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(54) Title: METHOD FOR PRODUCING MALIC ACID

(57) Abstract: There is provided is a method for producing malic acid from glycerol, which comprises culturing a specific bacterium capable of producing malic acid in a culture medium containing glycerol, or making cells of the bacterium, processed cells of the bacterium or immobilized product thereof contact with glycerol.

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DESCRIPTION

METHOD FOR PRODUCING MALIC ACID

5 TECHNICAL FIELD

The present invention relates to a method for producing malic acid from glycerol using a microorganism.

BACKGROUND ART

10 Bio Diesel Fuel (BDF) is a carbon-neutral alternative fuel for light oil, and has recently been receiving attention as a fuel contributing to resolving environmental problems such as depletion of energy resources, global warming, and air pollution.

Bio Diesel Fuel is produced from vegetable oils, animal fats, 15 waste oil, and the like. In this time, a waste liquid containing glycerol is produced as a by-product. There is no specific use for the waste liquid, so that it is currently just discarded.

As a method for utilizing Bio Diesel waste, Japanese Unexamined Patent Publication (Kokai) No. 2006-180782 discloses 20 a method for producing hydrogen and ethanol from glycerol in the waste using a bacterium of the genus Enterobacter.

Regarding production of organic acids, Japanese Unexamined Patent Publication (Kokai) No. 2003-235592 discloses a method for producing malic acid from glucose as a carbon source, using aerobic 25 bacteria (particularly, coryneform bacteria). PCT International Publication WO 02/00846 A1 discloses a bacterial strain (Mannheimia sp. 55E) which produces various organic acids from glucose.

Japanese Unexamined Patent Publication (Kokai) No. Hei

10-75772 discloses a method for producing malic acid by suspending bacterial cells of *Comamonas testosteroni* FERM P-15714 strain with maleic acid. Japanese Patent Publication No. Shou 44-14786 discloses a method for producing malic acid by culturing an aerobic
5 bacterium in a fumaric acid-containing medium. Biotechnology Letters (1993) 15(3): 272-276 discloses a method for producing D-malic acid by suspending bacterial cells with D, L-malic acid. However, it has not been known yet that the following bacteria can produce malic acid from glycerol: a bacterium of a species selected
10 from the bacterial species group consisting of *Achromobacter xylosoxydans*, *Microbacterium saperdae*, *Streptomyces moderatus* and *Pseudomonas fragi*; or *Comamonas testosteroni* ATCC 27911 strain.

DISCLOSURE OF INVENTION

15 It is desired to produce useful substances from Bio Diesel waste for effective utilization of resources.

An object of the present invention is to provide a method for manufacturing malic acid from glycerol, by culturing a bacterium of a species selected from the bacterial species group
20 consisting of *Achromobacter xylosoxydans*, *Microbacterium saperdae*, *Streptomyces moderatus* and *Pseudomonas fragi*; or *Comamonas testosteroni* ATCC 27911 strain (hereinafter, these bacteria are collectively referred to as malic acid-producing bacteria used for the present invention, or simply as malic acid-producing bacteria)
25 in a culture medium containing glycerol, particularly by culturing the bacterium under an aerobic condition, or by contacting to glycerol cells of the bacterium, processed cells of the bacterium, or immobilized product thereof.

Namely, the present invention can provide the followings:

1. A method for producing malic acid from glycerol, which comprises:

culturing in a culture medium containing glycerol, a
5 bacterium which has an ability to produce malic acid from glycerol and belongs to a species selected from the bacterial species group consisting of *Achromobacter xylosoxydans*, *Microbacterium saperdae*, *Streptomyces moderatus* and *Pseudomonas fragi*; or

contacting cells of the bacterium, processed cells of the
10 bacterium or immobilized product thereof with glycerol;

2. The method according to the above 1, wherein the bacterium belonging to a species selected from the bacterial species group consisting of *Achromobacter xylosoxydans*, *Microbacterium saperdae*, *Streptomyces moderatus* and *Pseudomonas fragi* is a bacterium of
15 *Achromobacter xylosoxydans* NBRC 15126 strain, *Microbacterium saperdae* JCM 1352 strain, *Streptomyces moderatus* NBRC 13432 strain or *Pseudomonas fragi* JCM 20552 strain;

3. A method for producing malic acid from glycerol, which comprises:

20 culturing a bacterium of *Comamonas testosteroni* ATCC 27911 strain in culture medium containing glycerol, or

contacting cells of the bacterium, processed cells of the bacterium or immobilized product thereof with glycerol;

