

LACTOBACILLUS DIOLIVORANS BIOTRANSFORMATION PROCESS

FIELD OF THE INVENTION

- 5 The invention relates to a method of culturing *Lactobacillus diolivorans* to produce cell metabolites, and new *Lactobacillus diolivorans* strains.

BACKGROUND

- 10 Chemical modification of small molecules by microorganisms is commonly known as biotransformation or biological synthesis. There is extensive biotransformation research ongoing for production of materials for industry use. Microbial production of alcohols, sugars or organic acids is a promising approach for obtaining final products or building-block chemicals from renewable carbon sources, e.g.
15 monomers for polymer synthesis. Among the most common chemicals produced by a biotransformation process there are 1,3-propanediol, citric, lactic or succinic acid, and sugar alcohols, like mannitol. The biotechnological conversion of glycerol to 1,3-propanediol is commonly achieved by bacteria under anaerobic conditions.

- Willke et al. (Eur. J. Lipid Sci. Technol. 2008, 110, 831-840) review the
20 biotransformation of glycerol into 1,3-propanediol without using fossil resources. Microbes of the species Clostridiaceae, Enterobacteriaceae and Lactobacilli were reported to be promising candidates for industrial 1,3-propanediol production.

- While microbial processes for the microbiological preparation of valuable chemicals from renewable resources are known, most of the production organisms rely
25 on one or a few carbon sources and most of the known production organisms require a high degree of purity of the carbon sources. Thus, the carbon source is a major cost factor.

- WO 2013064682 A2 discloses *Lactobacillus diolivorans* used in biotransformation processes using raw materials which are complex mixtures of
30 organic carbon sources comprising the carbohydrate to be biotransformed in a purity of less than Technical Grade, such as crude glycerol. In any case, cell culture employs nitrogen gassing to provide anaerobic conditions in the reactor.

 It would be desirable to reduce the cost of producing cell metabolites in a *L. diolivorans* cell culture.

Lactobacilli have been widely used in the food and feed industry. WO2010/122165A1 refers to a method for producing sour dough and baked goods having an extended shelf life by biological preservation through co-fermenting selected Lactobacilli, for example, *L. buchneri*, *L. parabuchneri* and *L. diolivorans*.

5 *L. diolivorans* sp. nov. (DSMZ14421, LMG19667), was identified as a 1,2-propane diol degrading bacterium and isolated from aerobically stable maize silage (Krooneman et al. International Journal of Systematic and Evolutionary Microbiology 2002, 52, 639-646).

WO2006/007395A1 and WO2007/103032A2 describe bacterial additives to
10 (sugar cane) silage employing an inoculum including *L. diolivorans*, in particular to reduce the dry matter content of the silage.

Vivek et al. (Bioresource Technology 2016, 213: 222-230) describe biological valorization of pure and crude glycerol into 1,3-propanediol using an isolate of *Lactobacillus brevis* employing anaerobic conditions.

15 Jolly et al. (Journal of Bioscience and Bioengineering 2014, 1182:188-194) disclose biosynthesis of 1,3-propanediol from glycerol with *L. reuteri* under anaerobic conditions (sparging nitrogen).

SUMMARY OF THE INVENTION

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It is the objective of the present invention to provide a biotransformation process suitable for producing cell metabolites in a *L. diolivorans* cell culture, which is simple and cost efficient, in particular to obtain the cell metabolites at a high yield.

The objective is solved by the subject matter as claimed, and as further
25 described herein.

The invention provides for a method of producing a cell metabolite by culturing *Lactobacillus diolivorans* cells in a cell culture comprising feeding the cell culture in an aerobic reactor system during a production phase using a feed medium comprising a carbon source for biotransformation into the cell metabolite, and isolating the cell
30 metabolite from said cell culture.

According to a specific aspect, the aerobic reactor system does not comprise or does not employ any device or means capable of protecting the cell culture from oxygen supply. Specifically, the aerobic reactor system does not comprise or employ means for sparging (or feeding or otherwise actively introducing) nitrogen or any

anaerobic gas, in particular gas other than air or oxygen. Though oxygen may be present in a feed medium, and therefore supplied to the cell culture by feeding the cell culture during the production phase, it turned out that the method as described herein can successfully produce the product of biotransformation at high yield without any
5 device limiting the oxygen content in the cell culture.

Specifically, the cell culture is carried out without protection from oxygen *e.g.*, without anaerobic gas feeding during the production phase. Specifically, the anaerobic gas feeding is less than 0.1 (vol/vol/min).

It has turned out that the *L. diolivorans* cell culture as used in the method
10 described herein successfully produces cell metabolites by biotransforming a carbon source despite of the absence of oxygen-limiting devices in the reactor system. Though the prior art describes the use the organism under oxygen protection (under strict anaerobic conditions) during a production phase, the cell culture as described herein was found to readily consume any oxygen present in the cell culture during the
15 production phase, thus, produced anaerobiosis *in situ* without any external measures to limit oxygen, was surprisingly insensitive to any such oxygen present in an aerobic reactor system as described herein, and still capable of producing products of biotransformation at a high yield.

Specifically, the dissolved oxygen in the cell culture during the production phase
20 is not more than 80% of the dissolved oxygen in the oxygen saturated liquid phase (100% being oxygen saturation), and may be less than the detection limit (as determined by a standard assay, such as an assay employing luminescence quenching *e.g.*, using a VisiFerm™ OD Sensor HAMILTON, according to the manufacturer's instructions). In an exemplary standard method, the luminescence of
25 certain organic pigments (luminophore) is quenched in the presence of oxygen. The luminophore absorbs the excitation light and releases a part of the absorbed energy by emission of fluorescence. In the presence of oxygen, energy transfer takes place from the excited luminophore to oxygen. The luminophore does not emit fluorescence and the measurable fluorescence signal decreases. ").

30 Specifically, the dissolved oxygen is in the range of 0.1 - 5%, preferably 0.1 - 1%.

According to a specific aspect, the cell culture is maintained at a pH in the range of 4 to 7, and at a temperature in the range of 25 to 40°C during the production phase.

Preferably, the pH is about 5.7 ("about" is understood as ± 0.1), and the temperature is about 30°C ("about" is specifically understood as $\pm 1^\circ$).

According to a specific aspect, the duration of the production phase is in the range of 0.1 – 200 hours. Preferably, the duration of the production phase is at least
5 any one of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 hours. Preferably, the duration of the production phase is less than 180 hours, even more preferably it is less than 100 hours.

According to a specific aspect, the carbon source comprised in the feed medium comprises one or more carbohydrates selected from the group consisting of glycerol,
10 sugars and sugar acids. Sugars include hexoses and pentoses. Among the preferred hexoses are glucose, mannose, galactose and rhamnose, among the preferred pentoses are xylose, arabinose and ribose. D- and L-forms of the sugars can be employed; furthermore the sugars might be derivatized such as acetylated. Among the preferred sugar acids are uronic acids such as glucuronic acid, galacturonic acid, or
15 derivatives from such acids like methylglucuronic acid.

According to a specific aspect, the carbon source comprised in the feed medium is selected from the group consisting of glycerol, crude glycerol, glucose, fructose, lactose, sucrose, starch, biomass hydrolysates, cellulose, lignocellulose, lignocellulosic hydrolysates, sugar beet extract, molasses, organic acid, organic salts, and a
20 combination of any of the foregoing, preferably wherein the carbon source comprises glycerol or a carbohydrate with a purity of less than Technical Grade and an ash content of at least 0.1% (w/w).

The method described herein specifically employs a raw material as a carbon source, which raw material comprises a complex mixture of organic carbon sources comprising the carbohydrate(s) to be biotransformed and impurities e.g., the
25 carbohydrate(s) with a purity of less than Technical Grade.

Specifically, the raw material is a complex mixture of organic carbon sources comprising an ash content of at least 0,1% (w/w), specifically at least 0,2% or at least 0,25% (w/w).

30 Specific embodiments refer to a raw material selected from the group consisting of raw glycerol (herein also referred to as crude glycerol), sugar cane, sugar beet, starchy plant, cellulose, hemicellulose, lignocellulose, including lignocellulosic plant material or lignocellulose hydrolysates, and chitin, e.g. chitin containing material derived from shell fish. Raw glycerol can be derived for example from fatty acid

production, from biodiesel production, from bio-ethanol production, or soap production. Other raw materials can be derived from the waste stream of paper, sugar or wood industries.

Specifically, the carbon source is crude glycerol and used in the concentration
5 of up to 90% (w/w) in the feed medium, specifically at least any one of 10%, 20%, 30%, 40%, 50%, 60%, 70%, or 80% (w/w) in the feed, preferably in the range of 40 to 60%, or about 50%.

Specifically, the crude glycerol comprises glycerol of a purity of less than Technical Grade and comprising an ash content of at least 0.1% (w/w), specifically at
10 least 0,2% or at least 0,25% (w/w).

Specifically, the crude glycerol is obtained as a by-product of fatty acid production, soap production, bio-ethanol production, or biodiesel production.

The method described herein specifically produces a cell metabolite of interest. In particular, the cell metabolite is not produced constitutively (or a primary metabolite)
15 *i.e.* to support growth of the bacteria, but a secondary metabolite or such metabolite which is produced by biosynthesis upon culturing while feeding with the carbon source to be biotransformed.

Specifically, the cell metabolite is a chemical substance or a low molecular weight organic molecule (small molecule).

Specifically, the cell metabolite is selected from the group consisting of 1,3-
20 propanediol, 1,2-propanediol, 2-amino-1,3-propanediol, 3-hydroxybutyrate, poly-3-hydroxybutyrate, ethanol, 1-butanol, 2-butanol, isobutanol, 2,3-butanediol, butanone, lactic acid, citric acid, propionic acid, 3-hydroxy propionaldehyde, 3-hydroxy propionic acid, butyric acid, valeric acid, hexanoic acid, adipic acid, succinic acid, fumaric acid,
25 malic acid, 2,5-furan dicarboxylic acid, aspartic acid, glucaric acid, gluconic acid, glutamic acid, itaconic acid, levulinic acid, acrylic acid, propanol, isopropanol, 1-butanol, 2-butanol, pentanol, hexanol, heptanol, octanol, butanediol, 2,3-butandiol, 3-hydroxybutyrolactone, xylitol, arabinitol, sorbitol, mannitol, vitamin C, riboflavin, thiamine, tocopherol, cobalamin, pantothenic acid, biotin, pyridoxine, niacin, folic acid,
30 3-hydroxybutyrolactone, diamino pentane, diamino hexane and dihydroxyacetone.

Specifically, the chemical substance is an organic acid or an alcohol; exemplary chemicals produced are 1,3-propanediol, lactic acid, 3-hydroxypropionic acid, and mannitol.

Specifically, biotransformation of glycerol, in particular crude glycerol, produces at least any one or more of 1,3-propanediol, 3-hydroxypropionaldehyde, and/or 3-hydroxypropionic acid.

