

DESCRIPTION

METHOD FOR PRODUCING 2,3-BUTANEDIOL

TECHNICAL FIELD

5 [0001]

The present invention relates to a method for producing 2,3-butanediol by microbial fermentation.

BACKGROUND ART

[0002]

10 2,3-Butanediol (which may hereinafter be referred to as “2,3-BDO”) is a useful compound which is used as an intermediate material for pharmaceuticals and cosmetics, and as a material for inks, perfumes, liquid crystals, insecticides, softening agents, explosives, plasticizers, and the like. More specifically, for example, 2,3-butanediol can be used as a material for methyl ethyl ketone (Non-
15 patent Document 1) or 1,3-butadiene (Non-patent Document 2). Industrially, 2,3-BDO is produced by a method in which 2-butene oxide is hydrolyzed in an aqueous perchloric acid solution. In recent years, in order to solve the problems of depletion of petroleum resources and global warming, achievement of a sustainable, recycling-oriented society is demanded. Also in the chemical industry, shifting from
20 petroleum materials to biomass-derived materials is being intensively studied. Under such circumstances, methods for producing 2,3-butanediol by microbial fermentation have begun attracting attention.

[0003]

Known examples of microorganisms capable of efficiently producing 2,3-
25 butanediol by fermentation include enterobacteria such as *Klebsiella pneumoniae* (Non-patent Document 3) and *Klebsiella oxytoca* (Non-patent Document 4). Since these microorganisms do not require a particular vitamin, amino acid, or the like for

the fermentation, their use is advantageous in that the processes required for isolation/purification of 2,3-butanediol from the fermentation liquid can be reduced. However, these microorganisms are pathogenic, and known to cause respiratory infections and the like. Therefore, in cases where large-scale culture of these fermentation microorganisms is carried out for the purpose of industrial production of 2,3-butanediol, facilities for strict control are required, and the cost increases as a result.

[0004]

There are disclosed methods using biologically safe 2,3-butanediol fermentation microorganisms showing high fermentation efficiency, such as bacteria belonging to the family Bacillaceae including *Bacillus licheniformis* (Non-patent Document 5) and *Bacillus amyloliquefaciens* (Non-patent Document 6). However, 2,3-butanediol fermentation by *Bacillus licheniformis* requires use of an animal extract, which is expensive, as a nutrient source for promoting the fermentation. 2,3-Butanediol fermentation by *Bacillus amyloliquefaciens* requires use of ammonium citrate for promotion of the fermentation. Since the boiling point of citric acid is close to that of 2,3-butanediol, isolation/purification of 2,3-butanediol is difficult in cases where citric acid remains in the culture liquid.

[0005]

On the other hand, as fermentation feedstocks for microbial fermentation, not only conventional sugars derived from edible biomass, but also sugars derived from non-edible biomass are attracting attention. In cases where a sugar derived from non-edible biomass is used as the fermentation feedstock, cellulose, hemicellulose, and the like contained in the non-edible biomass are decomposed into sugars using a saccharifying enzyme. In this process, pentoses such as xylose are obtained in addition to hexoses such as glucose. Therefore, development of an efficient fermentation technique for materials containing pentose has been demanded.

[0006]

Known examples of microorganisms capable of efficiently producing 2,3-butanediol from pentose by fermentation include enterobacteria such as *Klebsiella oxytoca* (Non-patent Document 3). Since these microorganisms do not require a particular vitamin, amino acid, or the like for the fermentation, their use is advantageous in that the processes required for purification of 2,3-butanediol from the fermentation liquid can be reduced. However, these microorganisms are pathogenic, and known to cause respiratory infections and the like. Therefore, in cases where large-scale culture of these fermentation microorganisms is carried out for the purpose of industrial production of 2,3-butanediol, facilities for strict control are required, and the cost increases as a result.

[0007]

On the other hand, there are disclosed methods using biologically safe microorganisms capable of producing 2,3-butanediol from pentose by fermentation, such as bacteria belonging to the family Bacillaceae including *Bacillus licheniformis* (Non-patent Document 9) and *Paenibacillus polymyxa* (Non-patent Document 10). However, in 2,3-butanediol fermentation using these microorganisms, an expensive auxiliary material such as a yeast extract or animal protein hydrolysate needs to be used, so that the cost is high.

[0008]

There are known transformed microorganisms belonging to the genus *Zymobacter* and having a capacity to metabolize pentose, which microorganisms were prepared by introducing exogenous genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase into microorganisms belonging to the genus *Zymobacter* (Patent Document 1, Patent Document 2). However, although Patent Documents 1 and 2 describe that these transformed microorganisms have a capacity to produce ethanol, there is neither description nor suggestion on a capacity

to produce 2,3-butanediol from pentose by fermentation.

