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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 March 2013 (14.03.2013)

WIPO | PCT

(10) International Publication Number
WO 2013/036968 A1

(51) International Patent Classification:
C12P 19/18 (2006.01)

(21) International Application Number:
PCT/US2012/054521

(22) International Filing Date:
10 September 2012 (10.09.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/532,714 9 September 2011 (09.09.2011) US

(71) Applicant *(for all designated States except US)*: **E. I. DU PONT DE NEMOURS AND COMPANY** [US/US]; 1007 Market Street, Wilmington, Delaware 19899 (US).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **O'BRIEN, John P.** [US/US]; 871 Saginaw Road, Oxford, Pennsylvania 19363 (US). **PAYNE, Mark, S.** [US/US]; 4617 Old Linden Hill Road, Wilmington, Delaware 19808 (US).

(74) Agent: **SARIASLANI, Fateme, Sima**; E. I. du Pont de Nemours and Company, Legal Patent Records Center, 4417 Lancaster Pike, Wilmington, Delaware 19805 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, 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, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

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

Published:

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

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

WO 2013/036968 A1

(54) **Title**: HIGH TITER PRODUCTION OF POLY (α 1, 3 GLUCAN)

(57) **Abstract**: A process for enzymatic preparation of poly (α 1, 3 glucan) from sucrose is disclosed. The glucosyltransferase enzyme (gtfJ) from *Streptococcus salivarius* is used to convert sucrose to fructose and poly (α 1, 3 glucan). Application of semi-permeable membranes to continuously remove fructose, a by-product of the gtf enzyme, thus increasing the poly (α 1, 3 glucan) liter, is disclosed.

TITLE

HIGH TITER PRODUCTION OF POLY (α 1, 3 GLUCAN)

FIELD OF INVENTION

This invention relates to the field of production of a structural polysaccharide. Specifically, it relates to production of poly (α 1, 3 glucan) via an enzymatic reaction. More specifically, it relates to increasing the titer of poly (α 1, 3 glucan) formed during the enzymatic reaction.

BACKGROUND

Cellulose, a polysaccharide formed from glucose via β (1, 4) glycoside linkages by natural processes (Applied Fiber Science, F. Happey, Ed., Chapter 8, E. Atkins, Academic Press, New York, 1979), has achieved commercial prominence as a fiber as a consequence of the many useful products derived therefrom. In particular, cotton, a highly pure form of naturally occurring cellulose, is well-known for its beneficial attributes in textile applications.

Cellulose exhibits sufficient chain extension and backbone rigidity in solution to form liquid crystalline solutions (U.S. Patent No. 4,501,886). However, sufficient polysaccharide chain extension has hitherto been achieved primarily in β (1, 4) linked polysaccharides. Any significant deviation from that backbone geometry in the glucan polysaccharide family lowers the molecular aspect ratio below that required for the formation of an ordered lyotropic phase. Additionally, it is well-known that important commercial cellulosic fibers such as cotton and rayon increasingly present sustainability issues with respect to land use and environmental imprint.

It is therefore highly desirable to discover other glucose-based polysaccharides with utility in films, fibers and resins largely because of the current emphasis on producing low cost, structural materials from renewable resources. In addition such polymers offer materials that are environmentally benign throughout their entire life cycle.

Poly (α 1, 3 glucan), a glucan polymer characterized by having α (1, 3) glycoside linkages, has been isolated by contacting an aqueous solution of sucrose with a glucosyltransferase (gtfJ) enzyme isolated from *Streptococcus salivarius* (Simpson et al., Microbiology, 141: 1451-1460, 1995). Glucan refers to a polysaccharide composed of D-glucose monomers linked by glycosidic bonds. Films prepared from poly (α1, 3 glucan) tolerated temperatures up to 150 ^{o}C and provided an advantage over polymers obtained from β (1, 4) linked polysaccharides (Ogawa et al., Fiber Differentiation Methods, 47: 353-362, 1980).

