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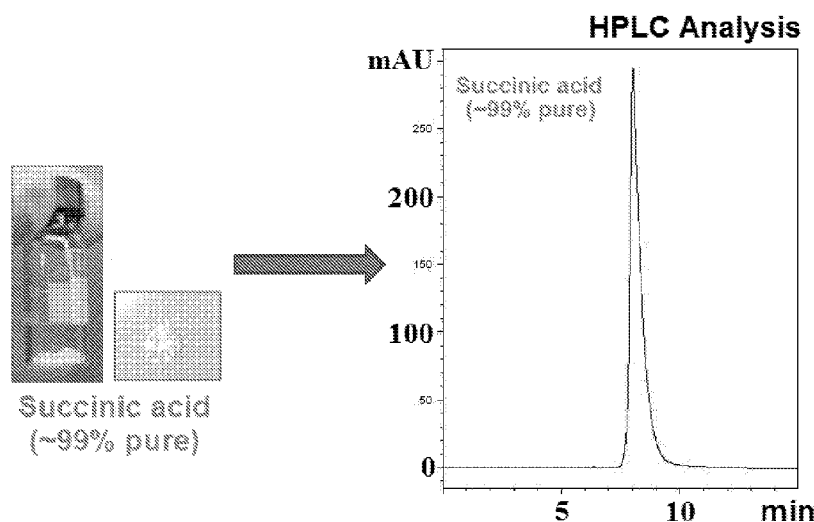
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(54) Title: BACTERIAL STRAIN

Figure 4



(57) **Abstract:** The present disclosure relates to a bacterium capable of fermenting plant biomass amongst other carbon sources and methods that use the bacterium in the production of succinate and/or hydrogen.



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- *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description (Rules 13bis.4(d)(i) and 48.2(a)(viii))* — *with sequence listing part of description (Rule 5.2(a))*

Bacterial Strain

Field of the Invention

The present disclosure relates to a bacterium capable of fermenting plant biomass amongst other carbon sources, to succinate; and includes methods that use the bacterium in the production of succinate for use in the pharmaceutical, chemical, automobile, food and beverage industries and hydrogen as a biofuel.

Background to the Invention

Plant biomass is one of the greatest untapped reserves on the planet and is largely composed of cell walls. For the production of useful products derived from plant biomass the cellulose and other polysaccharides must be converted to sugars by saccharification. Saccharification is a process by which plant lignocellulosic materials (e.g., lignin, cellulose, hemicellulose) are hydrolysed to glucose through chemical and enzymatic means. Typically, this involves the pre-treatment of plant material with alkali to remove lignin followed by enzyme digestion of cellulose. The enzymes currently available for industrial lignocellulose saccharification involve a cocktail of endoglucanases and cellobiohydrolases, the activity of which is severely limited by access to their substrates due to the lignification of the cell wall and cellulose crystallinity. In order to obtain efficient saccharification costly pre-treatments in the form of steam explosion, ammonia, or dilute acid hydrolysis at high temperature are required. Even after pre-treatment, commercial cellulases have to be used at loadings of several grams per kg of lignocellulose, and as a consequence, the cost and energy inputs of producing biologically useful products from plant biomass are currently too high to be economically competitive.

Lignocellulosic biomass is recalcitrant to digestion in the biological context but despite this a number of organisms have evolved the capability to live on a diet of this material. Indeed a wide range of microbes, especially fungi and bacteria are specialized at obtaining nutrition from lignocellulose. There is a continued need to identify new lignocellulose organisms with improved activity to address the demand for plant derived products.

Succinic acid, also known as amber acid, is an important chemical compound used in a wide range of industries such as pharmaceutical, chemical, automobile, food and beverage industries. Among the molecules that can be derived from succinic acid by known chemical processes are 1, 4 butanediol, Tetrahydrofuran, 2-pyrrolidone and γ -butyrolactone. These compounds are expensive and are required in large quantities in chemical, pharmaceutical, food, agricultural and textile industries (1-2). The worldwide market demand for succinic acid

and its derivatives are estimated about 30 million tons per year (2-3). Most of the commercially available succinic acid is produced petrochemically from n-butane through maleic anhydride, which has issues including dependency on non-renewable petrochemical maleic acid or anhydride as feed stock and other environmental issues including global warming (3-4). Moreover, the production costs are high.

Beside as an intermediate in the tricarboxylic acid (TCA) cycle, succinate can also be a fermentation end product when sugar or glycerol is used as a carbon source and several succinic acid producers including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens* have been isolated. However, cultivation of these bacteria is challenging and the yield of succinic acid is often low. Several key enzymes including pyruvate carboxylase, PEP carboxylase, malate dehydrogenase, fumarase and fumarate reductase, involved in the succinate fermentation pathway, are known and there has been a substantial effort to genetically engineer bacteria to improve succinate production.

WO2012103261 discloses genetically modified yeast strains comprising an active succinate fermentation pathway from phosphoenolpyruvate or pyruvate to succinate, which can be exported from the cell. Similarly, US2013203137 discloses recombinant bacteria engineered to produce fewer by-products such as acetate, lactate or ethanol and increasing the production rate of succinate and other components of the TCA cycle. However, although improvements in the production of succinate have been made, the microorganisms require glucose as a feedstock, which is with \$0.39 per kg glucose still considerable high.

In order to reduce costs further cheaper starting materials have been considered such as sucrose. WO2011163128 discloses engineered *E. coli* bacteria capable of utilising sucrose as a carbon source producing succinate. WO2010/115067 discloses engineered bacterial biocatalysts for the efficient production of succinic acid from carbohydrate feed stocks such as sucrose or hemicellulose. Although these methods reduce the costs of the feedstock, genetic manipulation of bacteria is of the associated with problems such as reduced protein expression, low productivity, difficulties in maintaining the genetically modified bacteria, high costs and the general acceptance of the genetically modified bacteria by the public and in industry.

Succinic diammonium salt is the product of the fermentation process which requires reactive distillation to remove both ammonia, in a one- or two-step distillation process and yielding solid succinic acid. Although this process results in succinic acid free of most impurities, further purification is often necessary to yield a white, odourless solid suitable for the

production of polymers. US2011237831 discloses an improved method to purify succinic acid from fermentation broth comprising distillation and cooling cycles to yield fairly pure succinic acid. US20130150621 discloses a distillation process to further purify the succinic acid obtained through the fermentation process to yield pure succinic acid. Although
5 commonly used purification methods yield succinic acid of high grade, distillation and cooling cycles are laborious and energy inefficient, and therefore, simpler, more affordable methods are greatly desired.

