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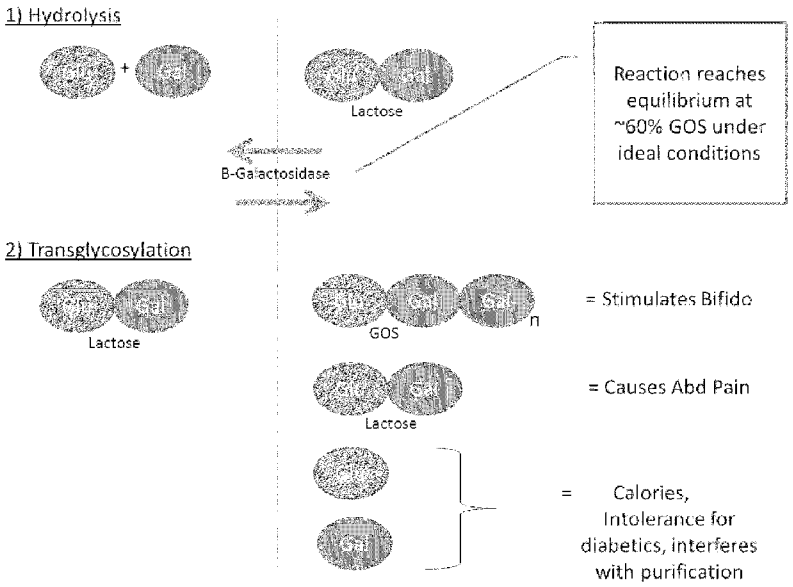
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*

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

[Continued on next page]

(54) Title: METHODS AND SYSTEMS FOR GALACTOOLIGOSAC-CHARIDES MANUFACTURE

FIGURE 1



(57) Abstract: Disclosed herein are methods and systems for the production of high purity galactooligosaccharide compositions. Also disclosed herein are genetically modified yeast strains for use in the production of high purity galactooligosaccharide compositions.

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METHODS AND SYSTEMS FOR GALACTOOLIGOSACCHARIDES MANUFACTURE

INCORPORATION BY REFERENCE

[0001] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BACKGROUND OF THE INVENTION

[0002] Higher purity GOS compositions can be manufactured from lower purity GOS compositions by removing digestible saccharides by fermentation with fermenting microorganisms such as yeast and/or bacteria. An important cost driver in GOS manufacturing processes is the time in the fermentation tanks. One factor that can affect the time of fermentation is the density of fermenting microorganisms verses the digestible saccharide utilization of the fermenting microorganisms. Another factor that can affect the time of fermentation is the number of fermentation steps required to remove the digestible saccharides. There is a need in the art to produce high purity GOS compositions more economically and efficiently.

[0003] Disclosed herein are improved methods and systems for the production of high purity GOS compositions. Also disclosed herein are genetically engineered yeast strains for use in the production of high purity GOS compositions.

SUMMARY OF THE INVENTION

[0004] Disclosed herein are continuous fermentation processes for increasing the concentration of galactooligosaccharides (GOS) and decreasing the concentration of digestible saccharides in a starting GOS compsition, the processes comprising: (a) contacting the starting GOS composition with one or more strains of fermenting microorganisms, wherein the starting GOS composition comprising one or more digestible saccharides; (b)