U.S. Patent 7,000,000 disclosed preparation of a polysaccharide fiber comprising hexose units, wherein at least 50% of the hexose units within the polymer were linked via α (1, 3) glycoside linkages using the glucosyltransferase enzyme gtfJ of *Streptococcus salivarius*. The disclosed polymer formed a liquid crystalline solution when it was dissolved above a critical concentration in a solvent or in a mixture comprising a solvent. From this solution continuous, strong, cotton-like fibers highly suitable for use in textiles were spun and used either in a derivatized form or as a non-derivatized (regenerated) form. The poly (α 1, 3 glucan) in U.S. Patent 7,000,000 was made in a batch process wherein the poly (α 1, 3 glucan) titers were typically less than 25 grams of poly (α 1, 3 glucan) per liter of the reactor volume.

It can be desirable to develop processes to increase the titer of the poly (α1, 3 glucan) formed by the enzymatic reaction.

SUMMARY OF INVENTION

This invention is a process for production of poly (α 1, 3 glucan) from a renewable feedstock, for applications in fibers, films, and pulps. The polymer is made directly in a one step enzymatic reaction using a recombinant glucosyltransferase (gtfJ) enzyme as the catalyst and sucrose as the substrate.

In one aspect, the disclosed invention is a process for producing poly (α 1, 3 glucan) in a reaction system comprising two chambers separated by a semi-permeable membrane, wherein:

a) a first chamber comprises an enzyme reaction solution comprising:

i) sucrose; and

ii) at least one glucosyltransferase enzyme; and

b) a second chamber, separated from the first chamber by a semi-permeable membrane in contact with the enzyme reaction solution wherein the semi-permeable membrane is permeable to fructose and other low molecular weight moieties but impermeable to poly (α 1, 3 glucan), facilitates continuous removal of fructose and other low molecular weight moieties while retaining poly (α 1, 3 glucan) and the at least one glucosyltransferase enzyme inside the first chamber.

In another aspect, the disclosed invention is a process wherein poly (α 1, 3 glucan), at a titer of 30 – 200 grams per liter, is produced from sucrose by at least one glucosyltransferase enzyme.

DESCRIPTION OF DNA SEQUENCES

SEQ NO. 1 is the sequence of the synthesized gene of the mature glucosyltransferase which has been codon optimized for expression in *E. coli*.

SEQ NO. 2 is the DNA sequence for the plasmid pMP52.

SEQ NO. 3 is the DNA sequence of the mature glucosyltransferase (gtfJ enzyme; EC 2.4.1.5; GENBANK® AAA26896.1) from *Streptococcus salivarius* (ATCC 25975).

DETAILED DESCRIPTION OF INVENTION

Poly (α 1, 3 glucan) is a potentially low cost polymer which can be enzymatically produced from renewable resources such as sucrose using the gtfJ enzyme of *Streptococcus salivarius*. It has been shown that selected polymers comprising hexose units with α (1, 3) glycoside linkages can form ordered liquid crystalline solutions when the polymer is dissolved in a solvent

under certain conditions (U. S. Patent No. 7,000,000). Moreover such solutions can be spun into continuous, high strength, cotton-like fibers. In U. S. Patent No. 7,000,000, batch enzymatic reactions were employed for conversion of sucrose to poly (α 1, 3 glucan) with gtfJ, and the by-products fructose and leucrose accumulated in the reactor. Since the accumulated fructose is known to compete for glucosyl moieties during enzymatic reaction, conversion of available glucose to poly (α 1, 3 glucan) was subsequently hindered thus limiting the final titer of the desired product per unit reactor volume.

The term "leucrose", as used herein, refers to a disaccharide consisting of glucose and fructose, linked by an α (1, 5) bond.

The term "glucosyltransferase (gtf) enzyme", as used herein, refers to an enzyme excreted by oral streptococci, such as *Streptococcus salivarius* which utilizes the high free energy of the glycosidic bond of sucrose to synthesize poly (α 1, 3 glucan). A glycosidic bond can join two monosaccharides to form a disaccharide. The glycosidic bonds can be in the α or β configuration and can generate, for example, α (1, 2), α (1, 3), α (1, 4), α (1, 6), β (1, 2), β (1, 3), β (1, 4) or β (1, 6) linkages. The term "α (1,3) glycoside linkage", as used herein, refers to a type of covalent bond that joins glucose molecules to each other through the ring carbons 1 and 3 on adjacent glucose rings.

