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(54) Title: LACTIC ACID-UTILIZING BACTERIA GENETICALLY MODIFIED TO SECRETE POLYSACCHARIDE-DEGRADING ENZYMES

(57) Abstract: Lactic acid (LA)-utilizing bacteria are provided, genetically modified to express and optionally secrete polysaccharide-degrading enzymes, such as cellulases, hemicellulases and amylases, and uses thereof. The polysaccharide-degrading enzymes are advantageous for processing organic waste so that the organic waste can be used as a substrate in industrial fermentation processes, particularly industrial production of discrete lactic acid enantiomer(s). Vectors and constructs useful for genetically modifying the LA- utilizing bacteria are also provided.



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LACTIC ACID-UTILIZING BACTERIA GENETICALLY MODIFIED TO SECRETE POLYSACCHARIDE-DEGRADING ENZYMES

FIELD OF THE INVENTION

5 The present invention relates to dual action lactic-acid (LA)-utilizing bacteria genetically modified to secrete polysaccharide-degrading enzymes such as cellulases, hemicellulases, and amylases, useful for processing organic waste both to eliminate lactic acid present in the waste and degrade complex polysaccharides, thus providing a substrate for industrial fermentation processes producing various biochemicals, including specific
10 lactic acid enantiomer(s).

BACKGROUND OF THE INVENTION

 Lactic acid fermentation, namely, production of lactic acid from carbohydrate sources via microbial fermentation, has been gaining interest in recent years due to the
15 ability to use lactic acid as a building block in the manufacture of bio-plastics. Lactic acid can be polymerized to form the biodegradable and recyclable polyester polylactic acid (PLA), which is considered a potential substitute for plastics manufactured from petroleum. PLA is used in the manufacture of various products including food packaging, disposables, fibers in the textile and hygiene products industries, and more.

20 Production of lactic acid by fermentation bioprocesses is preferred over chemical synthesis methods for various considerations, including environmental concerns, costs and the difficulty to generate enantiomerically pure lactic acid by chemical synthesis, which is desired for most industrial applications. The conventional fermentation process is typically based on anaerobic fermentation in batch, fed-batch, continuous or semi-continuous
25 reactors by lactic acid-producing microorganisms, e.g. bacteria of the *Lactobacillus* sp., which produce lactic acid as the major metabolic end product of carbohydrate fermentation (Abdel-Rahman et al. (2013) *Biotechnology Advances*, 31:877–902; Ghaffar et al. (2014) *Journal of Radiation Research and Applied Sciences*, 7(2): 222–229). For production of PLA, the lactic acid generated during the fermentation is separated from the
30 fermentation broth by various processes, for example processes involving solvent extraction, electro-dialysis, distillation or a combination of one or more processes. The purified lactic acid is then subjected to polymerization.

Lactic acid has a chiral carbon atom and therefore exists in two enantiomeric forms, D- and L-lactic acid. In order to generate PLA that is suitable for industrial applications, the polymerization process should utilize only one enantiomer. Presence of impurities or a racemic mixture of D- and L-lactic acid results in a polymer having undesired characteristics such as low crystallinity and low melting temperature. Thus, lactic acid bacteria that produce only L-lactate enantiomer or only D-lactate enantiomer are required.

In currently available commercial processes, the carbohydrate source is typically a starch-containing renewable source such as corn and cassava root. Additional sources, such as the cellulose-rich sugarcane bagasse, have also been proposed. Typically, lactic acid bacteria can utilize reducing sugars like glucose and fructose, but do not have the ability to degrade polysaccharides like starch and cellulose. Thus, to utilize such polysaccharides the process requires adding glycolytic enzymes, typically in combination with chemical treatment, to degrade the polysaccharides and release reducing sugars.

Sakai et al., 2004 *Journal of Industrial Ecology*, 7(3-4): 63-74, report about a recycling system for municipal food waste that combines fermentation and chemical processes to produce poly-L-lactate (PLLA). Food waste typically includes varied ratios of reducing sugars (glucose, fructose, lactose, etc.), starch and lignocellulosic material. The process in Sakai et al. involved treatment of minced and sterilized food waste with a glucoamylase to degrade starch in the food waste into soluble glucose, L-lactic acid fermentation using an L-forming lactic acid bacterium, and purification and polymerization steps to obtain PLLA. For the particular carbohydrate source used in Sakai et al., namely, food waste, the process is complicated due to the fact that food waste contains endogenous D,L-lactic acid (e.g., from dairy products) that need to be removed in order to obtain a final pure L- or D- lactic acid. Thus, the process in Sakai et al. further included removal of endogenous D,L-lactic acid from the food waste by a *Propionibacterium* that consumes lactic acid as a carbon source, prior to the lactic acid fermentation step. Only 50% of the carbon content in the food waste was utilized by the method of Sakai et al. and converted to lactic acid. In addition, although food waste contains reducing sugars, starch and lignocellulose, the latter was not utilized by the method of Sakai et al., since the lactic acid bacteria cannot directly utilize this carbohydrate source.

US 7,507,561 discloses a process for producing polylactic acid from fermentation of renewable agricultural feed-stocks comprising molasses or cane bagasse employed as starting material.

US 8,119,376 discloses a method for the production of lactic acid or a salt thereof
5 wherein starch is subjected to a process of simultaneous saccharification and fermentation, the method comprising saccharifying starch in a medium comprising at least a glucoamylase and simultaneously fermenting the starch using a microorganism, and optionally isolating lactic acid from the medium, characterized in that a moderately thermophilic lactic acid-producing microorganism is used. The invention further relates to
10 a method of performing said process in the presence of a moderately thermophilic lactic acid producing microorganism, which has been adapted to have its maximum performance at the working pH.

EP 2843039 discloses a method for preparing a D-lactic acid-producing strain modified to inhibit L-lactate dehydrogenase (L-LDH) activity and to introduce D-lactate
15 dehydrogenase (D-LDH) activity in an L-lactic acid-producing strain, a mutated D-lactic acid-producing strain prepared by the above method, and a method for producing D-lactic acid including the steps of culturing the strain and recovering D-lactic acid from the culture media.

WO 2011/098843 discloses a procedure for producing lactic acid or its salts. The
20 procedure describes a simultaneously conducted saccharification of starch from a starch material and fermentation of sugars thereof into lactic acid by selected bacterium that produces amylolytic enzymes.

Morais et al., 2013 *Applied and Environmental Microbiology*, 79(17): 5242–5249, report about the introduction of genes coding for a cellulase or a xylanase into
25 *Lactobacillus plantarum*, and establishment of a two-strain cell-based consortium secreting both cellulase and xylanase. The enzymatic activity of the cell consortium was assessed on wheat straw.

WO 2015011250 discloses vectors for producing and secreting substance of interest by bacteria and applications thereof.

30 WO 2015/097685 discloses lactic acid cell cultures for processing lignocellulose. The bacterial culture may comprise a biomass composition and a population of lactic acid bacteria which comprises: (i) a first population of lactic acid bacteria which has been genetically modified to express a secreted cellulase; and (ii) a second population of lactic

acid bacteria which has been genetically modified to express a secreted xylanase, wherein the ratio of the first population: second population is selected such that the specific activity of cellulase: xylanase in the culture is greater than 4:1 or less than 1:4.

WO 2015/097686 discloses lactic acid bacterial cultures, cell populations and articles of manufacture comprising same for generating ethanol from lignocellulose.

Hitherto described methods of producing lactic acid from renewable sources have a number of drawbacks, such as low carbon-to-lactic acid conversion rate, complicated procedure requiring many separate steps, relatively high cost, and limited sources of carbohydrates that can be utilized. In addition, some of the methods are disadvantages for using source materials that are of high value as human food.

There is thus a need for bacteria, compositions and methods for efficient processing of a variety of carbohydrate-containing sources, such as organic wastes comprising starch-rich material, lignocellulose-rich material or combinations thereof, to obtain large amounts of soluble reducing sugars suitable for use in industrial fermentation processes.

SUMMARY OF THE INVENTION

The present invention provides, according to some aspects, lactic acid (LA)-utilizing bacteria genetically-engineered to produce one or more polysaccharide-degrading enzyme, particularly glycoside hydrolases such as cellulases, hemicellulases and amylases. The genetically-engineered dual action LA-utilizing bacteria disclosed herein facilitate degradation of complex polysaccharides found in organic wastes, such as food and agricultural wastes, in addition to their natural ability to consume and metabolize lactic acid selectively, substantially without utilizing other reducing sugars, under certain conditions. Such genetically-engineered LA-utilizing bacteria surprisingly provide improved means for processing organic wastes, enabling saccharification of the waste together with removal of endogenous lactic acid present in the waste. The processed waste can then be used as a substrate in industrial fermentation processes, particularly in the production of specific discrete lactic acid enantiomer(s) by lactic acid-producing microorganisms.

The LA-utilizing bacteria disclosed herein are advantageous for processing a large variety of organic wastes, including various and diverse food waste as well as plant material, and man-made material, such as paper. In some embodiments, the LA-utilizing

bacteria disclosed herein are particularly suitable for use with mixed food waste of commercial, industrial and municipal origin. The LA-utilizing bacteria disclosed herein enable efficient and cost-effective processing of organic waste, and according to some embodiments efficient and cost-effective production of lactic acid, characterized by increased carbon-to-lactic acid conversion rate compared to other methods. Further advantages of the LA-utilizing bacteria disclosed herein are that when used for processing waste, the process does not call for addition of external degrading enzymes to the process, as such enzymes are produced by the bacteria. Thus, by avoiding the need to add such external enzymes (i.e., adding enzymes to the vessels in which the process occurs), an advantageous process is achieved, which is much more cost efficient and is much more robust, as the risk of contamination (involved in adding an external enzyme mixture to the process) is markedly reduced. Additionally, by utilizing the LA-utilizing bacteria disclosed herein for processing waste, decreased viscosity of the processed waste is obtained, as saccharification (which is achieved by the degrading enzymes produced by the bacteria) reduces viscosity. This allows for a more efficient mixing of the waste and results in increased yield production of end products, such as discrete enantiomers of lactic acid.

According to one aspect, the present invention provides a lactic-acid (LA)-utilizing bacterium genetically modified to express and secrete one or more exogenous polysaccharide-degrading enzyme, wherein the one or more exogenous polysaccharide-degrading enzyme comprises a cellulase, a hemicellulase, an amylase or combinations thereof.

As used herein, the term "exogenous", when referring to a polysaccharide-degrading enzyme expressed by a LA-utilizing bacterium, indicates that the polysaccharide-degrading enzyme is encoded by a foreign nucleic acid introduced into the bacterial cell. In some embodiments, the exogenous polysaccharide-degrading enzyme is encoded by a nucleic acid introduced into the bacteria and is capable of being secreted from the bacterial cell. In some embodiments, the nucleic acid may be transiently expressed in the bacteria (i.e. utilizing an expression vector introduced into the bacteria). In some embodiments, the nucleic acid may be integrated into the genome of the bacteria. In some embodiments, the exogenous polysaccharide-degrading enzyme is not naturally found in these bacteria or cannot naturally be secreted therefrom.

"Polysaccharide-degrading enzymes" as used herein refers to hydrolytic enzymes (or enzymatically-active portions thereof) that catalyze the breakdown of saccharides, including bi-saccharides, oligosaccharides, polysaccharides and glycoconjugates, selected from the group consisting of glycoside hydrolases, polysaccharide lyases and carbohydrate esterases. Each possibility represents a separate embodiment of the present invention. The polysaccharide-degrading enzymes for use with the present invention are selected from those that are active towards saccharides (such as polysaccharides) found in organic wastes, including food waste and plant material. In some embodiments, the Polysaccharide-degrading enzymes may be modified enzymes (i.e., enzymes that have been modified and are different from their corresponding wild-type enzymes). In some embodiments, the modification may include one or more mutations that result in improved activity of the enzyme. In some embodiments, the Polysaccharide-degrading enzymes are wild type (WT) enzymes.

In some embodiments, the LA-utilizing bacterium is selected from the group consisting of *Propionibacterium* species, *Megasphaera* species, *Selenomonas* species and *Veillonella* species. Each possibility represents a separate embodiment of the present invention. In some embodiments, the LA-utilizing bacterium is a *Propionibacterium* sp. In some particular embodiments, the LA-utilizing bacterium is *Propionibacterium freudenreichii*.

According to another aspect, the present invention provides a population of lactic-acid (LA)-utilizing bacteria genetically modified to express and secrete a plurality of exogenous polysaccharide-degrading enzymes comprising a cellulase, a hemicellulase, an amylase or combinations thereof, wherein the population comprises a plurality of sub-populations of LA-utilizing bacteria, wherein each sub-population is genetically modified to express and secrete a different polysaccharide-degrading enzyme.

For example, in some embodiment, there is provided a population of LA-utilizing bacteria genetically modified to express and secrete a plurality of polysaccharide-degrading enzymes, wherein the population comprises:

a first sub-population of LA-utilizing bacteria genetically modified to express and secrete a first polysaccharide-degrading enzyme;

a second sub-population of LA-utilizing bacteria genetically modified to express and secrete a second polysaccharide-degrading enzyme; and

a third sub-population of LA-utilizing bacteria genetically modified to express and secrete a third polysaccharide-degrading enzyme,

wherein the first polysaccharide-degrading enzyme is a cellulase, the second polysaccharide-degrading enzyme is a hemicellulase and the third polysaccharide-degrading enzyme is an amylase. In some embodiments, the hemicellulase is a xylanase.

In some embodiments, the sub-populations are sub-populations of the same bacterial species. In other embodiments, the sub-populations are sub-populations of different bacterial species.

In some embodiments, the LA-utilizing bacteria are selected from the group consisting of *Propionibacterium* species, *Megasphaera* species, *Selenomonas* species and *Veillonella* species. In some embodiments, the LA-utilizing bacteria are selected from *Propionibacterium* sp. In some particular embodiments, the *Propionibacterium* sp. is *Propionibacterium freudenreichii*.

Polysaccharide-degrading enzymes for use in accordance with the present invention may be bacterial enzymes. In some embodiments, the polysaccharide-degrading enzymes are from thermophilic bacteria. In some embodiments, the thermophilic bacteria are selected from the group consisting of *Clostridium* sp., *Paenibacillus* sp., *Thermobifida fusca*, *Bacillus* sp., *Geobacillus* sp., *Chromohalobacter* sp. and *Rhodothermus marinus*. Each possibility represents a separate embodiment of the present invention.

In other embodiments, the polysaccharide-degrading enzymes are from mesophilic bacteria. In some embodiments, the mesophilic bacteria are selected from the group consisting of *Klebsiella* sp., *Cohnella* sp., *Streptomyces* sp, *Acetivibrio cellulolyticus*, *Ruminococcus albus*; *Bacillus* sp. and *Lactobacillus fermentum*. Each possibility represents a separate embodiment of the present invention.

In additional embodiments, the polysaccharide-degrading enzymes are fungal enzymes. In some embodiments, the fungi are selected from the group consisting of *Trichoderma reesei*, *Humicola insolens*, *Fusarium oxysporum*, *Aspergillus oryzae*, *Penicillium fellutanum* and *Thermomyces lanuginosus*. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the polysaccharide-degrading enzymes engineered into the LA-utilizing bacteria are selected such that they have optimal activity at the same temperature and pH range that are optimal for growth of the LA-utilizing bacteria. As used herein, temperature and pH range that are optimal for growth of the LA-utilizing bacteria

indicates also temperature and pH in which the LA-utilizing bacteria selectively consumes lactic acid as a carbon source (if particular conditions are required for the selective consumption).

In other embodiments, the polysaccharide-degrading enzymes are selected such that they have optimal activity at a temperature and/or pH range that is different from the optimal temperature and pH range for growth of the LA-utilizing bacteria. For example, in some embodiments, the polysaccharide-degrading enzymes have optimal activity at a temperature higher than the optimal growth temperature of the LA-utilizing bacteria. In additional embodiments, the polysaccharide-degrading enzymes have optimal activity at a pH that is higher/lower than the optimal growth pH of the LA-utilizing bacteria. In some embodiments, the polysaccharide-degrading enzymes have optimal activity at a temperature and/or pH range in which the LA-utilizing bacteria are inactivated.

In some embodiments, the LA-utilizing bacteria can selectively consume lactic acid until the latter is substantially exhausted and only thereafter can consume (ferment) the reducing sugars.

According to an additional aspect, the present invention provides a method for processing organic waste, the method comprising contacting the organic waste with the LA-utilizing bacterium of the present invention genetically modified to express and secrete one or more exogenous polysaccharide-degrading enzyme, under conditions in which lactic acid is consumed by the LA-utilizing bacterium and which are suitable for expression and activity of said one or more polysaccharide-degrading enzyme, wherein said processing eliminates D-lactic acid, L-lactic acid or both from the organic waste and at least partially degrades the polysaccharides to release reducing sugars. In some embodiments, the processing is performed in one-vessel (one-pot process).

According to a further aspect, the present invention provides a method for processing organic waste, the method comprising contacting the organic waste with the population of LA-utilizing bacteria of the present invention genetically modified to express and secrete a plurality of polysaccharide-degrading enzymes, under conditions in which lactic acid is consumed by the LA-utilizing bacteria and which are suitable for expression and activity of said plurality of polysaccharide-degrading enzymes, wherein said processing eliminates D-lactic acid, L-lactic acid or both from the organic waste and at least partially degrades the polysaccharides to release reducing sugars. In some embodiments, the processing is performed in one-vessel (one-pot process).