allowing sufficient time for the one or more strains fermenting microorganisms to ferment the one or more digestible saccharides to produce a higher purity GOS composition; and (c) recovering the one or more strains of fermenting microorganisms from the higher purity GOS composition. In some embodiments, the one or more digestible saccharides comprise lactose, glucose, galactose, or a combination thereof. Some embodiments further comprise ceramic ultrafiltration, nanofiltration, decolorization with carbon, ion-exchange resin deionization, or vacuum concentration of the higher purity GOS composition. In some embodiments, the one or more strains of fermenting microorganism were recovered from a previous fermentation process. In some embodiments, the one or more strains of fermenting microorganisms are in a log growth phase when contacted with the starting GOS composition. In some embodiments, the one or more strains of fermenting microorganism comprise a yeast strain, a bacterial strain, or a combination thereof. In some embodiments, the one or more strains of fermenting microorganism comprise a yeast strain. In some embodiments, the one or more strains of fermenting microorganism, wherein the one or more strains of fermenting microorganism comprise a genetically modified yeast strain that expresses a B-galactosidase enzyme. In some embodiments, the B-galactosidase enzyme is a bacterial B-galactosidase enzyme. In some embodiments, the bacterial B-galactosidase enzyme is from a Streptococcus strain. In some embodiments, the bacterial B-galactosidase enzyme is from a *Streptococcus thermophilus* strain. In some embodiments, the B-galactosidase enzyme is genetically modified to be anchored to the cell surface. In some embodiments, expression of the B-galactosidase enzyme is controlled by a high-expression promoter. In some embodiments, the high-expression promoter is an inducible promoter. In some embodiments, the high-expression promoter is an alcohol dehydrogenase promoter. In some embodiments, the one or more fermenting microorganisms comprise a bacterial strain. In some embodiments, the bacterial strain is a *Streptococcus* strain. In some embodiments, the bacterial strain is a *Streptococcus thermophilus* strain. In some embodiments, the sufficient time is about: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, or 80% less than a time required in a batch fermentation process. In some embodiments, the batch fermentation process utilizes freeze dried yeast. Some embodiments further comprise contacting the higher purity GOS composition with one or more other fermenting microorganisms.

[0005] Also disclosed herein are continuous fermentation systems for increasing the concentration of galactooligosaccharides (GOS) and decreasing the concentration of digestible saccharides in a starting GOS mixture, the systems comprising: (a) a source of the starting GOS mixture, wherein the starting GOS mixture comprises one or more digestible saccharides; (b) a delivery device coupled to the source of the starting GOS mixture; (c) a fermentor coupled to the delivery device, wherein the fermentor is arranged to receive the starting GOS mixture from the delivery device; (d) one or more fermenting microorganisms, wherein the one or more fermenting microorganisms ferment the one or more digestible saccharides in the starting GOS mixture to produce a higher purity GOS composition; (e) a cell separator coupled to an outlet on the fermentor, wherein the cell separator recovers the one or more fermenting microorganisms from the higher purity GOS composition.

[0006] Some embodiments further comprise a second fermentor. In some embodiments, the second fermentor comprises one or more other fermenting microorganisms and is operatively coupled to the cell separator in order to receive the higher purity GOS composition.

BRIEF DESCRIPTION OF THE DRAWINGS

[0007] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[0008] **Figure 1** summarizes the enzyme activity of B-galactosidase.

[0009] **Figure 2** illustrates an exemplary manufacturing protocol.

[0010] **Figure 3** is a comparison between two exemplary GOS manufacturing protocols.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Summary

[0011] There are multiple methods that can be implemented to reduce costs of manufacturing high purity galactooligosaccharide (GOS) compositions from lower purity GOS compositions. One method is to improve the efficiency of the manufacturing process. Steps can also be taken to reduce starting material costs and end product formulations.

[0012] Many or all GOS products on the market are food grade, however, the methods and systems disclosed herein can be used in the manufacture of food grade or pharmaceutical grade GOS compositions.

Enzymatic Chemistry:

[0013] GOS (galactooligosaccharides) can be made by incubation of lactose, and the B-galactosidase enzyme (lactase or B-gal). As illustrated in Figure 1, B-gal has at least two activities: a) hydrolysis: at low concentrations of lactose, B-gal cleaves the lactose disaccharide into the two monosaccharides, glucose and galactose and b) transglycosylation: at high concentrations of lactose, B-gal adds additional galactose molecules to lactose to yield longer polymer lengths, i.e. GOS. The percentage of GOS, glucose, galactose and lactose that results can be varied by the origin of the B-gal, pH, lactose concentration etc.

[0014] The reaction of lactose and B-gal reaches an equilibrium; it can be difficult to push the reaction to greater than 60% GOS. If the reaction is left long enough, all of the GOS products can be converted to the monosaccharides glucose and galactose.

[0015] The relative efficiencies of the hydrolysis and transglycosylation activities can vary depending upon the particular B-gal that is used. It can be possible to bias towards the direction of the reaction depending on the source of the B-gal used. For example, see Song et al., Biosci. Biotch. Biochem 75(2) 268-278, 2011, which is hereby incorporated by reference in its entirety.