Specific embodiments refer to methods, wherein

- 5 (i) 1,3-propanediol is produced from crude glycerol; or
- (ii) 3-hydroxypropionic acid is produced from crude glycerol; or
- (iii) 3-hydroxypropionaldehyde is produced from crude glycerol; or
- (iv) lactic acid is produced from lignocellulose or lignocellulosic biomass hydrolysate; or
- 10 (v) mannitol is produced from sugar beet extract or hydrolysate.

According to a specific aspect, the feed medium is continuously added to the cell culture during the production phase.

According to a specific aspect, the production phase is carried out in a fed-batch mode or continuous mode.

- 15 Specifically, prior to the production phase, the cell culture is carried out in a batch mode using a growth medium to accumulate biomass.

Specifically, the growth medium allowing the accumulation of biomass comprises a carbon source, a nitrogen source, a source for sulphur and a source for phosphate. Typically, such a medium comprises furthermore trace elements, vitamins,
20 amino acids.

Typically, the growth medium comprises more than one carbon source to obtain a high concentration of biomass, e.g. at least 5 g/L, preferably at least 7.5 g/L and even more preferably at least 10 g/L biomass.

- 25 Specifically, the growth medium comprises a carbon source selected from hexoses, pentoses or heptoses. Specific carbon sources are selected from one or more of glucose, sucrose, fructose, xylose, arabinose and mannose.

Specifically, carbon sources comprised in the growth medium are selected from raw materials, e.g. selected from the group consisting of raw glycerol, hydrolysates of starchy plant material or hydrolysates of lignocellulosic plant material.

- 30 A specific method described herein comprises:

- a) inoculating *L. diolivorans* into a fermentation medium,
- b) culturing the *L. diolivorans* in a growth medium to accumulate biomass,
- c) culturing the *L. diolivorans* in a production medium to produce the cell metabolite or chemical substance, and

d) isolating and purifying the cell metabolite or chemical substance.

According to a specific embodiment, the fermentation medium may be the growth medium and/or the culturing steps b) and c) are employed in a single batch or separate batches.

5 According to a specific aspect, the method refers to producing 1,3-propanediol from raw glycerol, comprising

a) inoculating *L. diolivorans* into a fermentation medium with the raw glycerol as the carbon source,

b) culturing the *L. diolivorans* in a growth medium to accumulate biomass,

10 c) culturing the *L. diolivorans* in a production medium to produce 1,3-propanediol, and

d) isolating and purifying the 1,3-propanediol.

According to a specific aspect, lactic acid is produced from glucose and/or xylose derived from lignocellulose or lignocellosic hydrolysate, comprising

15 a) inoculating *L. diolivorans* into a fermentation medium with the lignocellulose or lignocellulose hydrolysate as the carbon source,

b) culturing the *L. diolivorans* in a growth medium to accumulate biomass,

c) culturing the *L. diolivorans* in a production medium to produce lactic acid, and

d) isolating and purifying the lactic acid.

20 According to a specific aspect, mannitol is produced from fructose of sugar beet (e.g., extract or hydrolysate), comprising

a) inoculating *L. diolivorans* into a fermentation medium with the sugar beet extract or hydrolysate as the carbon source,

b) culturing the *L. diolivorans* in a growth medium to accumulate biomass,

25 c) culturing the *L. diolivorans* in a production medium to produce mannitol, and

d) isolating and purifying the mannitol.

Specifically, the method described herein provides for the inoculation of the bacterium directly into the growth medium. The fermentation medium can be used as growth medium.

30 A specific embodiment refers to a batch culture of *L. diolivorans* growing on raw material, e.g. raw glycerol, lignocellulose or lignocellulose hydrolysates thereby accumulating the biomass, followed by the feed of purified or non-purified carbon sources, e.g. containing carbohydrates, such as glycerol, optionally concomitantly with

glucose and/or xylose, with a specific feed rate, thereby accumulating the cell metabolite or chemical substance, e.g. 1,3-propanediol or lactic acid.

Specifically, the strain of *L. diolivorans* is a native strain, which can be isolated from a natural source or otherwise obtained from a strain collection. Any such isolated strain is provided as pure strain without detectable cells of different origin. Such isolated strains are not naturally-occurring, and thus, artificial or man-made.

According to a specific aspect, the *Lactobacillus diolivorans* cells are of isolated naturally occurring *L. diolivorans* bacteria, strains, progeny or derivatives thereof.

Specifically, the *Lactobacillus diolivorans* cells are of any one of the *L. diolivorans* strains deposited as DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060, or progeny or derivatives of any of the foregoing.

Specific embodiments refer to *L. diolivorans* derived from a selection of strains, including DSM 14421 strain (Leibniz Institut DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, GERMANY; Genbank accession number: AF264701 (16S rRNA), e.g. AF264701.2), identical with LMG 19667, LMG 19668 strain (LMG from BCCM/LMG Laboratorium voor Microbiologie, Universiteit Gent (UGent) Netherlands), G77 strain (CUPV: Colección de la Universidad del Pais Vasco (Spain), Journal of Food Protection (2006) 69, 161-169), KKP 2057p (Collection of Industrial Microorganism, Wacław Dabrowski Institute of Agricultural and Food Biotechnology) described in Electronic Journal of Biotechnology Volume 26, March 2017, 60-63, strain 1Z described in Benef Microbes. 2014 Dec;5(4):471-81, e.g., employing native cell lines or recombinant production cell lines.

According to a specific embodiment, a genetically engineered or recombinant strain of *L. diolivorans* is used. Specific embodiments refer to the strain, which is subjected to mutagenesis and selected for the improved biotransformation.

Specifically, the *L. diolivorans* is used as a genetically engineered or recombinant strain, e.g. which is modified to produce a metabolite, including chemical substances, preferably processing raw or waste material, such as to produce an isolated fermentation product.

Specifically, a genetically engineered or recombinant strain of *L. diolivorans* can be used, preferably a host cell modified to overexpress homologous sequences and/or transformed with a vector comprising heterologous sequences, which are specifically coding and/or non-coding sequences, e.g. to overexpress either homologous and/or heterologous sequences. Specifically, said host cell is engineered to increase the yield

or the titer or the productivity of a biotransformation product e.g., by overexpressing one or more genes forming the cell metabolite of interest.

More specifically, the host cell is engineered to increase the yield of 1,3-propanediol biotransformed from raw glycerol and/or the productivity of the host cell, e.g. the specific and/or volumetric productivity.

As an example, the host cell is engineered to overexpress NADPH-dependent 1,3-propanediol oxidoreductase.

The invention further provides for a *L. diolivorans* strain which any one of the *L. diolivorans* strains deposited as DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060, or progeny or derivatives of any of the foregoing.

Specifically, derivatives of such strains comprise one or more mutations for overexpressing homologous sequences and/or transforming the bacterium with a vector comprising heterologous sequences, to improve production of a cell metabolite.

Such strains, progeny or derivatives are specifically used in a method described herein.

According to a specific aspect, the invention provides for the use of *L. diolivorans* cells in a cell culture for biotransformation of a carbon source into a cell metabolite by an aerobic feeding fermentation process.

Specifically, the carbon source is glycerol or a carbohydrate in a purity of less than Technical Grade and an ash content of at least 0.1% (w/w), specifically at least 0,2% or at least 0,25% (w/w).

Specifically, the use of *L. diolivorans* cells described herein is for biotransformation of glycerol into 1,3-propanediol, preferably wherein the glycerol is biodiesel-derived raw glycerol or glycerol of a purity of less than Technical Grade and comprising an ash content of at least 0.1% (w/w), specifically at least 0,2% or at least 0,25% (w/w).

According to a specific aspect, *L. diolivorans* is used for processing raw or waste material to produce an isolated fermentation product, e.g. the cell metabolite or chemical substance, such as metabolites produced by a (native, wild-type or recombinant) metabolic pathway of the organism.

According to a specific aspect, the cell metabolite is a chemical produced by biotransformation of a carbon source in the bacterial cell culture, and purified from the cell culture or a cell culture fraction.

Such chemical may be final products, Technical Grade chemicals or chemicals of higher purity, such as USP grade chemicals, or intermediates for producing derivatives, including chemical substances, solvents or polymers. Preferred chemicals are produced as purified chemicals by preparative separation and/or purification, preferable on a large or industrial scale. Specifically, chemicals may be produced in large amounts, called bulk material or bulk chemicals. Chemicals may also be produced for use as pharmaceutical ingredients, biocides, and specialty chemicals for technical applications

The cell metabolite may be obtained at high concentrations. For example, the 1,3-propanediol concentration at the end of the production phase or process is preferably at least 40 g/L, more preferably at least 60 g/L, more preferably at least 80 g/L and more preferably at least 100 g/L.

The method described herein specifically provides for the high yield production. Specifically a chemical, such as 1,3-propanediol, is produced at a high yield of at least 40% or 50%, which is particularly determined as follows.

In particular, the product yield can be calculated based on substrate consumption, in other words the amount of product produced on a molar basis divided by the amount of substrate consumed on a molar basis, is preferably at least 40%, more preferably at least any one of 50%, 60%, 70%, 80%, 85%, 90%, or 95%.

The product yield can be calculated based on substrate provision, in other words the amount of product produced on a molar basis divided by the amount of substrate fed into the process on a molar basis, is preferably at least 40%, more preferably at least any one of 50%, 60%, 70%, 80%, 85%, 90%, or 95%.

It is preferred that the cell metabolite is produced as a high purity material, specifically with at least 95% purity, preferably with at least 96% purity, preferably at least 97% purity, preferably at least 98%, even more preferably with 99% purity and most preferably with 99,5% purity, (w/w).

The preferred purity grade is at least Technical Grade, which is understood to be higher than 97% (w/w). A particularly preferred purity grade is Polymer Grade, which is understood to be higher than 98% (w/w).

Specifically preferred is the production and isolation of the cell metabolite on a large scale, e.g. at an industrial scale. Therefore, the term "isolation" as used herein specifically to the preparative separation and purification of sufficient quantities of the cell metabolite from the fermentation system to be further used. Such preparative

isolation is thus opposed to analytical determination of fermentation products, which is used for analysis purposes only.

Preferred cell lines exhibit maximum volumetric and specific productivities for producing a cell metabolite or chemical substance, such as 1,3-propanediol, which are
5 0,8 g/L/h and 0,15 g/g cell mass/h, respectively. Preferred cell lines exhibit mean volumetric and specific productivities for producing a cell metabolite or chemical substance, such as 1,3-propanediol, which are 0,5 g/L/h and 0,1 g/g cell mass/h, respectively.

According to a specific embodiment, the invention refers to the use of *L.*
10 *diolivorans* in one of a series of biotransformation processes, e.g. as platform micro-organism, wherein a carbohydrate from at least two different carbohydrate sources of raw material, e.g. including different types of carbohydrates of low purity, are transformed into cell metabolites or chemical substances. The *L. diolivorans* has proven to be tolerant in growing on different raw materials, possibly utilizing a variety of carbon
15 sources for growing and/or producing cell metabolites or chemical substances.