As used herein, "elimination", when referring to D-lactic acid, L-lactic or both from organic waste refers to reduction to residual amounts such that there is no interference with downstream processes of producing discrete lactic acid enantiomer(s) and subsequently polymerization to polylactic acid that is suitable for industrial applications. "Residual amounts" indicates less than about 5% lactic acid, preferably less than about 3%, more preferably less than about 1%, and even less than about 0.5 % lactic acid (w/w).

Organic waste for use with the methods of the present invention comprises complex polysaccharides including starch, cellulose, hemicellulose and combinations thereof. In some embodiments, the organic waste is selected from the group consisting of food waste, municipal waste, agricultural waste, plant material and combinations thereof. Food waste in accordance with the present invention encompasses food waste of plant origin. Food waste in accordance with the present invention encompasses household food waste, commercial food waste and industrial food waste. Plant material in accordance with the present invention encompasses agricultural waste and manmade products such as paper waste.

The reducing sugars typically comprise C5 sugars (pentoses), C6 sugars (hexoses) or a combination thereof. In some embodiments, said reducing sugars comprise glucose. In some embodiments, said reducing sugars comprise xylan.

In some embodiments, elimination of lactic acid from the waste is effected concomitant with saccharification (degradation of the polysaccharides to soluble reducing sugars). According to these embodiments, the LA-utilizing bacteria are genetically modified to express and secrete polysaccharide-degrading enzymes having optimal activity at the same temperature and pH range that are optimal for growth of the LA-utilizing bacteria. According to these embodiments, the method comprises contacting the organic waste with the LA-utilizing bacteria at a temperature and pH optimal for growth of the LA-utilizing bacteria and secretion of the polysaccharide-degrading enzymes. Contacting is performed for sufficient time to eliminate lactic acid (D-, L-, or both) from the waste and obtain desired level of reducing sugars.

In other embodiments, elimination of lactic acid from the waste is effected separately from (prior to) saccharification. According to these embodiments, the LA-utilizing bacteria are genetically modified to express and secrete polysaccharide-degrading

enzymes having optimal activity at a temperature and/or pH range that is different from the optimal temperature and/or pH range for growth of the LA-utilizing bacteria.

In some embodiments, the elimination of the D-lactic acid, L-lactic acid or a combination thereof present in the waste is performed concomitantly or sequentially with the degradation of the polysaccharides in the waste.

In some embodiments, the polysaccharide-degrading enzymes have optimal activity at a temperature higher than the optimal growth temperature of the LA-utilizing bacteria. According to these embodiments, the method comprises: (i) contacting the organic waste with the LA-utilizing bacteria at a first temperature, the first temperature being suitable for growth of the LA-utilizing bacteria and expression of the polysaccharide-degrading enzymes, for sufficient time to eliminate D-lactic acid, L-lactic acid or both from the waste; and (ii) increasing the temperature to a second temperature, the second temperature being optimal for activity of the polysaccharide-degrading enzymes. Typically, the LA-utilizing bacteria are inactivated at the second temperature.

In some embodiments, the polysaccharide-degrading enzymes may have optimal (or enhanced) activity at a pH that is higher/lower than the optimal (or otherwise suitable) growth pH of the LA-utilizing bacteria. According to these embodiments, the method comprises: (i) contacting the organic waste with the LA-utilizing bacteria at a first pH, the first pH being suitable for growth of the LA-utilizing bacteria and expression of the polysaccharide-degrading enzymes, for sufficient time to eliminate D-lactic acid, L-lactic acid or both from the waste; and (ii) lowering/increasing the pH to a second pH, the second pH being optimal for activity of the polysaccharide-degrading enzymes. Typically, the LA-utilizing bacteria are inactivated at the second pH.

In some embodiments, the methods further comprise determining the percentage of at least one of starch, cellulose and hemicellulose in the organic waste prior to said contacting, and selecting the LA-utilizing bacterium or the population of LA-utilizing bacteria to be contacted with the organic waste according to the determined percentage. In some embodiments, the determining further comprises determining the percentage of soluble reducing sugars.

According to another aspect, the present invention provides a method for producing discrete lactic acid enantiomer(s) from organic waste, the method comprising:

(i) processing the organic waste to eliminate D-lactic acid, L-lactic acid or a combination thereof present in the waste and at least partially degrade polysaccharides in

the waste to release soluble reducing sugars, by contacting the organic waste with the LA-utilizing bacterium of the present invention, or population of LA-utilizing bacteria of the present invention, genetically modified to express and secrete polysaccharide-degrading enzymes, under conditions suitable for lactic acid consumption by the LA-utilizing bacteria and for expression and activity of the polysaccharide-degrading enzymes;

(ii) inactivating the LA-utilizing bacteria;

(iii) fermenting the soluble reducing sugars obtained in (i) with a lactic acid-producing microorganism that produces only one of D-lactic acid and L-lactic acid, to obtain discrete enantiomer(s) of lactic acid; and

(iv) recovering the discrete lactic acid enantiomer(s) from the fermentation broth.

Typically, the fermenting step is carried out under anaerobic or microaerophilic conditions. The fermenting step is typically selected from the group consisting of batch, fed-batch, continuous and semi-continuous fermentation. Each possibility represents a separate embodiment of the present invention.

In some embodiments, the polysaccharide-degrading enzymes may have optimal (or otherwise suitable) activity at a temperature and/or pH range different from the temperature/pH range optimal (or otherwise suitable) for growth of the LA-utilizing bacteria. According to these embodiments, the conditions suitable for lactic acid consumption by the LA-utilizing bacteria and expression of the polysaccharide-degrading enzymes are different from the conditions suitable for activity of the enzymes. According to these embodiments, step (i) comprises: contacting the organic waste at a first temperature/pH, the first temperature/pH being suitable for lactic acid consumption by the LA-utilizing bacteria and expression of the polysaccharide-degrading enzymes, for sufficient time to eliminate D-lactic acid, L-lactic acid or both from the waste; and adjusting the temperature/pH to a second temperature/pH, the second temperature/pH being optimal for activity of the polysaccharide-degrading enzymes. Typically the LA-utilizing bacteria are inactivated at the second temperature/pH.

In some embodiments, the lactic acid-producing microorganism is a bacterium. In some embodiments, the lactic acid-producing bacterium is a *Lactobacillus* species. In other embodiments, the lactic acid-producing bacterium is a *Bacillus* species.

In some embodiments, the lactic acid-producing bacterium is a consortium of lactic acid bacteria of different species.

Recovering lactic acid typically includes separation of the lactic acid from the fermentation broth and purification of the lactic acid. Separation and purification of lactic acid can be performed by methods known in the art.

The method typically further comprises pretreatment of the organic waste to
5 decrease particle size and increase surface area, and also to inactivate endogenous bacteria within the waste. The pretreatment is carried out prior to processing the waste with the LA-utilizing bacteria. In some embodiments, the organic waste undergoes shredding, mincing and sterilization prior to processing with the LA-utilizing bacteria.

Sterilization may be carried out by methods known in the art, including for
10 example, high pressure steam, UV radiation or sonication.

In some embodiments, the method further comprises analyzing the composition of the organic waste, particularly the sugar content, prior to processing thereof. In some embodiments, the method comprises analyzing the percentage of at least one of starch, cellulose and hemicellulose in the organic waste. In additional embodiments, the method
15 comprises analyzing the percentage of reducing sugars.

In some embodiments, the method further comprises determining the quantity of total soluble reducing sugars released by the polysaccharide-degrading enzymes prior to the fermentation step.

In some embodiments, there is provided a method for producing stereo-specific
20 lactic acid from organic waste. As used herein, the term "stereo-specific" with respect to lactic acid relates to enantiomer(s) (or discrete enantiomer(s)) of lactic acid.

According to some embodiments, the LA-utilizing bacteria disclosed herein may further be advantageously utilized to increase the overall yield of lactic acid production from organic waste, by recycling residual lactic acid (which remained as a residual after
25 recovery of lactic acid from fermentation broth) to be re-processed by the LA-utilizing bacteria and used to cultivate LA-producing microorganism, to thereby produce high quantities of polysaccharide-degrading enzymes for the next Lactic-Acid producing cycle.

According to yet another aspect, the present invention provides a method for producing polylactic acid from organic waste, the method comprising producing discrete
30 lactic acid enantiomer(s) by the method described above, and further comprising polymerizing the discrete lactic acid enantiomer(s) to polylactic acid.

In some embodiments, the present invention provides a method for producing polylactic acid from organic waste, the method comprising producing stereo-specific lactic

acid by the method described above, and further comprising polymerizing the stereo-specific lactic acid to polylactic acid.

According to additional aspect, there is provided an expression vector for expressing and secreting an exogenous polysaccharide-degrading enzyme by a

5 *Propionibacterium*, said vector comprising:

at least one nucleotide sequence encoding for a *Propionibacterium* replication protein;

at least one *Propionibacterium* origin of replication nucleotide sequence; and an at least one expression cassette comprising:

10 at least one promoter nucleic acid sequence, which is operably linked to:

at least one nucleic acid sequence encoding for a *Propionibacterium* signal peptide (SP), which is translationally fused to:

at least one nucleic acid sequence encoding a polysaccharide degrading enzyme, selected from cellulase, hemicellulase, and amylase.

15 In some embodiments, the nucleotide sequence encoding for a *Propionibacterium* replication protein comprises the nucleic acid sequence of SEQ ID NO: 84. In some embodiments, the promoter nucleic acid sequence may be selected from the group consisting of SEQ ID NOs: 27-32 and homologs thereof. In some embodiments, the SP amino acid sequence may be selected from the group consisting of sequence of SEQ ID
20 NOs: 87-179. In some embodiments, the polysaccharide degrading enzyme sequence may be selected from the group consisting of SEQ ID NOs: 14-26, and homologs thereof. In some embodiments, the expression cassette may comprise or consist of a nucleic acid sequence as denoted by any one of SEQ ID NOs: 33-49, and/or homologs thereof.

25 In further embodiments, there is provided a *Propionibacterium* lactic-acid (LA)-utilizing bacteria comprising an expression vector.

In some embodiments, there is provided an expression cassette comprising or consisting of sequence as denoted by any one of SEQ ID NOs: 33-49, and/or homologs thereof. In further embodiments, there is provided a *propionibacterium* lactic-acid (LA)-utilizing bacteria comprising the expression cassette.

30

These and further aspects and features of the present invention will become apparent from the detailed description, examples and claims which follow.

BRIEF DESCRIPTION OF THE DRAWINGS

Exemplary embodiments are illustrated in referenced figures. Dimensions of components and features shown in the figures are generally chosen for convenience and clarity of presentation and are not necessarily shown to scale. The figures are listed below.

5

Fig. 1 – A schematic illustration of a fermentation system, for producing optically pure Lactic Acid and recycling of residual lactate waste, according to some embodiments;

Fig. 2A- A schematic illustration of a vector; according to some embodiments;

Fig. 2B- A schematic illustration of an expression vector, according to some
10 embodiments;

Fig. 3- A pictogram of a control Lugol assay - an agar plate containing YEL media and 0.5% starch after being exposed to Lugol solution. All the areas containing starch appear in black. A circle of degraded starch (by glucoamylase enzyme) appears colorless.

15

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses for the first time LA-utilizing bacteria genetically modified to produce various exogenous polysaccharide-degrading enzymes, particularly enzymes capable of degrading complex polysaccharides present in organic waste such as
20 food waste and lignocellulosic waste. The LA-utilizing bacteria disclosed herein are particularly advantageous for processing various sources of organic waste, prior to and for using the waste as a substrate for production of discrete enantiomer(s) of lactic acid. Such genetically-engineered LA-utilizing bacteria surprisingly provides improved means for processing organic wastes, enabling removal of endogenous lactic acid present in the
25 waste together with saccharification of the waste. The novel dual action bacteria of this invention thus enables carrying out two stages of the process together in a “one-vessel” (“one-pot”) process. The “one-vessel process” enables using one vessel (such as, reactor, fermenter or other suitable operating unit), instead of two, thus leading to a more economical industrial process. This makes possible carrying out the two stages
30 concomitantly in one reactor (one vessel). As the two stages may sometimes have different optimal reaction temperatures and pH, it may be recommendable sometimes to carry out the one-pot process in a sequential_manner, first the removal of endogenous lactic acid present in the waste (performed at its optimal temperature and pH), followed by

saccharification of the waste in the same reactor but at its own optimal temperature and pH.

Genetically-modified lactic acid (LA)-utilizing bacteria

5 LA-utilizing bacteria are engineered according to the present invention to produce one or more exogenous polysaccharide-degrading enzyme. As used herein "produce" with respect to the exogenous polysaccharide-degrading enzymes indicates expression (generation of the protein within the cells) and optionally secretion. In some embodiments, the one or more exogenous polysaccharide-degrading enzyme is engineered into the
10 bacteria as a secreted enzyme. According to these embodiments, the LA-utilizing bacteria are genetically modified to express and secrete one or more exogenous polysaccharide-degrading enzyme. In other embodiments, the one or more exogenous polysaccharide-degrading enzyme is engineered into the bacteria as a non-secreted enzyme, to be active towards polysaccharides in the organic waste only after lysis of the LA-utilizing bacterial
15 cells. According to these embodiments, the LA-utilizing bacteria are genetically modified to express one or more exogenous polysaccharide-degrading enzyme.

LA-utilizing bacteria for genetic modification according to the present invention may be selected from *Propionibacterium* species, *Megasphaera* species, *Selenomonas* species and *Veillonella* species. Each possibility represents a separate embodiment of the
20 present invention.

A suitable LA-utilizing bacterium for genetic modification according to the present invention is typically characterized by defined ranges of temperature and/or pH in which it consumes lactic acid selectively as a carbon source. As used herein, "selective" with respect to consumption of lactic acid as a carbon source by a LA-utilizing bacterium
25 indicates consumption of lactic acid by the bacterium without consuming reducing sugars available in its environment, or consuming available reducing sugars at a very slow rate such that insignificant levels are consumed over a period of several hours. Thus, when contacted with an organic waste under conditions in which it selectively consumes lactic acid, the LA-utilizing bacterium eliminates lactic acid from the waste substantially without
30 affecting the quantity of reducing sugars present in the waste. Alternatively, a suitable LA-utilizing bacterium consumes lactic acid selectively as a carbon source regardless of specific conditions and presence of reducing sugars in its environment.

In some particular embodiments, the LA-utilizing bacterium is *Propionibacterium freudenreichii*. *P. freudenreichii* is characterized by selective consumption of lactic acid as a carbon source under certain conditions, such as, acidic conditions (optimally at pH in the range of pH=5.5-6.5, such as, for example, pH=5.5).

5 LA-utilizing bacteria for genetic modification according to the present invention may be bacteria that consume only one of the enantiomer(s) of lactic acid, or bacteria that consume both.

In some embodiments, the genetically modified LA-utilizing bacteria of the present invention typically comprise an expression vector comprising a polynucleotide sequence
10 encoding a polysaccharide-degrading enzyme. In some embodiments, the expression vector comprises a polynucleotide sequence encoding a polysaccharide-degrading enzyme and a signal peptide fused in frame, such that upon transcription and translation of the polynucleotide sequence a secreted polysaccharide-degrading enzyme is generated. In some embodiments, the expression vector further comprises a suitable promoter of choice.
15 In some embodiments, the genetically modified LA-utilizing bacteria of the present invention comprise a polynucleotide sequence encoding a polysaccharide-degrading enzyme integrated into its genome. In some embodiments, the genetically modified LA-utilizing bacteria of the present invention comprise a polynucleotide sequence encoding a polysaccharide-degrading enzyme and a signal peptide fused in frame, integrated into its
20 genome.

In some embodiments, the terms “genetically modified”, “genetically-engineered” and “recombinant” may interchangeably be used. Thus, a genetically modified LA-utilizing bacteria is also referred to herein as a recombinant bacteria or recombinant LA-utilizing bacteria.

25 Genetic modification of the LA-utilizing bacteria can be performed using expression vectors, signal peptides and methods known in the art for LA-utilizing bacteria, described for example in Kiatpapan and Murooka (2001) *Appl Microbiol Biotechnol.*, 56(1-2):144-9; Brede et al., (2005) *Appl Environ Microbiol.*, 71(12):8077-84; Mukdsi et al., (2014) *Appl Environ Microbiol.*, 80(2): 751–756; and WO 2015/011250.

30 As referred to herein, the terms "nucleic acid", "nucleic acid sequence", "polynucleotide", "nucleotide" and "nucleotide sequence" may interchangeably be used. The terms are directed to polymers of deoxyribonucleotides (DNA), ribonucleotides (RNA), and modified forms thereof in the form of a separate fragment or as a component

of a larger construct. The terms further include oligonucleotides composed of naturally occurring bases, sugars, and covalent internucleoside linkages, as well as oligonucleotides having non-naturally occurring portions, which function similarly to respective naturally occurring portions. A DNA may include, for example, genomic DNA, plasmid DNA, recombinant DNA or complementary DNA (cDNA). An RNA may include, for example, messenger RNA (mRNA), ribosomal RNA (rRNA) or transfer RNA (tRNA). In some embodiments, the nucleic acid sequence may be a coding sequence (i.e., a sequence that can encode for an end product in the cell, such as, a protein or a peptide). In some embodiments, the nucleic acid sequence may be a regulatory sequence (such as, for example, a promoter).

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. The term "amino acid sequence" relates to a sequence composed of any one of naturally occurring amino acids, amino acids that have been chemically modified, or synthetic amino acids. The term relates to peptides and proteins, as well as fragments, analogs, derivatives and combinations of peptides and proteins.