4. The method according to any of the above 1 to 3, wherein
25 the bacterium is cultured under an aerobic condition;

5. The method according to any of the above 1 to 4, further comprises recovering malic acid from a culture obtained by culturing; and

6. The method according to any of the above 1 to 5, wherein glycerol is derived from Bio Diesel waste.

MODE FOR CARRYING OUT THE INVENTION

5 The malic acid-producing bacteria used for the present invention are exemplified by the bacterial genera, bacterial species and bacterial strains described in the above 1 to 3. Among these malic acid-producing bacteria, preferred bacteria are those being able to produce malic acid from glycerol in higher yield.
10 Examples of such bacteria include *Microbacterium saperdae* JCM 1352 strain, *Achromobacter xylosoxydans* NBRC 15126 strain, *Comamonas testosteroni* ATCC 27911 strain, *Streptomyces moderatus* NBRC 13432 strain and *Pseudomonas fragi* JCM 20552 strain, but they are not limited thereto. *Achromobacter xylosoxydans* NBRC 15126 strain,
15 *Comamonas testosteroni* ATCC 27911 strain, *Streptomyces moderatus* NBRC 13432 strain and *Pseudomonas fragi* JCM 20552 strain are preferred, and *Comamonas testosteroni* ATCC 27911 strain, *Streptomyces moderatus* NBRC 13432 strain and *Pseudomonas fragi* JCM 20552 strain are more preferred.

20

 Herein, malic acid being produced in "higher yield" denotes that malic acid is accumulated from glycerol in a culture medium in a yield of about 0.1 g/L or more. In general, the higher the yield, the better it is. For example, malic acid is accumulated
25 in a culture medium in a yield of about 0.2 g/L or more, and preferably about 0.5 g/L or more.

Achromobacter xylosoxydans NBRC 15126 strain and

Streptomyces moderatus NBRC 13432 strain are available from Biological Resource Center, Department of Biotechnology, Incorporated Administrative Agency National Institute of Technology and Evaluation, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818 Japan. Comamonas testosteroni ATCC 27911 strain is available from American Type Culture Collection, P.O.Box 1549 Manassas, VA 20108 USA. Microbacterium saperdae JCM 1352 strain and Pseudomonas fragi JCM 20552 strain are available from Microbe Division / Japan Collection of Microorganisms, RIKEN BioResource Center, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan.

The malic acid-producing bacteria used for the present invention may not only be their wild-type strains, but also be any given naturally-occurring or artificial mutants, including those obtained by treatment with X-ray irradiation, ultraviolet-ray irradiation or a chemical mutagen such as N-methyl-N'-nitro-N-nitrosoguanidine, or recombinants obtained through genetic engineering techniques such as cell fusion and gene recombination. A host of the recombinant can belong to any bacterial genera as long as it is a transformable microorganism. However, it is preferred that the host belongs to the same bacterial genus as a parent strain that a targeted gene is originated from. It is desirable to select a malic acid-producing bacterium with improved conversion capacity from glycerol into malic acid.

25

A culture medium containing glycerol may be a medium obtained by adding pure glycerol or a glycerol-containing mixture. Components other than glycerol in the glycerol-containing mixture

or their amount are preferably those which do not have harmful effects on the malic acid-producing bacteria used for the present invention. Origin of the glycerol-containing mixture is not particularly limited, but using Bio Diesel waste is preferable for
5 effective use of resources.

One of a method for producing Bio Diesel Fuel is to produce Fatty Acid Methyl Ester (FAME) by alcoholysis of triglyceride using an alkali catalyst. In this method, waste fluid containing
10 glycerol is produced as a by-product (called Bio Diesel waste). This waste fluid is usually contaminated with a catalyst, unconverted fatty acids (they differ depending on used oil) and the like. For example, the composition of a Bio Diesel waste used after-mentioned Examples is glycerol: 51%, methanol: 11%,
15 potassium hydroxide: 8%, water: 4%, others such as glyceride: 26%. Bio Diesel waste fluids having arbitrary composition, such as glycerol: 65%, potassium/sodium salt: 4 to 5%, methanol: 1%, water: 28% described in S. Papanikolaou et al., Bioresource Technology (2002) 82: 43-49, and glycerol: 65%, sodium salt: 5% or less
20 described in M. Gonzalez-Pajuelo et al., J IndMicrobiol Biotechnol (2004) 31: 442-446, can also be used.