FIGURE

Figure 1: Agarose gel of genomic DNA preparation of three *Lactobacillus*
20 *diolivorans* strains: DSM14421; LMG19668, and Vogelbusch1050 (deposited under DSM 33056).

DETAILED DESCRIPTION OF THE INVENTION

25 Specific terms as used throughout the specification have the following meaning.

The term “aerobic reactor system” as used herein specifically refers to a production unit comprising one or more reactors, including a production reactor (which can e.g., be a flow-through as used in fed-batch or continuous processes, or closed containment as used in batch processes) comprising the production cell culture, i.e.
30 the cell culture during the production phase. Such production reactor may be an air tight container. The air tight container may have one or more vents to allow exhaust gases to be released and ensure minimal pressure build up in the container. The aerobic reactor system is specifically equipped with one or more devices to introduce a feed medium into the production reactor to feed the cell culture during the production

phase, and to separate or harvest cell culture media or supernatant containing the cell metabolite of interest.

Specific reactor systems provide for the cell culture at commercial scale. In some embodiments, the size of the culture is at least about 100 L, or at least about 200 L, or at least about 500 L, or at least about 1,000 L, or at least about 10,000 L, or at least about 100,000 L, or at least about 500,000 L. In some embodiments, the culture is from about 300 L to about 1,000,000 L.

According to a cost-efficient cultivation strategy, the cell line is cultivated under aerobic cultivation conditions, and optionally employing an aerobic feeding fermentation regimen. For such purpose, the aerobic reactor system typically does not comprise means or devices to protect the cell culture from oxygen e.g., by sparging anaerobic gas.

When culturing the *L. diolivorans* cell culture in an aerobic reactor system, the cell culture may still comprise only a low amount of detectable dissolved oxygen measured in the cell culture medium during the production phase, or an amount below the detection limit. This is because *L. diolivorans* may have the ability to consume oxygen contained in the cell culture medium or feed during the production phase. The low level of oxygen typically denotes a level of oxygen which is lower than that in the oxygen saturated liquid phase of the cell culture. The low level of oxygen may be about 0.1% to about any one of 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, or 1% of a saturated dissolved oxygen concentration in the liquid phase (100% being oxygen saturation), or less than the detection limit. In a specific embodiment, the cell culture medium comprises, for example, about 4 ppb to about 8 ppb of dissolved oxygen in the medium.

The method described herein specifically refers to cell culture under aerobic cultivation conditions, which particularly differs from an anaerobic fermentation insofar that it employs an aerobic reactor system which does not comprise a vented production reactor or fermentation vessel, which would introduce an inert or anaerobic gas for sparging, or to purge the air (or oxygen) from the vessel. Anaerobic fermentation generally comprises a step of purging accomplished by introducing into a production chamber an anaerobic gas e.g., a gas selected from the group consisting of helium, nitrogen, a Noble gas, and combinations thereof. This is particularly avoided in an aerobic reactor system. By avoiding the use of such vented production reactor or

fermentation vessel, the production costs are significantly lower compared to anaerobic fermentation.

According to prior art methods, *L. diolivorans* was used for producing cell metabolites by biotransformation processes carried out under oxygen protection by using nitrogen for ventilation and sparging, thus, using an anaerobic reactor system. This was considered necessary, because in the cultivation of anaerobic bacteria belonging to the genus *Lactobacillus*, it was desirable to employ growing conditions conducive to the proliferation of anaerobes and particularly for the production phase as the key-enzyme glycerol dehydratase is considered highly oxygen sensitive.

It turned out that *L. diolivorans* can be cultivated in an aerobic reactor system without using ventilation with inert or anaerobic gas, thus, can be cultivated under aerobic conditions, and at the same time maintaining low oxygen conditions. Surprisingly, *L. diolivorans* tolerated such conditions when culturing in an aerobic reactor system according to the method described herein. Despite of dispensing ventilation for oxygen protection, the biotransformation turned out to be highly efficient and cell metabolites of interest were produced at high yields. Thereby, a cost effective production method can be provided, and expensive ventilation equipment and ventilation processes can be avoided.

Specific *L. diolivorans* strains are advantageously used, which are novel isolates and deposited as further described herein, in particular *L. diolivorans* strains deposited as DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060.

The deposited strains turned out to be easy to handle and are surprisingly not oxygen sensitive, which means they can be handled in fully aerated environments without losing their ability to grow or to produce. This oxygen insensitivity was even more surprising in a cell culture while producing a cell metabolite during a production phase, which provides a significant advantage. The deposited strains grow very well under the described conditions and readily convert glycerol of pharma grade or of less than Technical Grade into 1,3-propanediol.

The aerobic reactor system as used herein can take the form of a static fermentation or a continuous fermentation system which provides environments favorable for the method described herein such that optimum biotransformation and synthesis of cell metabolites of interest is maintained. In specific cases, the use of an inert or anaerobic gas is avoided to sparge the cell culture or to purge the reactor.

In general, the media used in such cell culture e.g., a growth medium or a feed medium can be used in the form of an aqueous solution, or slurry, and introduced into the reactor without providing an anaerobic environment.

As used herein, "reactor" can include a fermentor or fermentation unit, or any other reaction vessel and the term "reactor" is used interchangeably with "fermentor." For example, a production reactor or bioreactor unit can perform one or more, or all, of the following: feeding of nutrients and/or carbon sources, inlet and outlet flow of fermentation or cell culture medium, separation of gas and liquid phases, maintenance of temperature, maintenance of oxygen and CO₂ levels, maintenance of pH level, agitation (e.g., stirring), and/or cleaning/sterilizing. The exemplary production reactor as used in an aerobic reactor system typically does not perform injection of suitable inert gas. Exemplary, reactor systems may contain multiple reactors within the system, and a facility may contain multiple reactor systems. In various embodiments, the reactor can be suitable for batch, semi fed-batch, fed-batch, perfusion, and/or a continuous fermentation processes. Suitable reactors can be multi-use, single-use, disposable, or non-disposable and can be formed of any suitable material including metal alloys such as stainless steel and Inconel, plastics, and/or glass.

In embodiments, the devices, facilities, and methods described herein can also include any suitable unit operation and/or equipment not otherwise mentioned, such as operations and/or equipment for separation, purification, and isolation of the products of biosynthesis. Any suitable facility and environment can be used, such as traditional stick-built facilities, modular, mobile and temporary facilities, or any other suitable construction, facility, and/or layout. Additionally, and unless otherwise stated, the devices, systems, and methods described herein can be housed and/or performed in a single location or facility or alternatively be housed and/or performed at separate or multiple locations and/or facilities.

Suitable techniques may encompass culturing in a bioreactor starting with a batch phase, followed by a short exponential fed batch phase at high specific growth rate, further followed by a fed batch phase at a low specific growth rate. Another suitable culture technique may encompass a batch phase followed by a fed-batch phase at any suitable specific growth rate or combinations of specific growth rate such as going from high to low growth rate during the production phase. Another suitable culture technique may encompass a batch phase followed by a continuous culturing phase at a low dilution rate.

The term "biotransformation" as used herein is understood as a process of cellular biosynthesis of a compound such as an organic carbon source, or a carbohydrate into a cell metabolite. A compound can be biotransformed by a cell in a cell culture. Such production of the compound by a cell culture is also called "in vivo production" or "in vivo biotransformation". It is understood that such biotransformation in a cell culture is "ex vivo" meaning that it does not employ higher organism, animals or human beings. Specific biotransformation processes are performed in a bacterial cell culture as further described herein to produce cell metabolites, such as chemicals, and specifically the chemical substances as further described herein.

The term "carbon source" as used herein shall mean a carbon substrate, in particular a fermentable carbohydrate, such as used in a biotransformation process, specifically including a source carbohydrate, capable of being metabolized by host organisms or production cell lines. The term "carbohydrate" as used herein is understood in the broader sense, e.g. including sugars, sugar acids, and derivatives thereof, such as polyhydroxy aldehydes or ketones conforming to the general formula $(CH_2O)_n$ and their derivatives. Specifically included are aldehydes, such as glyceraldehydes, ketones, such as dihydroxyacetone, and hydrogenated derivatives, such as glycerol. Specifically included are polyols, preferably at least three-carbon polyols, such as sorbitol, mannitol or glycerol. Particular carbohydrates used as carbon source are selected from the group consisting of monosaccharides, oligosaccharides, polysaccharides, polyols, and derivatives thereof, in the purified form or provided in raw materials.

The carbon source as used for biotransformation described herein can be a purified carbon source or a raw material (such as a carbon source comprising a carbohydrate in a mixture with impurities), and is also understood as "fermentable carbon source" or "fermentable carbohydrate", because it is biotransformed into a cell metabolite by fermentation.

The term "cell line" as used herein shall refer to an established clone of a particular cell type that has acquired the ability to proliferate over a prolonged period of time. The term "host cell line" refers to a cell line as used for expressing an endogenous or recombinant gene or products of a metabolic pathway to produce polypeptides or cell metabolites mediated by such polypeptides. A "production host cell line" or "production cell line" is commonly understood to be a cell line ready-to-use for cultivation in a bioreactor to obtain the product of a production process. The applicable

production cell line may produce a number of useful intermediates and products by fermenting a low molecular weight sugar produced by saccharifying the treated biomass materials. The applicable production cell line may also produce the useful intermediates and products while saccharifying the treated biomass materials by
5 producing the necessary enzymes. For example, fermentation or other bioprocesses can produce alcohols, organic acids, hydrocarbons, hydrogen, proteins or mixtures of any of these materials.

The term "cell culture" or "culturing" or "cultivation" as used herein with respect to a *L. diolivorans* cell refers to the maintenance of the bacterial cells in an artificial,
10 e.g., an *in vitro* environment, under conditions favoring growth, differentiation or continued viability, in an active or quiescent state, of the cells, specifically in a controlled bioreactor according to methods known in the industry.

When culturing a cell culture using appropriate culture media, the cells are brought into contact with the media in a culture vessel or with substrate under
15 conditions suitable to support culturing the cells in the cell culture. As described herein, a culture medium is provided that can be used for the growth of cells. Standard cell culture techniques are well-known in the art.

The cell cultures as described herein particularly employ techniques which provide for the production of cell metabolite of interest by biotransformation, such as to
20 obtain the product in the cell culture medium, which is separable from the cellular biomass, herein referred to as "cell culture supernatant", and may be isolated and optionally purified to obtain the product at a higher degree of purity.

Cell culture media provide the nutrients necessary to maintain and grow cells in a controlled, artificial and *in vitro* environment. Characteristics and compositions of the
25 cell culture media may vary depending on the particular cellular requirements. Important parameters include osmolality, pH, and nutrient formulations. Feeding of nutrients may be done in a continuous or discontinuous mode according to methods known in the art.