In some embodiments, a sequence (such as, nucleic acid sequence and amino acid sequence) that is "similar" or "homologous" to a reference sequence refers herein to percent identity between the sequences. The percent identity may be at least 70%, at least 80%, at least 85%, at least 90%, at least 95%, at least 98% or at least 99%. The percent identity can be distributed randomly over the entire length of the sequences. Accordingly, homologous sequences can include, for example, variations related to mutations (such as, truncations, substitutions, deletions and/or additions of at least one amino acid or at least one nucleotide). Similar sequences can also include variations related to codon usage and degeneration of the genetic code. For example, a sequence is said to be homologous to another sequence if the homology (identity/similarity) is at least 80% over the entire length of the sequences.

30

The term "construct", as used herein, refers to an artificially assembled or isolated nucleic acid molecule which may include one or more nucleic acid sequences, wherein the nucleic acid sequences may include coding sequences (that is, sequence which encodes an

end product, such as, Polysaccharide-degrading enzymes), regulatory sequences (such as, promoters), non-coding sequences, or any combination thereof. The term construct includes, for example, plasmids and vectors but should not be seen as being limited thereto.

5 "Expression vector" and "recombinant vector" may interchangeably be used and refer to constructs that have the ability to express heterologous (exogenous) nucleic acid in the bacterial cell. In some embodiments, the expression vector can autonomously replicate in the cell. In some embodiments, the nucleic acid may be integrated into the genome of the bacterial cell.

10 As used herein, the terms "introducing" and "transformation" may interchangeably be used and refer to the transfer of molecules, such as, for example, nucleic acids, polynucleotide molecules, vectors, and the like into the bacterial cell(s), and more specifically into the interior of a membrane-enclosed space of a target cell(s). The molecules can be "introduced" into the target cell(s) by any means known to those of skill
15 in the art, for example as taught by Sambrook et al. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York (2001).

The term "promoter" is directed to a regulatory DNA sequence which can control or direct the transcription of a coding sequence to produce an mRNA in the cell. Usually, the promoter is located in the 5' region (that is, precedes, located upstream) of the coding
20 sequence. Promoters may be derived in their entirety from a native source, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. Promoters can be constitutive (i.e. promoter activation is not regulated by an inducing agent and hence rate of transcription is constant), or inducible (i.e., promoter activation is regulated by an inducing agent). In
25 some embodiments, various types and sequences of promoters may be utilized in the expression vectors of the current invention. In some embodiments, the promoters may be derived from various sources, species and organisms. In some embodiments, a combination of specific promoters and signal peptide sequences may be utilized in the expression vectors of the current invention.

30 In some exemplary embodiments, the promoter may be selected from, but not limited to a promoter having a nucleotide sequence as denoted by any one of SEQ ID NOs: 27-32, and/or homologs thereof. Each possibility is a separate embodiment.

In some embodiments, the promoter sequence comprises or consists of a nucleotide sequence as denoted by any one of SEQ ID NOs: 27-32, and/or homologs thereof. Each possibility is a separate embodiment.

In some exemplary embodiments, the promoter sequence comprises or consists of a nucleic acid sequence as denoted by SEQ ID NO. 31, or homologs thereof. In some exemplary embodiments, the promoter sequence comprises or consists of a nucleic acid sequence as denoted by SEQ ID NO. 27, or homologs thereof. In some exemplary embodiments, the promoter sequence comprises or consists of a nucleic acid sequence as denoted by SEQ ID NO. 28, or homologs thereof.

The terms “signal peptide” and “SP” refer to a short (usually 5-30 amino acids long) peptide that can be present at the N-terminus of a protein and direct the protein to be secreted from the bacterial cell. In some embodiments, the secretion is into the extracellular medium. In some embodiments, the SP sequence may be obtained from a natural source (as is, or modified). In some embodiments, the SP sequence may be designed in-silico. In some exemplary embodiments, the SP sequence is from *Propionibacterium* bacterial source. In some embodiments, the SP sequence is homologous to a *Propionibacterium* signal peptide. In some embodiments, the nucleic acid sequence encoding for the SP amino acid sequence may be included in an expression vector, such that it is in-frame with the sequence encoding the protein to be secreted. In some embodiments, amino acid sequences that are at least 60%, at least 70%, at least 80%, at least 90%, at least 95% homologous to the amino acid sequences of the SP disclosed in the current disclosure are also functional and are encompassed herein. In some embodiments, nucleic acid sequences that are at least 60%, at least 70%, at least 80%, at least 90%, at least 95% homologous to the nucleic acid sequences encoding for the SP disclosed in the current disclosure are also encompassed herein.

In some exemplary embodiments, the SP may be selected from, but not limited to an SP having an amino acid sequence as denoted by any one of SEQ ID NOs: 87-179 and/or homologs thereof. Each possibility is a separate embodiment.

In some embodiments, the nucleotide sequence encoding for a SP may be selected from a nucleic acid encoding for amino acid sequence as denoted by any one of SEQ ID NOs: 87-179, and/or homologs thereof. Each possibility is a separate embodiment.

In some embodiments, the nucleotide sequence encoding for a SP may be selected from a nucleic acid as denoted by any one of the nucleic acids as denoted by SEQ ID NOs: 180-189 and/or homologs thereof. Each possibility is a separate embodiment.

In some exemplary embodiments, the SP comprises or consists of an amino acid sequence as denoted by SEQ ID NO. 87, or homologs thereof. In some exemplary embodiments, the SP comprises or consists of an amino acid sequence as denoted by SEQ ID NO. 88, or homologs thereof. In some exemplary embodiments, a nucleic acid encoding for a SP comprises or consists of the nucleic acid sequence as denoted by SEQ ID NO. 180, or homologs thereof. In some exemplary embodiments, a nucleic acid encoding for a SP comprises or consists of the nucleic acid sequence as denoted by SEQ ID NO. 181, or homologs thereof.

In some embodiments, genetic modification of the LA-utilizing bacteria can be performed using suitable expression vectors (including suitable coding sequences for the proteins of interest (for example, polysaccharide degrading enzymes), suitable signal peptide sequences (if used) and/or suitable promoters), and methods known in the art for LA-utilizing bacteria, described, for example in Kiatpapan and Murooka (2001) *Appl Microbiol Biotechnol.*, 56(1-2):144-9; Brede et al., (2005) *Appl Environ Microbiol.*, 71(12):8077-84; Mukdsi et al., (2014) *Appl Environ Microbiol.*, 80(2): 751-756; and WO 2015/011250. In some embodiments, the expression vectors allow integration of the protein of interest (and SP, if used) to the genome of the bacteria.

In some embodiments, when determining the various nucleic acid sequences utilized to genetically engineer the LA-utilizing bacteria, suitable codon usage may be applied, to allow optimal expression and/or function of the polysaccharide degrading enzymes and/or SP in the bacterial cells.

According to some embodiments, there is provided an expression vector for expressing a polysaccharide degrading enzyme in a bacterial cell, the vector comprising an expression cassette (also referred to herein as "insert"), comprising at least one suitable promoter nucleic acid sequence, operably linked to at least one nucleic acid sequence encoding for a signal peptide (SP), which is translationally fused to least one nucleic acid sequence encoding a polysaccharide degrading enzyme, selected from cellulase, hemicellulase and amylase.

In some embodiments, the expression vector comprises a plasmid backbone, one or more suitable origin of replication (Ori) sequences, one or more antibiotic resistance gene sequences and one or more expression cassettes. In some embodiments, an expression vector without an expression cassette (insert) is referred to herein as a "shuttle vector". In
5 some embodiments, the shuttle vector is capable of being replicated in various bacterial cell types.

In some embodiments, the expression vector is suitable for expression of an exogenous polysaccharide-degrading enzyme in *Propionibacterium*. In some embodiments, the *Propionibacterium* is *Propionibacterium freudenreichii*. In some
10 embodiments, an expression vector suitable for expression of an exogenous polysaccharide degrading enzyme in *Propionibacterium* comprises a nucleic acid sequence encoding for a *Propionibacterium* replication protein.

In some exemplary embodiments, the expression vector is pOWR3 vector, as further exemplified below herein. In some embodiments, such expression vector
15 comprises a nucleic acid sequence encoding for a *Propionibacterium* replication protein (such as denoted by SEQ ID NO: 84). In some embodiments, such expression vector further comprises one or more chloramphenicol resistance genes (such as, *cmx(A)* and *cml(A)* from *Corynebacterium striatum* pT10 plasmid). In some embodiments, such expression vector further comprises a *Propionibacterium* origin of replication (Ori)
20 sequence.

In some embodiments, there is provided an expression vector for expressing and secreting an exogenous polysaccharide-degrading enzyme by a *Propionibacterium*, said vector comprising:

- at least one nucleotide sequence encoding for a *Propionibacterium* replication protein;

- at least one *Propionibacterium* origin of replication nucleotide sequence; and an at least one expression cassette comprising:

- at least one promoter nucleic acid sequence, which is operably linked to: at least one nucleic acid sequence encoding for a *Propionibacterium* signal peptide (SP) (or homologs thereof), which is translationally fused to at least one nucleic acid sequence encoding a polysaccharide degrading enzyme, selected from cellulase, hemicellulase, and amylase.

In some embodiments, as further exemplified herein, an expression vector for expressing an exogenous polysaccharide degrading enzyme in *Propionibacterium* is advantageous as it can be replicated both in *e. coli* (either as is, or without an expression cassette), and in *Propionibacterium*. Further, such vector is advantageous as it allows, due to its structure and compact size (about 5.6 kb, (without expression cassette)) improved expression (such as, high yield and quality) of polysaccharide-degrading enzymes in the bacteria. Further, the expressed polysaccharide degrading enzymes, are capable of being successfully secreted from the bacterial cells and be functional in degrading polysaccharides in the substrate. Further, being smaller than other vectors used for expression in *P. freudenreichii* is advantageous as it makes it easier to transform to bacterial cells and easier to use for cloning by the restriction free method. Moreover, the cloning region for inserting an expression cassette is bordered (upstream and downstream) by termination signals, which can prevent polar effect interruptions and stabilizes the mRNA transcript of the polysaccharide degrading enzyme produced in the bacterial cell, to thereby results in improved expression of the proteins in the cell.

In some embodiments, the expression vector may include more than one expression cassettes, each comprising the same or different promoter, same or different SP sequence and a different polysaccharide degrading enzyme sequence.

In some exemplary embodiments, an expression cassette may comprise or consist of a nucleotide sequence as denoted by any one of SEQ ID NOs: 33-49, and/or homologs thereof. Each possibility is a separate embodiment.

In some embodiments, there is provided an expression cassette, said expression cassette comprise or consist of a nucleotide sequence as denoted by any one of SEQ ID NOs: 33-49 and/or homologs thereof. Each possibility is a separate embodiment.

In some exemplary embodiments, the cassette comprise or consist of a nucleotide sequence as denoted by SEQ ID NO: 43.

In some embodiments, when said expression cassette is expressed in the target bacterial cell, under the control of the promoter sequence of the expression cassette, the signal peptide is translationally linked in-frame to the polysaccharide degrading enzyme. Thus, a chimeric protein which includes the signal peptide sequence and the polysaccharide degrading enzyme sequence is expressed in the bacterial cell and is capable of being secreted therefrom.

In some embodiments, there is provided a LA-utilizing bacteria comprising one or more expression vector(s) as disclosed herein.

In some embodiments, there is provided a LA-utilizing bacteria comprising one or more expression cassette(s), as disclosed herein.

5 In some embodiments, there is provided a *Propionibacterium* comprising one or more expression vectors wherein the expression vector comprises: at least one nucleotide sequence encoding for a *Propionibacterium* replication protein;

at least one *Propionibacterium* origin of replication nucleotide sequence; and an at least one expression cassette comprising:

at least one promoter nucleic acid sequence, which is operably linked to: at least one nucleic acid sequence encoding for a *Propionibacterium* signal peptide (SP), which is translationally fused to at least one nucleic acid sequence encoding a polysaccharide degrading enzyme, selected from cellulase, hemicellulase, and amylase.

In some embodiments, there is provided an isolated nucleic acid molecule comprising or consisting of a sequence selected from the group consisting SEQ ID NOs:
10 33-49, and homologs thereof.

In some embodiments, the polysaccharide degrading enzyme sequence is selected from the group consisting of SEQ ID NOs: 14-26, and homologs thereof.

In some embodiments, the nucleic acid sequence encoding the polysaccharide degrading enzyme is selected from the group consisting of SEQ ID NOs: 1-13, and
15 homologs thereof.

In some embodiments, a LA-utilizing bacterium is genetically-engineered to produce a single polysaccharide-degrading enzyme. In other embodiments, a LA-utilizing bacterium is genetically-engineered to produce a plurality of different polysaccharide-degrading enzymes. A "plurality" indicates at least two. In some embodiments, in order to
20 control the expression level of each polysaccharide-degrading enzyme when a plurality of enzymes is engineered into the bacterium, each enzyme is engineered with a Ribosome Binding Site (RBS) of different potency, as known in the art.

In some embodiments, each of the polysaccharide-degrading enzymes may be engineered with a similar or different promoter. In some embodiments, each of the

polysaccharide-degrading enzymes may be engineered with a similar or different signal peptide, if such is used.

In some embodiments, a population of LA-utilizing bacteria comprising a plurality of sub-populations is generated, each sub-population is genetically-engineered to express and secrete a different polysaccharide-degrading enzyme. A “plurality” of sub-populations indicates at least two sub-populations, for example two, three, four sub-populations. The sub-populations are for use as a co-culture in processing organic waste. In some embodiments, a population comprises substantially equal amounts of each sub-population. In other embodiments, a population comprises sub-populations at various ratios (i.e. each sub-population may constitute a different percentage within the population).

As noted above, the polysaccharide-degrading enzymes engineered into the LA-utilizing bacteria may be selected from glycoside hydrolases, polysaccharide lyases and carbohydrate esterases. The broad group of polysaccharide-degrading enzymes is divided into enzyme classes and further into enzyme families according to a standard classification system (Cantarel et al. 2009 *Nucleic Acids Res* 37: D233-238). An informative and updated classification of such enzymes is available on the Carbohydrate-Active Enzymes (CAZy) server (www.cazy.org).

In some embodiments, the polysaccharide-degrading enzymes are enzymes that degrade polysaccharides selected from starch and non-starch plant polysaccharides.

In some embodiments, the polysaccharide-degrading enzymes engineered into the LA-utilizing bacteria are glycoside hydrolases.

In some embodiments, the glycoside hydrolases comprise a cellulase. The cellulase may be an exocellulase or an endocellulase. In some embodiments, a LA-utilizing bacterium of the present invention is genetically modified to express and secrete a single cellulase. In other embodiments, the LA-utilizing bacterium is genetically modified to express and secrete a plurality of different cellulases. In some embodiments, a cellulase may be selected from, but not limited to: endo-(1,4)- β -D-glucanase, exo-(1,4)- β -D-glucanase, β -glucosidases, Carboxymethylcellulase (CMCase); endoglucanase; cellobiohydrolase; avicelase, celludextrinase, cellulase A, celulosin AP, alkali cellulase, and pancellase SS. Each possibility is a separate embodiment.

In some embodiments, a nucleic acid encoding for a cellulase may have a nucleic acid sequence as denoted by any one of SEQ ID Nos: 12-13, or homologs thereof. Each possibility is a separate embodiment. In some embodiments, a cellulase may have an

amino acid sequence as denoted by any one of SEQ ID NOs: 25-26, or homologs thereof. Each possibility is a separate embodiment. In some embodiments, a cellulase comprises or consists of an amino acid sequence as denoted by any one of SEQ ID NOs: 25-26, or homologs thereof. Each possibility is a separate embodiment.

5 In additional embodiments, the glycoside hydrolases comprise a hemicellulase. In some embodiments, a LA-utilizing bacterium of the present invention is genetically modified to express and secrete a single hemicellulase. In other embodiments, the LA-utilizing bacterium is genetically modified to express and secrete a plurality of different hemicellulases. In some embodiments, the hemicellulase is a xylanase. Non-limiting
10 examples of additional hemicellulases include arabinofuranosidases, acetyl esterases, mannanases, α -D-glucuronidases, β -xylosidases, β -mannosidases, β -glucosidases, acetyl-mannanesterases, α -galactosidases, α -L-arabinanases, and β -galactosidases. Each possibility represents a separate embodiment of the present invention.

In some embodiments, a nucleic acid encoding for a xylanase may have a nucleic
15 acid sequence as denoted SEQ ID No: 11, or homologs thereof. In some embodiments, a xylanase may have an amino acid sequence as denoted by SEQ ID No. 24, or homologs thereof. Each possibility is a separate embodiment.

In yet additional embodiments, the glycoside hydrolases comprise an amylase. In some embodiments, a LA-utilizing bacterium of the present invention is genetically
20 modified to express and secrete a single amylase. In other embodiments, the LA-utilizing bacterium is genetically modified to express and secrete a plurality of different amylases. In some embodiments, an amylase may be selected from, but not limited to: α -amylase; (1,4- α -D-glucan glucanohydrolase; glycogenase) β -Amylase; (1,4- α -D-glucan maltohydrolase; glycogenase; saccharogen amylase) γ -Amylase; (Glucan 1,4- α -
25 glucosidase; amyloglucosidase; Exo-1,4- α -glucosidase; glucoamylase; lysosomal α -glucosidase and 1,4- α -D-glucan glucohydrolase. Each possibility is a separate embodiment.