The term "poly (α 1, 3 glucan)", as used herein, refers to high molecular weight, linear polymers obtained from polysaccharide molecules resulting from linking glucose units via α (1,3) glycosidic linkages.

The present invention relates to a process for increasing the titer of the polysaccharide, poly (α 1, 3 glucan), produced from sucrose in an enzymatic reaction using one or more gtf enzymes. The term "enzymatic reaction" refers to a reaction that is performed by the gtf enzyme. An "enzyme reaction solution" of the present invention generally refers to a reaction mixture comprising at least one gtf enzyme in a buffer solution comprising sucrose and possibly one or more primers to convert sucrose to poly (α 1, 3 glucan).

The glucosyltransferase enzyme used in the present invention can be any gtf enzyme. The gtf enzyme used can be from any streprococci. Suitable gtf enzymes can be, for example, the gtfJ of *Streptococcus salivarius*, the gtfB and the gtfC from *Streptococcus mutans*, and the gtfI from *Streptococcus downei*. Particularly, the *Streptococcus* species can be *Streptococcus salivarius*. More particularly, the gtf enzyme can be the gtfJ (E.C. 2.4.1.5) enzyme of *Streptococcus salivarius*.

In one embodiment, the enzyme reaction solution can comprise only one gtf enzyme as described herein. In another embodiment, the enzyme reaction solution can comprise a combination of more than one type of gtf enzyme.

For purposes of this invention, sufficient quantities of the gtfJ enzyme can be produced using a recombinant *E. coli* strain for gtfJ production as described in the Examples. Methods for designing the codon optimized genes and expression in *E. coli* are well known in the art.

Methods for the growth of recombinant microorganisms are well known in the art. Recombinant microorganisms expressing the desired gtf enzyme to perform the instant reaction can be grown in any container, such as, for example: various types of flasks with and without indentations; any autoclavable container that can be sealed and temperature-controlled; or any type of fermenter. In one embodiment, production of the gtfJ enzyme for poly (α 1, 3 glucan) production in the present invention can be achieved by growing the recombinant *E. coli* MG1655/pMP52, expressing the gtfJ enzyme, in a fermenter.

The gtfJ enzyme of *Streptococcus salivarius,* used as the catalyst for conversion of sucrose to poly (α 1, 3 glucan) in the current invention, is a primer-dependent gtf enzyme. A primer-dependent gtf enzyme as referenced in the present application, refers to a gtf enzyme that requires the presence of an initiating molecule in the enzyme reaction solution to act as a primer for the enzyme during poly (α 1, 3 glucan) synthesis. Thus a “primer”, as the term is used herein, refers to any molecule that can act as the initiator for the primer-dependent glucosyltransferases. Many other glucosyltransferases are primer-independent enzymes. The primer-independent enzymes do not require the

presence of a primer to perform the reaction. For the purposes of the present invention, either or both a primer-independent enzyme, and/or a primer-dependent gtf enzyme can be used in the same enzyme reaction system during poly (α 1, 3 glucan) synthesis.

The gtfJ is a primer-dependent enzyme. In the present invention, dextran, which is a complex, branched glucan was used as a primer for the gtfJ enzyme. While gtf is a primer-dependent enzyme, conversion of sucrose to poly (α 1, 3 glucan) with this enzyme can also occur in the absence of a primer.