Hao *et al* [World Journal of Microbiology and Biotechnology Vol 24, 2008, p1731-1740] discloses *Klebsiella pneumonia* strain TUAC01 that produces 1,3-propanediol required for
10 the synthesis of polytrimethylene terephthalate (PTT) or other polyester fibres under aerobic conditions from glycerol. The commercial routes to produce 1,3 propanediol are chemical methods using compounds such as acrolein, ethylene oxide or glycerol. Moreover, various bacteria such as *Klebsiella* have the ability to convert glycerol to 1,3-propanediol anaerobically. The authors identified from soil samples *Klebsellia* strains capable of
15 conversion of glycerol to 1,3 propanediol under less labour intensive aerobic conditions, lowering the cost of commercial production. Some of the strains were also found to produce small amounts of succinic acid (approximately 0.012 g succinic acid per 30 g glycerol starting material).

US2012/231511 discloses a method for the preparation of bio-chemicals using fermentation waste generated in alcohol production and bacteria such as *Klebsellia* to reduce the costs of additional nutrients and conventional carbon sources such as glucose, sucrose or glycerol. The applicants produced succinic acid using *Actinobacillus succinogens* ATCC 55618. US2003017559 discloses a method of fermentatively producing succinic acid from
25 hydrolysates such as lignocellulosic hydrolysates or corn derived sugar solutions using a organism with genes ptsG, pflB and ldhA or mutations in these genes. An example of a suitable organism is *Klebsiella*. The disclosed method results in succinate to feedstock production of 1.3:1 to 0.9:1.

This disclosure relates to a bacterium capable of using solid lignocellulosic materials such as raw sugarcane bagasse (SB), the fibrous matter that remains after extraction of the liquids from sugarcane, for the production of high levels of succinic acid through a solid state fermentation process. This process improves the efficiency of the production process greatly and reduces costs since raw lignocellulosic biomass can be used directly without pre-
35 treatment. The amount of water used in the fermentation process is substantially reduced and the bacterium can produce very pure and concentrated succinic acid juice (~80%) within

72h which then can be extracted from SB directly by using mechanical squeezing, enabling purification of pure succinic acid by simple precipitation and drying without the energy-intensive distillation process to remove water and impurities.

Statement of the Invention

5 According to an aspect of the invention there is provided an isolated succinate producing bacterium of the genus *Klebsiella* sp wherein said bacterium is characterized by features selected from the group:

- i) a bacterium comprising a nucleic acid sequence that hybridizes under high stringency conditions with a nucleic acid probe complementary to the nucleotide sequence set forth in SEQ ID NO: 1; or
- 10 ii) a bacterium comprising a nucleotide sequence as set forth in SEQ ID NO: 1 and that has 95%, 96%, 97%, 98% or 99% nucleotide sequence identity to the full length sequence set forth in SEQ ID NO: 1; wherein said bacterium can use at least lignocellulosic material as a carbon source and metabolizes
- 15 said carbon source[s] to at least succinate.

Hybridization of a nucleic acid molecule occurs when two complementary nucleic acid molecules undergo an amount of hydrogen bonding to each other. The stringency of hybridization can vary according to the environmental conditions surrounding the nucleic acids, the nature of the hybridization method, and the composition and length of the nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001); and Tijssen, Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes Part I, Chapter 2 (Elsevier, New York, 1993). The T_m is the temperature at which 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following is an exemplary set of hybridization conditions and is not limiting:

Very High Stringency (allows sequences that share at least 90% identity to hybridize)

Hybridization:	5x SSC at 65°C for 16 hours
30 Wash twice:	2x SSC at room temperature (RT) for 15 minutes each
Wash twice:	0.5x SSC at 65°C for 20 minutes each

High Stringency (allows sequences that share at least 80% identity to hybridize)

Hybridization: 5x-6x SSC at 65°C-70°C for 16-20 hours

Wash twice: 2x SSC at RT for 5-20 minutes each

Wash twice: 1x SSC at 55°C-70°C for 30 minutes each

- 5 In a preferred embodiment of the invention said bacterium comprises a nucleotide sequence as set forth in SEQ ID NO: 1.

The bacterium according to the invention is of the genus *Klebsiella*. *Klebsiella* is a Gram-negative, non-motile, encapsulated, facultative anaerobic, rod-shaped bacterium. As an example of the bacterium according to the invention and isolated using the screening
10 method disclosed in the present disclosure, *Klebsiella* sp. strain XMR21 has a wide carbon substrate spectrum, which could utilize glucose, xylose, glycerol and sucrose, the common components that widely exist in lignocellulosic materials, industrial (e.g., waste product from biodiesel plant) or food wastes (e.g., waste product from molasses).

The *Klebsiella* cell designated XMR21 was deposited on 4 June 2014 at American Type
15 Culture Collection (ATCC®), Manassas, VA, USA; accession number PTA-121320; (Deposited under the Budapest Treaty on the International Recognition of the deposit of Micro-organisms as amended in 1980).

In a preferred embodiment of the invention said bacterium is able to use one or more carbon sources selected from the group consisting of: lignin containing plant biomass comprising
20 lignocellulose; sugars, for example glucose, xylose, glycerol or sucrose.

In a preferred embodiment of the invention said bacterium is a high succinate producing bacterium.

The production of high levels of succinate means greater than 5gm of succinate per 100gm of lignocellulosic material, or between 5gm and 50gm of succinate per 100gm of
25 lignocellulosic material, for example 10gm, 15gm, 20gm, 25gm, 30gm, 35gm, 40gm, 50gm or greater than 50gm succinate per 100gm of lignocellulosic material.

In a preferred embodiment of the invention said high succinate production is for example, at least 28+/- 2gm of succinic acid per 100gm of sugarcane bagasse.

In a further preferred embodiment of the invention said bacterium is a high hydrogen producing bacterium, for example, at least 3.9+/- 2L of hydrogen per 100g of sugarcane bagasse.

5 According to a further aspect of the invention there is provided a bacterial cell culture comprising a bacterium according to the invention.

According to a further aspect of the invention there is provided a bacterial cell culture container comprising a bacterial cell culture according to the invention.

In a preferred embodiment of the invention said cell culture container is a fermentation bioreactor.

10 According to a further aspect of the invention there is provided a bacterium or bacterial cell culture according to the invention for use in the saccharification of plant biomass.

According to a further aspect of the invention there is provided a method for the saccharification of plant biomass comprising the steps:

- 15
- i) providing particulate plant biomass comprising lignocellulose;
 - ii) contacting the particulate plant material with a bacterial culture according to the invention to provide a reaction mixture; and
 - iii) culturing said reaction mixture under cell culture conditions sufficient to convert the particulate plant biomass to sugar which is further metabolised by at least the bacterial cell according to the invention.