In some embodiments, a nucleic acid encoding for an amylase may have a nucleic
acid sequence as denoted by any one of SEQ ID Nos: 1-10, or homologs thereof. Each
30 possibility is a separate embodiment. In some embodiments, an amylase may have an amino acid sequence as denoted by SEQ ID NOs: 14-23, or homologs thereof. Each possibility is a separate embodiment. In some embodiments, an amylase comprises or

consists of an amino acid sequence as denoted by SEQ ID NOs: 14-23, or homologs thereof. Each possibility is a separate embodiment.

The polysaccharide-degrading enzymes engineered into LA-utilizing bacteria according to the present invention may be from a bacterial source. In some embodiments, the bacterial source is a thermophilic bacterium. The term “thermophilic bacterium” as used herein indicates a bacterium that thrives at temperatures higher than about 45⁰C, preferably above 50⁰C. Typically, thermophilic bacteria according to the present invention have optimum growth temperature of between about 45⁰C to about 75⁰C, preferably about 50-70⁰C. Non-limiting examples of thermophilic bacterial sources for polysaccharide-degrading enzymes include: Cellulases and hemicellulases - *Clostridium* sp. (e.g. *Clostridium thermocellum*), *Paenibacillus* sp., *Thermobifida fusca*; Amylases - *Bacillus* sp. (e.g. *Bacillus stearothermophilus*), *Geobacillus* sp. (e.g. *Geobacillus thermoleovorans*), *Chromohalobacter* sp., *Rhodothermus marinus*. Each possibility is a separate embodiment.

In additional embodiments, the bacterial source of the polysaccharide-degrading enzymes is a mesophilic bacterium. The term “mesophilic bacterium” as used herein indicates a bacterium that thrives at temperatures between about 20⁰C and 45⁰C. Non-limiting examples of mesophilic bacterial sources for polysaccharide-degrading enzymes include: Cellulases and hemicellulases - *Klebsiella* sp. (e.g. *Klebsiella pneumonia*), *Cohnella* sp., *Streptomyces* sp, *Acetivibrio cellulolyticus*, *Ruminococcus albus*; Amylases – *Bacillus* sp. (e.g. *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis*), *Lactobacillus fermentum*. A person of skill in the art understands that some mesophilic bacteria (e.g. several *Bacillus* sp.) produce thermostable enzymes.

The polysaccharide-degrading enzymes engineered into LA-utilizing bacteria according to the present invention may also be from a fungal source. Non-limiting examples of fungal sources for polysaccharide-degrading enzymes include: Cellulases and hemicellulases - *Trichoderma reesei*, *Humicola insolens*, *Fusarium oxysporum*; Amylases - *Aspergillus oryzae*, *Penicillium fellutanum*, *Thermomyces lanuginosu*.

Additional sources for polysaccharide-degrading enzymes for use in accordance with the present invention can be found, for example, at the CAZy server mentioned above.

In some embodiments, a LA-utilizing bacterium of the present invention is genetically-engineered to produce polysaccharide-degrading enzymes having suitable

activity at the same temperature and/or pH range that are suitable for growth of the LA-utilizing bacteria. In some embodiments, a suitable activity is optimal activity. In some embodiments, suitable growth is optimal growth. In some embodiments, a suitable condition is sub-optimal.

5 In some embodiments, a LA-utilizing bacterium of the present invention is genetically-engineered to produce polysaccharide-degrading enzymes having optimal activity at the same temperature and/or pH range that are optimal for growth of the LA-utilizing bacteria.

10 In some embodiments, a LA-utilizing bacterium of the present invention is genetically-engineered to produce polysaccharide-degrading enzymes having suitable activity at a temperature and/or pH range that is different from the suitable temperature and/or pH range for growth of the LA-utilizing bacteria.

15 In some embodiments, a LA-utilizing bacterium of the present invention is genetically-engineered to produce polysaccharide-degrading enzymes having optimal activity at a temperature and/or pH range that is different from the optimal temperature and/or pH range for growth of the LA-utilizing bacteria. For example, polysaccharide-degrading enzymes from a thermophilic source may be engineered into a LA-utilizing bacterium, which typically is a mesophile.

20 Processing of organic waste

In some embodiments, organic wastes to be processed typically comprise endogenous D,L-lactic acid.

25 In some embodiments, in order to utilize the organic waste as a substrate for discrete lactic acid enantiomer(s) production, it is required to selectively remove at least the unwanted enantiomer prior to lactic acid fermentation (in order to polymerize lactic acid into polylactic acid suitable for industrial applications it should be at least about 95% optically pure, preferably at least about 99% optically pure). Removal of at least the unwanted enantiomer from the organic waste should be performed with minimal impact on the feedstock total sugar content.

30 In some embodiments, organic wastes to be processed also comprise complex polysaccharides and reducing sugars at varying ratios. The composition depends on the source of the waste, where some organic wastes may be more starch-rich (e.g., food waste from bakeries, mixed food waste of municipalities) and others may be rich with

lignocellulosic material (e.g. agricultural waste). In some embodiments, the organic waste to be processed includes a combination of wastes from different sources.

In some embodiments, the composition of the organic waste in terms of reducing sugars and polysaccharides may be determined prior to processing using methods known in the art, including for example enzymatic assays (colorimetric, fluorometric) with glucose oxidase, hexokinase or phosphoglucose isomerase for fructose determination. Alternatively, HPLC and/or reducing sugars continuous sensors can be utilized. Total sugar analysis can be performed, for example, by phenol-sulfuric assay. The composition of the organic waste, for example percentage of at least one of starch, cellulose and hemicelluloses, may be used for selecting the LA-utilizing bacterium or the population of LA-utilizing bacteria to be contacted with the organic waste. For example, for organic wastes comprising a higher percentage of starch compared to cellulose, a cell consortium may be selected which comprises a first sub-population producing an amylase and a second sub-population producing a cellulase, where the ratio between the sub-populations is tailored to the ratio between starch and cellulose in the organic waste. A single bacterium producing all the necessary enzymes may also be used, and the enzyme dose and ratios can be altered and tailored using Ribosome Binding Sites (RBS) of different potencies, as noted above.

In some embodiments, processing of the organic waste according to the present invention typically begins with pretreatment of the organic waste to decrease particle size and increase surface area, and also to inactivate endogenous bacteria within the waste. The pretreatment may include, for example, shredding and sterilization by methods known in the art. Pretreatment may also include mincing with an equal amount of water using a waste mincer, such as, e.g., an extruder, sonicator, shredder or blender.

According to some embodiments, following pretreatment, the organic waste is mixed in a reactor (fermenter) with the LA-utilizing bacteria of the present invention and the bacterial culture is propagated under conditions suitable for lactic acid consumption by the LA-utilizing bacteria and for producing the engineered polysaccharide-degrading enzymes. In some embodiments, such conditions may include a temperature in the range of about 30-40°C and any subranges thereof. Each possibility is a separate embodiment. In some embodiments, such conditions may include a pH in the range of about 5.5-6.5 and any subranges thereof. Each possibility is a separate embodiment.

In some embodiments, when the polysaccharide-degrading enzymes are secreted and have suitable (such as, optimum) temperature and/or pH similar to those which are suitable (such as optimal) for growth of the LA-utilizing bacteria, the result is concomitant lactic acid elimination and organic waste saccharification.

5 In some embodiments, when the polysaccharide-degrading enzymes are secreted and have suitable (such as, optimal) temperature and pH different from those which are suitable (such as optimal) for growth of the LA-utilizing bacteria, the enzymes are secreted but remain inactive, or partially active, until the conditions are adjusted to those suitable for enzyme activity, resulting in separate lactic acid elimination and organic waste
10 saccharification.

In some embodiments, when the polysaccharide-degrading enzymes are non-secreted and released only after lysis of the LA-utilizing bacterial cells, the result is also separate lactic acid elimination and organic waste saccharification.

In some embodiments, the culture is maintained for sufficient time to eliminate D-
15 lactic acid, L-lactic acid or both from the waste (depending on the type of LA-utilizing bacteria), and optionally to obtain desired level of reducing sugars (in concomitant lactic acid elimination and organic waste saccharification).

In some embodiments, the time period may range from about 2 hours to about 15 hours or any amount therebetween, preferably between 2-12 hours, or 2-10 hours, such as 2
20 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours and 10 hours.

As used herein, the term "about", when referring to a measurable value, is meant to encompass variations of +/-10%, preferably +/-5%, more preferably, +/- 1%, and still more preferably +/-0.1% from the specified value.

Organic wastes typically include nitrogen sources and other nutrients needed for
25 the LA-producing bacteria, but such nutrients may also be supplied separately if needed.

In some embodiments, following the above processing, the amount of reducing sugars is determined. Such determination may be useful for downstream fermentation processes utilizing the reducing sugars, enabling control of the concentration of fed sugars.

30 Production of discrete lactic acid enantiomer(s)

In some embodiments, the reducing sugars of the organic waste (those originally found in the waste and those released by the action of the polysaccharide-degrading enzymes) may be fermented to lactic acid by LA-producing microorganisms. To generate

only one discrete enantiomer of lactic acid, the LA-producing microorganisms that are used produce only one of D-lactic acid and L-lactic acid enantiomers. The LA-producing microorganisms may produce only one enantiomer naturally, or may be genetically modified to produce only one enantiomer, for example by knocking out one or more enzymes involved in the synthesis of the undesired enantiomer.

In some embodiments, prior to lactic acid fermentation, the LA-utilizing bacteria are inactivated to avoid consumption of lactic acid produced during fermentation. Inactivation may be performed, for example, by increasing the temperature or changing the pH to a temperature/pH at which the LA-utilizing bacteria are irreversibly inactivated. Alternatively or additionally, inactivation of the LA-utilizing bacteria may be performed by cell lysis using, for example, sonication. The latter may be used when the LA-utilizing bacteria are engineered to produce non-secreted polysaccharide-degrading enzymes, and the cell lysis inactivates the bacteria concomitant with release of the enzymes. In some embodiments, inactivation encompasses sterilization or pasteurization.

As used herein, "inactivated", indicates dead or dying cells. Typically, at least 80% of the cells are inactivated, for example at least 85% of the cells are inactivated, at least 90% of the cells are inactivated, at least 95% of the cells are inactivated, or 100% of the cells are inactivated. Each possibility represents a separate embodiment of the invention.

In some embodiments, lactic acid fermentation is carried out in the same reactor (fermenter) where processing of the organic waste by the LA-utilizing bacteria and polysaccharide-degrading enzymes was carried out. In other embodiments, fermentation is carried out in a separate reactor. In some embodiments, the processed organic waste is filtered to remove solid materials and the filtered broth is mixed with LA-producing microorganisms for the fermentation stage.

LA-producing microorganisms include various bacteria (including for example *Lactobacillus* species and *Bacillus* species) and fungi.

Typically, the fermenting step is carried out under anaerobic or microaerophilic conditions, using batch, fed-batch, continuous or semi-continuous fermentation. Each possibility represents a separate embodiment of the present invention.

In batch fermentation, the carbon substrates and other components are loaded into the reactor, and, when the fermentation is completed, the product is collected. Except for neutralizing agents for pH control, other ingredients are not added to the reaction before it is completed. The inoculum size is typically about 5-10% of the liquid volume in the

reactor. The fermentation is kept at substantially constant temperature and pH, where the pH is maintained by adding a suitable neutralizing agent, such as an alkali, a carbonate or ammonia.

In fed-batch fermentation, the substrate is fed continuously or sequentially to the reactor without the removal of fermentation broth (i.e., the product(s) remain in the reactor until the end of the run). Common feeding methods include intermittent, constant, pulse-feeding and exponential feeding.

In continuous fermentation, the substrate is added to the reactor continuously at a fixed rate, and the fermentation products are taken out continuously.

In semi-continuous processes, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. Repeated fed-batch culture, which can be maintained indefinitely, is another name of the semi-continuous process.

Lactic acid fermentation is typically carried out for about 1-4 days or any amount therebetween, for example, 1-2 days, or 2-4 days, or 3-4 days.

According to some embodiments, the polysaccharide-degrading enzymes may have optimal temperature and/or pH that different from those optimal for growth of the LA-utilizing bacteria that secrete them, and accordingly, the enzymes may be secreted during processing with the LA-utilizing bacteria, but they remain inactive (or partially active) and substantially do not degrade polysaccharides in the organic waste, or the degradation rate is low. In some embodiments, the optimum temperature and/or pH of the polysaccharide-degrading enzymes are similar to those that are optimal for lactic acid fermentation by the LA-producing microorganisms. According to these embodiments, the enzymes may remain inactive in the broth until the LA-producing microorganisms are added and the conditions are adjusted to allow their activation. This may result in simultaneous saccharification and fermentation.

In some embodiments, the polysaccharide-degrading enzymes may be non-secreted and released into the medium only after lysis of the LA-utilizing bacterial cells. In some embodiments, the LA-producing microorganisms are added into the medium after lysis of the LA-utilizing bacterial cells, such that simultaneous saccharification and fermentation occurs.

According to the above embodiments, the method for producing discrete lactic acid enantiomer comprises: (i) processing the organic waste to eliminate D-lactic acid, L-lactic acid or a combination thereof present in the waste by contacting the organic waste with the

LA-utilizing bacterium of the present invention, or population of LA-utilizing bacteria of the present invention, under conditions suitable for lactic acid consumption by the LA-utilizing bacteria and for production of the polysaccharide-degrading enzymes; (ii) inactivating the LA-utilizing bacteria; (iii) degrading polysaccharides in the waste to release reducing sugars concomitant with fermenting the released sugars to discrete lactic acid enantiomer, by contacting the organic waste obtained in (i) with a lactic acid-producing microorganism that produces only one of D-lactic acid enantiomer and L-lactic acid enantiomer, under conditions suitable for activity of the polysaccharide-degrading enzymes produced in (i) and for lactic acid fermentation by the LA-producing microorganism; and (iv) recovering the discrete lactic acid enantiomer from the fermentation broth.

In other embodiments, the organic waste is saccharified prior to lactic acid fermentation (separate hydrolysis and fermentation).

According to these embodiments, the method for producing discrete lactic acid enantiomer comprises: (i) processing the organic waste to eliminate D-lactic acid, L-lactic acid or a combination thereof present in the waste and degrade polysaccharides in the waste to release soluble reducing sugars, by contacting the organic waste with the LA-utilizing bacterium of the present invention, or population of LA-utilizing bacteria of the present invention, under conditions suitable for lactic acid consumption by the LA-utilizing bacteria and for secretion and activity of the polysaccharide-degrading enzymes; (ii) inactivating the LA-utilizing bacteria; (iii) fermenting the soluble reducing sugars obtained in (i) with a lactic acid-producing microorganism that produces only one of D-lactic acid and L-lactic acid, to obtain discrete lactic acid enantiomer; and (iv) recovering the discrete lactic acid enantiomer from the fermentation broth.

After fermentation is completed, the broth containing lactic acid may be typically clarified by centrifugation or passed through a filter press to separate solid residue from the fermented liquid. The filtrate may be concentrated, e.g. using a rotary vacuum evaporator.

Separation and purification of lactic acid from the broth may be carried out by methods known in the art, including distillation, extraction, electrodialysis, adsorption, ion-exchange, crystallization and combinations of these methods. Several methods are reviewed, for example, in Ghaffar et al. (2014), *supra*; and López-Garzón et al. (2014)

Biotechnol Adv., 32(5):873-904). Alternatively, recovery and conversion of lactic acid to lactide in a single step may be used (Dusselier et al. (2015) *Science*, 349(6243):78-80).

In some embodiments, the systems and methods disclosed herein for processing waste are particularly suitable for use with mixed food waste of commercial, industrial and municipal origin. The use of mixed food waste as substrate is particularly suitable for large-scale industrial fermentation as it is heterogeneous and hence it would contain most of the required minerals and vitamins for fermentation with bacteria of ruminal origin. Further, the systems and methods disclosed herein are advantageous over currently used methods as they exhibit low fossil fuel usage, do not use valuable arable land to grow crops for feedstock, water usage is low, as is GHG emission and further, the products obtained are biodegradable.

According to some embodiments, the systems and methods disclosed herein are further advantageous as they allow combining unit operations for an industrial process, as no externally added polysaccharide-degrading enzymes are needed to utilize waste. Rather, production of such enzymes is performed in the same vessel in which fermentation occurs.

Production of polylactic acid (PLA)

In order to generate PLA that is suitable for industrial applications, the polymerization process should utilize only one enantiomer. Presence of impurities or a racemic mixture of D- and L-lactic acid results in a polymer having undesired characteristics such as low crystallinity and low melting temperature. Thus, lactic acid bacteria that produce only L-lactate enantiomer or only D-lactate enantiomer are required.

Polymerization of PLA may be carried out by methods known in the art. Known methods include polymerization via lactide (di-lactic acid) formation, and direct condensation of lactic acid monomers. Several methods are reviewed, for example, in Södergård and Stolt (2010) *Industrial Production of High Molecular Weight Poly(Lactic Acid)*, in: *Poly(Lactic Acid): Synthesis, Structures, Properties, Processing, and Applications* (eds R. Auras, L.-T. Lim, S. E. M. Selke and H. Tsuji), John Wiley & Sons, Inc., Hoboken, NJ, USA. In some embodiments, the PLA is Poly-L Lactic Acid (PLLA). In some embodiments, the PLA is Poly-D Lactic Acid (PDLA).

