polynucleotide comprising a nucleotide sequence encoding a polypeptide having a disaccharide biosynthetic activity selected from the group consisting of (i) a disaccharide phosphate synthase; (ii) a disaccharide phosphate phosphatase; and (iii) a disaccharide phosphate synthase and a disaccharide phosphate phosphatase; and

a transcriptional termination sequence;

wherein the transgenic photosynthetic microorganism cell accumulates increased levels of the disaccharide compared to a photosynthetic microorganism cell not comprising the DNA construct.

Claim 2. The cell of claim 1, wherein the polynucleotide comprising a nucleotide sequence encoding a polypeptide having a disaccharide biosynthetic activity is selected from:

(a) a polynucleotide comprising a nucleotide sequence encoding a polypeptide selected from:

SEQ ID NO: 2 or a sequence 95% identical thereto having sucrose phosphate synthase and sucrose phosphate phosphatase (ASF) activity;

SEQ ID NO: 4 or a sequence 95% identical thereto having sucrose phosphate synthase (SPS) activity;

SEQ ID NO: 6 or a sequence 95% identical thereto having a sucrose phosphate phosphatase (SPP) activity;

SEQ ID NO: 77 or a sequence 95% identical thereto having trehalose phosphate synthase (TPS) activity;

SEQ ID NO: 79 or a sequence 95% identical thereto having trehalose phosphate phosphatase (TPP) activity;

SEQ ID NO: 81 or a sequence 95% identical thereto having glucosylglycerol phosphate synthase (GPS) activity;

SEQ ID NO: 83 or a sequence 95% identical thereto having glucosylglycerol phosphate phosphatase (GPP) activity;

SEQ ID NO: 85 or a sequence 95% identical thereto having mannosylfructose phosphate synthase (MPS) activity; or

SEQ ID NO: 87 or a sequence 95% identical thereto having mannosylfructose phosphate phosphatase (MPP) activity;

(b) an isolated polynucleotide comprising a sequence selected from:

SEQ ID NO: 1 or a sequence 95% identical thereto encoding sucrose phosphate synthase / sucrose phosphate phosphatase (ASF) activity;

SEQ ID NO: 3 or a sequence 95% identical thereto encoding sucrose phosphate synthase (SPS) activity;

SEQ ID NO: 5 or a sequence 95% identical thereto encoding sucrose phosphate phosphatase (SPP) activity;

SEQ ID NO: 76 or a sequence 95% identical thereto encoding trehalose phosphate synthase (TPS) activity;

SEQ ID NO: 78 or a sequence 95% identical thereto encoding trehalose phosphate phosphatase (TPP) activity;

SEQ ID NO: 80 or a sequence 95% identical thereto encoding glucosylglycerol phosphate synthase (GPS) activity;

SEQ ID NO: 82 or a sequence 95% identical thereto encoding glucosylglycerol phosphate phosphatase (GPP) activity;

SEQ ID NO: 84 or a sequence 95% identical thereto encoding mannosylfructose phosphate synthase (MPS) activity; or

SEQ ID NO: 86 or a sequence 95% identical thereto encoding mannosylfructose phosphate phosphatase (MPP) activity;

(c) an isolated polynucleotide that hybridizes under stringent conditions to a nucleic acid sequence selected from:

SEQ ID NO: 1, wherein the isolated polynucleotide encodes a polypeptide having ASF activity;

SEQ ID NO: 3, wherein the isolated polynucleotide encodes a polypeptide having SPS activity;

SEQ ID NO: 5, wherein the isolated polynucleotide encodes a polypeptide having SPP activity;

SEQ ID NO: 76, wherein the isolated polynucleotide encodes a polypeptide having TPS activity;

SEQ ID NO: 78, wherein the isolated polynucleotide encodes a polypeptide having TPP activity;

SEQ ID NO: 80, wherein the isolated polynucleotide encodes a polypeptide having GPS activity;

SEQ ID NO: 82, wherein the isolated polynucleotide encodes a polypeptide having GPP activity;

SEQ ID NO: 84, wherein the isolated polynucleotide encodes a polypeptide having MPS activity; or

SEQ ID NO: 86, wherein the isolated polynucleotide encodes a polypeptide having MPP activity;

wherein said stringent conditions comprise incubation at 65°C in a solution comprising 6X SSC (0.9 M sodium chloride and 0.09 M sodium citrate); or

(d) an isolated polynucleotide complementary to the polynucleotide sequence of (a), (b), or (c).

Claim 3. The cell of claim 1 or 2 wherein monomers of the accumulated disaccharide are endogenous to the cell.

Claim 4. The cell of any one of claims 1-3 wherein the cell is a cyanobacterium cell, a photosynthetic bacteria; or a green algae.

Claim 5. The cell of any one of claims 1-4 wherein the cell is a cyanobacterium selected from the group consisting of Synechococcus and Synechocystis.

Claim 6. The cell of any one of claims 1-5 wherein the promoter is

an inducible promoter or

a promoter selected from *carB*, *nirA*, *psbAII*, *dnaK*, *kaiA*, or λ_{PR}.