20 According to a further aspect of the invention there is provided a method for the conversion of plant biomass to succinate comprising the steps:

- 25
- i) providing particulate plant biomass comprising lignocellulose;
 - ii) contacting the particulate plant material with a bacterial culture according to the invention to provide a reaction mixture;
 - iii) culturing said reaction mixture under cell culture conditions sufficient to convert the particulate plant biomass to at least succinate; and
 - iv) collecting the succinate from the reaction mixture and optionally purifying the succinate from the reaction mixture.

30 According to a further aspect of the invention there is provided a method for the production of hydrogen comprising the steps:

- i) providing particulate plant biomass comprising lignocellulose;
- ii) contacting the particulate plant material with a bacterial cell or culture according to the invention to provide a reaction mixture;
- iii) culturing said reaction mixture under cell culture conditions sufficient to convert plant biomass to sugar which is metabolised by at least the bacterial cell according to the invention; and
- iv) collecting and/or storing hydrogen gas formed during the process in (iii) above.

In a preferred method of the invention said particles are at least about 0.5mm in diameter.

Preferably said particles are between about 0.5mm to 1.5mm.

- 10 In a preferred method of the invention the succinate is precipitated from the reaction mixture.

In a preferred method of the invention said reaction mixture is substantially a solid state fermentation method.

“Solid state fermentation” is known in the art and is an alternative to liquid or submerged bacterial cell culture and has reduced moisture content.

- 15 In a preferred method of the invention said moisture levels are between about 80-90%.

According to a further aspect of the invention there is provided a cell culture substrate comprising: particulate lignocellulose material and a bacterial culture according to the invention.

- 20 In a preferred embodiment of the invention said cell culture substrate is contained within a cell culture vessel.

In a preferred embodiment of the invention said cell culture vessel is in fluid contact with an air sealed second vessel wherein said second vessel is a collecting vessel for containing and/or storing released hydrogen gas by the method according to the invention.

- 25 According to a further aspect of the invention there is provided a method for the identification and isolation of a bacterial strain having the properties of a bacterial cell according to the invention comprising:

- i) obtaining a sample comprising bacterial cells to be screened;
- ii) obtaining bacterial clones from said sample and forming a bacterial cell culture;
- 30 iii) providing anaerobic growth conditions and culturing said bacterial clones

- iv) measuring the production of succinate and/or hydrogen; and
- v) identifying bacterial clones that are high succinate and/or high hydrogen producers; and optionally comparing the production of succinate and/or hydrogen to the bacterial strain XMR21.

5 According to a further aspect of the invention there is provided a bacterial cell obtained or obtainable by the screening method according to the invention.

In a preferred embodiment of the invention said bacterial cell is of the genus *Klebsiella* sp.

10 The bacteria used in the processes according to the invention are grown or cultured in the manner with which the skilled worker is familiar. As a rule, bacteria are grown in a medium comprising a carbon source, usually in the form of complex polysaccharides and/or sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as salts of iron, manganese and magnesium and, if appropriate, vitamins.

15 The pH of the growth medium can either be kept constant, that is to say regulated during the culturing period, or not. The cultures can be grown batchwise, semi-batchwise or continuously. Nutrients can be provided at the beginning of the fermentation or fed in semi-continuously or continuously. In this process, the pH value is advantageously kept between pH 4 and 12, preferably between pH 6 and 9, especially preferably between pH 7 and 8.

20 An overview of known cultivation methods can be found in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess technology 1. Introduction to Bioprocess technology] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral equipment] (Vieweg Verlag, Brunswick/Wiesbaden, 1994)). Culture medium to be used must suitably meet the requirements of the strains in question.

25 As described above, these media which can be employed in accordance with the invention usually comprise one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements. Carbon sources are sugars, such as mono-, di- or polysaccharides. Examples of carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or complex polysaccharides such as
30 lignocellulose/hemicellulose. Sugars/complex polysaccharides can also be added to the media via complex compounds such as molasses or other by-products from sugar refining. The addition of mixtures of a variety of carbon sources may also be advantageous. Other possible carbon sources are oils and fats such as, for example, soya oil, sunflower oil,

peanut oil and/or coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and/or linoleic acid, alcohols and/or polyalcohols such as, for example, glycerol, methanol and/or ethanol, and/or organic acids such as, for example, acetic acid and/or lactic acid.

5 Nitrogen sources are usually organic or inorganic nitrogen compounds or materials comprising these compounds. Examples of nitrogen sources comprise ammonia in liquid or gaseous form or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex nitrogen sources such as cornsteep liquor, soya meal, soya protein, yeast extract, meat extract and others. The nitrogen sources can be used individually or as a
10 mixture.

Inorganic salt compounds which may be present in the media comprise the chloride, phosphorus and sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron. Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else
15 organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine. Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts may be used as sources of phosphorus. Chelating agents may be added to the medium in order to keep the metal ions in solution. Particularly
20 suitable chelating agents comprise dihydroxyphenols such as catechol or protocatechuate and organic acids such as citric acid.

In order to isolate bacteria capable to produce succinic acid, bacteria can be grown in medium comprising sucrose (20g/l), ammonium sulphate (6.6g/l), yeast extract (6.6g/L), dipotassium phosphate (10g/L), monobasic potassium phosphate (2g/L) and trace elements.
25 Fermentation proceeds ideally over a period of between 24 and 48h or longer after which the succinic acid production in culture supernatant can be analysed.

All media components are sterilized typically by heat (20 min at 1.5 bar and 121°C) or, when sterilizing medium containing no solid contents, by filter sterilization. The components may be sterilized either together or, if required, separately. All media components may be present
30 at the start of the cultivation or added continuously or batchwise, as desired.

The culture temperature is normally between 15°C and 45°C, preferably at from 25°C to 40°C, and even more preferably at around 30°C and may be kept constant or may be altered during the process. The pH of the medium should be in the range from 5 to 8.5, preferably

around 7.0. The pH for cultivation can be controlled during cultivation by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. When preparing seed cultures aerobic conditions are maintained by introducing oxygen or oxygen-containing gas mixtures such as, for example, ambient air into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C, and even more preferably at 30°C.

The biomass may, in order to extract the succinic acid, be removed completely or partially from the fermentation broth by separation methods comprising the compression of the solid substrate under pressure. It is advantageous to process the liquid extract after its separation, filtration or precipitation.

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”, means “including but not limited to”, and is not intended to (and does not) exclude other moieties, additives, components, integers or steps. “Consisting essentially” means having the essential integers but including integers which do not materially affect the function of the essential integers.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1 is a schematic illustration shows the solid state fermentation (SSF) process for the production of bio-succinic acid and hydrogen from sugarcane bagasse by a new bacterium XMR21. Brief description for the steps as follow, 1. Sugarcane bagasse (SB) collected and milled into small particles, 2. SB particles (<1mm) fermented by bacterium XMR21, 3. After

72h, the fermented biomass is squeezed to extract succinic acid produced, 4. Crude succinic acid (~80% purity), 5. $\text{Ca}(\text{OH})_2$ slurry is added to precipitate succinic acid, 6. Succinic acid (calcium salt) is washed 3-4 times with water, 7. The precipitate is collected and dried to obtain pure succinic acid (~99%);

5 Figure 2: illustrates hydrogen production from SB by bacterium XMR21;

Figure 3: illustrates crude succinic acid from fermented biomass of SB; and

Figure 4: illustrates pure succinic acid after salt precipitation.