According to some embodiments, the systems and methods disclosed herein can result in an end product, namely, poly-lactic acid (PLA) that is completely recycled (i.e., 100% recycled) from waste.

According to some embodiments, the LA-utilizing bacteria disclosed herein may be successfully utilized in various methods and systems for production of lactic acid and PLA, which can be implemented by various commercial settings, including, external outdoors, various fermenters and reactors, recycling factories, and the like.

In some embodiments, various steps in the methods may be performed in one discrete location. In some embodiments, various steps in the methods may be performed in one or more operational units, such as, fermenters and reactors. In some embodiments, various steps in the methods may be performed simultaneously or consecutively. For example, size reduction of the organic waste may be combined with sterilization step, temporally and/or spatially.

In some embodiments, steps in methods of obtaining polylactic acid from organic waste using the LA-utilizing bacteria of the present invention may include one or more of the steps of:

1. Obtaining organic waste. The organic waste may be selected from residential, commercial or industrial food waste. The organic waste may be source-separated or machine separated.
2. Size reduction of the organic waste –mincing and grinding of the organic waste can be applied to achieve a homogenous semi-solid feedstock. Further, sonication can be also be used to thereby combine size reduction with sterilization. This can be performed in the same operating unit.
3. Sterilization – can be achieved, for example, using sonication, heat, UV and/or pressure. This can be performed in-situ within a fermenter operating unit, or externally. In some embodiments, as mentioned above, this step can be combined with the step of size reduction.
4. Seeding of genetically engineered LA-utilizing bacteria– Genetically engineered LA-utilizing bacteria can be inoculated in a seed fermenter operating unit to produce enzymes. Additionally, lactate by-products from downstream processing can be added as feedstock at this step (as further detailed below). The LA-utilizing bacteria can also be cultivated on defined media, such as YEL.

5. Inoculation and co-generation of Polysaccharide degrading enzymes and CO₂ - and consumption of racemic lactate – The seed LA-utilizing bacteria is inoculated in a production fermenter to further produce enzymes and consume racemic lactate in the organic waste.
- 5 6. Inactivation of LA-utilizing bacteria – temperature in the fermenter may be increased (for example, to 45-55°C), to result in inactivation (sterilization) of the LA-utilizing bacteria. Advantageously, such elevated temperatures may further enhance the activity of secreted polysaccharide degrading enzymes, which have been secreted by the LA-utilizing bacteria providing improved conditions (such as optimal conditions) for their activity.
- 10 7. Inoculation with lactic acid producing organism (pure L or pure D producer) – consequently, fermentation media is depleted from racemic lactate and reducing sugars (formed by the secreted degrading enzymes) become available for the lactic acid fermentation process.
- 15 8. Recovery and purification of lactic acid – Various purification methods (or combinations thereof) can be utilized to purify the enantiomerically pure lactic acid. Such methods include, for example, micro/nano filtration, centrifugation, ion chromatography, precipitation, crystallization, and the like.
- 20 9. Polymerization to PLLA or PDLA- polymerization of the purified lactic acid to PLA is performed using methods known in the art, including polymerization via lactide (di-lactic acid) formation, and direct condensation of lactic acid monomers.

In some embodiments, recycling and reuse of downstream residual lactic acid or lactate waste formed in the process of obtaining pure lactic acid (and PLA), may be advantageously utilized using the LA-utilizing bacteria of the present invention. Such downstream residual lactic acid or lactate waste formed in the process may emerge from, for example, removing solids from fermentation broths (for example, using membrane filtration or centrifugation), the remaining solids contain useful lactate that did not separate. Additionally, when synthesizing lactides, some lactic acid molecules can undergo racemization. Such residual lactic acid and/or lactate waste fermentation can advantageously be recycled in the process, for example, by being utilized as feedstock for the LA-utilizing bacteria enzyme production. When organic acids such as lactic acid are

recovered from fermentation broths, normally the yield is not 100% and there is residual diluted lactic acid. Recovering the residual organic acid is costly so instead it can be recycled and used for another fermentation batch. Recycling the residue reintroduces minerals and salts also present in the residue, which might be required for a new fermentation batch. Continuous and semi-continuous fermentation lactic acid production processes require that a sample of the fermentation broth rich with bacteria and lactic acid, be used to seed incoming sterile fermentation feedstock. The problem of utilizing organic waste in a continuous or semi-continuous process for production of lactic acid, is the unwanted enantiomer which is present in the incoming raw material. If the feedstock seeded with the rich fermentation broth the production of lactic acid starts immediately, the unwanted enantiomer remains in the process as an impurity. Thus, the use of the lactate utilizing bacteria in the lactic acid production process allows for efficient recycling of all such resources. Residual lactic acid from the recovery process and/or pasteurized/sterilized lactic acid-rich broths can be used to cultivate the bacteria and produce high quantities of enzymes for another production cycle. This can be achieved either in situ, or in a separate smaller seed fermenter. In some embodiments, a sample from the lactic-acid rich fermentation broth can be split into two portions, one is pasteurized and used to cultivate enzyme producing bacteria. The second portion is not pasteurized and contains high cell mass of bacteria can be used to seed feedstock which has been treated with LA-utilizing bacteria to remove enantiomers and/or saccharified with enzymes.

Reference is now made to **Fig. 1**, which is a schematic illustration of a fermentation system for producing discrete enantiomers of lactic acid and recycling of residual lactate waste, according to some embodiments. As shown in Fig. 1, the fermentation system includes a seed fermenter (10), in which genetically engineered LA-utilizing bacteria is inoculated. The cultivating medium/broth can be size reduced and sterilized/pasteurized organic waste (14) of various sources as detailed above, defined media (such as, YEL) (16) and/or a combination of size reduced and sterilized organic waste and suitable nutrients beneficial for growth of the LA-utilizing bacteria (18). In the seed chamber, co-generation of secreted polysaccharide reducing enzymes (produced by the LA-utilizing bacteria) and CO₂ is achieved, as well as lactate consumption (by the LA-utilizing bacteria). The seed LA-utilizing bacteria is then transferred to a production

fermenter (12), to further produce and secrete polysaccharide reducing enzymes and consume racemic lactate. Additionally, further batches of size reduced and/or sterilized organic waste (20) may be added to the production fermenter. Next, the conditions (such as, temperature or pH) in the production fermenter are modified (for example, by
5 elevating the temperature to 45-65°C (for example, 55°C)) to thereby result in inactivation of the LA-utilizing bacteria and optionally, provide enhanced working conditions to the secreted polysaccharide degrading enzymes. This results in the fermentation media to be depleted from racemic lactate and to be enriched with reducing sugars available for fermentation. LA-producing microorganisms are inoculated with the medium to produce
10 an optically pure lactic acid enantiomer, which may then be purified and further processed, for example, by polymerization to PLA. Advantageously, downstream residual lactic acid and/or lactate waste (22) that has been formed in the process can be re-used and recycled, by being added to the seed fermenter and/or production fermenter (as indicated by the curved arrows).

15 According to some embodiments, there is thus provided a system and method of recycling residual lactic acid and/or lactate waste formed in the process of preparing optically pure lactic acid enantiomers.

In some embodiments, the system may include one or more sources of organic waste; one or more fermenters; a genetically modified LA-utilizing bacteria, modified to
20 express and secrete one or more exogenous polysaccharide-degrading enzyme, wherein the fermenter is configured to allow growth of the LA-utilizing bacteria under conditions in which lactic acid is consumed by the LA-utilizing bacteria and which are suitable for expression and activity of the polysaccharide-degrading enzymes, wherein said processing eliminates D-lactic acid, L-lactic acid or both from the organic waste and degrades
25 polysaccharides in the waste to release soluble reducing sugar; and further configured to allow conditions which inactivate the LA-utilizing bacteria, and LA-producing microorganisms that can be inoculated in the fermenter, said LA-producing microorganisms are capable of fermenting the soluble reducing sugars produced by the secreted polysaccharide-degrading enzymes to produce only one of D-lactic acid and L-
30 lactic acid, to obtain discrete enantiomers of lactic acid; wherein downstream residual lactic acid and/or lactate waste formed in the process are added back to the one or more fermenters, to be re-used as substrate in the process.

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to". The terms "comprises" and "comprising" are limited in some embodiments to "consists" and "consisting", respectively. The term "consisting of" means "including and limited to". The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed chemical structures and functions may take a variety of alternative forms without departing from the invention.

The following examples are presented in order to more fully illustrate some embodiments of the invention. They should, in no way be construed, however, as limiting the broad scope of the invention.

EXAMPLES

Example 1- Constructions of suitable vectors for genetically modifying bacteria

Construction of E. coli- P. freudenreichii shuttle vector:

The vectors constructed can be used in both E. coli and P. freudenreichii.

The pOWR3 vector (a schematic illustration is shown in **Fig. 2A**) was prepared as follows: Propionibacterium replication protein sequence (SEQ ID NO. 84) and

chloramphenicol resistance genes (cmx(A) (SEQ ID NO: 85) and cml(A) (SEQ ID NO: 86) from *Corynebacterium striatum* pT10 plasmid) were synthesized in tandem into a vector comprising *E. coli* origin of replication (ori) and ampicillin resistance cassette (AmpR). This results in a ~5.6 kbp plasmid able to replicate both in *E. coli* and *P. freudenreichii*. This vector further includes a PB origin of replication sequence. The resulting pOWR3 vector is smaller than other known *P. freudenreichii* expression vectors (5.6 kbp versus 6.2-8 kbp), which makes it easier to transform to bacterial cells (for example, by electroporation) and easier to use for cloning by the restriction free method. Moreover, the AmpR gene sequence region of pOWR3 is used as a cloning site, for inserting an expression cassette (as detailed below). This region is bordered (upstream and downstream) by termination signals which prevents polar effect interruptions and stabilizes the mRNA transcript (of the polysaccharide degrading enzyme) produced in the bacterial cell.

15 Transformation of pOWR3 vector to *P. freudenreichii*.

The pOWR3 vector was transformed into *P. freudenreichii* by electroporation. A Turbid culture of *P. freudenreichii* was diluted 1:125 to fresh YEL media (gr/l: 10 yeast extract, 10 peptone, 10 lactate) for overnight culture in 30°C under aerobic conditions. The culture (O.D 600 = 0.2 - 1.9) was placed on ice for 30 minutes, then washed twice in the same volume with ice cold water (3000 rpm 10 min). A 3rd wash was with same volume of cold 10% glycerol (3000 rpm 10 min). The pellet was concentrated (0.01% of the original culture) in 10% cold glycerol. 50 µl of electro-competent *P. freudenreichii* were mixed in a tube with 1000 ng of the pOWR3 vector. The bacteria and vector mix were transferred to a 0.1 cm gap cuvette (Biorad) and electroporated using the following conditions: field strength -20000 V/cm, resistance- 500 ohm, capacitor- 25uF. 900µl of YEL media was added and the bacteria were incubated for 3 hours in 30°C under aerobic conditions, followed by spreading on YEL media + 10ugr/ml Chloramphenicol agar plates.

To construct the suitable expression vectors, allowing expression and secretion of the polysaccharide degrading enzymes, the pOWR3 vector is modified by replacing the ampicillin resistance gene with an “expression cassette” which includes, i) a promoter sequence, operably linked to (ii) nucleic acid sequence encoding for a signal peptide (SP) which is translationally linked to (iii) a nucleotide sequence encoding for a polysaccharide degrading enzyme. (**Fig. 2B**). When said expression cassette is expressed in the target

cell, under the control of the promoter sequence, the signal peptide is translationally linked in-frame to the polysaccharide degrading enzyme. In other words, a chimeric protein which includes the SP peptide sequence and the polysaccharide degrading enzyme peptide sequence is expressed in the cell.

- 5 By utilizing such expression cassette, a matrix of suitable combinations of promoter-SP-polysaccharide degrading enzyme can be utilized.

Example 2- Construction of suitable expression cassettes and expression vectors for genetically modifying bacteria to express and secrete polysaccharide degrading enzyme

Listed below are exemplary polysaccharide degrading enzymes (Glucoamylases, cellulases and hemicellulases (Xylanases), signal peptides and promoters used for creating expression cassettes used in expression vectors.

The following exemplary enzymes listed below in Table 1, are used in expression cassettes and expression vectors for genetically modifying bacteria. The enzymes sequences are obtained from different sources (organisms). The original signal peptide of each of the listed enzymes is removed and is replaced by the foreign (different) signal peptide.

Table 1- Exemplary polysaccharide degrading enzymes

SEQ ID NO. (nt)	SEQ ID NO. (AA)	Organism name	origin	GI/accession number	Optimal pH	Optimal Temp. (°C)
1	14	Saccharomycopsis fibuligera	eukaryote	113795	5-6	45
2	15	Aspergillus niger	eukaryote	30025851	4-5	55
3	16	Clostridium SP.5 G000	Gram +	231542	5.5	60
4	17	Clostridium thermohydrosulfuricum	Gram+	490533596	5	70
5	18	Clostridium thermoamylolyticum	Gram+	34146785	5	65
6	19	Thermoanaerobacter tengcongensis MB4	Gram+	20516826	5	75

7	20	Picrophilus torridus DSM 9790	archaea	48431212	5	75
8	21	Picrophilus torridus DSM 9790	archaea	48430085	5	50
9	22	Caulobacter crescentus CB15	Gram-	AB813000	5	40
10	23	Bacillus licheniformis	Gram+	CAA01355	7	37
11	24	Thermobifida Fusca TM51 (Xylanase)	Gram+	EOR70069	5-6.5	50-60
12	25	Beta-1,4-Endoglucanase [Thermomonospora Fusca] (Cellulase)	Gram+	P26222	5-6.5	50-60
13	26	Endoglucanase [Clostridium Cellulolyticum] (Cellulase)	Gram+	WP_015924 277.1	7	50

The following promoter regions are utilized in the construction of various expression cassettes and expression vectors:

- PFREUD_04850 promoter from the *Propionibacterium frudenreichii* ATCC 9614 (SEQ ID NO: 27)
- PFRUD_04850 promoter from the *Propionibacterium frudenreichii* BIA118 (SEQ ID NO: 28)
- PFREUD_22150 promoter from the *Propionibacterium frudenreichii* ATCC 9614 (SEQ ID NO: 29)
- PFREUD_08450, promoter from *Propionibacterium frudenreichii* ATCC 9614. (SEQ ID NO: 30)
- PFREUD_18290 from *Propionibacterium frudenreichii* ATCC 9614. (SEQ ID NO: 31).
- pGRO promoter (pgro plasmid from *Propionibacterium acidipropionici*) (SEQ ID NO: 32).

The following exemplary signal peptides (SP) amino acid sequences are utilized in the construction of various of expression cassettes:

- > CBL57338 surface layer protein A (S-layer protein A) PFREUD_18290 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 87 (amino acid sequence), SEQ ID NO: 180 (nucleotide sequence)).
- 5 >CBL56016.1 cell-wall peptidases, NlpC/P60 family secreted protein PFREUD_04850 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 88 (amino acid sequence), SEQ ID NO: 181 (nucleotide sequence)).
- 10 >CBL56360.1 DSBA oxidoreductase [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 89 (amino acid sequence), SEQ ID NO: 182 (nucleotide sequence)).
- 15 CBL57333.1 Hypothetical protein PFREUD_18250 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 90 (amino acid sequence), SEQ ID NO: 183 (nucleotide sequence))
- 20 gil296921799|embl|CBL56359.1| thioredoxine like membrane protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO:91 (amino acid sequence), SEQ ID NO: 184 (nucleotide sequence))
- >gil296923311|embl|CBL57911.1| drug exporters of the RND superfamily [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO:92 (amino acid sequence), SEQ ID NO: 185 (nucleotide sequence))
- 25 >gil296921405|embl|CBL55958.1| Putative carboxylic ester hydrolase [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 93 (amino acid sequence), SEQ ID NO: 186 (nucleotide sequence)).
- 30 gil296921680|embl|CBL56237.1| Hypothetical secreted protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO:94 (amino acid sequence), SEQ ID NO: 187 (nucleotide sequence)).

- >gil296922532|embl|CBL57105.1| S-layer domain protein domain protein precursor
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 95 (amino acid sequence), SEQ ID NO: 188 (nucleotide sequence)).
- 5 >gil296922233|embl|CBL56805.1| ABC transporter [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1]
MRLARRVAAVLLASVLALTVASCAGAARSAPSL (SEQ ID NO:96 (amino acid sequence), SEQ ID NO: 189 (nucleotide sequence)).
- 10 >CBL57324.1 Hypothetical protein PFREUD_18170 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 97).
- >CBL57805.1 polar amino acid ABC transporter, binding protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 98).
- >CBL57413.1 Sortase family protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 99).
- 15 >CBL57271.1 Probable multidrug resistance transporter, MFS superfamily
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 100).
- >CBL57337.1 Hypothetical protein PFREUD_18280 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 101).
- 20 >gil296922302|embl|CBL56874.1| Putative peptidyl-prolyl cis-trans isomerase, FKBP-type (Precursor) [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] CIRM-BIA1 (SEQ ID NO: 102).
- >gil296923259|embl|CBL57856.1| Extracellular solute-binding protein precursor
- 25 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 103).
- >gil296923146|embl|CBL57733.1| Hypothetical protein PFREUD_22300
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 104).
- 30 >gil296921378|embl|CBL55931.1| Hypothetical protein PFREUD_03980
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 105).

gil296921927|embl|CBL56487.1| Hypothetical membrane protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 106).

gil296922947|embl|CBL57529.1| carboxypeptidase (serine-type D-Ala-D-Ala
5 carboxypeptidase) (D-alanyl-D-alanine-carboxypeptidase) [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 107).