Manufacturing Processes

[0016] Higher purity GOS compositions can be manufactured from lower purity GOS compositions. Lower purity GOS compositions typically comprise excess digestible saccharides (e.g., lactose, glucose, and galactose) that can be removed in one or more

fermentation processes. The fermentation processes can utilize yeast and/or bacteria. Additional reaction clean-up and/or purification processes can also be utilized in the manufacture of high purity GOS compositions.

[0017] An important cost driver in GOS manufacturing processes is the time in the fermentation tanks. One factor that can affect the time of fermentation is the density of fermenting microorganisms verses the digestible saccharide utilization of the fermenting microorganisms. Another factor that can affect the time of fermentation is the number of fermentation steps required to remove the digestible saccharides.

Continuous fermentation

[0018] High purity GOS manufacture can comprise one or more batch fermentation reactions. In such reactions, freeze dried fermenting microorganisms (e.g., yeast or bacteria), or dilute amounts of fermenting microorganism culture can be used. The cells typically go through a lag phase, then exponential log phase and eventually enter stationary phase as the sugars run out. Each fermentation step in a batch fermentation can take, e.g., 24 hours.

[0019] High purity GOS manufacture can comprise one or more continuous fermentation reactions. Such reactions can comprise starting with a seed culture in log phase, fermenting the solution containing a lower purity GOS composition and the seed culture for a sufficient time to remove the targeted digestible saccharide(s), followed by harvesting the culture and returning the cells to the next batch of lower purity GOS composition for another cycle of fermentation. By keeping the cells at an optimized density, a continuous fermentation scheme can minimize the time in the tank. For example, a 6 hour cycle would allow 4 times the yield in comparison to a 24 hour batch fermentation reaction.

[0020] Continuous fermentation can involve internal process controls such as a measurement of the amount of each digestible saccharide in the solution over time. Optimizing the fermentation cycles, based on time, cell density and using digestible saccharide levels as the process control method can be performed on a laboratory scale and then scaled to larger fermentation equipment.

Number of fermentation reactions

[0021] Removal of digestible saccharides from lower purity GOS compositions can comprise one or more fermentation reactions with one or more fermenting microorganisms. For example, in one process, yeast (e.g., a *Saccharomyces* species, e.g., *S. cerevisiae*) can be used in two fermentation cycles, and bacteria (e.g., a *Streptococcus* species, e.g., *S. thermophilus*) can be used in one cycle for a total 3 cycles. This would yield a total of 72 hours for fermentation, assuming each fermentation cycle required 24 hours. One reason that multiple fermentation cycles can be used is that the high glucose levels can suppress the LAC operon of a bacterium (e.g., a *Streptococcus* species) resulting in poor expression of B-gal and therefore inefficient conversion of the lactose to glucose and galactose.

[0022] Exogenous B-gal can be expressed in yeast to convert lactose to GOS via the transglycosylation reaction using a B-gal enzyme that is more efficient for transglycosylation versus hydrolysis. The B-gal expression can be maintained through multiple fermentation cycles and the yeast can use digestible saccharides such as glucose and galactose as an energy source.

[0023] Use of a single fermentation reaction can reduce the time in the tank, e.g., from 3 days for a three fermentation reaction process to 1 day for a single fermentation reaction process. By also combing this with a continuous fermentation schedule, an increased number of batches can be produced in the same time. For example, a process using a single continuous fermentation reaction can yield a 12 fold increase over a three fermentation batch process.

Genetic modification of yeast strains

[0024] Yeast strains can be genetically modified to produce B-gal. One consideration in genetically modifying the yeast strain is the selection of the B-gal gene. For example, the B-gal gene from a *Streptococcus* species (e.g., *S. thermophilus*) can be used because has efficient hydrolysis activity. In another example, a genetically modified B-gal gene with enhanced hydrolysis activity can be used.

[0025] A second consideration for genetically modifying a yeast strain to produce exogenous B-gal enzyme is the choice of promoter. In bacteria such as *S. thermophilus*, the

B-gal gene is under control of the Lac operon, which can be inhibited by glucose. Putting the B-gal gene under control of a constitutive promoter can increase the production of the B-gal enzyme. Examples of such promoters include the alcohol dehydrogenase promoter. The B-gal gene can also be genetically modified to produce an enzyme that is anchored to the cell surface, as opposed to being secreted into the media. For example, see Li et al., Appl. Environ. Microbiol., 2009 Sep; 75(18):5938-42 and Pepper et al. Comb. Chem. High Throughput Screen. 2008 February; 11(2): 127–134, each of which is hereby incorporated by reference in its entirety.