The production of poly (α 1, 3 glucan), by the gtfJ enzyme of *Streptococcus salivarius* is inhibited by its by-product, fructose. When fructose accumulates in the enzyme reaction solution it can inhibit the production of poly (α 1, 3 glucan) by the enzyme, presumably by competing for available glucosyl moieties which results in the formation of the disaccharide, leucrose. In the present invention, to reduce the effect on gtfJ of fructose, the fructose in the enzyme reaction solution can be continuously removed to prevent its accumulation to inhibitory levels in the enzyme reaction solution. For the purposes of the current invention the reaction system can comprise a semi-permeable membrane that separates the enzyme reaction solution, contained in the first chamber, comprising one or more gtf enzymes, one or more primers and sucrose, from the surrounding buffer contained in the second chamber. The term "chamber" as used herein, refers to any container that can hold the enzyme reaction solution or the products of the enzyme reaction solution. The chamber can be made of glass, plastic, metal, film, membrane or any other type of inert material that can hold the enzyme reaction solution. The term "semi-permeable membrane", as used herein, refers to a membrane that will allow passage of certain molecules or ions by diffusion while retaining some other molecules. Essentially any semi-permeable membrane, with a molecular cutoff between 12,000 and 100,000 Daltons that will allow fructose and other low molecular weight moieties to pass through while retaining the enzyme and poly (α 1, 3 glucan) can be suitable for use in the present invention. The term "other low molecular weight moieties" as used herein, refers to various compounds with

molecular weights below 1000 Dalton that can be present in the enzyme reaction solution. Due to the removal of the by-product fructose from the enzyme reaction solution contained in the first chamber, leucrose formation can be reduced. In one embodiment of the present invention, dialysis tubing can be used as the semi-permeable membrane to remove the by-product fructose from the enzyme reaction solution.

For the present invention, the enzyme reaction solution can be maintained at 20 °C to 25 °C.

The present invention provides for production of poly (α 1, 3 glucan), as a low cost material that can be economically obtained from readily renewable sucrose feedstocks for a variety of applications including fibers, films, and pulps. In particular, it is expected that poly (α 1, 3 glucan) fibers, for example, will functionally substitute for cotton and regenerated cellulose fibers, leading to new textile fibers with minimal environmental impact and excellent sustainability versus the aforementioned incumbents.

EXAMPLES

The advantageous attributes and effects of the composition and process disclosed herein can be more fully appreciated from the examples described below. The embodiments of the process on which the examples are based are representative only, and the selection of those embodiments to illustrate the invention does not indicate that materials, components, reactants, conditions, specifications, steps or techniques not described in these examples are unsuitable for practicing these processes, or that subject matter not described in these examples is excluded from the scope of the appended claims and equivalents thereof.

Materials

Dialysis tubing (Spectrapor 25225-226, 12000 molecular weight cut-off) was obtained from VWR (Radnor, PA).

Dextran and ethanol were obtained from Sigma Aldrich. Sucrose was obtained from VWR.

Suppressor 7153 antifoam was obtained from Cognis Corporation (Cincinnati, OH).

All other chemicals were obtained from commonly used suppliers of such chemicals.

Seed medium

The seed medium, used to grow the starter cultures for the fermenters, contained: yeast extract (Amberx 695, 5.0 grams per liter, g/L), K_2HPO_4 (10.0 g/L), KH_2PO_4 (7.0 g/L), sodium citrate dihydrate (1.0 g/L), $(NH_4)_2SO_4$ (4.0 g/L), $MgSO_4$ heptahydrate (1.0 g/L) and ferric ammonium citrate (0.10 g/L). The pH of the medium was adjusted to 6.8 using either 5N NaOH or H_2SO_4 and the medium was sterilized in the flask. Post sterilization additions included glucose (20 mL/L of a 50% w/w solution) and ampicillin (4 mL/L of a 25 mg/mL stock solution).

Fermenter medium

The growth medium used in the fermenter contained: KH_2PO_4 (3.50 g/L), $FeSO_4$ heptahydrate (0.05 g/L), $MgSO_4$ heptahydrate (2.0 g/L), sodium citrate dihydrate (1.90 g/L), yeast extract (Ambrex 695, 5.0 g/L), Suppressor 7153 antifoam (0.25 milliliters per liter, mL/L), NaCl (1.0 g/L), $CaCl_2$ dihydrate (10 g/L), and NIT trace elements solution (10 mL/L). The NIT trace elements solution contained citric acid monohydrate (10 g/L), $MnSO_4$ hydrate (2 g/L), NaCl (2 g/L), $FeSO_4$ heptahydrate (0.5 g/L), $ZnSO_4$ heptahydrate (0.2 g/L), $CuSO_4$ pentahydrate (0.02 g/L) and $NaMoO_4$ dihydrate (0.02 g/L). Post sterilization additions included glucose (12.5 g/L of a 50% w/w solution) and ampicillin (4 mL/L of a 25 mg/mL stock solution).