Figure 5 is a schematic illustration of the fermentation processes using XMR21;

10 Figure 6 illustrates a phylogenetic tree for strain XMR21 showing that this is a new strain belongs to *Klebsiella pneumoniae*; and

Table 1 illustrates production of succinic acid from various strains reported in literature as compared to XMR21.

Materials and Methods

15 *Screening of succinic acid- producing bacterium*

Initially, more than 50 anaerobic strains were isolated from soil sample for succinic acid production using anaerobic fermentation method. The culturing medium was prepared with sucrose (20g/l), ammonium sulphate (6.6g/l), yeast extract (6.6g/L), dipotassium phosphate (10g/L), monobasic potassium phosphate (2g/L) and trace elements. After 48h of
 20 fermentation, the succinic acid production in culture supernatant was analysed. Among the strains isolated, strain XMR21 supported high-level of succinic acid production. Later, XMR21 was slowly adopted for about 10-12 months to solid state fermentation (SSF) utilizing sugarcane bagasse as a substrate. Figure 5 shows the schematic illustration of how XMR21 was adapted to solid state fermentation of sugarcane bagasse. Initially, XMR21 was
 25 cultured in lower concentration (5-20g/100ml of 50mM potassium phosphate buffer, pH6.8±0.2) of sugarcane bagasse. Later, the concentration of sugarcane bagasse was gradually increased to 80-90g/100ml. In a slow process, the ability of XMR21 was promoted ferment high concentration of SB in a solid state fermentation. On the other side, the basic microbial and biochemical characterization analysis for isolate XMR21 supports that, it
 30 belongs to a gram-negative facultative anaerobic bacterium as a member of genus *Klebsiella*. Additional phylogenetic analysis based on 16s rDNA sequencing shows >95%

identity with *Klebsiella pneumoniae* strain. The phylogenetic tree for strain XMR21 is shown in Figure 6. The 16s rDNA sequence (Table 1) is given below.

Detected 16s rDNA sequence (1,169 bases) of bacterium XMR21

CACTGCGGCAGCTAAACATGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTG
 5 ACGAGCGGCGGACGGGTGAGTAATGTCTGGGAACTGCCTGATGGAGGGGGATAACT
 ACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGG
 GCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCA
 CCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAGTGAAGAC
 ACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC
 10 CTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTCAGC
 GGGGAGGAAGGCGATGAGGTTAATAACCTTGTGATTGACGTTACCCGCAGAAGAAGC
 ACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG
 AATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG
 GCTCAACCTGGGAACTGCATTGAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAG
 15 AATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGC
 GGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATT
 AGATACCCTGGTAGTCCACGCCGTAAACGATGTGATTTGGAGGTTGTGCCCTTGAGG
 CGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGT
 TAAACTCAAATGAATTGACGGGGGCCCGCACAAACCGGTGGACCATGTGGTTTAATTC
 20 CATCCAACCCCAAAAACCTTACCTGGTCTTGACATCCACAAAACCTTACCAAAAATGCTTT
 GGTGCCTTCGGGAACTTTAAACAGTTCTGCATGGCTTTCCTCACCTCCTGTTTTAAAT
 GTTGGTTAATTCCCCCAACAACCAACCCTTTATCCTTTTTTCCCACTTTCCCCCAAAA
 CTCAAAGAACTGCCATTATAAACTTGAAGATGTTGGGAATTGACCTCCAACCTTCATTCC
 ATGCCCC [SEQ ID NO: 1]

25 *Microorganism and culture medium*

Bacterium sp. XMR21 was maintained on nutrient agar plates by sub-culturing fortnightly and stored at 4°C for short term preservation. The nutrient medium contained glucose (10g/l), yeast extract (6.6g/l), ammonium sulphate (6.6g/l), dipotassium hydrogen phosphate (10g/l), potassium dihydrogen phosphate (2g/l) and trace elements. The strain is cultured in
 30 nutrient medium at 30°C for routine production of succinic acid.

Seed culture preparation

A loop of cells from plate culture was transferred into nutrient broth in serum bottle (30ml in 100ml) and incubated at 30°C with agitation (200 rpm) for 24h. The cell density of the culture determined as 3.5 ± 0.2 by measuring OD600.

Solid state fermentation (SSF)

- 5 SSF was carried out using sugarcane bagasse as growth substrates in 100ml serum bottle. The solid substrate can be screened to have particles smaller than 1mm using mechanical sieve. To each bottle, 5g of dry substrate was transferred and the moisture level can be adjusted between 80-90% with 50 mM potassium phosphate buffer. The content of the bottle was mixed and autoclaved at 121°C for 20 min. The flasks were inoculated with 10%v/v of seed culture and mixed for 30min at 150rpm and later incubated at 30°C for 72h (Figure 1- stage 1 and 2).

Hydrogen estimation

- The compositions of the gaseous products from fermentation were determined with another GC (model GC-17A; Shimadzu, Japan) equipped with a thermal conductivity detector (TCD) and a Supelco 80/100, Porapak-N column, (2 m × 1/8 inch stainless steel column). The oven temperature was kept constant at 110°C for 2.3 min and argon (15 ml/min) was used as the carrier gas. Standard gaseous mixtures consisting of hydrogen (5%), nitrogen (60%), carbon dioxide (15%), carbon monoxide (15%) and methane (5%) at known proportions were used to obtain a calibration curve. From the overall analysis, the cumulative hydrogen production was estimated as 3.9 ± 0.2 L from 100g sugarcane bagasse (Figure 2).

Succinic acid extraction:

- The solid culture content was mixed with 50 mM potassium phosphate buffer (pH 6) in a rotary shaker (180 rpm) for 1 h. The suspension is then centrifuged at 10000 rpm and 4 °C for 10 min. The clear supernatant was used to estimate the succinic acid produced. The solid pellet was dried in a hot air oven at 80 °C for 16 h and weighed. In parallel, the solid culture was mechanically squeezed, and the extract passes through a 0.45µm filter (Figure 1- stage 2 and 3). Eventually, a few drops of 20%w/v slurry of Ca(OH)₂ added to obtain succinic acid (calcium salt) by precipitation method (Figure1-stage 5 and 6).