[0026] A third consideration for genetically modifying a yeast strain to produce exogenous B-gal enzyme is the choice of the yeast strain to modify. Lab strains of *S. cerevisiae* (haploid or diploid) are typically auxotrophs, meaning that their growth is dependent upon media supplementation with a metabolic compound such as histidine or leucine. This has advantages during genetic engineering by enabling easy selection of transformants. However, lab strains may not be as robust as industrial strains used in beer and wine production (see, e.g., Nakao et al., DNA Res. 2009 Apr; 16(2):115–29, which is hereby incorporated by reference in its entirety). Industrial yeast strains are typically aneuploid and therefore not auxotroph's. This can make it difficult to select for transformants containing an artificial gene construct. However, in this case, expression of the B-gal gene can be detected by using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) which turns a colony of yeast blue when a cell expresses B-gal.

EXAMPLES

Example 1

[0027] This example details an 8 step method of manufacturing high purity GOS compositions from lower purity GOS compositions. Reference is made to Figure 2. The amounts given are exemplary amounts, and can be scaled up or down, or varied, as needed.

[0028] In the first step, glucose is removed from the lower purity GOS composition in a batch fermentation with yeast. The lower purity GOS composition (345 L) is diluted in demineralized water (3400 L), to which monobasic ammonium phosphate (1.25 L) and 30% NaOH (0.125 L) are added. The mixture is heated to approximately 37 ± 2°C.

Then, yeast (0.125 L) and polypropylene glycol (0.125 L) are added and air is injected into the fermentor. The levels of glucose are tested (e.g., by HPLC) during the fermentation and extra processing time is used as needed. Once the glucose levels have been reduced to within specification, the mixture is heated to 73 ± 5°C for 3 minutes, followed by cooling to 40 ± 2°C. Typical time for the fermentation reaction is approximately 24 hours.

[0029] In the second step, lactose is removed in a batch fermentation with a bacterial strain. Yeast extract in de-ionized water (18.75 L) and 15% NaOH (1.25L) are added to the mixture, followed by addition of the bacteria (e.g., *S. thermophilus*) (0.125 L). The fermentation reaction is agitated at 40 °C, during which lactose levels are tested (e.g., by HPLC). Extra processing time is used as needed. Once the lactose levels are reduced to within specification, the mixture is heated to 73 ± 5°C for 3 minutes, followed by cooling to 37 ± 2°C. Typical time for the fermentation reaction is approximately 24 hours.

[0030] In the third step, galactose is removed in a batch fermentation with yeast. Monobasic ammonium phosphate (0.125 L) is added to the mixture and the pH and temperature are determined and adjusted as necessary. Then, yeast (0.125 L) and polypropylene glycol (0.125 L) are added and air is injected into the fermentor. The purity of the GOS is monitored (e.g., by HPLC) and 50% sulphuric acid (1.25 L) are added once the GOS purity is within specification. Typical time for the fermentation reaction is approximately 24 hours.

[0031] In the fourth step, the mixture is clarified in one or more ultrafiltration steps (e.g., 4 steps), which removes cells and cell debris.

[0032] In the fifth step, nanofiltration is used to remove low molecular weight fermentation by-products such as lactic acid and glycerin.

[0033] In the sixth step, the solution was decolored with a granular decolorizing charcoal column.

[0034] In the seventh step, the solution is deionized with one or more ion-exchange columns.

[0035] In the eight step, the solution is microfiltered and concentrated under a vacuum.

Example 2

[0036] In this example, a method of manufacturing high purity GOS compositions from lower purity GOS compositions using 3 stages of continuous fermentation is detailed. The amounts given are exemplary amounts, and can be scaled up or down, or varied, as needed.