EXAMPLE 1

CONSTRUCTION OF GLUCOSYLTRANSFERASE (gtfJ) ENZYME EXPRESSION STRAIN

The gene encoding the mature glucosyltransferase enzyme (gtfJ; EC 2.4.1.5) from *Streptococcus salivarius* (ATCC 25975) as reported in GENBANK® (accession M64111.1) was synthesized using codons optimized for expression in *E. coli* (DNA 2.0, Menlo Park, CA). The nucleic acid product (SEQ ID NO: 1) was

subcloned into pJexpress404® (DNA 2.0, Menlo Park CA) to generate the plasmid identified as pMP52 (SEQ ID NO: 2). The plasmid pMP52 was used to transform *E. coli* MG1655 (ATCC 47076™) to generate the strain identified as MG1655/pMP52. All procedures used for construction of the glucosyltransferase enzyme expression strain are well known in the art and can be performed by individuals skilled in the relevant art without undue experimentation.

EXAMPLE 2

PRODUCTION OF RECOMBINANT gtfJ IN FERMENTATION

Production of the recombinant gtfJ enzyme in a fermenter was initiated by preparing a pre-seed culture of the *E. coli* strain MG1655/pMP52, expressing the gtfJ enzyme, constructed as described in Example 1. A 10 mL aliquot of the seed medium was added into a 125 mL disposable baffled flask and was inoculated with a 1.0 mL culture of *E. coli* MG1655/pMP52 in 20% glycerol. This culture was allowed to grow at 37 °C while shaking at 300 revolutions per minute (rpm) for 3 hours.

A seed culture, for starting the fermenter, was prepared by charging a 2 L shake flask with 0.5 L of the seed medium. 1.0 mL of the pre-seed culture was aseptically transferred into 0.5 L seed medium in the flask and cultivated at 37 °C and 300 rpm for 5 hours. The seed culture was transferred at optical density 550 nm (OD_{550}) >2 to a 14 L fermenter (Braun, Perth Amboy, NJ) containing 8 L of the fermenter medium described above at 37 °C.

Cells of *E. coli* MG1655/pMP52 were allowed to grow in the fermenter and glucose feed (50% w/w glucose solution containing 1% w/w $MgSO_4 \cdot 7H_2O$) was initiated when glucose concentration in the medium decreased to 0.5 g/L. The feed was started at 0.36 grams feed per minute (g feed/min) and increased progressively each hour to 0.42, 0.49, 0.57, 0.66, 0.77, 0.90, 1.04, 1.21, 1.41 1.63, 1.92, 2.2 g feed/min respectively. The rate remained constant afterwards. Glucose concentration in the medium was monitored using an YSI glucose analyzer (YSI, Yellow Springs, Ohio). When glucose concentration exceeded 0.1

g/L the feed rate was decreased or stopped temporarily. Induction of glucosyltransferase enzyme activity was initiated, when cells reached an OD_{550} of 70, with the addition of 9 mL of 0.5 M IPTG (isopropyl β-D-1-thiogalacto-pyranoside). The dissolved oxygen (DO) concentration was controlled at 25% of air saturation. The DO was controlled first by impeller agitation rate (400 to 1200 rpm) and later by aeration rate (2 to 10 standard liters per minute, slpm). The pH was controlled at 6.8. NH_4OH (14.5% weight/volume, w/v) and H_2SO_4 (20% w/v) were used for pH control. The back pressure was maintained at 0.5 bars. At various intervals (20, 25 and 30 hours), 5 mL of Suppressor 7153 antifoam was added into the fermenter to suppress foaming. Cells were harvested by centrifugation 8 hours post IPTG addition and were stored at -80 °C as a cell paste.