Succinic acid estimation

- 30 Succinic acid was analyzed by using high performance liquid chromatography (HPLC) with a rezex ROA-organic acid (Phenomenex; 300 × 7.8 mm) and UV-Vis-abs-variable wave length (210nm) detector manufactured by Agilent (USA). The mobile phase in HPLC is 5mM

of sulfuric acid with a flow rate of 1ml/min, and the temperature can be maintained at 50°C. Authentic HPLC grade succinic acid (Sigma, USA) was used as standard for identification and quantification. The crude succinic acid obtained from biomass shows the purity close to 80% (Figure 3). Further purification by salt precipitation and multiple washing by water shows the purity close to 99% (Figure 4).

16S rRNA gene

Genomic DNA of strain XMR21 was extracted and purified with DNeasy Tissue Kit (Qiagen GmbH, Germany) according to manufacturer's instructions. The genomic DNA was used as a template for PCR amplification of the 16S rRNA genes with a pair of universal bacterial primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1392R (5'-ACGGGCGGTGTGT-3'). The obtained PCR products were purified with a PCR purification kit (Qiagen GmbH, Germany) and sequenced using an ABI DNA Sequencer. Basic Local Alignment Search Tool (BLAST) analysis of the obtained 16S rRNA gene sequences were aligned to other related bacterial strains by using CLUSTAL X software.

Example

Succinic acid can be produced by biological process such as submerged (SmF) and solid state fermentation (SSF). Table 1 shows the list of strains reported in literature for production of succinic acid by SmF and SSF process. Most strains listed in the table (table 1) can produce succinic acid only when they cultured in SmF process. Interestingly, strains XMR21 and *Clostridium* BOH3 can produce succinic acid from SSF process. In fact, SSF has several advantages over SmF, in SSF (1) low cost, waste biomass such as sugarcane bagasse, rice straw can be used as substrate, (2) economic processing because simple tray or rotating drum can be used as fermenting vessel, (3) product (e.g. succinic acid) can be extracted easily in a concentrated-form, and (4) SSF generates relatively less secondary waste.

Though SSF has many advantages, succinic acid producing strains including *Actinobacillus succinogenes* (130Z, GXAS13, ATCC 55618, NJ113 and CGMCC1593), *Issatchenkia orientalis* SD108, genetically modified strains *Yarrowia lipolytica* SDH2 and metabolically engineered *Escherichia coli* (BS002, KMG111, BA305, K12 and SD121) were found incapable of utilizing solid substrates for their growth as well as succinic acid production. However, their succinic acid production in SmF process was reported between 6-64g/l (Table 1). Meanwhile, *Clostridium* BOH3 can produce 13.38g/l and 51.5mg/g of succinic acid from SmF and SSF process, respectively. Remarkably, strain XMR21 produces the highest level of succinic acid (0.28g/g) from sugarcane bagasse while culturing it in SSF process.

For succinic acid production in SmF process, several low cost substrates such as pine wood, corn stover and stalk hydrolysates, sugarcane molasses, and bagasse hydrolysate, microalgae hydrolysate were suggested in literature (Table 1). However, inevitably all these substrates needs pre-treatment steps such as acid/alkali/enzyme hydrolysis and removal of inhibitors to effectively modify the non-fermentable biomass into fermentable sugars. Notably, in case of succinic acid production from strains XMR21 and *Clostridium* BOH3, the solid substrates such as sesame oil cake, sugarcane bagasse does not require any pre-treatment, these strains (XMR21 and BOH3) can directly utilize solid substrates and produce relatively high yield of succinic acid.

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Table 1. Production of succinic acid from various strains reported in literature.

Microbe (type)	Fermentation type	Substrate (g/l)	Succinic acid yield	Reference
Metabolically engineered <i>Escherichia coli</i> BS002 (bacteria)	SmF	Macroalgae hydrolysate (Glucose-10.31g/l and mannitol-10.12g/l)	17.44g/l	Bai et al., 2015, Bioresource Technology, 185, pp 56-61
Metabolically engineered <i>Escherichia coli</i> strain KMG111 (bacteria)	SmF	Sugar/biomass hydrolysate (80g/l)	32.4g/l	Wang et al., 2014, Bioresource Technology, 170, pp 125-131
Metabolically engineered <i>Enterobacter aerogenes</i> AJ110637 (bacteria)	SmF	Glucose (40g/l)	5-5.5g/l	Tajima et al., 2014, Applied Microbiology and Biotechnology, 98(18), pp7803-13.
Metabolically engineered <i>Escherichia coli</i> strain BA305 (bacteria)	SmF	Sugarcane bagasse hydrolysate (fed-batch process)	39.3g/l	Liu et al., 2013, Bioresource Technology, 149, pp 84-91.
Metabolically engineered <i>Escherichia coli</i> strain SD121 (bacteria)	SmF	Corn stalk hydrolysate (sugar: >50g/l)	36.55g/l	Wang et al., 2011, Process Biochemistry, 46(1), pp 365-371.
Metabolically engineered <i>Escherichia coli</i> strain K12 (bacteria)	SmF	Glycerol (10g/l)	~6g/l	Matthew et al., 2010, Metabolic Engineering, 12(5), pp. 409-419
Genetically modified <i>Yarrowia lipolytica</i> SDH2 (yeast)	SmF	Glycerol (50g/l)	~45g/l	Tigran et al., 2010, Biotechnology and Bioengineering, 107(4), pp 673-682
<i>Actinobacillus succinogenes</i> 130Z (bacteria)	SmF	Industrial hemp (Cannabis sativa L.): Sugar content 73.5%	21.9g/l	Gunnarsson et al., 2015, Bioresource Technology, 182, pp58-66
<i>Actinobacillus succinogenes</i> GXAS13 (bacteria)	SmF	Sugarcane molasses (Sugar – 75g/l)	60-64g/l	Shen et al., 2015, Letters in Applied Microbiology, 60(6), pp 544-551
<i>Actinobacillus succinogenes</i> 130Z (bacteria)	SmF	Pinewood and corn stover hydrolysate (pretreated with	~20g/l	Wang et al., 2014, Bioresource Technology, 172, pp

		ionic liquid)		283-289
<i>Issatchenkia orientalis</i> SD108 (yeast)	SmF	Glucose (50g/l)	11.63g/l	Xiao et al., 2014, Microbial Cell Factories, 13(1), Art.# 121
<i>Actinobacillus succinogenes</i> ATCC 55618(bacteria)	SmF	Mixed food waste hydrolysate (Sugar: 470mg/g)	29.9g/l	Sun et al., 2014, Applied Biochemistry and Biotechnology, 174, pp1822–1833
<i>Actinobacillus succinogenes</i> ATCC 55618(bacteria)	SmF	Wheat hydrolysate (>110g/l of glucose)	16-64.2g/l	Du et al., 2008, Bioresource Technology, 99(17), pp8310-15 & Du et al., 2007, Applied Microbiology and Biotechnology, 76(6),pp 1263-1270
<i>Actinobacillus succinogenes</i> NJ113 (bacteria)	SmF	Cellobiose (50g/l)	30.3g/l	Jiang et al., 2013, Bioresource Technology, 135, pp469-474.
<i>Actinobacillus succinogenes</i> CGMCC1593 (bacteria)	SmF	Glucose (25-75g/l)	18-38g/l	Liu et al., 2008, Journal of Chemical Technology and Biotechnology, 83(5), pp 722-729
<i>Clostridium</i> strain BOH3 (bacteria)	SmF	Mannitol (60g/l)	13.38g/l	From our lab.
<i>Clostridium</i> strain BOH3 (bacteria)	SSF	Sesame oilcake and Sugaecane bagasse	160 and 51.5mg/g respectively	From our lab.
Strain XMR21 (bacteria)	SSF	Sugarcane bagasse	0.28g/g	From our lab.