Claim 7. The cell of any one of claims 1-6 wherein the DNA construct comprises a nucleotide sequence selected from: SEQ ID NO: 19 (pLybAL11 encoding *asf*); SEQ ID NO: 20 (pLybAL12 encoding *asf*); SEQ ID NO: 44 (pLybAL15 encoding *asf*); SEQ ID NO: 45 (pLybAL16 encoding *asf*); SEQ ID NO: 46 (pLybAL17 encoding *asf*); SEQ ID NO: 47 (pLybAL18 encoding *asf*); SEQ ID NO: 48 (pLybAL19 encoding *asf*); SEQ ID NO: 49 (pLybAL21 encoding *asf*); SEQ ID NO: 50 (pLybAL22 encoding *asf*); SEQ ID NO: 51 (pLybAL13f encoding *asf*); SEQ ID NO: 52 (pLyAL13r encoding *asf*); SEQ ID NO: 53 (pLybAL14f encoding *asf*); SEQ ID NO: 54 (pLybAL14r encoding *asf*); SEQ ID NO: 65 (pLybAL7f encoding *asf*); SEQ ID NO: 69 (pLybAL8f encoding *asf*); SEQ ID NO: 118 (pLybAL23 encoding *tps* and *tpp*); SEQ ID NO: 121 (pLybAL28 encoding *tps* and *tpp*); SEQ

ID NO: 122 (pLybAL29 encoding *tps* and *tpp*); SEQ ID NO: 123 (pLybAL30 encoding *tps* and *tpp*); SEQ ID NO: 124 (pLybAL31 encoding *tps* and *tpp*); SEQ ID NO: 125 (pLybAL36 encoding *tps* and *tpp*); SEQ ID NO: 126 (pLybAL37 encoding *tps* and *tpp*); SEQ ID NO: 130 (pLybAL24 encoding *tps* and *tpp*); or SEQ ID NO: 133 (pLybAL33 encoding *tps* and *tpp*).

Claim 8. The cell of any one of claims 1-7 wherein the cell accumulates

at least about 0.1 micrograms of the disaccharide per minute per gram dry biomass; or

at least about 0.1 micrograms of the disaccharide per minute per gram dry biomass up to about 10 micrograms of the disaccharide per minute per gram dry biomass.

Claim 9. The cell of any one of claims 1-8, wherein at least one of the following are satisfied:

(a) the cell does not comprise a nucleotide sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, and SEQ ID NO: 74, or a nucleotide variant thereof having at least 95% identity thereto and invertase activity or sucraseferridoxin activity;

(b) the cell does not express a polypeptide sequence selected from the group consisting of SEQ ID NO: 71, SEQ ID NO: 73, and SEQ ID NO: 75, or a polypeptide variant thereof having at least 95% identity thereto and invertase activity or sucraseferridoxin activity; or

(c) the cell expresses a small interfering RNA specific to a nucleotide sequence selected from the group consisting of SEQ ID NO: 70, SEQ ID NO: 72, and SEQ ID NO: 74, or a nucleotide variant thereof having at least 95% identity thereto and invertase activity or sucraseferridoxin activity;

(d) the cell comprises an isolated polynucleotide comprising SEQ ID NO: 94 or a sequence 95% identical thereto encoding an active porin polypeptide, wherein the accumulated disaccacharide is sucrose, the cell expresses porin, and the expressed porin secretes the accumulated sucrose from the cell;

(e) the cell comprises an isolated polynucleotide encoding a polypeptide comprising SEQ ID NO: 95 or a sequence 95% identical thereto and having porin activity, wherein the

accumulated disaccacharide is sucrose, the cell expresses porin, and the expressed porin secretes the accumulated sucrose from the cell; or

(f) the cell comprises an isolated polynucleotide comprising SEQ ID NO: 91 (pLybAL32 encoding a porin), wherein the accumulated disaccacharide is sucrose, the cell expresses porin, and the expressed porin secretes the accumulated sucrose from the cell.

Claim 10. An artificial DNA construct comprising at least one sequence selected from: SEQ ID NO: 19 (pLybAL11 encoding *asf*); SEQ ID NO: 20 (pLybAL12 encoding *asf*); SEQ ID NO: 44 (pLybAL15 encoding *asf*); SEQ ID NO: 45 (pLybAL16 encoding *asf*); SEQ ID NO: 46 (pLybAL17 encoding *asf*); SEQ ID NO: 47 (pLybAL18 encoding *asf*); SEQ ID NO: 48 (pLybAL19 encoding *asf*); SEQ ID NO: 49 (pLybAL21 encoding *asf*); SEQ ID NO: 50 (pLybAL22 encoding *asf*); SEQ ID NO: 51 (pLybAL13f encoding *asf*); SEQ ID NO: 52 (pLyAL13r encoding *asf*); SEQ ID NO: 53 (pLybAL14f encoding *asf*); SEQ ID NO: 54 (pLybAL14r encoding *asf*); SEQ ID NO: 65 (pLybAL7f encoding *asf*); SEQ ID NO: 69 (pLybAL8f encoding *asf*); SEQ ID NO: 118 (pLybAL23 encoding *tps* and *tpp*); SEQ ID NO: 121 (pLybAL28 encoding *tps* and *tpp*); SEQ ID NO: 122 (pLybAL29 encoding *tps* and *tpp*); SEQ ID NO: 123 (pLybAL30 encoding *tps* and *tpp*); SEQ ID NO: 124 (pLybAL31 encoding *tps* and *tpp*); SEQ ID NO: 125 (pLybAL36 encoding *tps* and *tpp*); SEQ ID NO: 126 (pLybAL37 encoding *tps* and *tpp*); SEQ ID NO: 130 (pLybAL24 encoding *tps* and *tpp*); SEQ ID NO: 133 (pLybAL33 encoding *tps* and *tpp*); SEQ ID NO: 91 (pLybAL32 encoding a porin); SEQ ID NO: 102 (pLybAL3f encoding SS-UPP); SEQ ID NO: 103 (pLybAL5f encoding SE-UPP); SEQ ID NO: 106 (pLybAL4f encoding SE-UPP); SEQ ID NO: 107 (pLybAL9f encoding SE-UPP); SEQ ID NO: 109 (pLybAL6fb encoding SE-UPP); SEQ ID NO: 110 (pLybAL10fb encoding SE-UPP); or SEQ ID NO: 91 (pLybAL32 encoding a porin).