EXAMPLE 3

PREPARATION OF GTFJ CRUDE ENZYME EXTRACT FROM CELL PASTE

The cell paste obtained above was suspended at 150 g/L in 50 mM potassium phosphate buffer pH 7.2 to prepare a slurry. The slurry was homogenized at 12,000 psi (Rannie-type machine, APV-1000 or APV 16.56) and the homogenate chilled to 4 °C. With moderately vigorous stirring, 50 g of a floc solution (Aldrich no. 409138, 5% in 50 mM sodium phosphate buffer pH 7.0) was added per liter of cell homogenate. Agitation was reduced to light stirring for 15 minutes. The cell homogenate was then clarified by centrifugation at 4500 rpm for 3 hours at 5-10 °C. Supernatant, containing crude gtfJ enzyme extract, was concentrated (approximately 5X) with a 30 kilo Dalton (kDa) cut-off membrane. The concentration of protein in the gftJ enzyme solution was determined by the bicinchoninic acid (BCA) protein assay (Sigma Aldrich) to be 4-8 g/L.

EXAMPLE 4

IMPROVING THE TITER OF POLY (α 1, 3 GLUCAN) BY USING A SEMI-PERMEABLE MEMBRANE

This Example demonstrates that removal and/or dilution of the by-product fructose, formed during conversion of sucrose to poly (α 1, 3 glucan), increases

poly (α 1,3 glucan) titer. Dialysis tubing was used as a semi-permeable membrane in this Example since it allows passage of the by-product fructose formed during the enzymatic reaction, from inside the tubing to outside of the dialysis tubes.

The enzyme reaction solution in this Example contained 8 L of the sucrose stock solution (Table 1), 24 g of dextran T-10, as the primer, and 1.0 volume % of the gtf enzyme.

TABLE 1

sucrose stock solution

Material	concentration
Sucrose	1200 g
KH_2PO_4 Buffer (pH 6.8 – 7.0)	50 mM
10% KOH sol'n	as needed for adjusting to pH 7
Ethanol	800 mL
De-ionized water	To 8 liters

Four individual dialysis tubes (50 mL capacity) were used as test samples and charged with 50 mL of the enzyme reaction solution and were sealed. The individual dialysis tubes were then suspended in polyethylene buckets holding different volumes of the sucrose stock solution (Table 1) as the surrounding buffer. These polyethylene buckets were then placed on a magnetic stirring plate and allowed to stir at 20-25 ^{o}C for 72 hours. A control sample was prepared, in a capped centrifuge tube, consisting of 50 mL of the enzyme reaction solution, in the same proportions as the test samples, and allowed to stand at 20-25 ^{o}C temperature for 72 hours without stirring. The control sample was not placed in the dialysis tube or the surrounding buffer.

After 72 hours, the test samples in the dialysis tubes were removed from the surrounding buffer, the tubes were cut open and the poly (α 1, 3 glucan) solids were collected on a Buchner funnel using 325 mesh screen over 40 micometers filter paper. The filter cake was resuspended in deionized water and filtered twice more as above to remove residual sucrose, fructose and other low molecular weight, soluble by-products. Finally two additional washes with methanol were carried out. The filter cake was pressed out thoroughly on the funnel and dried under vacuum at room temperature. The poly (α 1, 3 glucan) formed in the control sample was also isolated and weighed. Formation of poly (α 1, 3 glucan) in the tests and the control samples was confirmed using publically available information (Nakamura, T., et al., Biosci. Biotechnol. Biochem., 68: 868-872, 2004). The resulting dry weights of the poly (α 1, 3 glucan) obtained following conversion of sucrose to poly (α 1, 3 glucan) by the gtfJ enzyme, from the test and the control samples, are shown in Table 2.