PRIOR ART DOCUMENTS

[Patent Documents]

[0009]

5 [Patent Document 1] JP 2005-261421 A

[Patent Document 2] US 2007/0298476 A

[Non-patent Documents]

[0010]

10 [Non-patent Document 1] A. N. Bourns, The Catalytic Action Of Aluminium Silicates, Canadian J. Res. (1947)

[Non-patent Document 2] Nathan Shlechter, Pyrolysis of 2,3-butylene Glycol Diacetate to Butadiene, Indu. Eng. Chem. 905 (1945)

[Non-patent Document 3] Ma CQ, Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Appl Microbiol Biotechnol 2009; 82: 49-57

15 [Non-patent Document 4] Ji XJ, Engineering *Klebsiella oxytoca* for efficient 2,3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. Appl Microbiol Biotechnol 2010; 85: 1751-8.

[Non-patent Document 5] S. S. Nilegaonkar, Potential of *Bacillus licheniformis* for the production of 2,3-butanediol. Journal of Fermentation and Bioengineering Volume 82, Issue 4, 1996, Pages 408-410

20 [Non-patent Document 6] Yang T, Optimization and scale-up of 2,3-butanediol production by *Bacillus amyloliquefaciens* B10-127. World J Microbiol Biotechnol. 2012 Apr; 28 (4): 1563-74

25 [Non-patent Document 7] Okamoto T, *Zymobacter palmae* gen. nov., sp. nov., a new ethanol-fermenting peritrichous bacterium isolated from palm sap. Arch Microbiol. 1993; 160 (5): 333-7.

[Non-patent Document 8] Jansen NB, Production of 2,3-butanediol from D-

xylose by *Klebsiella oxytoca* ATCC 8724. Biotechnol Bioeng. 1984 Apr; 26 (4): 362-9.

[Non-patent Document 9] Wang Q, Metabolic engineering of thermophilic *Bacillus licheniformis* for chiral pure D-2,3-butanediol production. Biotechnol Bioeng. 2012 Jul; 109 (7): 1610-21.

[Non-patent Document 10] Marwoto B, Metabolic analysis of acetate accumulation during xylose consumption by *Paenibacillus polymyxa*. Appl Microbiol Biotechnol. 2004 Mar; 64 (1): 112-9.

SUMMARY OF THE INVENTION

10 PROBLEMS TO BE SOLVED BY THE INVENTION

[0011]

As described above, in cases where a biologically safe microorganism is used in a conventional method for producing 2,3-butanediol by microbial fermentation, the cost is high, or separation from a by-product is difficult, which is problematic.

15 [0012]

Accordingly, an object of the present invention is to establish a method for producing 2,3-butanediol by microbial fermentation utilizing a biologically safe microorganism. Another object of the present invention is to establish an economical method for producing 2,3-butanediol from a fermentation feedstock containing pentose as a carbon source, by microbial fermentation utilizing a

20 biologically safe microorganism.

MEANS FOR SOLVING THE PROBLEMS

[0013]

As a result of intensive study to solve the problems described above, the present inventors discovered that bacteria belonging to the genus *Zymobacter*, which have not been known as 2,3-butanediol fermentation microorganisms, have a capacity of 2,3-butanediol fermentation, thereby completing the present invention.

25

In addition, the present inventors discovered that 2,3-butanediol can be produced from pentose by using a *Zymobacter* bacterium having a capacity to metabolize pentose.

[0014]

5 That is, the present invention is as described in (1) to (16) below.

(1) A method for producing 2,3-butanediol, comprising the steps of:
 culturing a microorganism belonging to the genus *Zymobacter* in a
fermentation feedstock containing a carbon source (Step A); and
 purifying 2,3-butanediol from culture liquid obtained in the above step (Step
10 B).

(2) The method according to (1), wherein the microorganism is *Zymobacter palmae*.

(3) The method according to (1) or (2), wherein the Step A is culturing under aerated conditions.

15 (4) The method according to any one of (1) to (3) wherein the Step A is culturing at a volumetric oxygen transfer coefficient (kLa) of not less than 9 h^{-1} .

(5) The method for producing 2,3-butanediol according to any one of (1) to (4), wherein the Step A is culturing at a pH of 5 to 7.

(6) The method according to any one of (1) to (5), wherein the Step A is
20 culturing in a medium whose total sugar concentration is not less than 20 g/L.