>gil296923326|embl|CBL57926.1| ABC transporter, substrate-binding protein
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 108).

10

>gil296923322|embl|CBL57922.1| Hypothetical protein PFREUD_24060
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 109).

>gil296922617|embl|CBL57194.1| Hypothetical protein PFREUD_16830
15 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 110).

>gil296922915|embl|CBL57497.1| secreted glycosyl hydrolase [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 111).

20 >gil296921377|embl|CBL55930.1| Hypothetical protein PFREUD_03970
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 112).

>gil296922301|embl|CBL56873.1| peptidyl-prolyl cis-trans isomerase [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 113).

25

>gil296922866|embl|CBL57446.1| Hypothetical protein PFREUD_19530
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 114).

>gil296922346|embl|CBL56918.1| Hypothetical protein PFREUD_14190
30 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 115).

>gil296921344|embl|CBL55897.1| Hypothetical protein PFREUD_03630
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 116).

- >gil296922653|embl|CBL57230.1| Hypothetical protein PFREUD_17190
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 117).
- 5 >gil296921113|embl|CBL55660.1| extracellular protein without function
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 118).
- >gil296921687|embl|CBL56244.1| Hypothetical protein PFREUD_07130
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 119).
- 10 >gil296921593|embl|CBL56147.1| Hypothetical protein PFREUD_06170
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 120).
- >gil296922523|embl|CBL57096.1| Hypothetical protein PFREUD_15980
15 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 121).
- >gil296921018|embl|CBL55556.1| large surface protein A [Propionibacterium
freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 122).
- 20 >gil296922853|embl|CBL57433.1| Penicillin-binding protein
(Transglycosylase/transpeptidase) [Propionibacterium freudenreichii subsp. shermanii
CIRM-BIA1] (SEQ ID NO: 123).
- >gil296921190|embl|CBL55739.1| Hypothetical protein PFREUD_02240
25 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 124).
- >gil296922847|embl|CBL57427.1| multicopper oxidase [Propionibacterium freudenreichii
subsp. shermanii CIRM-BIA1] (SEQ ID NO: 125).
- 30 >gil296923003|embl|CBL57585.1| Regulator of chromosome condensation
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 126).

- >gil296923209|embl|CBL57803.1| polar amino acid ABC transporter, binding protein
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 127).
- >gil296923210|embl|CBL57804.1| polar amino acid ABC transporter, binding protein
5 component [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID
NO: 128).
- >gil296921884|embl|CBL56444.1| ABC-transporter metal-binding lipoprotein
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 129).
- 10 >gil296921649|embl|CBL56206.1| iron ABC transport system, solute-binding protein
precursor [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO:
130).
- 15 >gil296922500|embl|CBL57073.1| Peptidase M23B family / metalloendopeptidase
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 131).
- >gil296922061|embl|CBL56625.1| transporter [Propionibacterium freudenreichii subsp.
shermanii CIRM-BIA1] (SEQ ID NO: 132).
- 20 >gil296921977|embl|CBL56539.1| Hypothetical protein PFREUD_10150
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 133).
- >gil296921310|embl|CBL55863.1| Hypothetical protein PFREUD_03320
25 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 134).
- >gil296921967|embl|CBL56527.1| ABC transporter, substrate binding protein
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 135).
- 30 >gil296921196|embl|CBL55745.1| Hypothetical protein PFREUD_02300
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 136).

>gil296921666|embl|CBL56223.1| Hypothetical secreted protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO:137).

5 >gil296921230|embl|CBL55780.1| ABC transporter, binding lipoprotein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 138).

>gil296923102|embl|CBL57689.1| polar amino acid ABC transporter, binding protein component [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 139).

10

>gil296923079|embl|CBL57663.1| Sulfate-binding protein precursor [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 140).

15 >gil296921569|embl|CBL56123.1| solute binding protein of the ABC transport system [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 141).

>gil296922906|embl|CBL57488.1| Phosphate-binding transport protein of ABC transporter system [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 142).

20

>gil296920974|embl|CBL55511.1| membrane protein (s-layer) [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 143).

25 >gil296922551|embl|CBL57124.1| Hypothetical secreted protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 144).

>gil296923279|embl|CBL57879.1| penicillin-binding protein (peptidoglycan glycosyltransferase) [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 145).

30

>gil296921112|embl|CBL55659.1| ATP-binding region, ATPase-like:Histidine kinase, Histidine kinase A-like precursor [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 146)

>gil296922314|embl|CBL56886.1| Cobalt permease [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 147).

5 >gil296922614|embl|CBL57191.1| Hypothetical protein PFREUD_16800 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 148).

>gil296921959|embl|CBL56519.1| Secreted protease with a PDZ domain [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 149).

10

>gil296921835|embl|CBL56395.1| membrane protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 150).

15 >gil296921079|embl|CBL55620.1| secreted transglycosydase [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 151).

>gil296922047|embl|CBL56611.1| Leucine-, isoleucine-, valine-, threonine-, and alanine-binding protein [Precursor] (LIVAT-BP) [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 152).

20

>gil296922667|embl|CBL57244.1| Hypothetical protein PFREUD_17340 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 153).

25 >gil296922395|embl|CBL56967.1| Hypothetical protein PFREUD_14680 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 154).

>gil296921224|embl|CBL55774.1| Hypothetical secreted protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 155).

30 >gil296921129|embl|CBL55676.1| ABC transporter permease [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 156).

- >gil296921974|embl|CBL56536.1| Exopolysaccharide biosynthesis protein precursor (related to N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase precursor) [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 157).
- 5 >gil296921168|embl|CBL55717.1| Hypothetical protein PFREUD_02020 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 158).
- >gil296921340|embl|CBL55893.1| Hypothetical secreted protein [Propionibacterium
10 freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 159).
- >gil296923123|embl|CBL57710.1| Hypothetical membrane protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 160).
- 15 >gil296922282|embl|CBL56854.1| Hypothetical secreted protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 161).
- >gil296921324|embl|CBL55877.1| Hypothetical secreted protein [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 162).
- 20 >gil296922572|embl|CBL57145.1| ABC transporter glycine betaine [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 163).
- >gil296922949|embl|CBL57531.1| Hypothetical protein PFREUD_20380
25 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 164).
- >gil296921638|embl|CBL56194.1| ABC transport system component [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 165).
- 30 >gil296922507|embl|CBL57080.1| Hypothetical protein PFREUD_15820 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 166).

- >gil296921133|embl|CBL55680.1| Hypothetical protein PFREUD_01650
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 167).
- >gil296921157|embl|CBL55704.1| Hypothetical secreted protein [Propionibacterium
5 freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 168).
- >gil296921592|embl|CBL56146.1| ABC transporter-associated permease
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 169).
- 10 >gil296923131|embl|CBL57718.1| Hypothetical protein PFREUD_22150
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 170).
- >gil296921360|embl|CBL55913.1| Hypothetical outer membrane protein
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 171).
- 15 >gil296921416|embl|CBL55969.1| Hypothetical protein PFREUD_04350
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 172).
- >gil296923120|embl|CBL57707.1| Hypothetical protein PFREUD_22040
20 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 173).
- >gil296921006|embl|CBL55544.1| Hypothetical protein PFREUD_00440
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 174).
- 25 >gil296921415|embl|CBL55968.1| Carboxylic ester hydrolase [Propionibacterium
freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 175).
- >gil296922150|embl|CBL56718.1| ABC transporter substrate-binding protein
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 176).
- 30 >gil296921167|embl|CBL55716.1| Hypothetical secreted protein [Propionibacterium
freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 177).

>gil296923127|embl|CBL57714.1| Hypothetical protein PFREUD_22110
[Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 178).

>gil296921375|embl|CBL55928.1| Hypothetical protein PFREUD_03950
5 [Propionibacterium freudenreichii subsp. shermanii CIRM-BIA1] (SEQ ID NO: 179).

Example 3- Preparation of expression cassettes for expressing various polysaccharide degrading enzymes in PB, utilizing different combinations of signal peptides and promoters

The nucleotide sequences inserts of the various promoters and polysaccharide degrading enzymes were obtained using PCR reactions utilizing suitable primers (listed in Table 2) and a corresponding template. A Q5 High-Fidelity DNA Polymerase (New England Biolabs, M0491) was used for the PCR reaction, according to manufacture
15 instructions. PCR conditions were as follows: initial denaturation - 98°C for 1 minute, secondary denaturation - 98°C for 30 seconds, annealing -60°C for 30 seconds, elongation - 72°C for 50 seconds. PCR programs run for 30 cycles (secondary initiation step to elongation step). PCR products were tested on agarose gel and purified using Wizard PCR cleanup kit (Promega).

20

Example 3.1- Expression of Glucoamylase gil13795 from Saccharomycopsis fibuligera with a PFREUD_18290 promoter and a PFREUD_18290 signal peptide

The sequence of the promoter and signal peptide were amplified from P. freudenreichii using primers 1 and 2 (Table 2, below). The glucoamylase was amplified
25 using primers 3 and 4 (Table 2). All glucoamylases were synthesized with an addition of 21 nucleotides after the stop codon, to enable the use of a shared reverse primer for their amplification. (primer 4). The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 33.

30 **Example 3.2 -** Expression of Glucoamylase gi30025851 from Aspergillus niger combined with a PFREUD_18290 promoter and a PFREUD_18290 signal peptide:

The sequence of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 1 and 5 (Table 2). The glucoamylase was amplified using primers 6 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 34.

5

Example 3.3 - Expression of Glucoamylase gi231542 from *Clostridium* SP.5 G000 combined with a PFREUD_18290 promoter and a PFREUD_18290 signal peptide:

The sequence of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 1 and 7 (Table 2). The glucoamylase was amplified using primers 8 and 4.

10 The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 35.

Example 3.4- Expression of Glucoamylase gi490533596 from *Clostridium thermohydrosulfuricum* combined with a PFREUD_18290 promoter and a PFREUD_18290 signal peptide:

15

The sequence of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 1 and 9 (Table 2). The glucoamylase was amplified using primers 10 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 36.

20

Example 3.5- Expression of Glucoamylase gi34146785 from *Clostridium thermoamylolyticum* combined with a PFREUD_18290 promoter and a PFREUD_18290 signal peptide:

The sequence of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 1 and 11 (Table 2). The glucoamylase was amplified using primers 12 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 37.

25

Example 3.6- Expression of Glucoamylase gi48431212 from *Picrophilus torridus* DSM 9790 combined with a PFREUD_18290 promoter and a PFREUD_18290 signal peptide:

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The sequence of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 1 and 13 (Table 2). The glucoamylase was amplified using primers 14 and

4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 38.

Example 3.7- Expression of Glucoamylase gi113795 from *Saccharomycopsis fibuligera* combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 15 and 16 (Table 2). The glucoamylase was amplified using primers 3 and 4. All glucoamylases were synthesized with an addition of 21 nucleotides after the stop codon, to enable the use of a shared reverse primer for their amplification. (primer 4). The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 39.

Example 3.8- Expression of Glucoamylase gi30025851 from *Aspergillus niger* combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide was amplified from *P. freudenreichii* using primers 15 and 17 (Table 2). The glucoamylase was amplified using primers 6 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 40.

Example 3.9- Expression of Glucoamylase gi231542 from *Clostridium* SP.5 G000 combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequences of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 15 and 18 (Table 2). The glucoamylase was amplified using primers 8 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 41.

Example 3.10- Expression of Glucoamylase gi490533596 from *Clostridium thermohydrosulfuricum* combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequences of the promoter and signal peptide were amplified from *P. freudenreichii* using primers 15 and 19 (Table 2). The glucoamylase was amplified using primers 10 and

4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 42.

Example 3.11- Expression of Glucoamylase gi34146785 from *Clostridium thermoamylolyticum* combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide was amplified from *P. freudenreichii* using primers 15 and 20 (Table 2). The glucoamylase was amplified using primers 12 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 43.

Example 3.12- Expression of Glucoamylase gi48431212 from *Picrophilus torridus* DSM 9790 combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide was amplified from *P. freudenreichii* using primers 15 and 21 (Table 2). The glucoamylase was amplified using primers 14 and 4. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 44.

Example 3.13- Expression of Glucoamylase gi30025851 from *Aspergillus niger* combined with a PFREUD_08450 promoter and a PFREUD_04850 signal peptide:

The sequence of PFREUD_08450 promoter was amplified from *P. freudenreichii* using primers 22 and 23 (Table 2). The amplified oligo sequence is cloned using restriction free cloning method into a pOWR3 vector already containing a PFREUD_04850 signal peptide and the glucoamylase sequence (from example 3.8). The resulting vector is inserted and amplified in DH5alpha bacteria. Then transferred to dam⁻dcm⁻ *E. coli* strain, propagated and purified, to produce non-methylated vector. The DNA sequence of the insert is as denoted by SEQ ID NO: 45.

Example 3.14- Expression of glucoamylase gi30025851 from *Aspergillus niger* combined with a PFREUD_04850 promoter and PFREUD_18250 signal peptide.

The sequence of PFREUD_18250 signal peptide was amplified from *P. freudenreichii* using primers 24 and 25 (Table 2). The amplified oligo sequence is cloned using

restriction free cloning method into a pOWR3 vector already containing a PFREUD_04850 promoter and the glucoamylase sequence (from example 3.8). The resulting vector is inserted and amplified in DH5 α bacteria. Then transferred to dam⁻ E.coli strain, propagated and purified, to produce non-methylated vector. The

5 DNA sequence of the insert is as denoted by SEQ ID NO: 46.

Example 3.15- Expression of Xylanase EOR70069-F from *Thermobifida fusca* TM51 combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide was amplified from *P. freudenreichii*

10 using primers 15 and 26 (Table 2). The Xylanase was amplified using primers 27 and 28. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 47.

Example 3.16- Expression of Cellulase P26222 from *Thermomonospora fusca* combined

15 with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide was amplified from *P. freudenreichii* using primers 15 and 29 (Table 2). The glucoamylase was amplified using primers 30 and 31. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted

by SEQ ID NO: 48.

20 **Example 3.17-** Expression of Cellulase WP_015924277.1 from *Clostridium cellulolyticum* combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide:

The sequence of the promoter and signal peptide was amplified from *P. freudenreichii*

25 using primers 15 and 32 (Table 2). The glucoamylase was amplified using primers 33 and 34. The DNA sequence of the expression cassette (i.e., insert) thus obtained is as denoted by SEQ ID NO: 49.

Table 2. Primers

#	PCR primer		Nucleotide Sequence
1	P18290-F	Forward	GATCGGCACGTAAGAGGTTCCAACCTTTCACCCCGAG GTCCACACGCCGG (SEQ ID NO: 50)

2	P18290-R1	Reverse	GGC GCG CTT GTT CAG GGG GCC CTC CTG CAG GGC ATT CGC AGG CGC CAT G (SEQ ID NO: 51)
3	seq1-F	Forward	CTGCAGGAGGGCCCCCTGAACAAGCGCGCC (SEQ ID NO: 52)
4	Amylase -R	Reverse	CTA TTT CGT TCA TCC ATA GTT GCC TGA CTC ATG GGA TTC AGT TGT AGG TG (SEQ ID NO: 53)
5	p18290-R2	Reverse	CCA CGA GTC CCA GGT GGC ACG CTT CGA GAT GGC ATT CGC AGG CGC CAT G (SEQ ID NO: 54)
6	seq2-F	Forward	ATCTCGAAGCGTGCCACCTGGGACTCGTGG (SEQ ID NO: 55)
7	p18290-R3	Reverse	CAC GGC GGA GAT GTT GTT GAA GCG GTC GAT GGC ATT CGC AGG CGC CAT G (SEQ ID NO: 56)
8	seq3-F	Forward	ATCGACCGCTTCAACAACATCTCCGCCGTG (SEQ ID NO: 57)
9	pP18290-R4	Reverse	CTT GAT CGA CTT CTC GAC CAG GTT CTC CAT GGC ATT CGC AGG CGC CAT G (SEQ ID NO: 58)
10	seq4-F	Forward	ATGGAGAACCTGGTCGAGAAGTCGATCAAG (SEQ ID NO: 59)
11	p18290-R5	Reverse	CAG GCG CTC GAT CTT GAT GTT GGA GAT GTT GGC ATT CGC AGG CGC CAT G (SEQ ID NO: 60)
12	seq5-F	Forward	AACATCTCCAACATCAAGATCGAGCGCCTG (SEQ ID NO: 61)
13	p18290-R7	Reverse	GCC GTT GCC CAT GGG GAT GTA GCG CAC CAT GGC ATT CGC AGG CGC CAT G (SEQ ID NO: 62)
14	seq7-F	Forward	ATGGTGCGCTACATCCCCATGGGCAACGGC (SEQ ID NO: 63)
15	p04850-F	Forward	GATCGGCACGTAAGAGGTTCCAACCTTTCACCCCACC GTGAGCTGCACCT (SEQ ID NO: 64)
16	p04850-R1	Reverse	GGC GCG CTT GTT CAG GGG GCC CTC CTG CAG GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 65)
17	p04850-R2	Reverse	CCA CGA GTC CCA GGT GGC ACG CTT CGA GAT GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 66)
18	p04850-R3	Reverse	CAC GGC GGA GAT GTT GTT GAA GCG GTC GAT GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 67)
19	p04850-R4	Reverse	CTT GAT CGA CTT CTC GAC CAG GTT CTC CAT GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 68)
20	p04850-R5	Reverse	CAG GCG CTC GAT CTT GAT GTT GGA GAT GTT GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 69)
21	p04850-R7	Reverse	GCC GTT GCC CAT GGG GAT GTA GCG CAC CAT GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 70)
22	P08450-F	Forward	GATCGGCACGTAAGAGGTTCCAACCTTTCACCTCGGTC GGGTCCGGACGGG (SEQ ID NO: 71)
23	P08450-R	Reverse	CAC GCG CGC CGT CTT GAC GAA GGG ATT CAC