Claim 11. A method of producing a fermentable sugar using the transgenic photosynthetic microorganism cell, the method comprising:

inoculating a cultivation support with the transgenic photosynthetic microorganism cell of any one of claims 1-9;

cultivating the photosynthetic microorganisms on the inoculated cultivation support;

isolating accumulated fermentable sugar; and

optionally, one or more of the following features:

(a) the fermentable sugar accumulates within the photosynthetic microorganisms;

(b) isolating the accumulated fermentable sugar comprises (1) harvesting at least a portion of the cultivated photosynthetic microorganisms from the cultivation support; and (2) recovering the fermentable sugars from the harvest.;

(c) the accumulated fermentable sugar is secreted from the photosynthetic microorganisms and isolated from a cultivation media;

(d) isolating the accumulated fermentable sugar comprises isolating the accumulated fermentable sugar from a cultivation media;

(e) releasably sealing a physical barrier around the cultivation support after the inoculation such that all or a substantial portion of the cultivation of the photosynthetic microorganisms occurs while the physical barrier is sealed;

(f) at least one of (1) supplying fluid to the cultivation support; (2) supplying nutrients to the cultivation support; or (3) supplying gas to the cultivation support;

(g) conveying the cultivation support to at least one of an inoculation station, a cultivation station, and a harvesting station;

(h) inducing synthesis of the fermentable sugar by the photosynthetic microorganisms;

(i) inducing synthesis of the fermentable sugar comprises exposing the photosynthetic microorganism to an inducing agent selected from the group consisting of temperature, pH, a metabolite, light, an osmotic agent, a heavy metal, and an antibiotic;

(j) inducing synthesis of the fermentable sugar comprises treating the photosynthetic microorganisms with a salt compound;

(k) inducing synthesis of the fermentable sugar comprises treating the photosynthetic microorganisms with sodium chloride;

(l) inducing synthesis of the fermentable sugar comprises treating the photosynthetic microorganisms with a salt compound, wherein the salt compound is added at a concentration of between about 0.01 mM and 1.5 M or between about 0.2 and 0.9 M;

(m) inducing synthesis of the fermentable sugar comprises exposing the photosynthetic microorganism to an inducing agent applied to the growth surface by aerosol spray;

(n) the photosynthetic microorganisms are cultivated to a density of at least about 50 grams of dry biomass per liter equivalent;

(o) the fermentable sugar comprises at least one sugar selected from the group consisting of glucose, fructose, sucrose, trehalose, glucosylglyerol, and mannosylfructose;

(p) the fermentable sugar comprises at least one sugar selected from the group consisting of sucrose and trehalose;

(q) the photosynthetic microorganisms comprise naturally occurring photosynthetic microorganisms; or

(r) the photosynthetic microorganisms are cultivated in or on a photobioreactor or device of any one of claims 12-15.

Claim 12. A photobioreactor for cultivating photosynthetic microorganisms, the photobioreactor comprising:

a non-gelatinous, solid cultivation support suitable for providing nutrients and moisture to photosynthetic microorganisms on at least a portion of a surface thereof, wherein said portion of the surface has a topography that allows photosynthetic microorganisms to adhere thereto when said portion of the surface is oriented non-horizontally; and

a physical barrier covering at least said portion of the surface of the cultivation support, wherein the physical barrier is configured so as to allow inoculation of said portion of the surface of the cultivation support, formation and maintenance of an environment suitable for the cultivation of such photosynthetic microorganisms, and harvesting of such cultivated photosynthetic microorganisms.

Claim 13. The photobioreactor of claim 12, wherein the cultivation support has one or more of the following features:

(a) the cultivation support is flexible;

(b) the cultivation support comprises one or more rigid materials;

(c) the cultivation support comprises at least two layers, a first layer adjacent to a second layer, wherein material of the at least two layers is the same material or different materials;

(d) the cultivation support comprises at least two layers, a first layer adjacent to a second layer, wherein the first layer comprises a high surface area growth material and the second layer a permeable type material;

(e) the cultivation support comprises flexibly connected rigid portions, wherein the rigid portions are comprised of the one or more rigid materials;

(f) the cultivation support comprises a fabric;

(g) the cultivation support comprises a fabric comprised of fibers that are natural, modified natural, synthetic, or a combination thereof;

(h) the cultivation support comprises a fabric selected from the group consisting of a woven fabric, a knitted fabric, a felt, and a mesh of cross-linked fiber polymers, or a combination thereof;

(i) the cultivation support comprises a natural fiber selected from the group consisting of cotton, wool, hemp, tree fiber, and other cellulosic fibers, or combinations thereof;

(j) the cultivation support comprises a modified natural fiber selected from the group consisting of nitrocellulose, cellulose acetate, cellulose sulfonate, and crosslinked starches, or a combination thereof;