Whereas a batch process is a cell culture mode in which all the nutrients
30 necessary for culturing the cells are contained in the initial culture medium, without additional supply of further nutrients during fermentation, in a fed-batch process, after a batch phase, a feeding phase takes place in which one or more nutrients are supplied to the culture by feeding. Although in most processes the mode of feeding is

critical and important, the cell culture and methods described herein are not restricted with regard to a certain mode of cell culture.

In certain embodiments, the cell culture process is a fed-batch process.

In another embodiment, host cells described herein are cultured in a continuous mode. A continuous fermentation process is characterized by a defined, constant and continuous rate of feeding of fresh culture medium into a bioreactor, whereby culture broth is at the same time removed from the bioreactor at the same defined, constant and continuous removal rate. By keeping culture medium, feeding rate and removal rate at the same constant level, the cell culture parameters and conditions in the bioreactor remain constant.

A stable cell culture as described herein is specifically understood to refer to a cell culture maintaining the genetic properties of the cells, specifically keeping the biosynthesis rate and production level high, e.g. even after about 20 generations of cultivation, preferably at least 30 generations, more preferably at least 40 generations, most preferred of at least 50 generations. Specifically, a stable *L. diolivorans* cell culture is provided which is considered to be a great advantage when used to produce cell metabolites of interest on an industrial scale.

The term "cell metabolite" or "cell metabolite of interest" as used herein shall particularly refer to a product of biosynthesis by a cell by biotransforming a carbon source to produce a metabolite e.g., via a metabolic pathway, such as to produce a primary or secondary metabolite.

The products of biotransformation produced by a method described herein are specifically secondary metabolites. Secondary metabolites are referred to as organic compounds produced by cells such as bacteria which are not directly involved in the normal growth, development, or reproduction of the organism. Unlike primary metabolites, absence of secondary metabolites does not result in immediate death, but rather in long-term impairment of the organism's survivability, fecundity, or aesthetics, or perhaps in no significant change at all. Specific secondary metabolites are often restricted to a narrow set of species within a phylogenetic group.

The term "chemical" as used herein shall refer to a chemical substance or compound, in particular organic molecules of low molecular weight (known as "small organic molecules"), such as used in the chemical industry, pharmaceutical industry, the agricultural industry, the cosmetics industry, food and feed industry.

The compounds specifically produced according to the method described herein may comprise chemicals produced in small or large quantities, of low or high purity, including bulk chemicals, fine chemicals, and specialty chemicals. The term includes final products, but also intermediates to produce derivatives, such as reaction products, including monomers for polymer synthesis.

The term "crude glycerol" also referred to as "raw glycerol" is herein understood as the by-product from a fatty acid, bio-ethanol, or bio-diesel production process or from soap production, also called industrial glycerol stemming (or originating) from the production of biodiesel. Crude glycerol can be used as raw or source material and as a carbon substrate in several biotechnological applications. It is containing glycerol (typically in the range of 40-88%, in some cases up to 95%, e.g. about 50% (w/w)) together with salts, soaps and other impurities, including water and methyl alcohol. A typical composition for raw glycerol, such as the crude glycerol stream obtained from the biodiesel production process is provided by NOVAOL S.r.l. (Milano, Italy) Raw Glycerin from Diesel-Bi® production, with following composition: glycerol 85 – 95% w/w, salts of organic/inorganic acids 5 - 10% w/w and methyl alcohol 0 - 1.5% w/w. A further example of crude glycerol is thin stillage (a raw material resulting from ethanol distillation, or obtained as a by-product from bioethanol distillation), or thick stillage (which is concentrated thin stillage, e.g. obtained by evaporation of thin stillage into thick stillage).

In particular, raw glycerol is used in a method described herein, which is obtained as waste material of bio-diesel, bio-ethanol, or fatty acid production, e.g., from different seed oil feedstocks, including palm oil, jatropha, mustard, rapeseed, canola, crambe, soybean, and waste cooking oils.

Specifically, the crude glycerol is recovered from production of fatty acid, bio-diesel, bio-ethanol, or soap, without further processing e.g., upon separation from the fatty acid, biodiesel fuel and soap, respectively. Still, the term also encompasses fractionated or processed crude glycerol, such as including material treated and refined through any of filtration, ion exchange, chemical additions, and/or fractional vacuum distillation to yield various commercial grades. According to a specific embodiment, crude glycerol can be treated to neutralize the material, e.g., to obtain a pH ranging between 6 and 8, preferably about 7. Thereby an organic phase is obtained containing fatty acids and an aqueous phase is obtained containing the glycerol besides impurities. Such fractionated crude glycerol still comprises glycerol of a purity

which is less pure compared to Technical Grade Glycerol, and specifically comprises an undesired amount of ashes, but still can be employed as carbon source in a method described herein.

According to a certain embodiment, the crude glycerol is treated for
5 pasteurization or sterilization, e.g. by autoclaving the material at 121°C, for a period of 10 to 100 min, e.g. about 20 min, before using it as a carbon source for biotransformation. According to another embodiment, the crude glycerol is treated for pasteurization by heating the material to at least 80°C, for a period of 10 to 100 min, e.g. about 15 min ("about" meaning +/- 1 or 2°C), before using it as a carbon source for
10 biotransformation.

Technical Grade Glycerol typically is of a purity which is greater than 97% (w/w).

Specifically, the crude glycerol used as a carbon source for biotransformation is characterized by a glycerol purity which is less than the purity of Technical Grade Glycerol e.g., less than 97%, or less than 96%, specifically less than 95%, more
15 specifically less than 94%, more specifically less than 93%, more specifically less than 92%, more specifically less than 91%, more specifically less than 90% purity (w/w).

Specifically, crude glycerol is used that is characterized by the content of impurities, such as ashes, as determined by a standard assay.

The ash content in crude glycerol is mainly sodium from catalysts used in the
20 biodiesel production process, and typically is contained in crude glycerol within the range of 0.25 to 5.50%, in some cases up to 8%, depending on the oil source and the biodiesel production process (Thompson et al. Applied Engineering in Agriculture 2006, Vol. 22(2): 261-265).

The term "derivative" with respect to a *L. diolivorans* strain as used herein refers
25 to a strain that is derived from its parent strain by e.g., mutagenesis and selection, or by directed mutagenesis.

Specifically, derivatives of *L. diolivorans* strains referred to herein can be produced by methods of limited mutagenesis such as to maintain a certain sequence similarity throughout the genome e.g., at least any one of 60%, 70%, 80%, 90%, 95%,
30 96%, 97%, 98%, or 99% sequence identity.

It is understood that the biotransformation product produced by the method described herein is a cell metabolite or chemical substance, or a derivative of any of the foregoing, which is conveniently produced upon isolation and optionally purification. A "derivative" of a cell metabolite or chemical substance is specifically

understood as a compound derivable from the product of the biotransformation process e.g., by chemical, physical or biological synthesis, including polymerization, acetylation, methylation, or phosphorylation.

5 The term "*Lactobacillus diolivorans*" or "*L. diolivorans*" as used herein shall refer to a species of *Lactobacillus* as first described by Krooneman et al. (International Journal of Systematic and Evolutionary Microbiology 2002, 52, 639-646), as isolated strain, a wild-type bacterium or derivative thereof, e.g. a genetically engineered production cell line.

"Deposited strains" of *L. diolivorans* referred to herein are as follows.

10 The following *L. diolivorans* strains have been isolated in Austria and deposited by Vogelbusch GmbH, Vienna, Austria, on 26th February 2019 at the DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen, Mascheroder Weg 1b / Inhoffenstraße 7B, 38124 Braunschweig (DE).

15 The deposits refer to *L. diolivorans* strains and cultures, and are characterized as follows.

DSM 33056 (also referred to as *Lactobacillus diolivorans* Vogelbusch1050) is characterized as follows: the strain is easy to handle and is not oxygen sensitive, which means it can be handled in fully aerated environments without losing the ability to grow or to produce. Particularly on mixture of glycerol with any one or more of
20 glucose, fructose or mannose, the strain has an outstanding performance of 1,3-propanediol production.

DSM 33057 (also referred to as *Lactobacillus diolivorans* HH), is characterized as follows: the strain is easy to handle and is not oxygen sensitive, which means it can be handled in fully aerated environments without losing the ability to grow or to
25 produce. Particularly on mixture of glycerol with any one or more of glucose or fructose the strain has an outstanding performance of 1,3-propanediol production. Especially the strain is one of the best performers on a mixture of glycerol and sucrose for 1,3-propanediol production.

30 DSM 33058 (also referred to as *Lactobacillus diolivorans* HM), is characterized as follows: the strain is easy to handle and is not oxygen sensitive, which means it can be handled in fully aerated environments without losing the ability to grow or to produce. Particularly on mixture of any one or more of glycerol with glucose, fructose or mannose the strain has an outstanding performance of 1,3-propanediol production.

DSM 33059 (also referred to as *Lactobacillus diolivorans* HS), is characterized as follows: the strain is easy to handle and is not oxygen sensitive, which means it can be handled in fully aerated environments without losing the ability to grow or to produce. Particularly on mixture of glycerol with any one or more of glucose or fructose
5 the strain has an outstanding performance of 1,3-propanediol production. Especially the strain is the best performer on a mixture of glycerol and xylose for 1,3-propanediol production. Furthermore it is also one of the best performers on a mixture of glycerol and mannose.

DSM 33060 (also referred to as *Lactobacillus diolivorans* HF), is characterized
10 as follows: the strain is easy to handle and is not oxygen sensitive, which means it can be handled in fully aerated environments without losing the ability to grow or to produce. Particularly on mixture of glycerol with any one or more of glucose, fructose or mannose the strain has an outstanding performance of 1,3-propanediol production.

The term "final product" as used herein refers to a cell metabolite or chemical
15 substance of controlled quality that is manufactured and optionally finished to a product ready-to-use for further manufacturing or industrial purposes or as consumer product.

The term "genetically engineered" as used herein shall refer to a recombinant organism that is used for producing a fermentation product. The organism is typically a
20 production cell line engineered to improve a production process or to enable the production of a new product. The term is understood in contrast to the "wild-type" organism, which is typically not genetically engineered. The term "wild-type" as used within this disclosure generally means "as isolated from natural sources" such as *L. diolivorans* DSM 14424 or DSM 14421, or *L. diolivorans* LMG 19668, or *L. diolivorans*
25 G77, or any of the deposited strains DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060, including any progeny thereof.

The term "intermediate" as used herein shall refer to a chemical substance which is formed as a product of the biotransformation process, which may be separated from the fermentation broth and further processed to form a derivative or
30 final product.