(7) The method according to any one of (1) to (6), wherein the carbon source contains pentose, and the microorganism belonging to the genus *Zymobacter* has a capacity to metabolize pentose.

(8) The method according to any one of (1) to (7), wherein the microorganism is
25 a transformed microorganism belonging to the genus *Zymobacter* in which an exogenous gene(s) encoding at least one enzyme selected from the group consisting of xylose isomerase, xylulokinase, transaldolase, and transketolase is/are introduced.

- (9) The method according to (8), wherein the microorganism is a transformed microorganism belonging to the genus *Zymobacter* in which exogenous genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase are introduced.
- 5 (10) The method according to any one of (7) to (9), wherein the pentose contained in the fermentation feedstock in the Step A is xylose.
- (11) The method according to any one of (7) to (10), wherein the abundance of xylose with respect to total sugar in the fermentation feedstock is 5 to 100%.
- (12) The method according to any one of (7) to (11), wherein the fermentation
10 feedstock contains a sugar liquid derived from a biomass.
- (13) The method according to any one of (1) to (12), wherein the fermentation feedstock in the Step A contains corn steep liquor.
- (14) The method according to any one of (1) to (13), wherein the Step B comprises a distillation process.
- 15 (15) The method according to (14), comprising a desalting process before the distillation process.
- (16) The method according to (15), comprising an ion-exchange process as the desalting process.

EFFECT OF THE INVENTION

20 [0015]

According to the present invention, in a method for producing 2,3-butanediol from a fermentation feedstock by microbial fermentation, 2,3-butanediol can be more safely and economically obtained compared to conventional techniques. Even in cases where the fermentation feedstock contains pentose as a carbon source, 2,3-
25 butanediol can be safely and economically obtained from the pentose.

MODE FOR CARRYING OUT THE INVENTION

[0016]

Step A: 2,3-Butanediol Fermentation Step

The present invention is characterized in that a bacterium belonging to the genus *Zymobacter* is used as a 2,3-butanediol (which may hereinafter be referred to as “2,3-BDO”) fermentation microorganism. The microorganism used may be a
5 microorganism isolated from the natural environment, or may be a microorganism whose properties are partially modified by mutation or genetic recombination. Specific examples of the bacteria belonging to the genus *Zymobacter* having a 2,3-BDO fermentation capacity include *Zymobacter palmae*, which is preferably used from the viewpoint of the 2,3-BDO fermentation capacity.

10 [0017]

The fermentation feedstock is preferably an ordinary liquid medium containing, as appropriate, one or more of metabolizable carbon sources, metabolizable nitrogen sources, inorganic salts, and, if necessary, amino acids and organic micronutrients such as vitamins. The fermentation feedstock may also
15 contain an antifoaming agent as appropriate for the purpose of efficient fermentation.

[0018]

Specific examples of the metabolizable carbon sources include sugars such as glucose, mannose, galactose, fructose, sucrose, starch, waste molasses, and starch; organic acids such as citric acid, succinic acid, and acetic acid; and alcohols such as
20 glycerol and ethanol. Sugars are preferred. In cases where a sugar is used, the concentration of the sugar in the medium is usually not less than 15 g/L, preferably not less than 20 g/L from the viewpoint of efficiently carrying out the later step of purifying 2,3-butanediol. The total sugar concentration in the fermentation feedstock as the medium is usually 15 to 500 g/L, preferably 20 to 300 g/L. In
25 cases where the total sugar concentration is not more than 15 g/L, the effect of increasing the yield from the carbon source may decrease. In cases where the total sugar concentration is low, the production efficiency of 2,3-BDO also decreases.

[0019]

Specific examples of the metabolizable nitrogen sources include ammonia gas, aqueous ammonia, ammonium salts, urea, nitrates, and amino acids; and organic nitrogen sources which can be supplementarily used such as oil cakes, soybean hydrolysates, casein hydrolysates, animal tissue hydrolysates, corn steep liquor, yeasts or yeast extracts, meat extracts, and fermentation microorganism cells and hydrolysates thereof. Among these, as the organic nitrogen sources, soybean hydrolysates and corn steep liquor are preferred. Corn steep liquor is more preferred. The concentration of the corn steep liquor contained in the fermentation feedstock is not limited. For example, the concentration is usually 2.5 g/L to 35 g/L, preferably 5 g/L to 25 g/L, more preferably 10 g/L to 20 g/L in terms of the solid content contained in the corn steep liquor. The method for measuring the solid content contained in the corn steep liquor is not limited. The solid content can be calculated by, for example, determining the water content based on the difference between the weight before heat drying and the weight after heat drying, and subtracting the water content from the weight before drying. Examples of inorganic salts which may be added as appropriate include phosphoric acid salts, magnesium salts, calcium salts, iron salts, and manganese salts.