(k) the cultivation support comprises a synthetic fiber selected from the group consisting of polyester, polyacrylate, polyamine, polyamide, and polysulfone, or combinations thereof;

(l) the cultivation support is coated with a moisture absorbent polymer;

(m) the cultivation support comprises a fabric; the fabric comprises fibers; and the fabric, the fiber of the fabric, or both, are coated with a moisture absorbent polymer;

(n) the cultivation support comprises a fabric; the fabric comprises fibers; and the fabric, the fiber of the fabric, or both, are coated with a moisture absorbent polymer selected from the group consisting of agar, polyacrylate, polyamide, polyamine, polyethylene glycol, and modified starches, or a combination thereof;

(o) the photobioreactor comprises a single cultivation support; or

(p) the photobioreactor comprises a plurality of cultivation supports.

Claim 14. The photobioreactor of claim 12 or 13, wherein the physical barrier has one or more of the following features:

(a) the physical barrier is at least substantially impermeable to solid particulate and liquid but does not prevent the transport of gas or vapor to and from the space proximate to said portion of the surface of the cultivation support nor actinic irradiation of said portion of the surface of the cultivation support;

(b) the photobioreactor further comprises a source of actinic radiation situated between the cultivation support and the physical barrier;

(c) the physical barrier is between the cultivation support and a source of actinic radiation and is sufficiently transparent to such actinic radiation and sufficiently gas permeable to allow for photosynthesis by the photosynthetic microorganisms during cultivation;

(d) the physical barrier is sufficiently impermeable to water vapor so that the cultivation support upon being moistened will retain enough of the moisture so the photosynthetic microorganisms remain adequately hydrated during cultivation;

(e) the physical barrier is configured to enclose the cultivation support and any photosynthetic microorganisms thereon, and to be releasably sealed during at least a portion of the cultivation of the photosynthetic microorganisms;

(f) the physical barrier is flexible;

(g) the physical barrier further comprises a first portion that is at least substantially impermeable to solid particulate, liquid, gas, and vapor, and a second portion that is permeable to gas and vapor but at least substantially impermeable to solid particulate and liquid;

(h) the physical barrier further comprises a first portion that is at least substantially impermeable to solid particulate, liquid, gas, and vapor, and a second portion that is permeable to gas and vapor but at least substantially impermeable to solid particulate and liquid, wherein the second portion of the barrier has (1) a gas or vapor exchange rate that is from at least about 5 Gurley seconds to no greater than about 10,000 Gurley seconds or (2) comprises a selective membrane comprising olefin fiber or polyethylene fiber material, polytetrafluoroethylene filtration media, cellulosic filter material, fiberglass filter material, polyester filter material, polyacrylate filter material, polysulfone membranes, or nylon membranes; or

(i) the physical barrier further comprises a first portion that is at least substantially impermeable to solid particulate, liquid, gas, and vapor, and a second portion that is permeable to gas and vapor but at least substantially impermeable to solid particulate and liquid, wherein the first portion is at least substantially transparent to actinic radiation and the second portion is

not at least substantially transparent to actinic radiation, and the configuration of the first and second portions relative to each other and at least said portion of the surface of the cultivation support is such that there a sufficient amount of actinic radiation and gas exchange to support photosynthesis by photosynthetic microorganisms.

Claim 15. A device for cultivating photosynthetic microorganisms, comprising:

at least one photobioreactor of any one of claims 12-14;

a structure to which the at least one photobioreactor is attached that orientates at least one cultivation support of the at least one photobioreactor non-horizontally; and

optionally, one or more of the following features:

(a) the at least one photobioreactor is suspended from the structure;

(b) the structure is substantially covered by the physical barrier;

(c) the structure comprises a conveyor system or a component thereof such that the at least one cultivation support is capable of being conveyed along the path of the conveyor system;

(d) the device further comprises (1) an inoculation station such that each cultivation support as it is conveyed along the path of the conveyor system may be inoculated with photosynthetic microorganisms; (2) a cultivating station such that the photosynthetic microorganisms on each inoculated cultivation support are cultivated as each cultivation support is conveyed along the path of the conveyor system; and (3) a harvesting station to which the cultivation support is conveyed so that at least a portion of the cultivated photosynthetic microorganisms may be harvested from each cultivation support;

(e) the device further comprises (1) an inoculation station such that each cultivation support as it is conveyed along the path of the conveyor system may be inoculated with photosynthetic microorganisms; (2) a cultivating station such that the photosynthetic microorganisms on each inoculated cultivation support are cultivated as each cultivation support is conveyed along the path of the conveyor system; and (3) a harvesting station to which the cultivation support is conveyed so that at least a portion of the cultivated photosynthetic microorganisms may be harvested from each cultivation support; wherein the inoculation station and the harvesting station are substantially adjacent to each other or are substantially coextensive;

(f) the device further comprises an inducing station for inducing the synthesis of fermentable sugar by photosynthetic microorganisms on each cultivation support;

(g) the device further comprises at least one of a fluid supply system, a nutrient supply system, a gas supply system, or a microorgansim supply system; or

(h) the device further comprises a cell according to any one of claims 1-9, wherein the cell is adhered to the solid cultivation support.