SmF – Submerged Fermentation; SSF – Solid State Fermentation

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Claims

1 An isolated succinate producing bacterium of the genus *Klebsiella sp* wherein said bacterium is characterized by features selected from the group:

- 5 i) a bacterium comprising a nucleic sequence that hybridizes under high stringency conditions with a nucleic acid probe complementary to the nucleotide sequence set forth in SEQ ID NO: 1; or
- 10 ii) a bacterium comprising a nucleotide sequence as set forth in SEQ ID NO: 1 and that has 95%, 96%, 97%, 98% or 99% nucleotide sequence identity to the full length sequence set forth in SEQ ID NO: 1; wherein said bacterium can use at least lignocellulosic material as a carbon source and metabolizes said carbon source[s] to at least succinate wherein said bacterium is a high succinate producing bacterium.

2. The bacterium according to claim 1 wherein said bacterium comprises:

15 i) a nucleotide sequence that hybridizes under high stringency conditions with a nucleic acid probe complementary to the nucleotide sequence set forth in SEQ ID NO: 1; or

20 ii) a nucleotide sequence as set forth in SEQ ID NO: 1 and that has 95%, 96%, 97%, 98% or 99% nucleotide sequence identity to the full length sequence set forth in SEQ ID NO: 1; or

iii) a nucleotide sequence as set forth in SEQ ID NO: 1.

3. The bacterium according to claim 1 wherein said bacterium is able to use one or more carbon sources selected from the group consisting of: lignin containing plant biomass comprising lignocellulose; sugars, for example glucose, xylose, glycerol or sucrose.

25 4. The bacterium according to any one of claims 1 to 3 wherein said high succinate production is at least 28 ± 2 g succinate from 100g of sugarcane bagasse.

5. The bacterium according to any one of claims 1 to 4 wherein said bacterium is a high hydrogen producing bacterium.

30 6. The bacterium according to claim 5 wherein said high hydrogen production is at least 3.9 ± 0.2 L from 100g of sugarcane bagasse.

7. A bacterial cell culture comprising a bacterium according to any one of claims 1 to 6.
8. A bacterium or bacterial cell culture according to any one of claims 1 to 7 for use in the saccharification of plant biomass.

9. A method for the saccharification of plant biomass comprising the steps:

- 5 i) providing particulate plant biomass comprising lignocellulose;
 ii) contacting the particulate plant material with a bacterial culture according to claim 7 to provide a reaction mixture; and
 iii) culturing said reaction mixture under cell culture conditions sufficient to convert the particulate plant biomass to sugar which is further
10 metabolised by at least the bacterial cell according to the invention.

10. A method for the conversion of plant biomass to succinate comprising the steps:

- i) providing particulate plant biomass comprising lignocellulose;
 ii) contacting the particulate plant material with a bacterial culture according to claim 7 to provide a reaction mixture;
15 iii) culturing said reaction mixture under cell culture conditions sufficient to convert the particulate plant biomass to at least succinate; and
 iv) collecting the succinate from the reaction mixture and optionally purifying the succinate from the reaction mixture.

11. A method for the production of hydrogen comprising the steps:

- 20 i) providing particulate plant biomass comprising lignocellulose
 ii) contacting the particulate plant material with a bacterial cell or culture according to claim 7 to provide a reaction mixture;
 iii) culturing said reaction mixture under cell culture conditions sufficient to convert plant biomass to sugar which is metabolised by at least the
25 bacterial cell according to the invention; and
 iv) collecting and/or storing hydrogen gas formed during the process in (iii) above.

12. The method according to any one of claims 9 to 11 wherein said cell culture method substantially a solid state fermentation method.

- 30 13. A cell culture substrate comprising: particulate lignocellulose material and a bacterial culture according to claim 7.

14. The cell culture substrate according to claim 13 wherein said cell culture substrate is contained within a cell culture vessel.

15. The cell culture vessel according to claim 14 wherein said cell culture vessel is in fluid contact with an air sealed second vessel wherein said second vessel is a collecting vessel for containing and/or storing released hydrogen gas

16. A method for the identification and isolation of a bacterial strain having the properties of a bacterial cell according to any one of claims 1 to 6 comprising:

- i) obtaining a sample comprising bacterial cells to be screened;
- ii) obtaining bacterial clones from said sample and forming a bacterial cell culture;
- iii) providing anaerobic growth conditions and culturing said bacterial clones;
- iv) measuring the production of succinate and/or hydrogen; and
- v) identifying bacterial clones that are high succinate and/or high hydrogen producers; and optionally comparing the production of succinate and/or hydrogen to the bacterial strain XMR21