[0020]

In addition, nicotinic acid may be added to the medium as appropriate depending on the growth of the microorganism. An antifoaming agent may also be added as appropriate.

[0021]

The culture temperature is not limited as long as it is a temperature within the range which allows the growth of the microorganism. The culturing is carried out at a temperature of usually 20 to 50°C, preferably 25 to 40°C. The culture period may be a period during which 2,3-BDO accumulates, in the medium, in an amount

which allows purification of the 2,3-BDO. The culture period can be appropriately set by monitoring the concentration of the carbon source or the concentration of 2,3-BDO produced in the medium with time. The culture period is not limited, and usually 1 hour to 300 hours, preferably 4 hours to 150 hours.

5 [0022]

The pH of the medium during the culturing is maintained within the range of, usually, 4 to 8 by using an inorganic or organic acid, alkaline substance, urea, calcium carbonate, ammonia gas, and/or the like. The pH during the culturing is preferably adjusted to 5 to 7. The pH is more preferably not less than 5 and less
10 than 6.

[0023]

The mode of the culturing of the microorganism is not limited. The culturing is carried out by, for example, batch culture, fed-batch fermentation, or continuous culture.

15 [0024]

In the present invention, the microorganism is preferably cultured under aerated conditions. The aeration in the present invention means that a gas containing oxygen is allowed to flow into a culture vessel. Examples of the method of aeration include, but are not limited to, a method in which a gas containing oxygen
20 is passed through the culture medium, and upper surface aeration in which the air layer on the culture liquid is replaced with a gas containing oxygen. Examples of the upper surface aeration include a method in which a gas containing oxygen is blown into the culture vessel, and a method in which an air flow is generated on the culture liquid by stirring the culture liquid in a state where the culture liquid is
25 exposed to the atmosphere, thereby supplying oxygen into the culture liquid. In the present invention, the aeration is preferably carried out by a method in which a gas containing oxygen is passed through the culture liquid.

[0025]

In culturing of a microorganism, conditions for supplying oxygen into the medium can be appropriately set by combination of aeration and stirring. The conditions are expressed by the volumetric oxygen transfer coefficient, kLa (h^{-1}) (hereinafter simply referred to as kLa) for water at normal pressure at 30°C. kLa represents a capacity to produce dissolved oxygen by transferring oxygen from a gas phase to a liquid phase in a unit time under aeration and stirring, and is defined according to the Equation (1) below (Laboratory Manual for Bioengineering. The Society for Biotechnology, Japan ed., Baifukan Co., Ltd., p. 310 (1992)).

10 $dC/dt = kLa \times (C^* - C) \dots$ Equation (1)

In this equation, C represents the dissolved oxygen level DO (ppm) in the culture liquid; C^* represents the dissolved oxygen level DO (ppm) in the equilibrium state with the gas phase in the absence of oxygen consumption by the microorganism; and kLa represents the volumetric oxygen transfer coefficient (hr^{-1}).

15 Since the following Equation (2) is derived from the above Equation (1), kLa can be determined by plotting the logarithm of $C^* - C$ against the aeration period.

$\ln(C^* - C) = -kLa \times t \dots$ Equation (2)

[0026]

The kLa in the present invention is a value measured by the gas replacement method (dynamic method). In the gas replacement method (dynamic method), water is fed to an aeration/agitation culture apparatus into which a dissolved-oxygen level electrode is inserted, and oxygen in the liquid is replaced with nitrogen to reduce the oxygen concentration in the liquid. Subsequently, the nitrogen gas is switched to compressed air, and the process of increase in the dissolved oxygen is measured at a predetermined aeration rate, stirring rate, and temperature, thereby calculating kLa .

[0027]

The kLa set for the culturing in the present invention is not limited, and preferably not less than 9 h^{-1} . The upper limit of the kLa is not limited, and the kLa is preferably not more than 200 h^{-1} .

[0028]

5 In the method of the present invention, the fermentation feedstock contains pentose as a metabolizable carbon source. By culturing a microorganism belonging to the genus *Zymobacter* having a capacity to metabolize pentose, 2,3-butanediol can be produced using the pentose as a material. In such a case, the bacterium belonging to the genus *Zymobacter* may be a bacterium isolated from the natural
10 environment, or may be a bacterium whose properties were partially modified by mutation or genetic recombination. Specific examples of the bacterium belonging to the genus *Zymobacter* having a capacity of 2,3-butanediol fermentation include *Zymobacter palmae*, which is preferably used from the viewpoint of the 2,3-butanediol fermentation capacity. Normally, *Zymobacter palmae* does not
15 metabolize pentose. However, the capacity to metabolize pentose can be given thereto by introduction of a gene(s) involved in pentose metabolism.