Claim 16. A method of cultivating a photosynthetic microorganism using the photobioreactor or device of any one of claims 12-15, the method comprising:

inoculating the cultivation support with photosynthetic microorganisms;

cultivating the photosynthetic microorganisms on the inoculated cultivation support;

harvesting at least a portion of the cultivated photosynthetic microorganisms from the cultivation support; and

optionally, one or more of the following features:

(a) sealing the physical barrier of the photobioreactor after the inoculation of the cultivation support such that all or a substantial portion of the cultivation of the photosynthetic microorganisms occurs while the physical barrier is sealed;

(b) releasably sealing the physical barrier of the photobioreactor after the inoculation of the cultivation support such that all or a substantial portion of the cultivation of the photosynthetic microorganisms occurs while the physical barrier is sealed;

(c) conveying each cultivation support to an inoculation station, a cultivation station, and a harvesting station;

(d) at least one of (1) supplying fluid to the cultivation support; (2) supplying nutrients to the cultivation support; or (3) supplying gas to the cultivation support;

(e) the photosynthetic microorganisms are cultivated to a density of at least about 50 grams of dry biomass per liter equivalent; or

(f) the photosynthetic microorganisms comprise a cell according to any one of claims 1-9.

FIG. 1

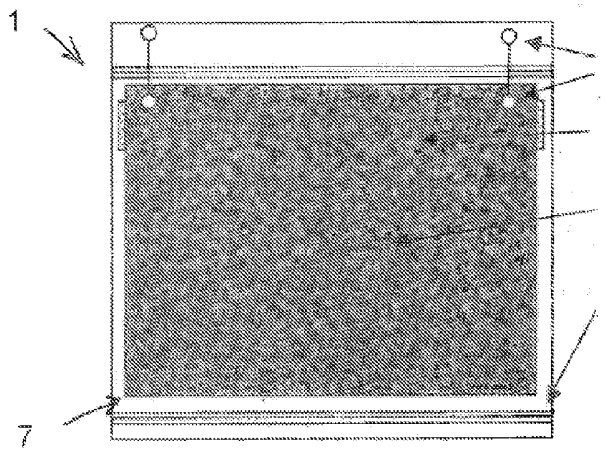


FIG. 2

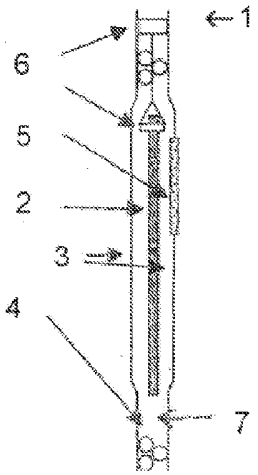


FIG. 3

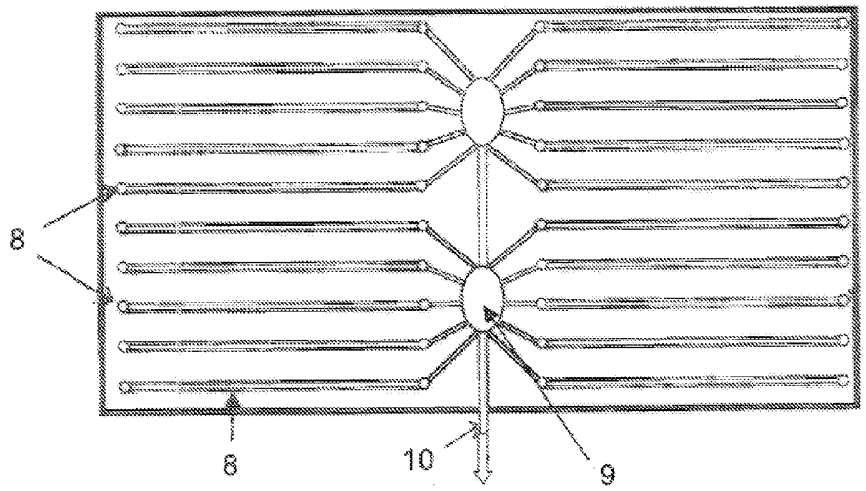


FIG. 4

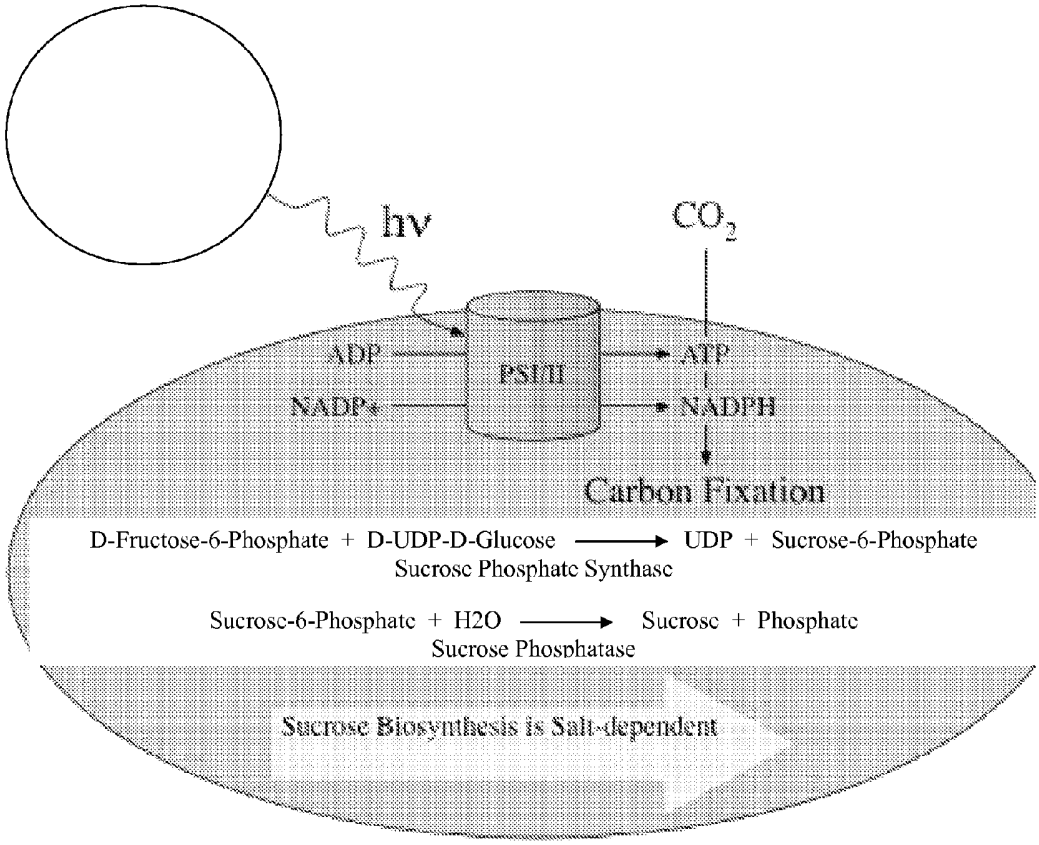

hv
CO_2
ADP
ATP
PSI/II
NADP+
NADPH
Carbon Fixation
D-Fructose-6-Phosphate + D-UDP-D-Glucose → UDP + Sucrose-6-Phosphate
Sucrose Phosphate Synthase
Sucrose-6-Phosphate + H2O → Sucrose + Phosphate
Sucrose Phosphatase
Sucrose Biosynthesis is Salt-dependent

FIG. 5

FIG. 6

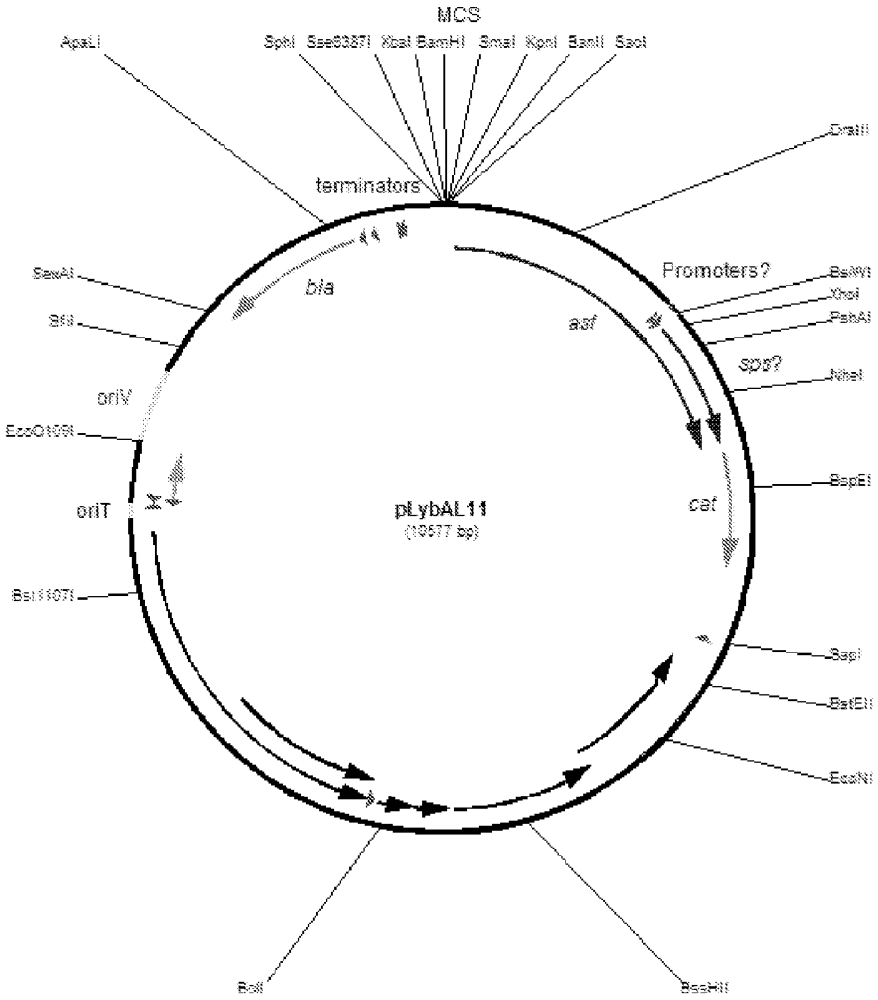

MCS
ApaLI
SphI Sse8387I XbaI BamHI SmaI KpnI BanII SacI
DraIII
terminators
Promoters?
BsiWI
XhoI
PstAI
NheI
SexAI
BlnI
bla
asf
sps?
oriV
EcoO109I
oriT
pLybAL11
(10577 bp)
cat
BspEI
Bst1107I
SapI
BstEII
EcoNI
BclI
BssHII