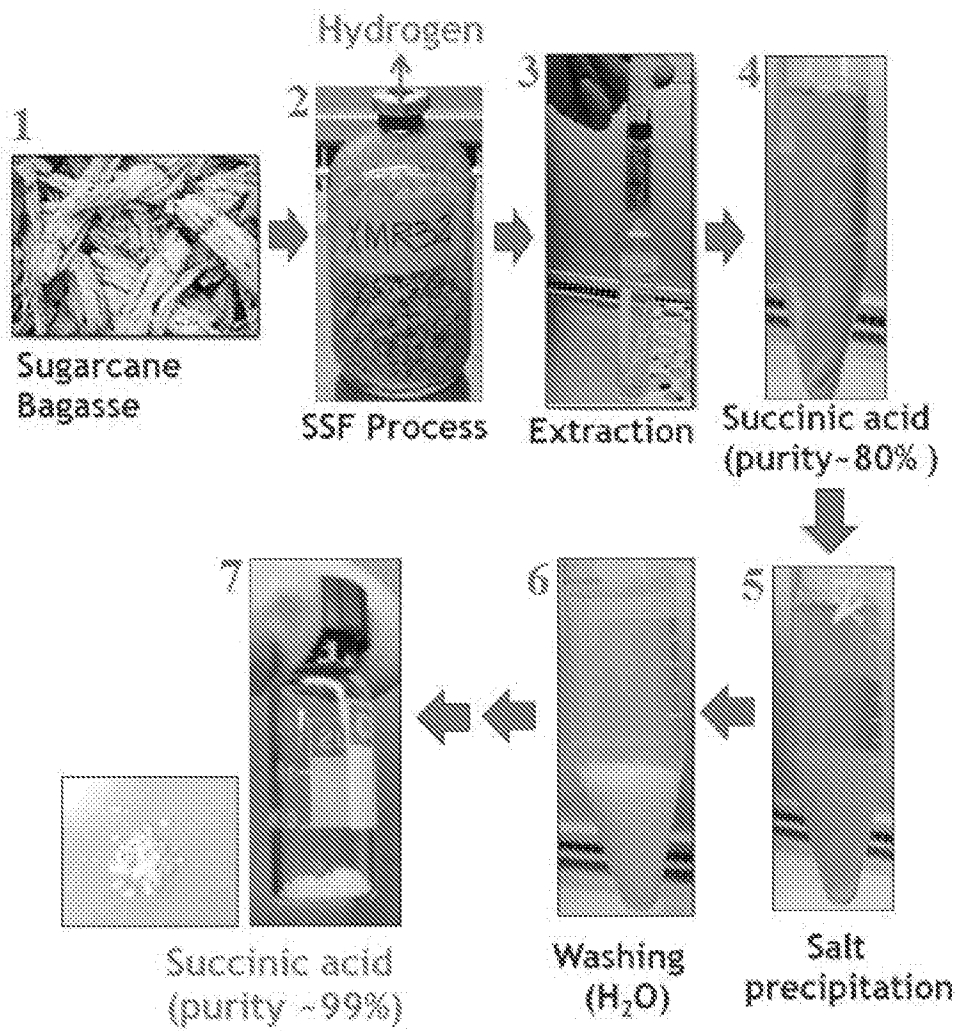
17. The method according to claim 16 wherein the bacterial cell culture comprises plant lignocellulosic material, for example sugar cane bagasse.

18. A bacterium obtained or obtainable by the screening method according to claim 16 or 17.

19. The bacterium according to claim 18 wherein said bacterium is of the genus *Klebsiella* sp.

20. The bacterium according to claim 18 wherein said bacterium is of the genus *Clostridium* sp.

Figure 1



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Figure 2

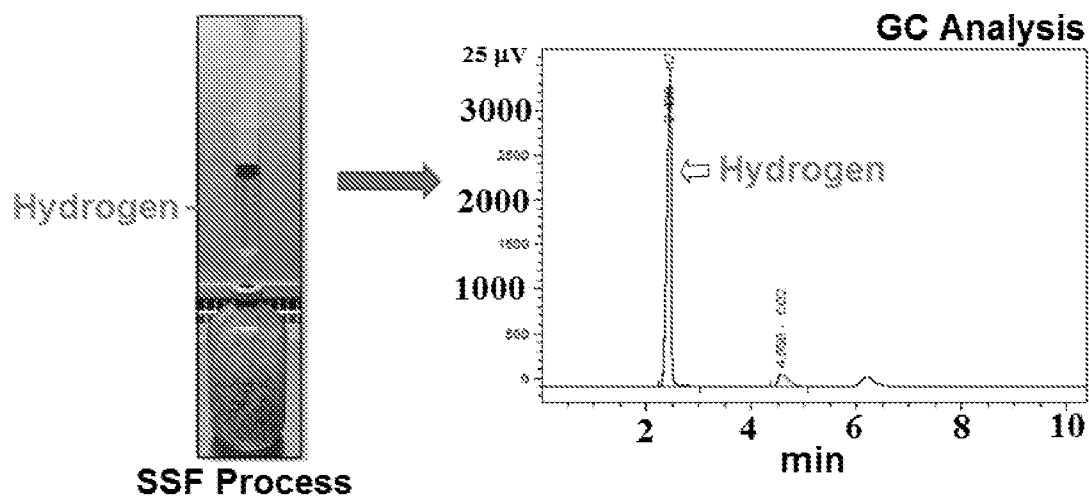
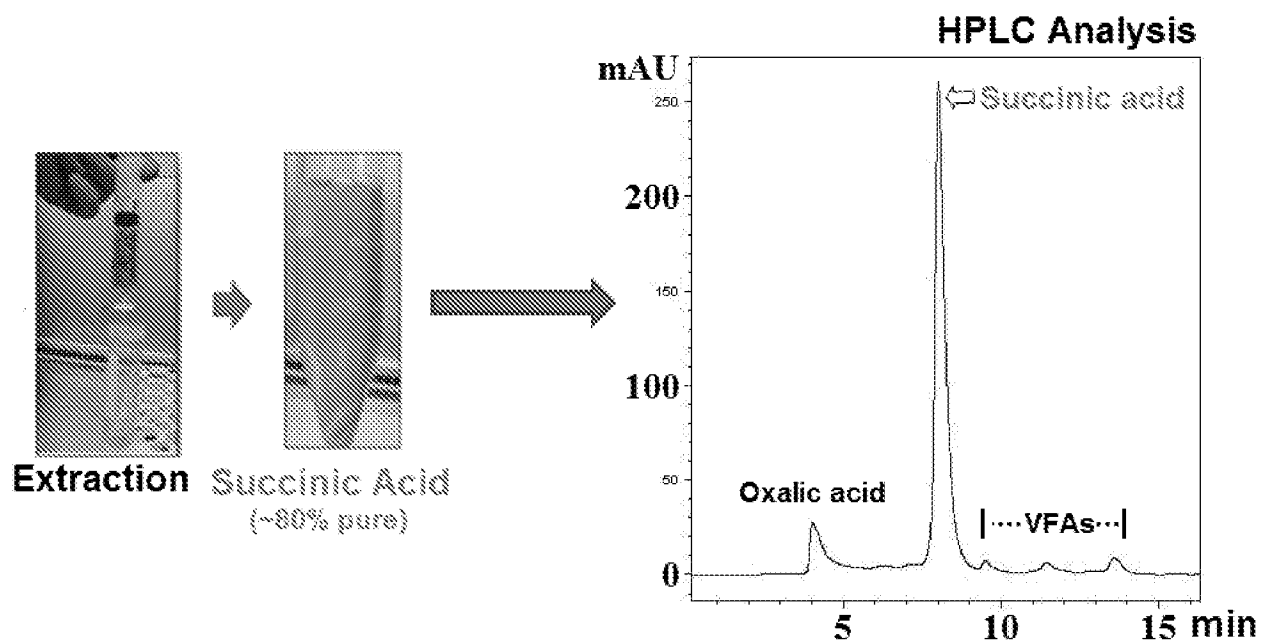
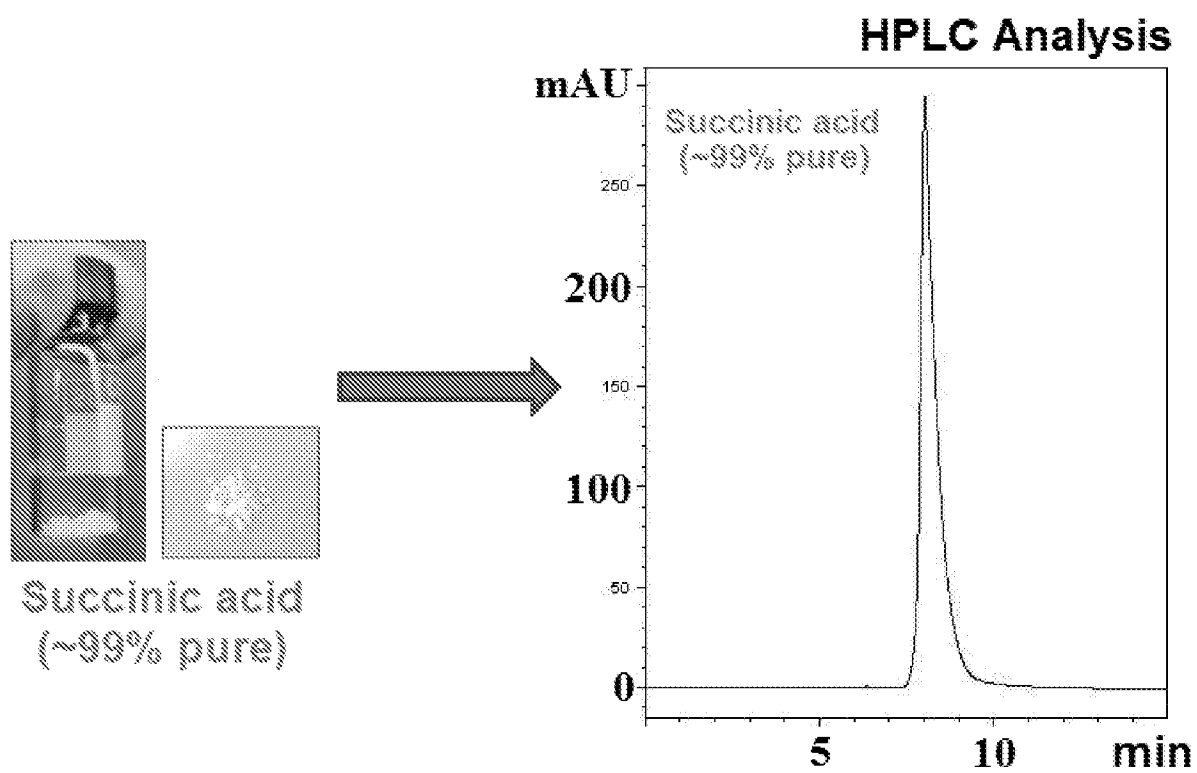