When a Bio Diesel waste is added to a medium in a method of the present invention, it is possible to produce malic acid in the
25 same or higher level of yield and conversion efficiency as in the case of adding pure glycerol.

The medium used for the method of the present invention may

be any kind as long as it contains common components necessary for culture of bacteria, and is not limited to a particular medium. In the present invention, it is possible to obtain malic acid even in a medium with plain composition containing carbon sources,
5 nitrogen sources and inorganic salts.

The medium used for the method of the present invention contains glycerol as a carbon source. The concentration of glycerol contained in the medium can be properly selected within
10 a range which exerts no harmful effect on growth of bacteria, and production of malic acid. However, it is usually from about 0.1 to 500 g/L, and preferably from about 1 to 300 g/L. When the Bio Diesel waste is used as a glycerol source, it is allowed to dilute the waste liquid, or to add glycerol thereto, until the amount of
15 glycerol in the medium falls within the above range, depending on the concentration of glycerol contained in the waste.

The medium may contain substances other than glycerol as carbon sources, but the amount thereof should be limited to the
20 extent which does not interfere production of malic acid from glycerol. The carbon sources used for the present invention are exemplified by glucose, fructose, starch, lactose, arabinose, xylose, dextrin, molasses and malt extract, but are not limited thereto. The amount of other carbon sources is preferably about
25 10% by weight or less of glycerol, and more preferably about 1% by weight or less. It is most preferable that the medium contains glycerol as a single carbon source.

Examples of a nitrogen source include inorganic nitrogen compounds such as ammonia, ammonium sulfate, ammonium chloride and ammonium nitrate, urea, and the like. It is also allowed to add to a culture medium organic nitrogen sources such as gluten flour, cottonseed flour, soybean flour, corn steep liquor, dried yeast, yeast extract, peptone, meat extract and casamino acid.

It is advantageous to use carbon sources and nitrogen sources in combination. Since the sources of low purity, containing a trace amount of growth factors and a great deal of inorganic nutrients, are also suitable for the use, there is no need to use them in a pure form.

Upon request, it is allowed to use inorganic salts, such as potassium phosphate monobasic, potassium phosphate dibasic, sodium chloride, magnesium sulfate, manganese sulfate, calcium carbonate, calcium chloride, sodium iodide, potassium iodide, and cobalt chloride. It is also allowed to add defoaming agents, such as liquid paraffin, higher alcohol, vegetable oil, mineral oil and silicon, as needed, particularly when the medium foams markedly.

Other components such as various vitamins may be added to the medium, as needed.

The nitrogen sources, inorganic salts and other components are known by those skilled in the art.

In the present invention, it is allowed to culture malic acid-producing bacteria under an anaerobic condition, but preferably it is performed under an aerobic condition. The aerobic

condition denotes culture in the presence of molecular oxygen. Ventilation, stirring and shaking can be performed for supplying oxygen. It is available to use any common devices for culture of microorganisms. In the case of culturing under an aerobic
5 condition, the method of the present invention allows to culture bacteria, and to produce malic acid, by a simple manner without using any devices necessary for bringing about an anaerobic condition.

10 Culture of bacteria under an anaerobic condition can be performed by introducing carbon dioxide or inert gas (nitrogen argon, etc.), or without ventilation.

It is preferred for mass production of malic acid to be
15 performed under a submerged culture condition. When bacteria are propagated in a large tank, it is preferred to inoculate bacteria in a vegetative period into a production tank, so as to avoid delay in propagation in a malic acid producing process. That is, it is preferred that bacteria are first inoculated to a relatively small
20 amount of medium, and cultured to produce seed bacteria in a vegetative period, and then the seed bacteria is transferred into the large tank in a sterile manner.

Stirring and ventilation of the culture solution can be
25 performed in various manners. Stirring can be performed using a propeller or a mechanical stirring device similar to a propeller, rotation or shake of a fermenter, or a pumping device. Ventilation can be performed by allowing sterilized air to pass through in the

culture solution. In doing so, the ventilation operation may provide stirring effect as well.

In the case of culture in a submerged medium, a culture method
5 such as batch culture, fed batch culture and continuous culture can be properly selected and used.

The culture conditions are discretionary as long as they are suitable for culture of malic acid-producing bacteria used for the
10 present invention. For example, the culture temperature is from about 4 to 40°C, preferably from about 20 to 37°C. The pH of the medium is from about 5 to 9, and preferably from about 6 to 8. When the pH of the medium declines along with production of malic acid, it is adjusted to be fallen within the above range, by adding alkali
15 such as an aqueous ammonia solution, calcium carbonate, sodium hydroxide and potassium hydroxide to the culture system, as needed.