The term "isolate" with respect to a cell metabolite, chemical substance or a product of a biosynthesis or biotransformation process as used herein shall mean to separate or purify the substance from at least one impurity, specifically by preparative means, to obtain an isolated product which is sufficiently separated from the

environment with which it would be associated before such isolation, so as to exist in "substantially pure" form. "Isolated" does not necessarily mean the exclusion of artificial or synthetic mixtures with other compounds or materials, or the presence of impurities that do not interfere with the fundamental activity, and that may be present, 5 for example, due to incomplete purification.

The term "isolate" as used herein with respect to a bacterial strain,

The term "metabolic pathway" as used herein shall refer to biochemical reactions which take place in an organism, e.g. the metabolism of a particular compound or a microbial enzyme pathway to produce cell metabolites, in particular small organic 10 molecules. This refers to all biosynthetic, modification and degradation pathways of a compound in the cell. Metabolic engineering is specifically through the introduction, deletion, and modification of metabolic pathways in microorganisms by using suitable recombination techniques, which are widely applied for the efficient production of desired metabolites and biomolecules. Even novel synthetic products can be obtained 15 e.g. by metabolic engineering of host cells.

The term "mutagenesis" as described herein shall refer to a recombinant construct or organism with a mutated nucleic acid, so to obtain variants thereof with at least one change in the non-coding or coding region. Mutagenesis may be through random, semi-random or site directed mutation.

20 The term "overexpression" as used herein with respect to a host cell, specifically a recombinant host cell, is intended to encompass increasing the expression of a polypeptide or protein (such as enzymes used in the biotransformation process) to a level greater than the cell normally produces. It is intended that the term encompass overexpression of homologous (endogenous), as well as heterologous sequences or 25 proteins, specifically aiming at the increase of the productivity of the cell to produce a biotransformation product, e.g. at least 1.5-fold, preferably at least 2-fold, more preferred at least 3-fold as compared to a wild-type host cell of the same type.

The term "production phase" as used herein refers to a certain phase of a cell culture during which the cell culture produces a biotransformation product. The 30 production phase may specifically follow a growth phase during which the cell culture is accumulating biomass by growing the cells. Cultivation during growth and production phases may be conveniently performed by batch, fed-batch and continuous cultivation process. A carbon source suitably used as a substrate for biotransformation into a cell metabolite may be contained in the feed of a fed-batch process e.g., under carbon

substrate limited conditions, *i.e.* using the carbon source in a limited amount to keep a production cell line under growth-limited conditions, and in the production mode. Such a limited amount may be employed in a fed-batch process, where the carbon source is contained in a feed medium and supplied to the culture at low feed rates for sustained
5 energy delivery, *e.g.* to produce a biosynthesis or fermentation product, while keeping the biomass at low specific growth rates. A feed medium is typically added to a fermentation broth during the production phase of a cell culture.

The term "raw material" as used herein specifically refers to any complex carbon source or carbohydrate materials, in particular carbohydrate enriched biomass,
10 including carbohydrate of low purity in the monomeric or polymeric form, such as derived from bioenergy crops, industrial crops, agricultural residues, municipal waste, industrial waste, yard waste, wood, straw, chitin containing residues from shellfish. Specific examples of raw materials are raw (crude) glycerol, sugar cane, sugar beet, starchy plant, cellulose, hemicellulose, lignocellulose, lignocellulose hydrolysate, or
15 chitin.

The complex carbohydrate mixture of a raw material typically comprises at least two different organic carbon sources in significant amounts *e.g.*, each in amounts of at least 2%, specifically at least 3%, more specifically at least 4%, more specifically at least 5% (w/w).

20 Specifically, a raw material is used in a method described herein, which is characterized by the fermentable carbohydrate content with a purity of less than Technical Grade (Technical Grade >97% (w/w) purity), *e.g.*, less than 96%, specifically less than 95%, more specifically less than 94%, more specifically less than 93%, more specifically less than 92%, more specifically less than 91%, more specifically less than
25 90% purity (w/w).

Specifically, a raw material is used in a method described herein which is characterized by the content of impurities, such as ashes, as determined by a standard assay. An exemplary standard assay determines the residue left after ignition of organic matter. Ashes are commonly understood as the approximate measure of the
30 mineral content and other inorganic matter in biomass.

A typical standard assay for the determination of the ash content in raw material such as crude glycerol, is a gravimetric method *e.g.*, as described in INTERNATIONAL UNION OF PURE AND APPLIED CHEMISTRY, APPLIED CHEMISTRY DIVISION, COMMISSION ON OILS, FATS AND DERIVATIVES, "STANDARD METHODS FOR

THE ANALYSIS OF OILS, FATS AND DERIVATIVES", 6th Edition, 1st Supplement: Part 2 (1980), SECTION III. GLYCERINES, Prepared for publication by A. HAUTFENNE, Université Catholique de Louvain, Louvain-la-Neuve, Belgium.

According to this method, the test involves combustion of a test portion, ignition
5 of the organic matter and weighing of the residue. The ash content is defined as the quantity of ash and expressed as a percentage by mass (n/m).

The raw material specifically used in a method of biotransformation described herein is characterized by the ash content of at least 0,1% (w/w), specifically at least 0,2% or at least 0,25%, more specifically at least 0,50%, more specifically at least
10 0,75%, more specifically at least 1%, even more specifically at least 2% (w/w).

More specifically, raw material is used which comprises fermentable carbohydrates with a purity of less than Technical Grade, and a content of impurities, such as ashes, as further described herein.

Specifically, the raw material is crude glycerol which is characterized by the
15 glycerol purity of less than the purity of Technical Grade Glycerol and the content of impurities, such as ashes, as further described herein.

Specifically, the raw material used for biotransforming a carbon source into a cell metabolite as described herein is a crude material as it is recovered from various sources without further processing. Still, the term also encompasses fractionated or
20 processed crude material, such as including material generally treated and refined through filtration, centrifugation, ion exchange, chemical additions, treatment with enzymes, and distillation, such as fractional vacuum distillation to yield various commercial grades. It is preferred that raw material is treated or processed before its use in a method described herein. Preferable treatment includes pasteurization,
25 sterilization, *e.g.* by autoclaving, treatment with chemicals, *e.g.* acids or bases, such as to obtain a pH value ranging between 6 and 8, preferably about 7, or fractionation, *e.g.* to reduce the amount of undesired organic acids or alcohols.

Such fractionated or processed crude material still comprises the fermentable carbohydrate at a purity which is less than Technical Grade and an undesired amount
30 of ashes, thus is employed as raw material for use in a method described herein.

According to a certain embodiment, the raw material is treated for pasteurization or sterilization *e.g.*, by heat treatment at 80°C, for a period of 10 to 100 min, *e.g.* about 15 min ("about" meaning +/- 1 or 2°C) or by autoclaving the material at 121°C, for a period of 10 to 100 min, *e.g.* about 20 min ("about" meaning +/- 1 or 2°C).

Specifically, the method described herein comprises an additional step of sterilizing the raw material before adding said material to the cell culture.

The term "sequence identity" as used herein indicates that two or more nucleotide sequences have (to a certain degree, up to 100%) the same or conserved
5 base pairs at a corresponding position.

"Percent (%) identity" with respect to the nucleotide sequence of a gene or genome is defined as the percentage of nucleotides in a candidate DNA sequence that is identical with the nucleotides in the DNA sequence to which the candidate sequence shall be compared, after aligning the sequence and introducing gaps, if necessary, to
10 achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent nucleotide sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software. Those skilled in the art can determine appropriate parameters for measuring alignment,
15 including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "recombinant" as used herein shall mean "being prepared by or the result of genetic engineering". Thus, a "recombinant microorganism" comprises at least one "recombinant nucleic acid". A recombinant microorganism may be a mutant
20 produced by a suitable method mutagenesis, and/or specifically comprises an expression vector or cloning vector, or it has been genetically engineered to contain a recombinant nucleic acid sequence or recombinant metabolic pathway to produce cell metabolites at high yield or to produce novel cell metabolites which the wild-type cell (before recombination) would not produce in significant amounts.

25 As described herein, *L. diolivorans* surprisingly turned out to be tolerant to aerobic cultivation conditions, such that a cell culture of a *L. diolivorans* strain could produce cell metabolites by biotransformation at high yields, even without nitrogen gassing or otherwise protecting from oxygen. A variety of carbon sources could be used as substrates for biotransformation, even those which are raw or waste materials
30 and of low purity. Therefore, the species could be used as a production cell line to produce products of a biotransformation process in a cost efficient way. Specific *L. diolivorans* strains are provided which are preferably used. Specifically the *L. diolivorans* strains turned out to readily metabolize a range of substrate sugars or polyols or other carbohydrates to be converted (biotransformed) into a cell metabolite

or chemical substance. A specifically preferred carbohydrate is glycerol, in particular crude glycerol.

A specifically preferred method comprises the steps of

- (i) raw material pre-treatment for purification and/or sterilization,
- 5 (ii) raw material fermentation for cell growth and accumulation of the cell metabolite or chemical substance,
- (iii) isolation of the cell metabolite or chemical substance, and
- (iv) purification of the cell metabolite or chemical substance.

In particular, the production process for producing 1,3-propanediol from raw
10 glycerol comprises the steps of

- (i) raw glycerol pre-treatment for purification and/or sterilization, e.g. by pH adjustment and fractionation to separate a non-aqueous phase, and/or by sterilization through autoclaving,
- (ii) raw glycerol fermentation for cell growth and accumulation of 1,3-
15 propanediol,
- (iii) isolation of 1,3-propanediol, and
- (iv) purification of 1,3-propanediol.

Specifically, the raw material can be used to culture *L. diolivorans* to grow the organism to increase the biomass in preparation of a biotransformation process, and
20 may further be used during the production phase e.g., as a substrate of biotransformation or concomitantly with another carbon source for biotransformation. Typically biomass is accumulated, and the cell metabolite produced, either consecutively or simultaneously. For growing the organism, the raw material is optionally pre-processed, e.g. to remove a lipid phase, and may be supplemented with
25 one or more further carbon sources, such as glucose, xylose, arabinose, fructose, sucrose or others.

In specific cases, more than one cell metabolite is produced by the fermentation process in significant amounts, which cell metabolites may be isolated and purified. Thus, a specific embodiment refers to the production of at least two different cell
30 metabolites in the purified form by biotransforming a fermentable carbohydrate of a raw material, in particular during the same fermentation process.

Renewable resources to be converted include glycerol, such as raw (crude) glycerol, e.g. thin stillage (a raw material obtained as a by-product from bioethanol distillation) or thick stillage (concentrated thin stillage, e.g. obtained by evaporation into

thick stillage). Raw glycerol is preferably processed before use in the fermentation process, e.g. by autoclaving and separating the aqueous phase containing the glycerol, thereby reducing the content of fatty acids.

Other preferable renewable resources include sugars. Examples for sugars are
5 hexoses such as glucose as well as pentoses such as xylose or arabinose. Possible sources for glucose could be starchy plant material or sugar cane or sugar beet in various grades of purification.