[0029]

As the gene to be introduced in the present invention, a gene for an enzyme which isomerizes aldopentose to ketopentose may be used. Examples of the gene
20 include genes for pentose isomerase, pentose reductase, and/or pentol dehydrogenase.

[0030]

Pentose isomerase is defined as an enzyme which catalyzes isomerization of aldopentose and ketopentose, which are structural isomers of pentose. The pentose isomerase used in the present invention is not limited as long as it has an activity to
25 catalyze direct isomerization of pentose. Examples of the pentose isomerase include xylose isomerase (EC 5.3.1.5) and arabinose isomerase (EC 5.3.1.3).

Among these, xylose isomerase is an enzyme which catalyzes direct isomerization of

xylose (aldopentose) to xylulose (ketopentose), and/or the reverse reaction thereof, and also known as xylose ketoisomerase. The direct isomerization means one-step isomerization catalyzed by pentose isomerase, which is different from two-step conversion through a sugar alcohol intermediate catalyzed by pentose reductase and pentol dehydrogenase.

[0031]

Pentose reductase is a reducing enzyme, and means an enzyme having an activity to convert an aldopentose into a sugar alcohol using NADH or NADPH as a coenzyme. For example, EC 1.1.1.307 and EC 1.1.1.21 correspond to xylose reductase, which is an enzyme that converts xylose to xylitol. For example, EC 1.1.1.21 corresponds to arabinose reductase, which is an enzyme that converts arabinose to arabitol. Enzymes that are not categorized into the EC numbers described above are also included in the pentose reductase in the present invention as long as the enzymes have the activities described above.

[0032]

Pentol dehydrogenase is a dehydrogenase, and means an enzyme that converts pentol to ketopentose using NAD⁺ as a coenzyme. For example, EC 1.1.1.9 and EC 1.1.1.10 correspond to xylitol dehydrogenase, which is an enzyme that converts xylitol to xylulose. For example, EC 1.1.1.11 and EC 1.1.1.12 correspond to arabitol dehydrogenase, which is an enzyme that converts arabitol to ribose. Enzymes that are not categorized into pentol dehydrogenase are also included in the pentol dehydrogenase in the present invention as long as the enzymes have the activities described above.

[0033]

The reaction catalyzed by pentose reductase and pentol dehydrogenase isomerizes aldopentose to ketopentose, similarly to the reaction by pentose isomerase.

[0034]

Among enzymes which catalyze isomerization of aldopentose and ketopentose, pentose isomerase of pentose may be preferably used. Xylose isomerase is more preferably used.

[0035]

5 The gene for the xylose isomerase used is not limited, and may be in the form of, for example, genomic DNA or cDNA. The gene may be derived from any of organisms including animals, plants, fungi (yeasts, molds, and the like), and bacteria. Information on such a gene can be easily known by searching a database published on a web site of, for example, NCBI. A base sequence of the xylose isomerase gene
10 in *Escherichia coli* which may be preferably used in the present invention is described in, for example, GenBank Accession No. NC_007779 REGION: 3909650..3910972.

[0036]

The activity of xylose isomerase can be confirmed by performing enzyme
15 reaction in the presence of xylose, and then measuring, by HPLC or the like, xylulose produced by isomerization. The activity of xylose isomerase can be measured by, for example, the method disclosed in JP 2008-79564 A.

[0037]

Examples of organisms which may be used as the donor of the xylose
20 isomerase include microorganisms belonging to the family Enterobacteriaceae such as the genera *Enterobacter*, *Escherichia*, *Klebsiella*, *Erwinia*, and *Salmonella*; actinomycetes such as the genera *Actinoplanes*, *Arthrobacter*, and *Streptomyces*; and the genera *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Staphylococcus*,
Thermoanaerobacter, *Thermus*, *Xanthomonas*, *Rhizobium*, *Pseudomonas*,
25 *Clostridium*, and *Bacteroides*. Microorganisms belonging to the genus *Escherichia* are preferred. *Escherichia coli* is more preferred.

[0038]

As the gene to be introduced in the present invention, a gene for an enzyme having an activity to catalyze phosphorylation of ketopentose may be used.

Examples of the enzyme having an activity to catalyze phosphorylation of ketopentose include xylulokinase (EC 2.7.1.17) and ribulokinase (EC 2.7.1.16).