The composition of the medium and other culture conditions are appropriately adjustable by those skilled in the art. It will
20 also be considered to adjust the conditions, in order to further enhance yield of malic acid.

The bacteria used for the method of the present invention may take a bacterial cell, processed bacterial cell or immobilized
25 product thereof. Herein, the processed bacterial cell denotes a disrupted bacterial cell or an enzyme extracted from cultured substances (include a bacterial cell and culture supernatant). Examples of the processed bacterial cell include that obtained by

treating a cultured bacterial cell with an organic acid (such as acetone and ethanol), freeze dry treatment or alkali treatment, that obtained by physically or enzymatically disrupting a bacterial cell, or a crude enzyme separated or extracted therefrom.

5 Specifically, cultured bacteria are subjected to a centrifugal treatment, and the cells to be collected are disrupted by a physical milling method such as an ultrasonic, Dyno-mill and French press treatment, or a chemical disrupting method using a surfactant or a lytic enzyme such as lysozyme. The resultant solution is
10 subjected to centrifuge or membrane filtration to remove insoluble materials, and the resultant cell-free extract is subjected to a separation/purification method, such as cation exchange chromatography, anion exchange chromatography, hydrophobic chromatography, gel filtration chromatography and metal chelate
15 chromatography, to fractionate and purify the enzyme.

Examples of a carrier used for the chromatography include insoluble polymer carriers such as cellulose, dextrin and agarose introduced with a carboxymethyl (CM) group, diethylaminoethyl (DEAE) group, phenyl group or butyl group. It is also allowed to
20 use a commercially available carrier-packed column. Disruption of the bacterial cell and extraction of the enzyme can be performed by a known method by those skilled in the art, as well as the above method.

25 Examples of contacting the bacterial cell, processed bacteria cell or immobilized thereof to glycerol are given below.

The method for producing malic acid from glycerol using a bacterial cell or processed bacterial cell is exemplified by a

method that the bacterial cell is suspended and reacted in a glycerol-containing substrate solution. The bacterial cell can be prepared by culturing malic acid-producing bacteria, followed by centrifuge thereof. It is preferred that the concentration of glycerol in the substrate solution is approx. from 0.01 to 50% by weight. The reaction temperature is usually from about 4 to 40°C, and preferably from about 20 to 37°C. The pH of the reaction solution is usually from about 5 to 9, and preferably from 6 to 8. When the pH of the medium declines along with production of malic acid, it is adjusted to be fallen within the above range, by adding alkali such as an aqueous ammonia solution, calcium carbonate, sodium hydroxide and potassium hydroxide to the culture system, as needed.

The method for producing malic acid from glycerol using an immobilized bacterial cell or immobilized processed-bacterial cell is exemplified by a method that the immobilized bacterial cell or immobilized processed-bacterial cell is filled in a column, and a glycerol-containing substrate solution is allowed to pass it through. The bacterial cell or processed bacterial cell is obtained by culturing the malic acid-producing bacteria, followed by centrifuge thereof. The method for immobilizing the bacterial cell is exemplified by a comprehensive immobilization means using a gel, and immobilization means by supporting an ion exchange material. Examples of the gel to be used include carrageenan, agar, mannan, PVA and polyacrylamide gels. The proper particle size of the gel is from about 1 to 10 mm in diameter, although the size varies depending on a kind of gel. Examples of the ion exchange

material include a cellulose-based material,
styrene-divinylbenzene-based material and phenol-formalin-based
ion exchange material. It is preferred that the concentration of
glycerol in the substrate solution is from about 0.01 to 50% by
5 weight. It is also allowed to add a SH compound such as
mercaptoethanol, cysteine and glutathione, reducing agent such as
sulfite, and enzyme activator such as a magnesium ion and manganese
ion. The velocity of the solution passing through varies depending
on the column size and amount of the immobilized substance. It
10 is proper that the space velocity (ml/ml resin·hr) is from 0.05
to 10, as an index of velocity for treating a solution.