Cellulosic glucose is another preferred carbon source. A preferred source of pentoses is hemicellulosic plant material. Specifically the degree of purification of the
10 hemicellulosic plant material may be low.

The conversion of the carbon source specifically takes place in a liquid phase, preferably in an aqueous environment. The conversion of the carbon source can take place concomitantly with the growth of the microorganisms or can be independent of the growth of the microorganisms. Therefore, the cell metabolite can be accumulated
15 in the medium while the microorganisms are growing or after the microorganisms ceased growing or both.

Inoculation of the microorganism may be into a fermentation medium, which is either a medium to provide for the bacterial growth, such as the growth medium, or directly into a production medium or feed.

20 Growth and/or production can suitably take place in batch mode, fed-batch mode or continuous mode.

A preferred embodiment includes a batch culture to provide biomass followed by a fed-batch culture for efficient conversion of the carbon source into the desired product. The carbon source used for biomass production can, but need not be identical
25 as the carbon source for producing the cell metabolite.

For example, the strain may be grown on glucose or xylose for biomass provision followed by a feed of (raw) glycerol or raw glycerol with glucose, which is converted into 1,3-propanediol.

A growth medium allowing the accumulation of biomass typically comprises a
30 carbon source, a nitrogen source, a source for sulphur and a source for phosphate. Typically, such a medium comprises furthermore trace elements, vitamins, amino acids. The medium may or may not contain antifoam agents.

Preferred nitrogen sources include ammonia, ammonium salts, such as NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$, $(\text{NH}_4)_2\text{CO}_3$ nitrate salts, such as NaNO_3 , KNO_3 , urea, amino acids.

Preferred sulphur sources include sulphuric acid or sulphates, such as $(\text{NH}_4)_2\text{SO}_4$, Na_2SO_4 , NaHSO_4 , MgSO_4 , MnSO_4 , methionine, cysteine.

Preferred phosphate sources include phosphoric acid or phosphates, such as Na_2HPO_4 , NaH_2PO_4 , K_2HPO_4 , KH_2PO_4 .

5 Further typical medium components include yeast extract, peptone, meat extract, malt extract, corn steep liquor, corn steep powder or fish meal.

Preferably the medium is supplemented with vitamin B_{12} .

A typical growth medium for *L. diolivorans* comprises casein peptone (tryptic digest), meat extract, yeast extract, Tween 80, K_2HPO_4 , Na-acetate, $(\text{NH}_4)_2\text{H-citrate}$,
10 $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, $\text{MnSO}_4 \times \text{H}_2\text{O}$.

Another typical growth medium for *L. diolivorans* comprises K_2HPO_4 , $(\text{NH}_4)_2\text{H-citrate}$, KH_2PO_4 , sodium chloride, ascorbic acid, potassium acetate, Tween 80, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$, $\text{MnSO}_4 \times \text{H}_2\text{O}$, $\text{CoSO}_4 \times 7 \text{H}_2\text{O}$, calcium lactate, DL-alanine, DL-aminobutyric acid, Glycine, L-histidine HCl, L-lysine HCl, L-phenylalanine, L-proline, L-serine,
15 L-threonine, L-cysteine, L-arginine, L-aspartic acid, L-asparagine, L-glutamic acid, L-isoleucine, L-leucine, L-methionine, L-tyrosine, L-tryptophan, L-valine, nicotinic acid, calcium pantothenate, cyanocobalamin, para-aminobenzoic acid, myo-inositol, pyridoxal HCl, riboflavin, biotin, folic acid, and $\text{FeSO}_4 \times 7 \text{H}_2\text{O}$.

A typical production medium comprises the carbon source for biotransformation,
20 and besides a sugar and optionally vitamins.

The feed to the fermentation typically is a carbohydrate solution comprising up to 50 wt % carbohydrates. The feed rate may limit the effects of product inhibition on the cell growth, thus a high product yield based on substrate provision will be possible.

The fermentation is preferably carried out in a slightly acidic medium, e.g. of
25 pH 5.5-6.0, preferably at a pH of about 5.7 ("about" is specifically understood as ± 0.1).

Typical fermentation times are about 24 to 180 hours with temperatures in the range of 26°C to 40°C, preferably about 30 °C ("about" is specifically understood as $\pm 1^\circ$).

30 The production cell line is specifically used on a large scale or industrial scale.

A specific process scale preferably employs volumina of at least 50 L, preferably at least 1 m³, more preferably at least 10 m³, more preferably at least 100 m³, most preferably at least 500 m³.

Production conditions in industrial scale refer to e.g. fed batch cultivation in reactor volumes of 100 L to 100 m³ or larger, employing typical process times of several days, or continuous processes in fermenter volumes of appr. 50 – 1000 L or larger, with dilution rates of appr. 0.05 – 0.15 h⁻¹.

5 Suitable cultivation techniques may encompass cultivation in a bioreactor starting with a batch phase, followed by a fed batch phase at a low specific growth rate. Another suitable cultivation technique may encompass a batch phase followed by a continuous cultivation phase at a low dilution rate.

Specifically the *L. diolivorans* strain may be employed as in a homofermentative
10 process, wherein the produced cell metabolite or the final product (which may be a derivative of the cell metabolite) is mostly one chemical substance, such as lactic acid or lactate. Alternatively the process may be heterofermentative, wherein some other products of metabolic pathways are produced. Genetically engineered variants of *L. diolivorans* may comprise metabolic pathways, which are not naturally present in *L.*
15 *diolivorans*, which allows producing a variety of cell metabolites.

Applicable metabolic pathways mediating the biotransformation or metabolism of a carbon source, such as glycerol, a hexose, such as glucose, fructose, galactose, or a pentose, such as xylose or arabinose in *L. diolivorans* under aerobic conditions, provide for effective conversion of the source carbon or source carbohydrate into the
20 cell metabolite. The cell metabolite so produced may be a typical negligible product of the metabolic pathway in wild-type strains. A homofermentative route may be engineered by overexpressing pathways involved in the conversion of the source carbohydrate to the desired cell metabolite, and/or blocking those leading to the synthesis of competing by-products.

25 For example, the overexpression of specific enzymes involved are a glycerol transporter, 1,3-propanediol oxidoreductase, glycerol kinase, glycerol-3-phosphate dehydrogenase, glycerol dehydrogenase, dihydroxyacetone kinase and triosephosphate isomerase. Further, the synthesis of undesired by-products, such as succinate, acetate, and ethanol may be minimized by knock-out, deletion or knock-
30 down of the respective genes.

Specific examples relate to the fermentation of glucose and glycerol into 1,3-propanediol with the wild-type *L. diolivorans* in a fed-batch process, e.g. where the biomass is accumulated upon growing on glucose, and fermentation is started upon feeding with glycerol.

Another specific example relates to the fermentation of glucose and glycerol into 1,3-propanediol with the wild-type *L. diolivorans* in a fed-batch process, where the biomass is accumulated upon growing on glucose in the presence of glycerol, and fermentation is started upon feeding with glycerol and glucose.

5 A further specific example refers to fermentation of the wild-type *L. diolivorans* with xylose and glycerol into 1,3-propanediol, e.g. with xylose as batch medium for growing biomass, and glycerol as feed medium to start the biotransformation process.

 According to a specific further example, the wild-type *L. diolivorans* is cultivated with glucose and raw glycerol in a batch process, employing both, glucose and raw
10 glycerol, in the batch medium to grow biomass and to effect biotransformation of glycerol into 1,3-propanediol.

 A further example refers to the cultivation of wild-type *L. diolivorans* with glucose in a batch process, to grow biomass and to effect biotransformation of glucose into lactic acid.

15 Yet, a further example refers to the cultivation of wild-type *L. diolivorans* with xylose in a batch process, to grow biomass and to effect biotransformation of xylose into lactic acid.

 It was further shown that wild-type *L. diolivorans* could be fermented with arabinose in a batch process, to grow biomass and to effect biotransformation of
20 arabinose into lactic acid.

 According to a further example, wild-type *L. diolivorans* was fermented with fructose in a batch process, to grow biomass and to effect biotransformation of fructose into mannitol.

 According to further examples,

- 25 a. 1,3-propanediol can be produced from glucose and/or mannose in industrial glycerol (crude glycerol, a by-product from fatty acid, bio-diesel, bio-ethanol, or soap production) in various mixtures. 1,3-propanediol production is not limited to using crude glycerol as the raw material, but can as well be produced using a mixture of crude glycerol and thin
30 stillage (a raw material resulting from alcohol distilleries or thick stillage (thin stillage evaporated into thick stillage) as the raw material.
- b. 3-Hydroxypropionic acid can be produced as a further chemical substance, using raw materials like any of crude glycerol, a mixture of crude glycerol and thin stillage, or thick stillage.

c. Lactic Acid can be produced as a further chemical substance, using raw materials such as any of crude glycerol, a mixture of crude glycerol and thin stillage, thick stillage or lignocellulosic biomass hydrolysate (*Miscantus* hydrolysate).

5 d. 3-Hydroxypropionaldehyde can be produced as a further chemical substance, using crude glycerol as raw material.

A recombinant strain of *L. diolivorans* LMG 19668 was described to employ an expression construct such as a plasmid comprising endogenous genetic elements, including a promoter of the *Lactobacillus diolivorans* gene for the glyceraldehyde-3-phosphate dehydrogenase and an origin of replication isolated from the endogenous
10 plasmid from *Lactobacillus diolivorans*. The recombinant *L. diolivorans* LMG 19668, has been engineered to overexpress the 1,3-propanediol oxidoreductase. Thereby the yield of biotransformation and 1,3-propanediol biosynthesis could be increased.

According to a specific embodiment, a *L. diolivorans* strain is subjected to
15 mutagenesis and/or selection procedures, e.g. to obtain a strain with improved productivity, improved substrate range, improved product or substrate tolerance, improved product range or an improved glycerol uptake rate.

Preferred methods for mutagenesis include contacting *L. diolivorans* with mutagenizing substances such as nitrosoguanidine (NTG), 5-bromo-deoxyuridine (5BU),
20 ethyl methane sulfonate (EMS), methyl methane sulfonate (MMS), or diethylsulfate (DES). Another preferred method for mutagenesis includes UV irradiation of a culture of *L. diolivorans*.

Preferred methods for selection include plating of bacterial cultures on agar plates exhibiting the selective pressure of choice. For example, selection might be
25 plating of the bacterial cultures on agar plates comprising an increasing amount of product or substrate impurities for selection of strains exhibiting increased tolerance to the product or substrate impurities. Another preferred method for selection of desired bacterial strains includes sorting procedures by flow cytometry.