5 Xylulokinase may be preferably used.

[0039]

The gene for the xylulokinase used is not limited, and may be in the form of, for example, genomic DNA or cDNA. The gene may be derived from any of organisms including animals, plants, fungi (yeasts, molds, and the like), and bacteria.

10 Information on such a gene can be easily known by searching a database published on a web site of, for example, NCBI. A base sequence of the xylulokinase gene in *Escherichia coli* which may be preferably used in the present invention is described in, for example, GenBank Accession No. NC_007779 REGION: 3911044..3912498.

[0040]

15 The activity of xylulokinase can be confirmed by performing enzyme reaction in the presence of xylulose, and then measuring, by HPLC or the like, xylulose-5-phosphate produced by phosphorylation, or measuring an increase in NADPH or the like using an auxiliary enzyme reaction. For example, the measurement can be carried out by the method disclosed in Feldmann SD, Sahm H, Sprenger GA.

20 Cloning and expression of the genes for xylose isomerase and xylulokinase from *Klebsiella pneumoniae* 1033 in *Escherichia coli* K12. Mol Gen Genet. 1992 Aug; 234 (2): 201-10.

[0041]

25 Examples of organisms which may be used as the donor of the xylulokinase include microorganisms belonging to the family Enterobacteriaceae such as the genera *Enterobacter*, *Escherichia*, *Klebsiella*, *Erwinia*, and *Salmonella*; lactic acid bacteria such as the genera *Bacteroides* and *Lactobacillus*; actinomycetes such as the

genera *Actinoplanes*, *Arthrobacter*, and *Streptomyces*; and the genera *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Thermoanaerobacter*, *Xanthomonas*, *Rhizobium*, *Pseudomonas*, and *Clostridium*. Microorganisms belonging to the genus *Escherichia* are preferred. *Escherichia coli* is more preferred.

5 [0042]

As the gene to be introduced in the present invention, a gene for an enzyme having an activity of transketolase and/or transaldolase may be used. Transketolase is defined as an enzyme which catalyzes a reaction to convert xylulose-5-phosphate and erythrose-4-phosphate to fructose-6-phosphate and glyceraldehyde-3-phosphate, and the reverse reaction thereof, and/or catalyzes a reaction to convert ribose-5-phosphate and xylulose-5-phosphate to sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate, and the reverse reaction thereof. The transketolase used in the present invention is not limited as long as it has the catalytic activity described above. For example, EC 2.2.1.1 corresponds the transketolase.

15 [0043]

The transketolase gene and/or transaldolase gene used is/are not limited, and may be in the form of, for example, genomic DNA or cDNA. Each gene may be derived from any of organisms including animals, plants, fungi (yeasts, molds, and the like), and bacteria. Information on such genes can be easily known by searching a database published on a web site of, for example, NCBI. A base sequence of the transketolase gene in *Escherichia coli* which may be preferably used in the present invention is described in, for example, GenBank Accession No. NC_007779 REGION: complement (3078300..3080291). A base sequence of the transaldolase gene in *Escherichia coli* which may be preferably used in the present invention is described in, for example, GenBank Accession No. NC_007779 REGION: 8238..9191.

[0044]

The activity of transketolase can be confirmed by performing enzyme reaction in the presence of xylulose-5-phosphate and erythrose-4-phosphate, and measuring, by HPLC or the like, glyceraldehyde-3-phosphate produced, or measuring a decrease in NADH or the like using an auxiliary enzyme reaction. For example, the measurement

5 can be carried out by the method disclosed in Sprenger GA, Schorken U, Sprenger G, Sahm H. Transketolase A of *Escherichia coli* K12. Purification and properties of the enzyme from recombinant strains. Eur J Biochem. 1995 Jun 1; 230 (2): 525-32.

[0045]

Transaldolase is defined as an enzyme which catalyzes a reaction to convert

10 sedoheptulose-7-phosphate and glyceraldehyde-3-phosphate to erythrose-4-phosphate and fructose-6-phosphate, and the reverse reaction thereof. The transaldolase used in the present invention is not limited as long as it has the catalytic activity described above. For example, EC2.2.1.2 corresponds the transaldolase.

[0046]

The activity of transaldolase can be confirmed by performing enzyme reaction in the presence of fructose-6-phosphate and erythrose-4-phosphate, and then measuring, by HPLC or the like, glyceraldehyde-3-phosphate produced, or measuring a decrease in NADH or the like using an auxiliary enzyme reaction. For example, the measurement

15 can be carried out by the method disclosed in GA. Sprenger, U. Schorken, G. Sprenger & H. Sahm Transaldolase B of *Escherichia coli* K-12: cloning of its gene, talB, and characterization of the enzyme from recombinant strains (J. Bacteriol., 1995, 177: 20: 5930-6).