Separation and purification of malic acid are performed in
accordance with a conventional known method. For example, malic
15 acid can be separated and purified by methods such as solvent
extraction, ion exchange chromatography, fractional precipitation
by an insolubilizing treatment, fractional crystallization by
crystallization, membrane separation by a reverse osmosis membrane,
and concentrated crystallization method. Specifically, for
20 example, filtration or centrifugation is performed to a culture
solution after completion of culture, to obtain a supernatant.
From the supernatant, malic acid can be purified by concentrated
crystallization, or by using activated carbon after as-needed acid
treatment of the supernatant, but methods for separation and
25 purification of malic acid are not limited thereto. Malic acid
can be separated and purified according to a method described in
Japanese Unexamined Patent Publication (Kokai) No. 2001-197897.

The present invention is further illustrated by the following examples. It is to be understood that the present invention is not limited to the examples, and various variations can be made within a range of the present invention.

5

EXAMPLES

Example 1

Achromobacter xylosoxydans NBRC 15126 strain was spread on an agar medium A, being a medium for plate culture, (composition: 10 3 g of potassium phosphate monobasic, 6 g of sodium phosphate dibasic, 0.5 g of sodium chloride, 1 g of ammonium chloride, 492 mg of magnesium sulfate heptahydrate, 147 mg of calcium chloride dihydrate, 100 mg of yeast extract, 10 g of glycerol, 20 g of agar and 1 L of distilled water (final pH 7.4)), and allowed to stand 15 at 30°C for 4 days. The bacterial strain grown on the above plate was inoculated with a platinum loop in 3 mL of a medium B, being a medium for test tube culture (composition: the same composition as the agar medium A, except for containing no calcium chloride, yeast extract and agar), and subjected to shaking culture 20 (pre-culture) at 30°C at 200 rpm for 24 hours. The bacterial strain culture solution of 30 µL grown above was transferred to 3 mL of a medium C, being a medium for test tube culture (composition: the same composition as the above medium B for test tube culture, except for containing 19.6 g of a glycerol fraction (glycerol: 51%, 25 methanol: 11%, potassium hydroxide: 8%, water: 4%, and others including glyceride: 26%) which was by-produced upon production of Bio Diesel Fuel, instead of 10 g of glycerol), and subjected to shaking culture (main culture) at 30°C at 200 rpm. Four (4)

days after initiation of the reaction, 0.4 g of glycerol was consumed and 0.1 g of malic acid was accumulated per one litter.

Example 2

5 *Achromobacter xylosoxydans* NBRC 15126 strain was spread on an agar medium A, being a medium for plate culture, (composition: 3 g of potassium phosphate monobasic, 6 g of sodium phosphate dibasic, 0.5 g of sodium chloride, 1 g of ammonium chloride, 492 mg of magnesium sulfate heptahydrate, 147 mg of calcium chloride
10 dihydrate, 100 mg of yeast extract, 10 g of glycerol, 20 g of agar and 1L of distilled water (final pH 7.4)), and allowed to stand at 30° C for 4 days. The bacterial strain grown on the above plate was inoculated with a platinum loop in 3 mL of a medium D, being a medium for test tube culture (composition: the same composition
15 as the agar medium A, except for containing no agar), and subjected to shaking culture (pre-culture) at 30° C at 200 rpm for 6 days. The bacterial strain culture solution of 30 µL grown above was transferred to 3 mL of a medium E, being a medium for test tube culture, (composition: the same composition as the above medium
20 D for test tube culture, except for containing 19.6 g of a glycerol fraction (glycerol: 51%, methanol: 11%, potassium: hydroxide: 8%, water: 4%, and others including glyceride: 26%) which was by-produced upon production of the Bio Diesel Fuel, instead of 10 g of glycerol), and subjected to shaking culture (main culture)
25 at 30° C at 200 rpm. Six (6) days after initiation of the reaction, 1.3 g of glycerol was consumed and 0.4 g of malic acid was accumulated per one litter.

Example 3

Comamonas testosteroni ATCC 27911 strain was reacted in the same manner as in Example 1. As a result, 2.6 g of glycerol was consumed and 0.5 g of malic acid was accumulated per one liter.