Figure 3



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Figure 4



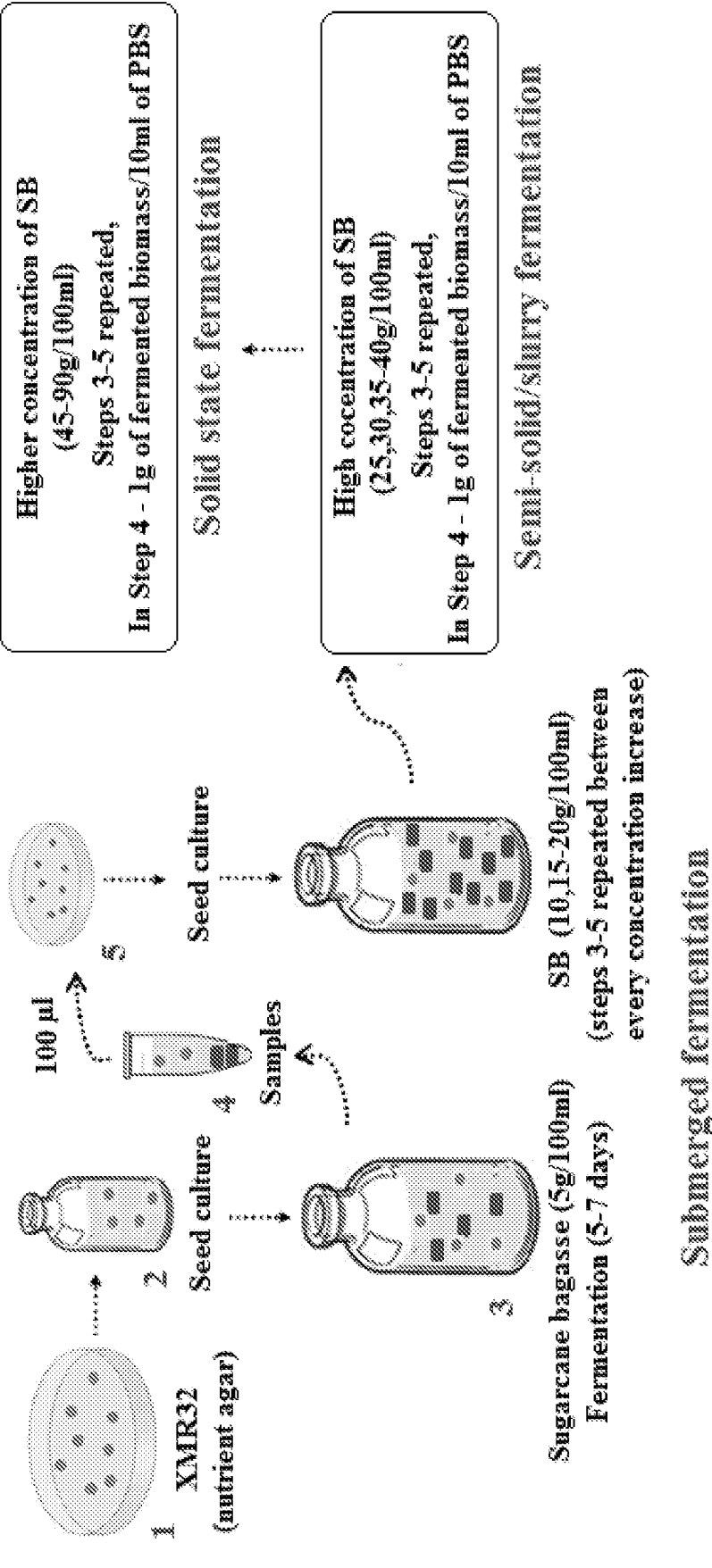


Figure 5

Figure 6