			GTA GTA GGG CTC CGA TGT TGG ATG (SEQ ID NO: 72)
24	SP18250-F	Forward	CGGCCGCGGGAAGGACCAGACATGCTCACTCGCAAG AGAGTGG (SEQ ID NO: 73)
25	SP18250-R	Reverse	CCAGGTGGCACGCTTCGAGATGGGGCCGGTGGCGGC GCTG (SEQ ID NO: 74)
26	SeqX-SP	Reverse	GTT CTG GGC GGC CAG CTC GCG CAG GGT CGA CTC GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 75)
27	SeqX-F	Forward	GAGTCGACCCTGCGCGAGCTGGCCGCCCAGAAC (SEQ ID NO: 76)
28	seqX-R	Reverse	TCT ATT TCG TTC ATC CAT AGT TGC CTG ACT CGA GCA GGT GGC GCC GTT CAG GGT G (SEQ ID NO: 77)
29	SeqY-SP	Reverse	CAT GTT GGG GTT CAC GTA GAA GGG CGA GTC GTT GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 78)
30	SeqY-F	Forward	AACGACTCGCCCTTCTACGTGAACCCCAACATG (SEQ ID NO: 79)
31	SeqY-R	Reverse	TCT ATT TCG TTC ATC CAT AGT TGC CTG ACT CGA GGC GGC GCA GGT CAG GGT GGG C (SEQ ID NO: 80)
32	seqZ-SP	Reverse	CTG CAG GGC CTC GCC GTA GTT GTA GGT GCC GGC GGC CTG TGC AAG TGG TGC AAA C (SEQ ID NO: 81)
33	seqZ-F	Forward	GCCGGCACCTACAACCTACGGCGAGGCCCTGCAG (SEQ ID NO: 82)
34	SeqZ-R	Reverse	TCT ATT TCG TTC ATC CAT AGT TGC CTG ACT GCC CTG GGC CAG CTG GGT GAT (SEQ ID NO: 83)

Example 4 - Cloning expression cassettes (inserts) into pOWR3 vector, expression in *P. freudenreichii*, and examining polysaccharide degrading activity in various substrates

5 The various amplified nucleotides sequences (expression cassettes) of example 3 (examples 3.1-3.17), were cloned into the pOWR3 vector using restriction free cloning method (Peleg Y, Unger T. "Application of the Restriction-Free (RF) cloning for multicomponents assembly". Methods Mol Biol. 2014; 1116:73-87). The resulting vector was inserted and amplified in DH5alpha bacteria and then transferred to dam\dcn- E.coli
10 strain, propagated and purified, to produce non-methylated vector.

The purified vector was inserted into *P. freudenreichii* using the electroporation process as described in Example 1. The resulting recombinant *P. freudenreichii* is examined for its ability to express and secrete polysaccharide degrading enzymes, and the resulting activity in degrading a suitable saccharide (such as, starch, cellulose and xylan),

Examining starch degrading activity in various substrates- The resulting recombinant *P. freudenreichii* is examined for its ability to express and secrete glucoamylase, and the resulting glucoamylase activity of degrading starch.

The recombinant *P. freudenreichii* colony is transferred to agar plates containing
5 YEL media (gr/l: 10 yeast extract, 10 peptone, 10 lactate) and 0.5% starch, for overnight culture. Degradation of starch is detected by pouring Lugol solution on the agar plate to visualize the starch.

In the presence of starch, the Lugol forms black precipitate. In the presence of glucoamylase the starch is degraded and colorless halo is observed. A control Lugol assay
10 is demonstrate in **Fig. 3**, which show an agar plate containing YEL media and 0.5% starch that is exposed to Lugol solution. All the areas containing starch are visualized in black, while a colorless circle, indicating that no starch is present can be seen in the area in which glucoamylase enzyme has diffused in the agar plate.

Additionally, the resulting recombinant (genetically engineered) *P. freudenreichii*
15 is transferred to liquid YEL media (gr/l:10 yeast extract, 10 peptone, 10 lactate) containing 0.5-5% starch, for overnight culture in 30°C-37°C, followed by culture in 55°C for 2 hours. First, 0.5 mL of the post-incubations culture is tested by a colorimetric assay for its glucose content. In some cases, the assay is compared to a control culture of wild-type (non-modified) *P. freudenreichii* culture treated under the same conditions and/or to the
20 same media, which does not include added bacteria. The secreted glucoamylase degrades the starch in the media and produces glucose that can be detected by a colorimetric method. Second, a similar assay is done, in which the post-incubations medium is being centrifuged and only the supernatant is examined for its glucose content. Further, the activity of the glucoamylase activity under optimal conditions is tested: 0.1 mL of the
25 overnight culture is incubated with 0.4 mL of 1% starch solution for 2 hours, after which the tube is centrifuged and the supernatant is tested for its glucose content by a colorimetric method.

Additionally, 10^8 CFU/ml of the recombinant *P. freudenreichii* is inoculated into a shredded organic waste (pH 5.5) for overnight culture in 30°C-37°C, followed by culture
30 in 55°C for about 2 hours. 0.5 mL of the overnight culture is centrifuged and the supernatant is tested for its glucose content by a colorimetric method.

Example 4.1- Expression of Glucoamylase gi34146785 from *Clostridium thermoamylolyticum* combined with a PFREUD_04850 promoter and a PFREUD_04850 signal peptide in *P. freudenreichii*, and examination of its starch degrading activity.

The expression cassette described in example 3.11 (SEQ ID NO: 43), was cloned
5 into the pOWR3 vector and transformed into *P. freudenreichii* as described above.

The resulting recombinant *P. freudenreichii* was examined for its ability to express and secrete the Glucoamylase, and to test the glucoamylase activity of degrading starch.

10^6 - 10^9 CFU/ml of the resulting recombinant (genetically engineered) *P. freudenreichii*, or of corresponding wild-type bacteria (*P. freudenreichii* control), were
10 transferred to liquid YEL media (gr/l:10 yeast extract, 10 peptone, 10 lactate) containing starch, for culture in 30-37°C, followed by additional culture in 55°C.

After the incubation periods, the cultures were verified for similar CFU content in the recombinant and control *P. freudenreichii* (for each temperature and/or CFU/ml bacteria). Each culture was tested by a colorimetric assay for its glucose content. A significant
15 increase (over 35 fold) in glucose content was observed in the recombinant *P. freudenreichii* treated media, compared to the wild-type *P. freudenreichii* control.

These results indicate that the genetically engineered bacteria can successfully express and secrete an active polysaccharide degrading enzyme (Glucoamylase in this example) and that the secreted enzyme is active, as it is able to successfully degrade starch in the
20 substrate to produce glucose.

Example 5- Sequential lactic acid utilization and saccharification of organic waste

The LA-utilizing bacteria are used to utilize lactic acid and saccharify polysaccharide in organic waste, in a sequential mode of operation.

25 Grinding of mixed food waste is performed in an Optimum Commercial Blender in multiple batches that include 1000 gr food waste in 1000 ml of water. A semi-solid mixture of 10 L (working volume) is introduced into the fermenter (New Brunswick 15L capacity). pH was measured at 5.2.

In-situ sterilization of the media (by autoclaving) is performed for 30 minutes at
30 121 °C, with media agitation at 200 rpm. The fermenter is then cooled to 37°C and temperature is maintained. Airflow is kept in head space to maintain positive pressure in the fermenter.

Lactic acid utilizing bacteria Starter preparation - inoculum for fermentation and lactic acid utilization by lactic acid utilizing bacteria (in this example, *Propionibacterium freudenreichii* (ATCC 9614)) is performed in a 1L Shake flask, in Yeast-Extract Lactate (YEL) medium at low 100 rpm at 37°C. The cells are grown up to cell density of 1×10^8 .

5 Fermentation - 15ml is inoculated for 10.8L fermentation, at 37°C, pH at 5.5 (controlled by 10% ammonia solution), until substantially complete utilization of lactic acid present in the media. Measurements of lactic acid and total glucose and fructose is performed. Broth sample are centrifuged at 6000g for 10 min in 15°C and supernatant is analyzed in a Reflectoquant analysis system (RQflex plus 10).

10 The fermenter is then heated to 55 °C for two hours to allow optimal conditions for activity of the polysaccharide degrading enzyme secreted by the bacteria (for example, Glucoamylase) and to allow sterilization of the bacteria (*Propionibacterium freudenreichii*). Following saccharification, the fermenter is cooled to 37 °C, to allow further processing and production of discrete enantiomers of Lactic acid, by utilizing lactic acid producing bacteria (LAB).

15 LAB starter preparation - inoculum for L-lactate fermentation by *Lactobacillus rhamnosus* (ATCC 11443): 1 Liter Shake flask, conditions include MRS medium at 100 rpm agitation at 37°C. Cells are grown to a density of 1×10^9 . 15ml of starter culture in inoculated for 10 L fermentation. Control temperature at 37 °C, and pH at 6.5 by ammonia solution (10 %). L-lactate fermentation is terminated as glucose is depleted from the media, typically after 24 to 48 hours.

Example 6 - Concomitant utilization of lactic acid and saccharification of organic waste

25 The LA-utilizing bacteria are used to concomitantly utilize lactic acid and saccharify polysaccharides in organic waste.

Grinding of mixed food waste is performed in an Optimum Commercial Blender in multiple batches that include 1000 gr food waste in 1000 ml of water. A semi-solid mixture of 10 L (working volume) is introduced into the fermenter (New Brunswick 15L capacity). pH is measured at 5.2.

30

In-situ sterilization of the media (by autoclaving) is performed for 30 minutes at 121 °C, with media agitation at 300 rpm. The fermenter is then cooled to 37°C and temperature is maintained.

Lactic Acid utilizing bacteria Starter preparation - inoculum for fermentation and
5 lactic acid utilization by lactic acid utilizing bacteria (in this example, *Propionibacterium freudenreichii* (ATCC 9614)) is performed in a 1L Shake flask, in Yeast-Extract Lactate (YEL) medium at low 100 rpm at 37°C. The cells are grown up to cell density of 1×10^8 .

Fermentation - 15ml is inoculated for 10.8L fermentation, at 37°C, pH at 5.5 (controlled by 10% ammonia solution), until substantially complete utilization of lactic
10 acid presented in the media concomitant with saccharification of polysaccharide by the polysaccharide degrading enzyme secreted by the bacteria (for example, Glucoamylase). Measurements of lactic acid and total glucose and fructose is performed. Broth sample are centrifuged at 6000g for 10 min in 15°C and supernatant is analyzed in a Reflectoquant analysis system (RQflex plus 10).

15 The fermenter is heated to 55 °C for two hours to allow sterilization of the bacteria (*Propionibacterium freudenreichii*).

Thereafter, the fermenter is cooled to 37 °C, to allow further processing and production of discrete enantiomers of Lactic acid, by utilizing lactic acid producing bacteria (LAB).

20 LAB starter preparation - inoculum for L-lactate fermentation by *Lactobacillus rhamnosus* (ATCC 11443): 1 Liter Shake flask conditions include MRS medium at 100 rpm agitation at 37°C. Cells are grown to density of 1×10^9 . 15ml of starter culture is inoculated for 10 L fermentation. Control temperature at 37 °C, and pH at 6.5 by ammonia solution (10 %). L-lactate fermentation is terminated as glucose is depleted from the
25 media, typically after 24 to 48 hours.

CLAIMS

1. A lactic acid (LA)-utilizing bacterium genetically modified to express and secrete one or more exogenous polysaccharide-degrading enzyme, wherein the one or more
5 exogenous polysaccharide-degrading enzyme comprises a cellulase, a hemicellulase, an amylase or combinations thereof.
2. The LA-utilizing bacterium of claim 1, wherein the polysaccharide-degrading enzyme is a bacterial enzyme.
3. The LA-utilizing bacterium of claim 2, wherein the bacterial polysaccharide-
10 degrading enzyme is from a thermophilic bacterium.
4. The LA-utilizing bacterium of claim 3, wherein the thermophilic bacterium is selected from the group consisting of *Clostridium* sp., *Paenibacillus* sp., *Thermobifida fusca*, *Bacillus* sp., *Geobacillus* sp., *Chromohalobacter* sp. and *Rhodothermus marinus*.
5. The LA-utilizing bacterium of claim 2, wherein the bacterial polysaccharide-
15 degrading enzyme is from a mesophilic bacterium.
6. The LA-utilizing bacterium of claim 5, wherein the mesophilic bacterium is selected from the group consisting of *Klebsiella* sp., *Cohnella* sp., *Streptomyces* sp, *Acetivibrio cellulolyticus*, *Ruminococcus albus*; *Bacillus* sp. and *Lactobacillus fermentum*.
7. The LA-utilizing bacterium of claim 1, wherein the polysaccharide-degrading
20 enzyme is a fungal enzyme.
8. The LA-utilizing bacterium of claim 7, wherein the fungal enzyme is from fungi selected from the group consisting of *Trichoderma reesei*, *Hemicola insolens*, *Fusarium oxysporum*, *Aspergillus oryzae*, *Penicillium fellutanum* and *Thermomyces lanuginosu*.
9. The LA-utilizing bacterium of claim 1, wherein the polysaccharide-degrading
25 enzyme has suitable activity at the same temperature and pH range suitable for growth of the LA-utilizing bacterium.

10. The LA-utilizing bacterium of claim 1, wherein the polysaccharide-degrading enzyme has suitable activity at a temperature higher than the suitable growth temperature of the LA-utilizing bacterium.

11. The LA-utilizing bacterium of claim 1, wherein the polysaccharide-degrading
5 enzyme has suitable activity at a pH higher or lower than the suitable growth pH of the LA-utilizing bacterium.

12. The LA-utilizing bacterium of claim 1, wherein the LA-utilizing bacterium is selected from the group consisting of *Propionibacterium* species, *Megasphaera* species, *Selenomonas* species and *Veillonella* species.

10 13. The LA-utilizing bacterium of claim 12, wherein the LA-utilizing bacterium is a *Propionibacterium* species.

14. The LA-utilizing bacterium of claim 13, wherein the *Propionibacterium* species is *Propionibacterium freudenreichii*.

15 15. A population of lactic-acid (LA)-utilizing bacteria genetically modified to express and secrete a plurality of exogenous polysaccharide-degrading enzymes comprising a cellulase, a hemicellulase, an amylase or combinations thereof, wherein the population comprises a plurality of sub-populations of LA-utilizing bacteria, each sub-population being genetically modified to express and secrete a different polysaccharide-degrading enzyme.

20 16. The population of LA-utilizing bacteria of claim 15, wherein the polysaccharide-degrading enzymes are bacterial enzymes.

17. The population of LA-utilizing bacteria of claim 16, wherein the polysaccharide-degrading enzymes are from thermophilic bacteria.

25 18. The population of LA-utilizing bacteria of claim 17, wherein the thermophilic bacteria are selected from the group consisting of *Clostridium* sp., *Paenibacillus* sp., *Thermobifida fusca*, *Bacillus* sp., *Geobacillus* sp., *Chromohalobacter* sp. and *Rhodothermus marinus*.

19. The population of LA-utilizing bacteria of claim 16, wherein the polysaccharide-degrading enzymes are from mesophilic bacteria.

20. The population of LA-utilizing bacteria of claim 19, wherein the mesophilic bacteria are selected from the group consisting of *Klebsiella* sp., *Cohnella* sp.,
5 *Streptomyces* sp, *Acetivibrio cellulolyticus*, *Ruminococcus albus*; *Bacillus* sp. and *Lactobacillus fermentum*.

21. The population of LA-utilizing bacteria of claim 15, wherein the polysaccharide-degrading enzymes are fungal enzymes.

22. The population of LA-utilizing bacteria of claim 21, wherein the fungal enzyme
10 is from fungi selected from the group consisting of *Trichoderma reesei*, *Humicola insolens*, *Fusarium oxysporum*, *Aspergillus oryzae*, *Penicillium fellutanum* and *Thermomyces lanuginosu*.

23. The population of LA-utilizing bacteria of claim 15, wherein the sub-populations are sub-populations of the same bacterial species.

15 24. The population of LA-utilizing bacteria of claim 15, wherein the sub-populations are sub-populations of different bacterial species.

25. The population of LA-utilizing bacteria of claim 15, wherein the LA-utilizing bacteria are selected from *Propionibacterium* species.

20 26. The population of LA-utilizing bacteria of claim 25, wherein the *Propionibacterium* species is *Propionibacterium freudenreichii*.

27. A method for processing organic waste, the method comprising contacting the organic waste with the LA-utilizing bacterium of claim 1, or with the population of lactic acid utilizing bacteria of claim 15, under conditions in which lactic acid is consumed by the LA-utilizing bacteria and which are suitable for expression and activity of the
25 polysaccharide-degrading enzymes, wherein said processing eliminates D-lactic acid, L-lactic acid or both from the organic waste and degrades polysaccharides in the waste to release soluble reducing sugars.

28. The method of claim 27, wherein elimination of lactic acid from the waste is effected concomitant with degradation of polysaccharides into soluble reducing sugars.