[0037] In the first step, glucose is removed from the lower purity GOS composition in a fermentation with yeast. In the first fermentor, the lower purity GOS composition (345 L) is diluted in demineralized water (3400 L), to which monobasic ammonium phosphate (1.25 L) and 30% NaOH (0.125 L) are added. The mixture is heated to approximately 37 ± 2°C. Then, yeast in a continuous log growth phase (either newly added or returned from a previous fermentation) and polypropylene glycol (0.125 L) are added and air is injected into the fermentor. The levels of glucose are tested (e.g., by HPLC) during the fermentation. Once the glucose levels have been reduced to within specification, the media is recovered from the fermentor while the cells are separated and returned to the fermentor for used to ferment the next batch.

[0038] In the second step, lactose is removed in a fermentation with a bacterial strain. In a second fermentor, yeast extract in de-ionized water (18.75 L) and 15% NaOH (1.25L) are added to the media recovered from the first fermentor, followed by addition of the bacteria (e.g., *S. thermophilus*) in a log growth phase (either newly added or returned from a previous fermentation). The fermentation reaction is agitated at 40 °C, during which lactose levels are tested (e.g., by HPLC). Once the lactose levels are reduced to within specification, the media is recovered from the fermentor while the cells are separated and returned to the fermentor for used to ferment the next batch.

[0039] In the third step, galactose is removed in a fermentation with yeast. In a third fermentor, monobasic ammonium phosphate (0.125 L) is added to the media recovered from the second fermentor and the pH and temperature are determined and adjusted as necessary. Then, yeast in a continuous log growth phase (either newly added or returned from a previous fermentation) and polypropylene glycol (0.125 L) are added and air is injected into the fermentor. The purity of the GOS is monitored (e.g., by HPLC) and, once the GOS purity is within specification, the media is recovered from the fermentor while the cells are

separated and returned to the fermentor for used to ferment the next batch. The media from the third fermentor can be further processed as detailed in Example 1.

Example 3

[0040] In this example, a genetically engineered yeast strain is produced that expresses a bacterial B-galactosidase gene. The bacterial B-galactosidase gene is amplified using standard PCR techniques and inserted into a propagation vector. The propagation vector contains a desired promoter sequence to ensure high levels of enzyme expression in the yeast strain. The propagation vector is then transformed into a host bacterial strain and plated on selective media (e.g., agar plates with an antibiotic). A number of colonies are selected, grown in culture, and the propagation vector is purified by standard microbiology techniques. The propagation vector is confirmed by DNA sequencing.

[0041] Then, the propagation vector, optionally linearized by restriction digest, is transformed into the host yeast strain under conditions to promote insertion into the yeast genome. The transformed yeast is plated on media containing X-gal, and transformants are selected based upon the blue color of the transformed yeast strains. A number of colonies are selected, and grown in culture. The correct insertion of the B-galactosidase gene and promoter are verified by DNA sequencing.

Example 4

[0042] In this example, a genetically engineered yeast strain is produced that expresses a recombinant B-galactosidase gene that is anchored to the cell surface upon expression in the yeast strain. The recombinant B-galactosidase gene is amplified using standard PCR techniques and inserted into a propagation vector. The propagation vector contains a desired promoter sequence to ensure high levels of enzyme expression in the yeast strain. The propagation vector is then transformed into a host bacterial strain and plated on selective media (e.g., agar plates with an antibiotic). A number of colonies are selected, grown in culture, and the propagation vector is purified by standard microbiology techniques. The propagation vector is confirmed by DNA sequencing.

[0043] Then, the propagation vector, optionally linearized by restriction digest, is transformed into the host yeast strain under conditions to promote insertion into the yeast

genome. The transformed yeast is plated on media containing X-gal, and transformants are selected based upon the blue color of the transformed yeast strains. A number of colonies are selected, and grown in culture. The correct insertion of the recombinant B-galactosidase gene and promoter are verified by DNA sequencing.

Example 5

[0044] In this example, a method of manufacturing high purity GOS compositions from lower purity GOS compositions using a single continuous fermentation reaction is detailed. Reference is made to Figure 3.