According to a specific aspect, the *L. diolivorans* strain is genetically
30 engineered, such as that the natural genetic setup of the organism has been changed. This includes for example:

- Deletion or inactivation or attenuation of genes naturally present in the organism
- Overexpression of genes naturally present in the organism
- Mutation of genes naturally present in the organism

- Addition or expression of genes naturally not present in the organism
- Or any combinations therefrom

Specific methods of genetically engineering of *L. diolivorans* comprise:

- The provision of a recombinant DNA fragment, and/or
- 5 • Transformation of the organism with said DNA fragment

In general, the recombinant nucleic acids or organisms as referred to herein may be produced by recombination techniques well known to a person skilled in the art. In accordance with the present invention conventional methods of molecular biology, microbiology, and recombinant DNA techniques within the skill of the art may
10 be employed. Such techniques are explained in detail e.g., in Maniatis, Fritsch & Sambrook, Molecular Cloning: A Laboratory Manual (1982).

Examples for DNA fragments for genetic engineering of *L. diolivorans* include plasmids, linear DNA fragments, such as PCR products, and others well-known in the art. A specific plasmid comprises the replication origin of the native *L. diolivorans*
15 plasmid, native promoters, selectable markers and expression cassettes for the genes of interest. The nucleotide sequences that could be used for engineering a cell line as used in a method described herein, which would provide for an improved biotransformation process, can be obtained from a variety of sources. The origin of a promoter is preferably from genomic DNA of *L. diolivorans* DSM 14421, DSM 33056, DSM
20 33057, DSM 33058, DSM 33059, DSM 33060, LMG 19668 or G77.

The promoter may be any suitable DNA sequence which shows transcriptional activity in the host cell and may be derived from genes encoding proteins either homologous or heterologous to the host. The promoter is preferably derived from a gene encoding a protein homologous to the host cell. The promoter can be an endogenous
25 promoter or heterologous to the host cell.

Suitable promoter sequences for use with prokaryotic host cells may include but are not limited to promoters obtained from *Lactobacillus* sp., *Lactococcus* sp., *Staphylococcus* sp., *Pediococcus* sp., *Enterobacteria* sp., *Streptococcus* sp. *Oenococcus* sp. The promoter is not limited to any particular species provided that
30 they can function in prokaryotic host cells and in particular in *Lactobacilli*.

Further suitable promoter sequences for use with bacterial host cells may include but are not limited to promoters obtained from genes that code for metabolic enzymes which are known to be present at high concentration in the cell, e.g. glycolytic

enzymes like glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, phosphofructokinase, or enolase.

In a preferred expression system the promoter is an inducible or a constitutive promoter.

5 Appropriate expression vectors typically comprise regulatory sequences suitable for expression of DNA encoding a heterologous polypeptide or protein in a prokaryotic host cell. Examples of regulatory sequences include promoters, operators, and enhancers, ribosomal binding sites, and sequences that control transcription and translation initiation and termination. The regulatory sequences may be operably linked to
10 the DNA sequence to be expressed. For example, a promoter sequence is said to be operably linked to a coding sequence, if the promoter controls the transcription of the coding sequence.

 A preferred method employs a plasmid, which is pSHM. Expression vectors may include but are not limited to cloning vectors, modified cloning vectors and specifically
15 designed plasmids. A preferred expression vector used for producing recombinant *L. diolivorans* may be any expression vector suitable for expression of a recombinant gene in a bacterial host cell. The recombinant expression vector may be any vector which is capable of replicating in or integrating into the genome of the host organisms.

 It may be preferred to use a plasmid originating from wild-type *L. diolivorans* as
20 an expression vector.

 To allow expression of a recombinant nucleotide sequence in a host cell, the expression vector may provide the recombinant nucleotide sequence with a functional promoter adjacent to the 5' end of the coding sequence. The transcription is thereby regulated and initiated by this promoter sequence.

25 According to a specific embodiment, a recombinant construct is obtained by ligating the relevant genes into a vector. These genes can be stably integrated into the host cell genome or replicated as an episomal plasmid. The vector may be transferred into the host cell by transformation. Preferred methods of transformation for the uptake of the recombinant DNA fragment by the microorganism include chemical
30 transformation, electroporation or transformation by protoplastation. Transformants can be obtained by introducing such a vector DNA, e.g. plasmid DNA, into a host and selecting transformants which express the relevant protein or host cell metabolite with high yields.

The polypeptides encoded by the genes can be produced using the recombinant host cell line by culturing a transformant, thus obtained in an appropriate medium, isolating the expressed product or metabolite from the culture, and optionally purifying it by a suitable method.

5 There are several preferred approaches for the production of one or more cell metabolites using the strains and methods described herein. Substances may be expressed, processed and optionally secreted by transforming the bacterial organism with an expression vector harbouring recombinant DNA encoding a relevant protein and at least one of the regulatory elements as described above, preparing a culture of
10 the transformed organism, growing the culture and fermenting a carbohydrate source to recover the product of the biotransformation process.

A preferred host cell line can stably maintain its genetic properties, and the production level remains high, e.g. at least at a μg level, even after about 20 generations of cultivation, preferably at least 30 generations, more preferably at least
15 40 generations, most preferred of at least 50 generations. A stable host cell is considered a great advantage when used for producing chemicals at industrial scale.

A cell metabolite or chemical substance produced by the method or strains described herein can be isolated and purified using state of the art techniques, including the increase of the concentration of the desired cell metabolite or chemical
20 substance and/or the decrease of the concentration of at least one impurity.

As isolation and purification methods the following are preferred, specifically for preparative isolation: distillation, chromatography, crystallization, filtration, centrifugation, decanting, reprecipitation or electrodialysis. The cell metabolite or chemical substance may be e.g. obtained from the fermented broth that is clarified using a
25 centrifuge.

A highly purified product may be produced which is essentially free from contaminating proteins, and preferably has a purity of at least 90%, more preferred at least 95%, or even at least 98%, up to about 100%. The purified products may be obtained by purification of the cell culture supernatant or else from cellular debris.

30 The isolated and purified product can be identified by conventional methods such as HPLC, GC, MS, NMR, infrared spectroscopy.

The foregoing description will be more fully understood with reference to the following examples. Such examples are, however, merely representative of methods of

practicing one or more embodiments of the present invention and should not be read as limiting the scope of the invention.

EXAMPLES

5

Example 1: Preparation of MRS-Medium used for the isolation and cultivation of *Lactobacillus diolivorans*:

Table 1: MRS Medium

Component	Concentration [g/L]
Casein peptone, tryptic digest	10
Meat extract	10
Yeast extract	5
K ₂ HPO ₄	2
Na-acetate	5
(Na) ₃ -citrate x 2 H ₂ O	2,6
(NH ₄) ₂ HPO ₄	1,17
MgSO ₄ x 7 H ₂ O	0,20
MnSO ₄ x H ₂ O	0,05
Dissolve in H ₂ O	

10

30 g/L of the desired carbon source is added (typically glucose) and if desired additionally 10 g/L glycerol is added. For solid media 20 g/L of agar was added. The pH-value of the medium was adjusted to 5.7 with concentrated hydrochloric acid (HCl). Then the medium was sterile filtered or autoclaved.

15

Example 2: Preparation of OS-Medium used for the isolation of *Lactobacillus diolivorans*:

Table 2: OS Medium

Component	Concentration [g/L]
Fresh Orange Juice (filtered)	200
Casein peptone	10
Yeast Extract	3
K ₂ HPO ₄	2.5
Agar	17

The pH-value of the medium was adjusted to 5.5 with concentrated hydrochloric acid (HCl). Then the medium was autoclaved. Then 40 g/l of Glucose were added as
 5 10x stock solution which had been autoclaved separately.

Example 3: Rifampicin selection of *Lactobacillus diolivorans*:

L. diolivorans DSM14421 was cultured overnight in 5 ml MRS Medium
 10 (Glucose+Glycerol) + 0.03125 µg/ml Rifampicin in a sterile glass eppovette, shaking
 (180 rpm) at 30°C in an anaerobic jar. This amount of rifampicin significantly inhibits
 the growth of *L. diolivorans*. The cells were streaked on a MRS agar plate (MRS
 medium +Glucose + 1.5% agar) and incubated for 3 days at 30°C in an anaerobic jar.
 Single colonies were restreaked, cultured again on MRS agar plates and then
 15 inoculated into liquid MRS medium (+ Glucose) for plasmid isolation using the Qiagen
 DNeasy Blood & Tissue Kit (Cat No./ID: 69504). One clone was found (designated *L.*
diolivorans Vogelbusch1050, deposited under DSM 33056) which has a distinctive
 different DNA pattern compared to that of the parental clone (DSM14421) as can be
 seen in Figure 1.

20

Example 4: Isolation of new *Lactobacillus diolivorans* strains:

Six samples of maize silage, five samples of grass silage, two samples of
 mountain herbs from different parts of Austria, and one sample of a fermented dough
 made from lentils and rice have been used for isolation of bacteria. To this end 30 g
 25 (dry weight) of each sample was suspended in a shake flask with 100 ml of sterile
 0,9% NaCl solution. After two minutes of shaking the supernatant was used to prepare
 serial dilutions up to 10⁻⁹ with sterile 0,9% NaCl solution. 100 µL from each dilution
 were spread onto MRS and OS plates, both containing 50 mg/L of Vancomycin. The

plates were incubated at 30°C for three days in anaerobic conditions. At the end of the incubation the colonies have been selected according to the morphological appearance. Only small colonies (1–4 mm) with defined margins, convex, smooth, glistening, and opaque without pigment were selected and restreaked on the same kind of plates where they have been isolated from. The plates were incubated at 30°C for three days in anaerobic jars supplemented with Oxoid™ AnaeroGen™ 2.5L Sachet from Thermo Scientific and then used for species analysis by MALDI – TOF mass spectroscopy using a MALDI Biotyper (Bruker). The identification was performed immediately after the incubation in order to analyse fresh cultures and increase the quality of the spectra. For the preparation of HCCA Matrix solution 250 µL of standard solvent (OS-Solution) was added to a tube of portioned HCCA undissolved Matrix (Bruker, Nr. 8255344). HCCA Matrix was dissolved by vortexing at room temperature until the solution became clear. For identification, a small amount from a single colony was spread directly onto a spot on a MALDI target plate. The spots were overlaid with 1 µL of 70% formic acid (FA) and left to dry at room temperature. Then, the spots were overlaid with 1 µL of HCCA solution and left drying at room temperature. Subsequently, the prepared target was measured at the MALDI Biotyper using bacterial test standard mode (BTS). Out of about 786 colonies sent for analysis 579 could be identified, out of which 4 strains have been identified as *Lactobacillus diolivorans*.

The strains have been designated as *Lactobacillus diolivorans* HF deposited under DSM 33060, HM deposited under DSM 33058, HH deposited under DSM 33057 and HS deposited under DSM 33059, respectively.

Example 5: Anaerobic co-fermentation of glucose and glycerol into 1,3-propanediol with *Lactobacillus diolivorans* Vogelbusch1050.