FIG. 7

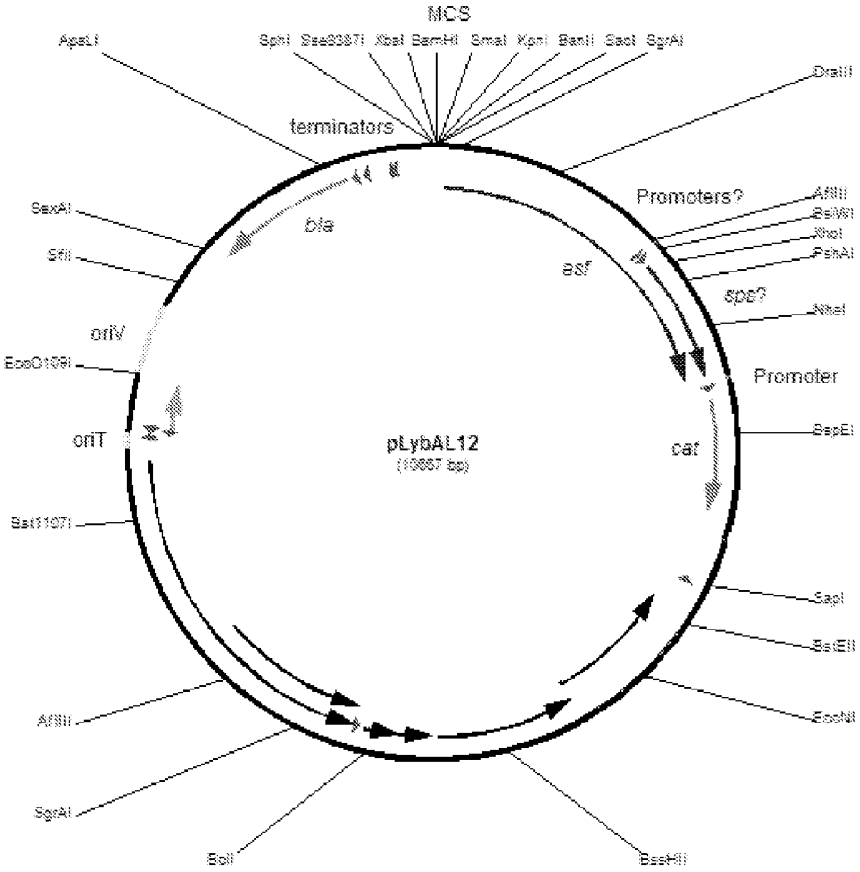

MCS
SphI Sse83ΔTI XbaI BamHI SmaI KpnI BanII SacI SgrAI
ApaLI
DraIII
terminators
Promoters?
AflII
BsiWI
XhoI
PshAI
NheI
SexAI
SfiI
bla
asf
sps?
oriV
EcoO109I
Promoter
BspEI
oriT
pLybAL12
(10667 bp)
cat
Bst1107I
SapI
BstEII
EcoNI
AflIII
SgrAI
BclI
BssHII

FIG. 8

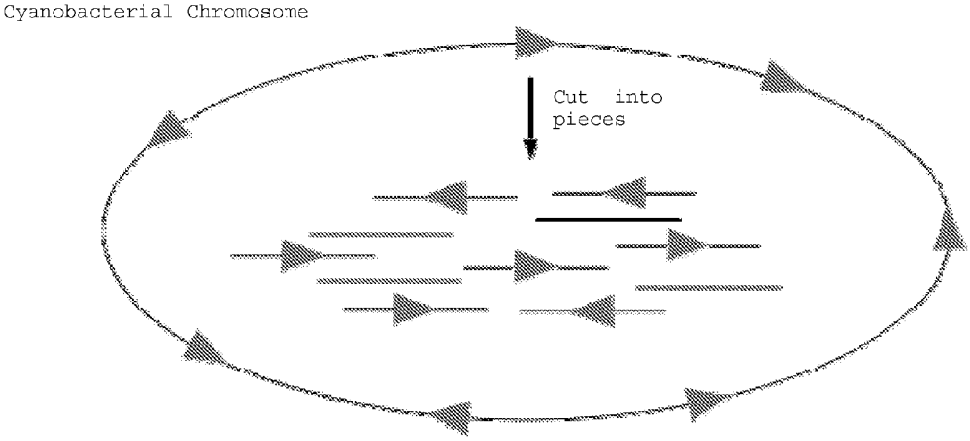


FIG. 9

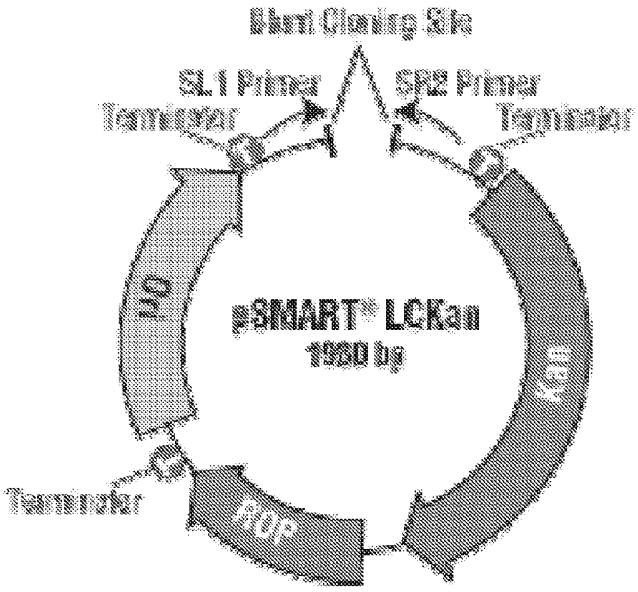

Blunt Cloning Site
SL1 Primer
SP2 primer
Terminator
Terminator
pSMART® LCKan
1980 bp
Kan
Ori
Terminator