20

[0047]

Examples of organisms which may be used as the donor of the transaldolase

25 and/or transketolase include microorganisms belonging to the family Enterobacteriaceae such as the genera *Enterobacter*, *Escherichia*, *Klebsiella*, *Erwinia*,

and *Salmonella*; actinomycetes such as the genera *Actinoplanes*, *Arthrobacter*, and *Streptomyces*; and the genera *Bacillus*, *Paenibacillus*, *Lactobacillus*, *Staphylococcus*, *Thermoanaerobacter*, *Thermus*, *Xanthomonas*, *Rhizobium*, *Pseudomonas*, *Clostridium*, and *Bacteroides*. Microorganisms belonging to the genus *Escherichia* are preferred. *Escherichia coli* is more preferred.

[0048]

As long as the bacterium belonging to the genus *Zymobacter* has a capacity to metabolize pentose, it may have at least any one of the genes described above introduced thereto. Preferably, exogenous genes encoding (1) xylose isomerase, (2) xylulokinase, and (3) at least one of transaldolase and transketolase, are introduced. More preferably, exogenous genes encoding (1) xylose isomerase, (2) xylulokinase, and (3) both transaldolase and transketolase, are introduced.

[0049]

A microorganism other than those described above may also be used as a donor in cases where the microorganism has a capacity to decompose xylose, or in cases where the microorganism lacks a capacity to decompose xylose due to, for example, abnormality in a promoter site or ribosome binding site, but has DNA encoding a xylose isomerase, xylulokinase, transaldolase, and/or transketolase gene(s).

[0050]

A combination of genes derived from a plurality of donors may be introduced. Such genes may be incorporated into the genomic DNA of the bacterium belonging to the genus *Zymobacter* to be used as the host, or may be present in a vector(s).

[0051]

The method for introducing the gene(s) is not limited. For example, as the method for introducing the gene(s) present in the vector(s), the method disclosed in Patent Document 1 and Patent Document 2 may be used. That is, each gene is

inserted into the multicloning site of a commercially available plasmid cloning vector for *E. coli* (e.g., pUC118), and *E. coli* is then transformed with the resulting plasmid, followed by allowing the growth of the transformant producing the product of each gene. From the transformant grown, the plasmid is recovered, and the region in which each gene is introduced is cleaved out, followed by inserting the resulting fragment into the multicloning site of a wide host-range vector plasmid (e.g., pMFY31 (Agric. Biol. Chem., Vol.49(9), 2719-2724, 1985)). By transforming a bacterium belonging to the genus *Zymobacter* with the resulting recombinant plasmid vector, a transformant belonging to the genus *Zymobacter* having a capacity to metabolize pentose can be prepared. As the wide host-range vector plasmid, a commercially available product may also be used. Methods of transformation per se are well known. For example, as described in Patent Document 1 and Patent Document 2, competent cells may be prepared by culturing in a medium such as T medium supplemented with magnesium ions (2.0 wt% glucose; 1.0 wt% Bacto-yeast extract; 1.0 wt% potassium dihydrogen phosphate; 0.2 wt% ammonium sulfate; 0.05 wt% magnesium sulfate heptahydrate, pH6.0). Thereafter, transformation can be carried out by a conventional method such as electroporation. A transformant having a capacity to metabolize xylose can be obtained by culturing, in a medium containing xylose as the only carbon source, the bacteria subjected to the transformation treatment.

[0052]

In cases where each gene to be introduced is incorporated into the genomic DNA, a method disclosed in, for example, Kirill A. Datsenko and Barry L. Wanner. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A. Jun 6, 2000; 97(12): 6640-6645. may be used.

[0053]

Even in cases where the fermentation feedstock contains pentose, the

fermentation feedstock may contain hexose in addition to the pentose.

[0054]

Pentose has five carbons constituting the sugar, and is also called five-carbon sugar. Pentose can be divided into aldopentose, which has an aldehyde group at the 1-position, and ketopentose, which has a ketone group at the 2-position. Examples of aldopentose include xylose, arabinose, ribose, and lyxose, and examples of ketopentose include ribulose and xylulose. The pentose used in the present invention may be any pentose as long as it can be metabolized by a microorganism, and, in view of the abundance in nature, availability, and the like, xylose and arabinose are preferred, and xylose is more preferred.