TABLE 2

Comparison of poly (α 1, 3 glucan) formed in the presence and absence of a semi-permeable membrane

Volume of 15% sucrose solution (the surrounding buffer)	poly (α 1, 3 glucan) (g)
5 L	1.395g
2.5 L	1.515g
250 mL	1.132g
250 mL	1.114g
Control	**0.696g**

The above results show clearly that the titer of poly (α 1, 3 glucan) was significantly enhanced when the enzyme reaction solution was placed in a dialysis tube and was placed in the surrounding buffer that allowed continuous

passage of the by-product fructose from inside the dialysis tube to outside, thus diluting the concentration of fructose, formed by the enzyme, inside the tube. The highest polymer titers were obtained at higher volumes of the surrounding buffer which can result in higher dilution of the by-product fructose.

EXAMPLE 5

DETERMINATION OF THE AMOUNT OF POLY (α 1, 3 GLUCAN) FORMED AT TIMED INTERVALS

To 3 L of the sucrose stock solution (Table 1), 9 g of dextran T-10, as the primer and 2.0% volume% of gtf were added to prepare the enzyme reaction solution.

Seven individual dialysis tubes were used as test samples and charged with 50 mL of the enzyme reaction solution and were sealed. The individual dialysis tubes were then suspended in a polyethylene bucket containing 3 L of the sucrose stock solution as the surrounding buffer. The buckets were placed on a magnetic stirring plate and allowed to stir at 20-25 ^{o}C for 72 hours. Individual dialysis tubes were removed at timed intervals shown in Table 3. Since the titer of the poly (α 1, 3 glucan) formed in control samples, as described in the Example 4, was consistently around 0.6 g after 72 hours, no control samples were used in this experiment.

At each timed interval, the poly (α 1, 3 glucan) solids formed in the dialysis tubes were isolated as described in Example 4. The resulting dry weights of the poly (α 1, 3 glucan) obtained enzyme reaction solution at various time intervals are shown in Table 3.

TABLE 3

Weight of the Poly (α 1, 3 glucan) formed at various timed intervals

Time (hour)	poly (α1, 3 glucan) (g)
6	0.48
21	1.21
28	1.33
36	1.52
48	1.71
60	2.07
72	2.50

The above results clearly showed that production of poly (α1, 3 glucan) was significantly enhanced as the enzyme reaction was allowed to proceed for a longer period of time (e.g., 72 hours).

CLAIMS

What is claimed is:

1. A process for producing poly (α 1, 3 glucan) in a reaction system comprising two chambers, separated by a semi-permeable membrane, wherein:

a) a first chamber comprises an enzyme reaction solution comprising:

i) sucrose; and

ii) at least one glucosyltransferase enzyme; and

b) a second chamber, separated from the first chamber by a semi-permeable membrane in contact with the enzyme reaction solution wherein the semi-permeable membrane is permeable to fructose but impermeable to poly (α 1, 3 glucan), facilitates continuous removal of fructose and other low molecular weight moieties while retaining poly (α 1, 3 glucan) and the at least one glucosyltransferase enzyme inside the first chamber.

2. The process of claim 1 wherein the enzyme reaction solution is maintained at a temperature of from 20 ^{o}C to 25 ^{o}C.

3. The process of claim 1 further comprising at least one primer.

4. The process of claim 1 wherein the semi-permeable membrane facilitates accumulation of poly (α 1, 3 glucan) to a concentration ranging from 30 grams per liter to 200 grams per liter.

5. The process of claim 1 wherein the semi-permeable membrane has a molecular weight cut-off from 12,000 to 100,000 Daltons.

6. The process of claim 5 wherein the semi-permeable membrane is a dialysis tubing.

7. The process of claim 1 wherein the at least one glucosyltransferase enzyme is derived from streptococci.

8. The process of claim 7 wherein the at least one glucosyltransferase enzyme is selected from the group consisting of gtfJ, gtfB, gtfC and gtfI.