[0045] Digestible saccharides such as glucose, galactose, and lactose are removed from the lower purity GOS composition in a continuous fermentation process with a genetically engineered yeast strain that produces a bacterial or recombinant B-galactosidase gene. The yeast strain can be produced as detailed in Example 3 or Example 4. The lower purity GOS composition is diluted in demineralized water, to which monobasic ammonium phosphate and 30% NaOH are added. The mixture is heated to approximately 37 ± 2°C. Then, a culture of yeast in log phase growth and polypropylene glycol (0.125 L) are added and air is injected into the fermentor. The levels of digestible saccharides are monitored (e.g., by HPLC) during the fermentation. Once the digestible saccharide levels have been reduced to within specification, the media is recovered from the fermentor while the cells are separated and returned to the fermentor for used to ferment the next batch of low purity GOS. The separated media can be further processed according to any of steps 4-8 as detailed in example 1.

[0046] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

WHAT IS CLAIMED IS:

1. A continuous fermentation process for increasing the concentration of galactooligosaccharides (GOS) and decreasing the concentration of digestible saccharides in a starting GOS compsition, the process comprising:

contacting the starting GOS composition with one or more strains of fermenting microorganisms, wherein the starting GOS composition comprising one or more digestible saccharides;

allowing sufficient time for the one or more strains fermenting microorganisms to ferment the one or more digestible saccharides to produce a higher purity GOS composition; and

recovering the one or more strains of fermenting microorganisms from the higher purity GOS composition.

2. The continuous fermentation process of claim 1, wherein the one or more digestible saccharides comprise lactose, glucose, galactose, or a combination thereof.

3. The continuous fermentation process of claim 1, further comprising ceramic ultrafiltration, nanofiltration, decolorization with carbon, ion-exchange resin deionization, or vacuum concentration of the higher purity GOS composition.

4. The continuous fermentation process of claim 1, wherein the one or more strains of fermenting microorganism were recovered from a previous fermentation process.

5. The continuous fermentation process of claim 1, wherein the one or more strains of fermenting microorganism comprise a yeast strain, a bacterial strain, or a combination thereof.

6. The continuous fermentation process of claim 1, wherein the one or more strains of fermenting microorganism comprise a yeast strain.

7. The continuous fermentation process of claim 1, wherein the one or more fermenting microorganisms comprise a bacterial strain.

8. The continuous fermentation process of claim 7, wherein the bacterial strain is a Streptococcus strain.

9. The continuous fermentation process of claim 7, wherein the bacterial strain is a Streptococcus thermophilus strain.

10. The continuous fermentation process of claim 1, wherein the sufficient time is about: 10%, 20%, 30%, 40%, 50%, 60%, 70%, 75%, or 80% less than a time required in a batch fermentation process.

11. The continuous fermentation process of claim 10, wherein the batch fermentation process utilizes freeze dried yeast.

12. The continuous fermentation process of claim 1, further comprising contacting the higher purity GOS composition with one or more other fermenting microorganisms.

13. A continuous fermentation system for increasing the concentration of galactooligosaccharides (GOS) and decreasing the concentration of digestible saccharides in a starting GOS composition, the system comprising:

a source of the starting GOS composition, wherein the starting GOS composition comprises one or more digestible saccharides;

a delivery device coupled to the source of the starting GOS composition;

a fermentor coupled to the delivery device, wherein the fermentor is arranged to receive the starting GOS composition from the delivery device;

one or more fermenting microorganisms, wherein the one or more fermenting microorganisms ferment the one or more digestible saccharides in the starting GOS composition to produce a higher purity GOS composition;

a cell separator coupled to an outlet on the fermentor, wherein the cell separator recovers the one or more fermenting microorganisms from the higher purity GOS composition.

14. The continuous fermentation system of claim 13, further comprising a second fermentor.

15. The continuous fermentation system of claim 14, wherein the second fermentor comprises one or more other fermenting microorganisms and is operatively coupled to the cell separator in order to receive the higher purity GOS composition.