29. The method of claim 27, wherein elimination of lactic acid from the waste is effected prior to degradation of polysaccharides into soluble reducing sugars.

5 **30.** The method of claim 29, wherein the polysaccharide-degrading enzymes have suitable activity at a temperature higher than the suitable growth temperature of the LA-utilizing bacteria, and wherein the method comprises: (i) contacting the organic waste with the LA-utilizing bacteria at a first temperature, the first temperature being suitable for growth of the LA-utilizing bacteria and expression of the polysaccharide-degrading
10 enzymes, for sufficient time to eliminate D-lactic acid, L-lactic acid or both from the waste; and (ii) increasing the temperature to a second temperature, the second temperature being suitable for activity of the polysaccharide-degrading enzymes.

31. The method of claim 29, wherein the polysaccharide-degrading enzymes have suitable activity at a pH higher or lower than the suitable growth pH of the LA-utilizing
15 bacteria, and wherein the method comprises: (i) contacting the organic waste with the LA-utilizing bacteria at a first pH, the first pH being suitable for growth of the LA-utilizing bacteria and expression of the polysaccharide-degrading enzymes, for sufficient time to eliminate D-lactic acid, L-lactic acid or both from the waste; and (ii) adjusting the pH to a second pH, the second pH being suitable for activity of the polysaccharide-degrading
20 enzymes.

32. The method of claim 27, further comprising determining the percentage of at least one of starch, cellulose and hemicellulose in the organic waste prior to said contacting, and selecting the LA-utilizing bacterium or the population of LA-utilizing bacteria to be contacted with the organic waste according to the determined percentage.

25 **33.** The method of claim 32, wherein said determining further comprises determining the percentage of soluble reducing sugars.

34. The method of claim 27, wherein said processing is performed in one vessel.

35. A method for producing discrete lactic acid enantiomer from organic waste, the method comprising:

(i) processing the organic waste to eliminate D-lactic acid, L-lactic acid or a combination thereof present in the waste and degrade polysaccharides in the waste to release soluble reducing sugars, by contacting the organic waste with the LA-utilizing bacterium of claim 1, or with the population of LA-utilizing bacteria of claim 15, under conditions suitable for lactic acid consumption by the LA-utilizing bacteria and for expression and activity of the polysaccharide-degrading enzymes;

(ii) inactivating the LA-utilizing bacteria;

(iii) fermenting the soluble reducing sugars obtained in (i) with a lactic acid-producing microorganism that produces only one of D-lactic acid and L-lactic acid, to obtain discrete lactic acid enantiomer; and

(iv) recovering the discrete lactic acid enantiomer from the fermentation broth.

36. The method of claim 35, wherein the elimination of the D-lactic acid, L-lactic acid or a combination thereof present in the waste is performed concomitantly or sequentially with the degradation of the polysaccharides in the waste.

37. A method for producing polylactic acid from organic waste, the method comprising producing discrete lactic acid enantiomer by the method of claim 35, and further comprising polymerizing the discrete lactic acid enantiomer to polylactic acid.

38. An expression vector for expressing and secreting an exogenous polysaccharide-degrading enzyme by a *Propionibacterium*, said vector comprising:

at least one nucleotide sequence encoding for a *Propionibacterium* replication protein;

at least one *Propionibacterium* origin of replication nucleotide sequence; and an at least one expression cassette comprising:

at least one promoter nucleic acid sequence, which is operably linked to:

at least one nucleic acid sequence encoding for a *Propionibacterium* signal peptide (SP), which is translationally fused to:

at least one nucleic acid sequence encoding a polysaccharide degrading enzyme, selected from cellulase, hemicellulase, and amylase.

39. The expression vector of claim 38, wherein the nucleotide sequence encoding for a *Propionibacterium* replication protein comprises the nucleic acid sequence of SEQ ID NO: 84.

40. The expression vector of claim 38, wherein the promoter nucleic acid sequence is selected from the group consisting of SEQ ID NOs: 27-32 and homologs thereof.

41. The expression vector of claim 38, wherein the SP amino acid sequence is selected from the group consisting of sequence of SEQ ID NOs: 87-179.

42. The expression vector of claim 38, wherein the polysaccharide degrading enzyme sequence is selected from the group consisting of SEQ ID NOs: 14-26, and homologs thereof.

43. The expression vector of claim 38, wherein the expression cassette comprises a nucleic acid sequence as denoted by any one of SEQ ID NOs: 33-49, or homologs thereof.

44. The expression vector of claim 38, wherein the expression cassette consists of a nucleic acid sequence as denoted by any one of SEQ ID NOs: 33-49.

45. A *Propionibacterium* lactic-acid (LA)-utilizing bacteria comprising the expression vectors of any one of claims 38-44.

Fig. 1

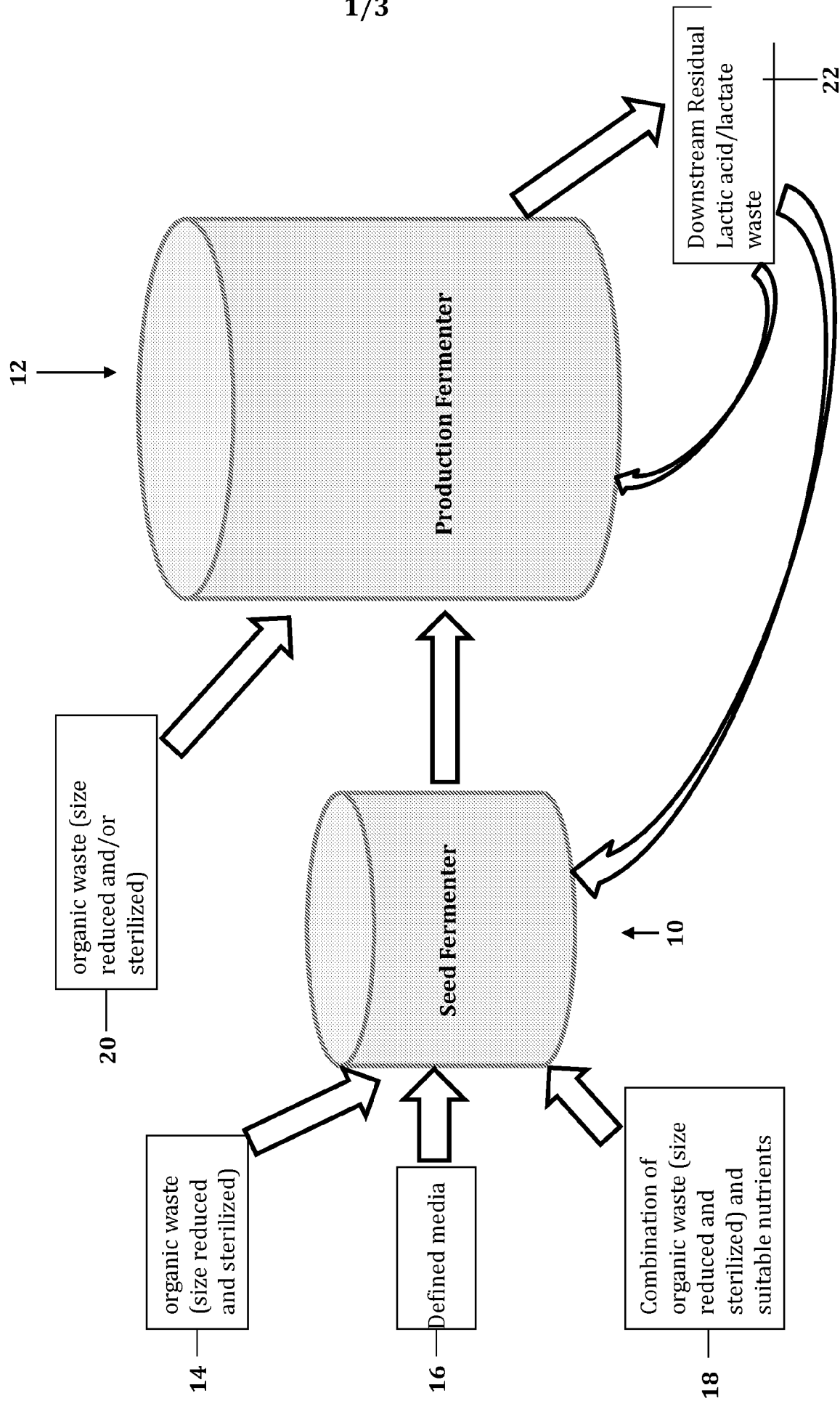


Fig. 2A

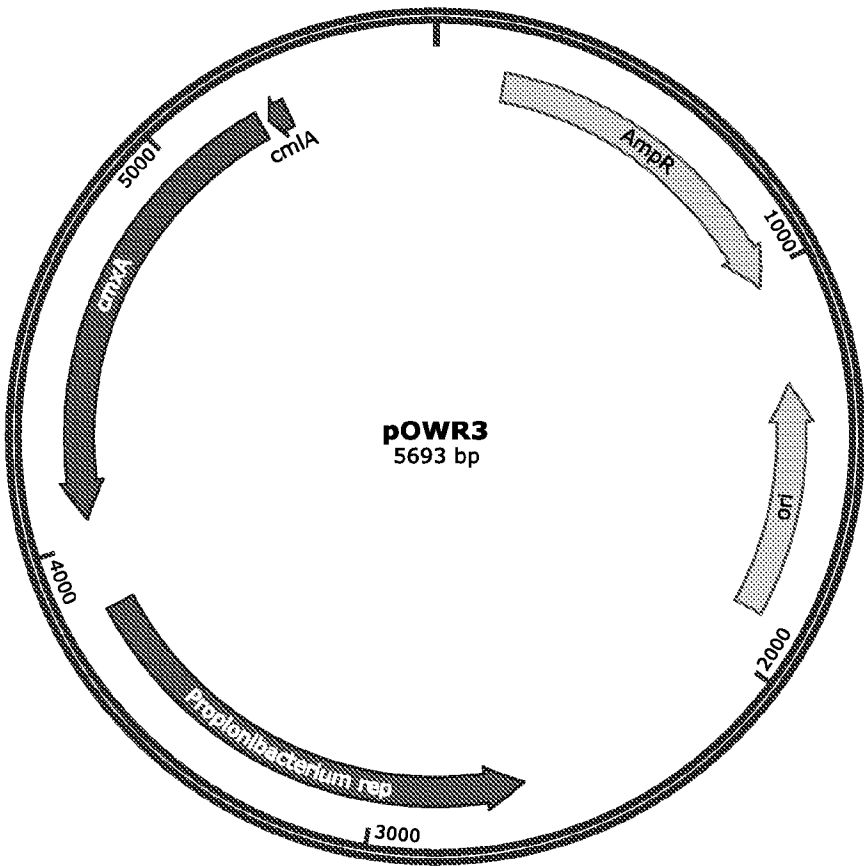


Fig. 2B

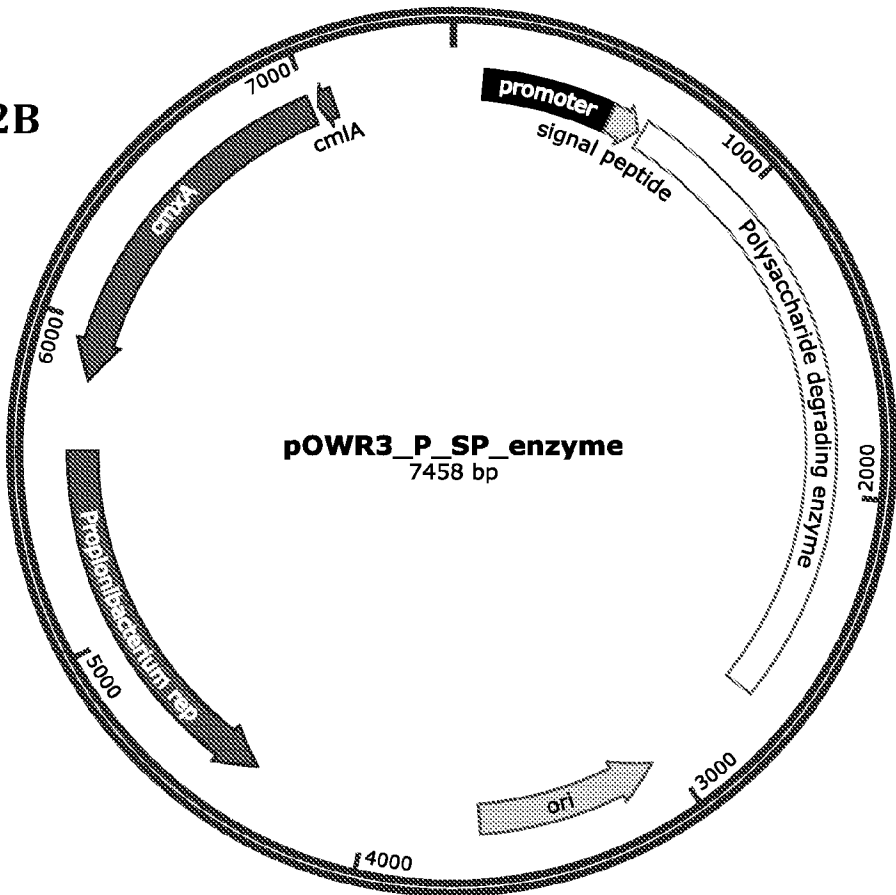
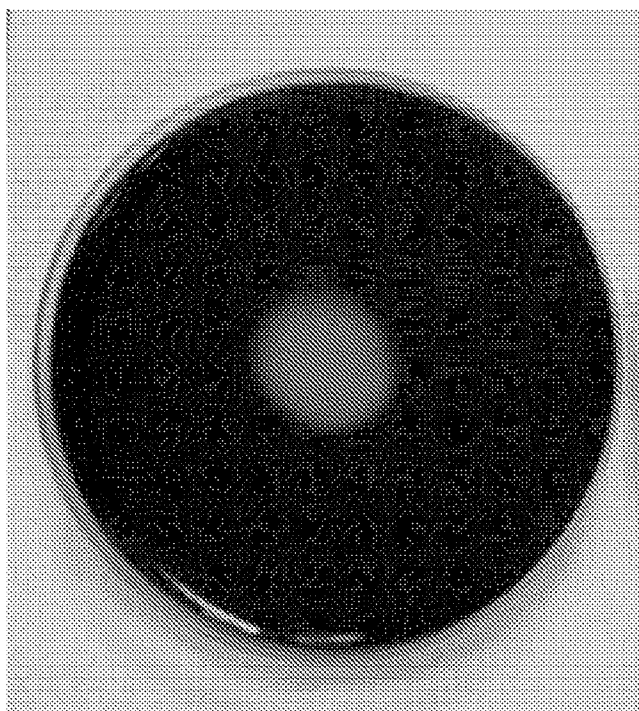


Fig. 3



INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050031

A. CLASSIFICATION OF SUBJECT MATTER IPC (2017.01) C12N 1/21, C12N 1/22, C12N 9/28, C12N 9/42, C12N 15/31, C12N 15/56, C12N 15/74 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 (2017.01) C12N 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) See extra sheet.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BREDE, Dag Anders, et al. Heterologous production of antimicrobial peptides in <i>Propionibacterium freudenreichii</i> . Applied and environmental microbiology, 2005, 71.12: 8077-8084.DOI: 10.1128/AEM.71.12.8077-8084.2005. http://aem.asm.org/content/71/12/8077.full.pdf+html 31 Dec 2005 (2005/12/31) the whole document	27-37
X	The whole document	1-26,38-45
Y	WO 2015011250 A1 INSTITUT NATIONAL DE LA RECHERCHE AGRONOMIQUE [RE/FR]; 29 Jan 2015 (2015/01/29) page 1 lines 1-15, page 3 lines 15-27, page 13 line 31 - page 14 line 11, page 27 line 8 - page 34 line 25	27-37
X	Page 1 lines 1-15, page 3 lines 15-27, page 13 line 31 - page 14 line 11, page 27 line 8 - page 34 line 25	1-26,38-45
☒ Further documents are listed in the continuation of Box C. ☒ 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 15 Mar 2017		Date of mailing of the international search report 15 Mar 2017
Name and mailing address of the ISA: Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616		Authorized officer AXELRAD Itschak Telephone No. 972-2-5651648

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050031

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAKAI, Kenji, et al. Making plastics from garbage. Journal of industrial ecology, 2003, 7.3?4: 63-74.DOI: 10.1162/108819803323059406 http://onlinelibrary.wiley.com/doi/10.1162/108819803323059406/epdf 31 Jul 2017 (2017/07/31) abstract, Fig 1, paragraph bridges pages 66 and 67, table 1, page 70 left column lines 1 - 2	27-37

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IL2017/050031

B. FIELDS SEARCHED:

* Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases consulted: NCBI, THOMSON INNOVATION, CAPLUS, BIOSIS, EMBASE, MEDLINE, PubMed, Google Scholar, DWPI

Search terms used: Lactic acid utilizing bacteria, cellulase, hemicellulase, amylase, vector, secrete, Propionibacterium, Propionibacterium freudenreichii, Garbage, plastic, lactate, plasmid, Megasphaera, Veillonella, modifi, Shapira, Oranim, 3PLW

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/IL2017/050031

Patent document cited search report			Publication date	Patent family member(s)			Publication Date
WO	2015011250	A1	29 Jan 2015	WO	2015011250	A1	29 Jan 2015
				EP	2829274	A1	28 Jan 2015
				EP	3024469	A1	01 Jun 2016