9. The process of claim 7 wherein the glucosyltransferase enzyme is from *Streptococcus salivarius*.

10. The process of claim 9 wherein the glucosyltransferase enzyme of *Streptococcus salivarius* is gtfJ.

11. The process of claim 3 wherein the at least one primer is dextran.

12. The process if claim 1 wherein the glucosyltransferase enzyme is a primer-independent enzyme.

13. The process of claim 1 wherein the glucosyltransferase enzyme is a primer-dependent enzyme.

14. The process of claim 1 wherein more than one glucosyltransferase enzyme is present in the enzyme reaction solution.

15. The process of claim 14 wherein the more than one glucosyltransferase enzyme comprises a mixture of at least one primer-dependent enzyme and at least one primer-independent enzyme.

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/054521

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P19/18
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	WO 99/40217 A1 (NEOSE TECHNOLOGIES INC [US]) 12 August 1999 (1999-08-12) page 4, paragraph 9 - page 5, paragraph 1 page 8, paragraph 2-4 page 9, paragraph 2-5 page 11, paragraph 3-6 page 12, paragraph 1 page 15, last paragraph - page 17, paragraph 2; figure 3 claims 13-20 ----- -/--	1,2,5,7, 12,14 8-15

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 November 2012	28/11/2012

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Mateo Rosell, A

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2012/054521

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SIMPSON C ET AL: "Four glucosyltransferases, GtfJ, GtfK, GtfL, and GtfM from Streptococcus salivarius ATCC 25975", MICROBIOLOGY AND IMMUNOLOGY, CENTER FOR ACADEMIC PUBLICATIONS JAPAN , JP, vol. 141, no. 6, 1 June 1995 (1995-06-01), pages 1451-1460, XP002082272, ISSN: 0385-5600 cited in the application abstract page 1454, left-hand column, paragraph 4 - page 1457, left-hand column, paragraph 2; table 2	8-15
Y	US 7 000 000 B1 (O'BRIEN JOHN P [US]) 14 February 2006 (2006-02-14) cited in the application column 2, line 58 - column 3, line 2 column 6, line 55 - column 8, line 5	8-11
Y	WO 2010/024887 A1 (CORNING INC [US]; MARTIN GREGORY R [US]; TANNER ALLISON J [US]) 4 March 2010 (2010-03-04) paragraph [0008] paragraph [0059] figures 1,14	6
Y	LINARDOS T I ET AL: "MONOCLONAL ANTIBODY PRODUCTION IN DIALYZED CONTINUOUS SUSPENSION CULTURE", BIOTECHNOLOGY AND BIOENGINEERING, WILEY & SONS, HOBOKEN, NJ, US, vol. 39, no. 5, 1 January 1992 (1992-01-01), pages 504-510, XP002443305, ISSN: 0006-3592, DOI: 10.1002/BIT.260390505 abstract page 504, right-hand column, paragraph 3 - page 505, left-hand column, paragraph 1	6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2012/054521

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9940217 A1	12-08-1999	AR 015226 A1	18-04-2001
		AR 024034 A2	04-09-2002
		AR 024035 A2	04-09-2002
		AU 762500 B2	26-06-2003
		AU 2566199 A	23-08-1999
		BR 9904774 A	08-03-2000
		CA 2286256 A1	12-08-1999
		CN 1255947 A	07-06-2000
		EP 0973931 A1	26-01-2000
		HU 0002415 A2	28-11-2000
		JP 2001520525 A	30-10-2001
		NZ 337701 A	28-01-2000
		TW I243209 B	11-11-2005
		US 5952205 A	14-09-1999
		US 6242225 B1	05-06-2001
		US 2001055793 A1	27-12-2001
		WO 9940217 A1	12-08-1999
		ZA 9900932 A	05-08-1999
US 7000000 B1	14-02-2006	AU 2509700 A	07-08-2000
		DE 60009886 D1	19-05-2004
		DE 60009886 T2	31-03-2005
		EP 1165867 A1	02-01-2002
		JP 2002535501 A	22-10-2002
		TW 504525 B	01-10-2002
		US 7000000 B1	14-02-2006
		WO 0043580 A1	27-07-2000
WO 2010024887 A1	04-03-2010	EP 2324107 A1	25-05-2011
		JP 2012501176 A	19-01-2012
		US 2010055764 A1	04-03-2010
		WO 2010024887 A1	04-03-2010