FIGURE 1

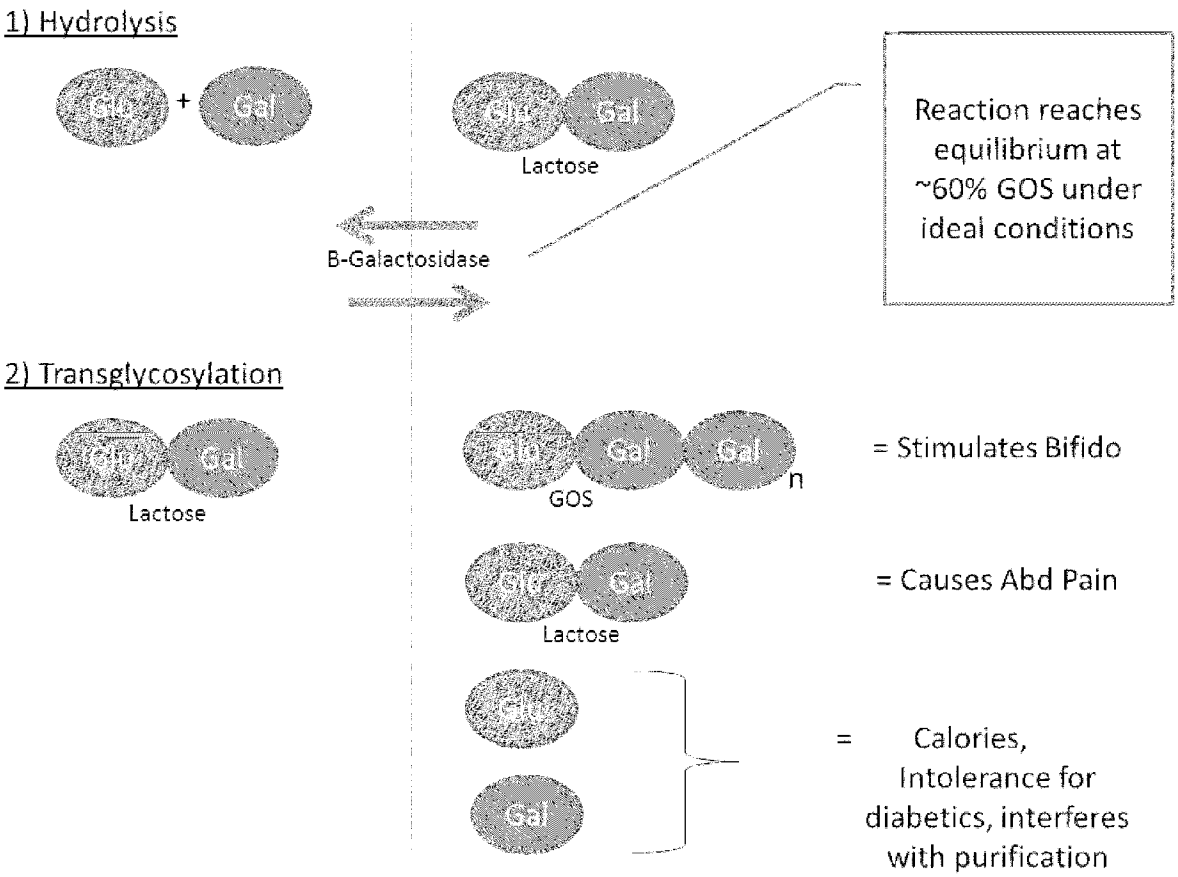
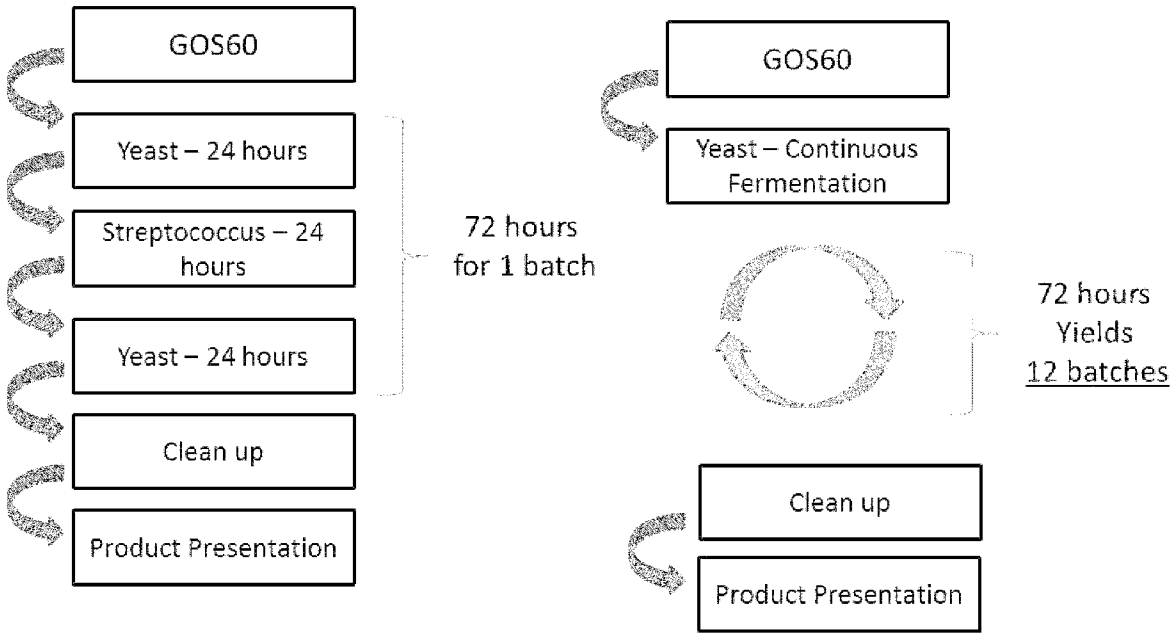