5

Example 4

Microbacterium saperdae JCM 1352 strain was spread on an agar medium A, being a medium for plate culture, (composition: 3 g of potassium phosphate monobasic, 6 g of sodium phosphate dibasic, 0.5 g of sodium chloride, 1 g of ammonium chloride, 492 mg of magnesium sulfate heptahydrate, 147 mg of calcium chloride dihydrate, 100 mg of yeast extract, 10 g of glycerol, 20 g of agar and 1L of distilled water (final pH 7.4)), and allowed to stand at 30° C for 4 days. The bacterial strain grown on the above plate was inoculated with a platinum loop in 3 mL of a medium D, being a medium for test tube culture (composition: the same composition as the agar medium A, except for containing no agar), and subjected to shaking culture (pre-culture) at 30° C at 200 rpm for 2 days. The bacterial strain culture solution of 30 µL grown above was transferred to 3 mL of a medium D, being a medium for test tube culture, and subjected to shaking culture (main culture) at 30° C at 200 rpm. Four (4) days after initiation of the reaction, 0.4 g of glycerol was consumed, and 0.1 g of malic acid was accumulated per one liter.

25

Example 5

Comamonas testosteroni ATCC 27911 strain was reacted in the same manner as in Example 3, except for changing the pre-culture

period from 2 days to 6 days, and the main culture period from 4 days to 6 days. As a result, 2.3 g of glycerol was consumed and 0.2 g of malic acid was accumulated per one liter.

5 Example 6

Pseudomonas fragi JCM 20552 strain was reacted in the same manner as in Example 1. As a result, 4.4 g of glycerol was consumed and 0.3 g of malic acid was accumulated per one liter.

10 Example 7

Pseudomonas fragi JCM 20552 strain was reacted in the same manner as in Example 2. As a result, 4.5 g of glycerol was consumed and 0.8 g of malic acid was accumulated per one liter.

15 Example 8

Pseudomonas fragi JCM 20552 strain was reacted in the same manner as in Example 4. As a result, 8.8 g of glycerol was consumed and 0.4 g of malic acid was accumulated per one liter.

20 Example 9

Streptomyces moderatus NBRC 13432 strain was reacted in the same manner as in Example 2. As a result, 6.3 g of glycerol was consumed and 0.5 g of malic acid was accumulated per one liter.

25 INDUSTRIAL APPLICABILITY

According to the present invention, malic acid is produced from glycerol (Bio Diesel waste) using a microorganism by a simple manner. Malic acid is used as food additives, deodorant, cleaning

aid, pH adjuster, bath powder, and the like. Thus, the present invention serves for producing useful substances from a waste material.

CLAIMS

1. A method for producing malic acid from glycerol, which comprises:

5 culturing in a culture medium containing glycerol, a bacterium which has an ability to produce malic acid from glycerol and belongs to a species selected from the bacterial species group consisting of *Achromobacter xylosoxydans*, *Microbacterium saperdae*, *Streptomyces moderatus* and *Pseudomonas fragi*; or

10 contacting cells of the bacterium, processed cells of the bacterium or immobilized product thereof with glycerol.

2. The method according to claim 1, wherein the bacterium belonging to a species selected from the bacterial species group
15 consisting of *Achromobacter xylosoxydans*, *Microbacterium saperdae*, *Streptomyces moderatus* and *Pseudomonas fragi* is a bacterium of *Achromobacter xylosoxydans* NBRC 15126 strain, *Microbacterium saperdae* JCM 1352 strain, *Streptomyces moderatus* NBRC 13432 strain or *Pseudomonas fragi* JCM 20552 strain.

20 3. A method for producing malic acid from glycerol, which comprises:

 culturing a bacterium of *Comamonas testosteroni* ATCC 27911 strain in culture medium containing glycerol, or

25 contacting cells of the bacterium, processed cells of the bacterium or immobilized product thereof with glycerol.

4. The method according to any of claims 1 to 3, wherein the

bacterium is cultured under an aerobic condition.

5. The method according to any of claims 1 to 4, further comprises recovering malic acid from a culture obtained by
5 culturing.

6. The method according to any of claims 1 to 5, wherein glycerol is derived from Bio Diesel waste.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/058979

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12P7/46

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

B. FIELDS SEARCHED

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

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

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

EPO-Internal, WPI Data, BIOSIS, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 3 980 520 A (DEGEN LUDWIG ET AL) 14 September 1976 (1976-09-14) the whole document	1, 3



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

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- * & * document member of the same patent family

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report		Publication date	Patent family member(s)		Publication date
US 3980520	A	14-09-1976	BE	808849 A1	16-04-1974
			CA	1000221 A1	23-11-1976
			CH	606417 A5	31-10-1978
			DE	2363285 A1	11-07-1974
			FR	2212431 A1	26-07-1974
			GB	1419668 A	31-12-1975
			IT	972897 B	31-05-1974
			LU	69054 A1	22-02-1974
			NL	7317522 A	01-07-1974