Co-fermentation of glucose and glycerol for the conversion of glycerol to 1,3-propanediol (1,3-PD) using *Lactobacillus diolivorans* Vogelbusch1050 (deposited under DSM 33056) is given in Table 3. The fermentation was carried out as fed-batch process using 700 ml MRS-medium supplemented with 5 mg/L Vitamin B₁₂, 30 g/L glucose and 10 g/L glycerol as batch medium (see example 1) and a glucose/glycerol solution (0.1 mol glucose/1 mol glycerol), supplemented with 5 mg/L vitamin B₁₂, with a glycerol concentration of 500 g/L, as feed medium. The feed was started after glucose and glycerol were consumed from the batch culture medium (t= 20 h). A starting rate of

2.4 mL/h was used to add the feed medium to the culture. The feed rate was decreased continuously following the formula 1 until the end of the process (t= 188 h).

$$Feed [ml/h] = 0.0106 * t [h] - 2.61$$

Formula 1

5

The pH-value was adjusted to 5.7 with 8 M sodium hydroxide (NaOH) throughout the whole process. Nitrogen (N₂) was used for gassing (2 L/h) during the fermentation to avoid contamination with oxygen and to avoid accumulation of carbon dioxide.

10

Table 3: Conversion of glycerol to 1,3-propanediol (1,3-PD) using *Lactobacillus diolivorans* Vogelbusch1050 in a fed-batch process co-fermenting glucose and glycerol

Time [h]	OD600 [AU]	Glucose [g/L]	Glycerol [g/L]	1,3-PDO [g/L]	Lactic acid [g/L]	Acetic acid [g/L]	Ethanol [g/L]
0	0,1	30,9	10,2	0	0,1	0,2	0,1
187,8	24	0	13,2	81,1	0	28,7	1,1

The yield of 1,3-propanediol was 62.8 wt % (g 1,3-propanediol/g glycerol total), and 70.0 wt % (g 1,3-propanediol/g glycerol used) and a titer of 81.1 g/L 1,3-propanediol was obtained. The maximal specific productivity was 7.2 g/L per g biomass.

Example 6: Co-fermentation of glucose and glycerol into 1,3-propanediol with *Lactobacillus diolivorans* Vogelbusch1050 without nitrogen sparging

Co-fermentation of glucose and glycerol for the conversion of glycerol to 1,3-propanediol (1,3-PD) using *Lactobacillus diolivorans* Vogelbusch1050 (deposited under DSM 33056) without nitrogen sparging is given in Table 4. The fermentation was carried out as fed-batch process using 700 ml MRS-medium supplemented with 5 mg/L Vitamin B₁₂, 30 g/L glucose and 10 g/L glycerol as batch medium (see example 1) and a glucose/glycerol solution (0.1 mol glucose/1 mol glycerol), supplemented with 5 mg/L Vitamin B₁₂ and with a glycerol concentration of 500 g/L, as feed medium. When the batch was started the dissolved oxygen value was at 80 % (100% being oxygen saturation). The dissolved oxygen concentration continuously decreased for

16 h to reach 0 % (undetectable). The feed was started after glucose and glycerol were consumed from the batch culture medium (t=28 h). A starting rate of 2.4 mL/h was used to add the feed medium to the culture. The feed rate was decreased continuously following formula 1 (see example 5) until the end of the process (t= 185 h). The pH-value was adjusted to 5.7 with 8 M sodium hydroxide (NaOH) throughout the whole process. The culture was not sparged with any gas.

The yield of 1,3-propanediol was 62.4 wt % (g 1,3-propanediol/g glycerol total), 69.6 wt % (g 1,3-propanediol/g glycerol used), and a titer of 80.4 g/L 1,3-propanediol was obtained. The maximal specific productivity was 7.9 g/L per g biomass. The values are very similar to the values obtained with the traditional process using nitrogen gas for sparging (example 5).

Table 4: Conversion of glycerol to 1,3-propanediol (1,3-PD) using *Lactobacillus diolivorans* Vogelbusch1050 in a fed-batch process co-fermenting glucose and glycerol without nitrogen sparging.

Time [h]	OD600 [AU]	Glucose [g/L]	Glycerol [g/L]	1,3-PDO [g/L]	Lactic acid [g/L]	Acetic acid [g/L]	Ethanol [g/L]
0	0,1	30,2	9,8	0	0,1	0,1	0
184,7	24,2	0	14	80,4	0	27,6	1

Surprisingly, the strain grew well and produced 1,3-propanediol also under aerobic conditions without any nitrogen sparging. The titer and yield were comparable (80.4 g/L as compared to 81.1 g/L with nitrogen sparging and 62.4% as compared to 62.8% with nitrogen sparging). Particularly surprising was that the maximal specific productivity was with 7.9 g/L per g biomass without nitrogen sparging higher than with nitrogen sparging (7.2 g/L per g biomass).

Example 7: Co-fermentation of different sugars with glycerol by new isolates of *Lactobacillus diolivorans*

Co-fermentation of glucose xylose, fructose, arabinose, mannose, or sucrose respectively, with glycerol using newly isolated *Lactobacillus diolivorans* strains is given in Table 5. The fermentation was carried out as batch process in glass eprouvettes using 2 ml MRS-medium supplemented with 0.005 g/L Vitamin B₁₂, 30 g/L of the respective sugar and 10 g/L of glycerol as batch medium (see example 1). The eprouvettes were incubated 72 h shaking at 30°C in anaerobic jars supplemented with Oxoid™ AnaeroGen™ 2.5L Sachet from Thermo Scientific then the culture supernatant was analysed by HPLC.

Table 5: 1,3-Propanediol concentration in g/L after 72 h of fermentation on MRS medium with the indicated carbon sources

	CARBON SOURCE MIXTURES		
STRAINS	GLUCOSE GLYCEROL	XYLOSE GLYCEROL	FRUCTOSE GLYCEROL
DSM 33056	8,72	3,75	8,3
DSM 33057	8,69	4,01	8,33
DSM 33058	8,66	3,89	8,2
DSM 33059	8,74	4,36	8,3
DSM 33060	8,68	1,2	8,25
	CARBON SOURCE MIXTURES		
STRAINS	ARABINOSE GLYCEROL	MANNOSE GLYCEROL	SUCROSE GLYCEROL
DSM 33056	0,21	6,92	0,76
DSM 33057	0,24	-	3,87
DSM 33058	0,24	6,88	0,87
DSM 33059	0,24	8,27	1,86
DSM 33060	0,24	7,06	1,03

All the strains are of the same efficiency of 1,3-propanediol production on glucose and glycerol. Very interesting is a significant difference on sucrose and glycerol. While Vogelbusch1050 (deposited under DSM 33056), HF (deposited under DSM 33060) and HM (deposited under DSM 33058) are less efficient, strains HS
5 (deposited under DSM 33059) and particularly HH (deposited under DSM 33057) are efficiently producing 1,3-propanediol also on sucrose and glycerol. Strain HS (deposited under DSM 33059) is particularly efficient on xylose and mannose. All of the strains are growing on arabinose but they do not accumulate significant amounts of 1,3-propanediol on arabinose and glycerol.

CLAIMS

1. A method of producing a cell metabolite by culturing *Lactobacillus diolivorans* cells in a cell culture comprising feeding the cell culture in an aerobic reactor system
5 during a production phase using a feed medium comprising a carbon source for biotransformation into the cell metabolite, and isolating the cell metabolite from said cell culture.

2. The method of claim 1, wherein the aerobic reactor system does not employ
10 means to protect the cell culture from oxygen.

3. The method of claim 1 or 2, wherein the carbon source comprised in the feed medium is selected from the group consisting of glycerol, crude glycerol, glucose, fructose, lactose, sucrose, starch, biomass hydrolysates, cellulose, lignocellulose,
15 lignocellulosic hydrolysate, sugar beet extract, molasses, organic acid, organic salts, and a combination of any of the foregoing.

4. The method of claim 3, wherein the carbon source comprises a carbohydrate with a purity of less than Technical Grade and an ash content of at least 0.1% (w/w).
20

5. The method of any one of claims 1 to 4, wherein the cell metabolite is selected from the group consisting of 1,3-propanediol, 1,2-propanediol, 2-amino-1,3-propanediol, 3-hydroxybutyrate, poly-3-hydroxybutyrate, ethanol, 1-butanol, 2-butanol, isobutanol, 2,3-butanediol, butanone, lactic acid, citric acid, propionic acid, 3-hydroxy propionaldehyde, 3-hydroxy propionic acid, butyric acid, valeric acid, hexanoic acid, adipic acid, succinic acid, fumaric acid, malic acid, 2,5-furan dicarboxylic acid, aspartic acid, glucaric acid, gluconic acid, glutamic acid, itaconic acid, levulinic acid, acrylic acid, propanol, isopropanol, 1-butanol, 2-butanol, pentanol, hexanol, heptanol, octanol, butanediol, 2,3-butanediol, 3-hydroxybutyrolactone, xylitol, arabinitol, sorbitol, mannitol,
25 vitamin C, riboflavin, thiamine, tocopherol, cobalamin, pantothenic acid, biotin, pyridoxine, niacin, folic acid, 3-hydroxybutyrolactone, diamino pentane, diamino hexane and dihydroxyacetone.
30

6. The method of any one of claims 1 to 5, wherein the production phase is carried out in a fed-batch mode or continuous mode, preferably wherein the feed medium is continuously added to the cell culture during the production phase.

5 7. The method of any one of claims 1 to 6, wherein prior to the production phase, the cell culture is carried out in a batch mode using a growth medium to accumulate biomass.

10 8. The method of any one of claims 1 to 7, wherein the *Lactobacillus diolivorans* cells are of naturally occurring *L. diolivorans* bacteria, progeny or derivatives thereof.

15 9. The method of any one of claims 1 to 8, wherein the *Lactobacillus diolivorans* cells are of any one of the *L. diolivorans* strains deposited as DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060, or progeny or derivatives of any of the foregoing.

20 10. A *L. diolivorans* strain which any one of the *L. diolivorans* strains deposited as DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060, or progeny or derivatives of any of the foregoing.

25 11. The *L. diolivorans* strain of claim 10, wherein said derivatives comprise one or more mutations for overexpressing homologous sequences and/or transforming the bacterium with a vector comprising heterologous sequences, to improve production of a cell metabolite.

 12. Use of *L. diolivorans* cells in a cell culture for biotransformation of a carbon source into a cell metabolite by an aerobic feeding fermentation process.

30 13. The use of claim 12, wherein the carbon source is glycerol or a carbohydrate with a purity of less than Technical Grade and an ash content of at least 0.1% (w/w).

 14. The use of claim 13, for biotransformation of glycerol into 1,3-propanediol, preferably wherein the glycerol is biodiesel-derived crude glycerol.

15. The use of any one of claims 11 to 13, wherein the *L. diolivorans* cells are of any one of the *L. diolivorans* strains deposited as DSM 33056, DSM 33057, DSM 33058, DSM 33059, or DSM 33060, or progeny or derivatives of any of the foregoing.