1) Hydrolysis
Glu
Gal
Glu
Gal
Lactose
B-Galactosidase
Reaction reaches equilibrium at ~60% GOS under ideal conditions
2) Transglycosylation
Glu
Gal
Lactose
Glu
Gal
Gal
n
GOS
= Stimulates Bifido
Glu
Gal
Lactose
= Causes Abd Pain
Glu
Gal
= Calories, Intolerance for diabetics, interferes with purification

FIGURE 2

Step 1: Glucose removal	**Step 4: Ultrafiltration**
Demineralized water	Filtration #1
Low Purity GOS composition (e.g., GOS 60)	Filtration #2
Monobasic ammonium phosphate	Filtration #3
30% NaOH	Filtration #4
Heat to 37 ± 2°C	**Step 5: Nanofiltration**
Yeast	Concentration #1
Polypropylene glycol	Concentration #2
Air injection	Concentration #3
Test for glucose	Concentration #4
Extra processing time	**Step 6: Decolorization**
Heat to 73 ± 5°C for 3 min.	Granular decolorizing charcoal column
Cool to 40 ± 2°C	**Step 7: Deionization**
Step 2: Lactose Removal	75% phosphoric acid
Yeast extract in DI water	**Step 8: Concentration**
15% NaOH	Microfiltration
Streptococcus thermophilus	Vacuum concentration
Agitation at 40°C	
Test for Lactose	
Extra processing time	
Heat to 73 ± 5°C for 3 min.	
Cool to 37 ± 2°C	
Step 3: Galactose removal	
Monobasic ammonium phosphate	
Check pH and temperature	
Yeast	
Polypropylene glycol	
Air injection	
Check GOS purity ≥ 95%	
50% sulphuric acid	

FIGURE 3



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US15/30164

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12P 19/04 (2015. 01)
CPC - C12P 19/04
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (8): C12P 19/04; C07H 03/06; A61K 31/715 (2015.01)
CPC: C12P 19/04; C12Y 302/01023; C07H 03/06; A61K 31/715; USPC: 426/61; 435/72, 101

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)
PatSeer (US, EP, WO, JP, DE, GB, CN, FR, KR, ES, AU, IN, CA, INPADOC Data), ProQuest, IEEE, Google Scholar, Google Patents GOS, galactooligosaccharides, saccharide, lactose, glucose, galactose, microorganism, enzyme, bacteria, yeast, pur*, concentrat*, recover, filter

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 272 095 A2 (KABUSHIKI KAISHA YAKULT HONSHA) 22 June 1988; entire document	1-3, 5-7, 12
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Y		4, 8-11, 13-15
Y	US 2011/0189342 A1 (JEONG, H et al.) 04 August 2011; paragraphs [0012]-[0013]	8-11
Y	JP 02-207796 A (IKUMAS, O et al.) 17 August 1990; see English Abstract	4
Y	CN 102471792 A (INALCO) 23 May 2012; see English translation; abstract; paragraph [0097]	11
Y	US 2009/0202589 A1 (MUELLER, G et al.) 13 August 2009; paragraph [0037]; figure 1	13-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
7 July 2015 (07.07.2015)	05 AUG 2015

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