FIG. 10

MfeI
SD
Stop
Start
-35? Promoter 1?
-10?
-35? Promoter 2?
-10?
SD?
Start?
Stop

FIG. 11

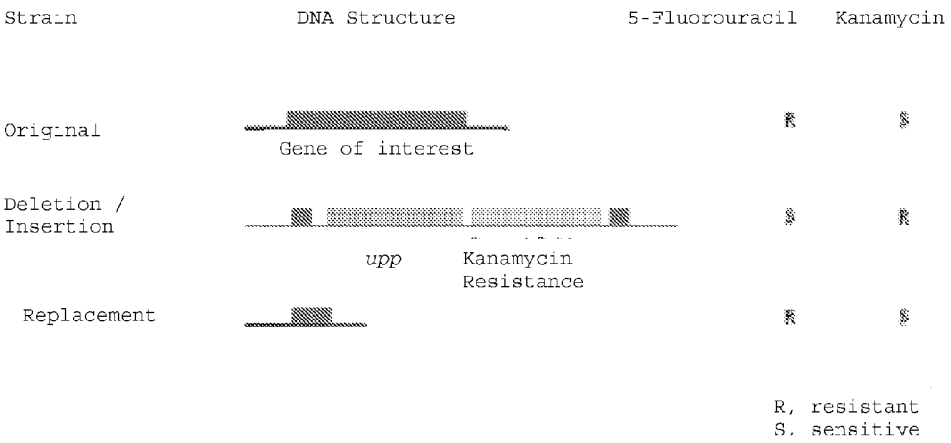
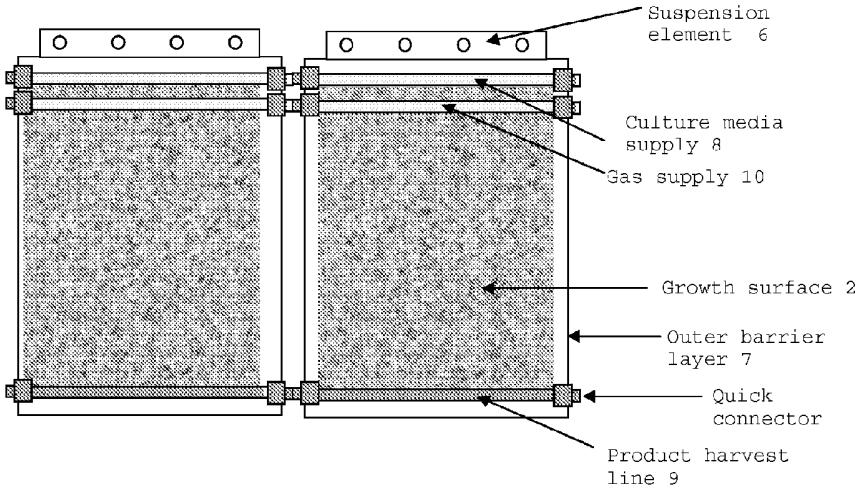

Strain
DNA Structure
5-Fluorouracil
Kanamycin
Original
Gene of interest
R
S
Deletion / Insertion
upp
Kanamycin Resistance
S
R
Replacement
R
S
R, resistant
S, sensitive

FIG. 12

A


Suspension element 6
Culture media supply 8
Gas supply 10
Growth surface 2
Outer barrier layer 7
Quick connector
Product harvest line 9

B

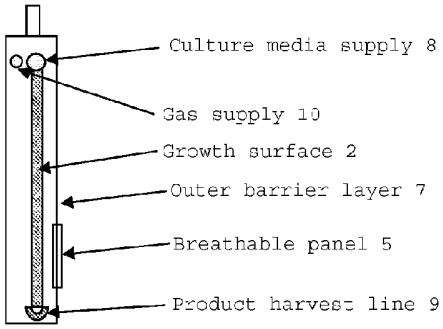
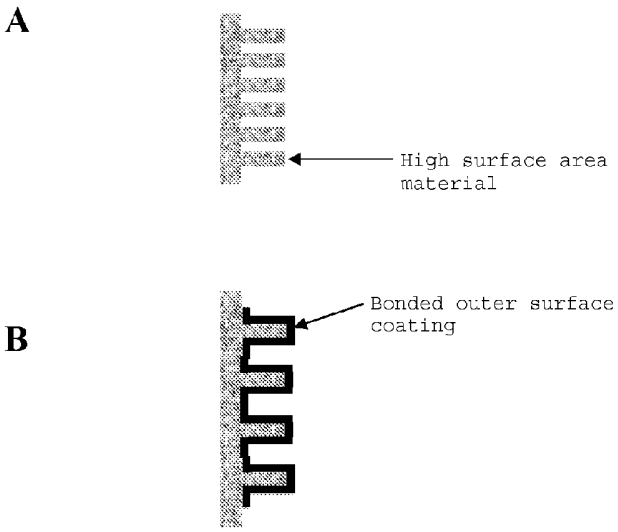

Culture media supply 8
Gas supply 10
Growth surface 2
Outer barrier layer 7
Breathable panel 5
Product harvest line 9

FIG. 13


A
High surface area material
B
Bonded outer surface coating