[0055]

Hexose has six carbons constituting the sugar, and is also called six-carbon sugar. Hexose can be divided into aldose, which has an aldehyde group at the 1-position, and ketose, which has a ketone group at the 2-position. Examples of aldose include glucose, mannose, galactose, allose, gulose, and talose, and examples of ketose include fructose, psicose, and sorbose. The hexose used in the present invention may be any hexose as long as it can be metabolized by a microorganism, and, in view of the abundance in nature, availability, and the like, glucose, mannose, and galactose are preferred, and glucose is more preferred.

[0056]

The fermentation feedstock containing pentose and hexose is not limited, and it is preferably a sugar liquid derived from a cellulose-containing biomass that is known to contain both hexose and pentose. Examples of the cellulose-containing biomass include herbaceous biomasses such as bagasse, switchgrass, corn stover, rice straw, and wheat straw; and woody biomasses such as trees and waste building materials. Cellulose-containing biomasses contain cellulose and/or hemicellulose, which are polysaccharides produced by dehydration condensation of sugars. By

hydrolyzing such polysaccharides, sugar liquids which may be used as fermentation feedstocks can be produced. The method per se for preparing a sugar liquid derived from a cellulose-containing biomass is well known, and may be any method.

Examples of disclosed methods for producing such a sugar include a method in which a sugar liquid is produced by acid hydrolysis of a biomass using concentrated sulfuric acid (Japanese Translated PCT Patent Application Laid-open No. 11-506934, JP 2005-229821 A), and a method in which a biomass is subjected to hydrolysis treatment using dilute sulfuric acid and then enzymatically treated with cellulase and/or the like to produce a sugar liquid (A. Aden et al., "Lignocellulosic Biomass to Ethanol Process Design and Economics Utilizing Co-Current Dilute Acid Prehydrolysis and Enzymatic Hydrolysis for Corn Stover" NREL Technical Report (2002)). Further, examples of disclosed methods in which acids are not used include a method in which a biomass is hydrolyzed using subcritical water at about 250 to 500°C to produce a sugar liquid (JP 2003-212888 A), a method in which a biomass is subjected to subcritical water treatment and then enzymatically treated to produce a sugar liquid (JP 2001-95597 A), and a method in which a biomass is subjected to hydrolysis treatment with pressurized hot water at 240°C to 280°C and then enzymatically treated to produce a sugar liquid (JP 3041380 B). These treatments may be followed by purification of the obtained sugar liquid. An example of the method is disclosed in WO 2010/067785.

[0057]

The weight ratio between the pentose and hexose contained in the mixed sugar is not limited, and preferably 1:9 to 9:1 as represented by the ratio of (pentose):(hexose) in terms of the weight ratio between pentose and hexose in the mixed sugar. Such a sugar ratio is expected in cases where the mixed sugar is a sugar liquid derived from a cellulose-containing biomass.

[0058]

As long as the ranges of the total sugar concentration and the ratio between pentose and hexose described above are satisfied, the concentration of hexose contained in the fermentation feedstock used in the present invention is not limited. By use of the method for producing 2,3-BDO of the present invention, a favorable yield can be obtained even with a mixed sugar liquid containing hexose at a concentration of not less than 5 g/L.

[0059]

Even in cases where the fermentation feedstock contains pentose, the fermentation feedstock may contain, as described above, in addition to the sugars, one or more of metabolizable carbon sources, metabolizable nitrogen sources, inorganic salts, and, if necessary, amino acids and organic micronutrients such as vitamins which favorably act on the fermentation by the 2,3-butanediol fermentation microorganism, as appropriate. The fermentation feedstock may also contain an antifoaming agent for the purpose of efficient fermentation.

[0060]

Even in cases where the fermentation feedstock contains pentose, specific examples of the metabolizable nitrogen sources include, as described above, ammonia gas, aqueous ammonia, ammonium salts, urea, nitrates, and amino acids; and organic nitrogen sources which can be supplementarily used such as oil cakes, soybean hydrolysates, casein digests, corn steep liquor, yeasts or yeast extracts, meat extracts, peptides including peptone, and fermentation microorganism cells and hydrolysates thereof. Examples of inorganic salts which may be added as appropriate include phosphoric acid salts, magnesium salts, calcium salts, iron salts, manganese salts, and zinc salts. For production of 2,3-butanediol by fermentation of pentose, the fermentation feedstock preferably contains, among these components, one or more of corn steep liquor; ammonium salts such as ammonium sulfate and ammonium hydrogen phosphate; phosphoric acid salts such as potassium dihydrogen

phosphate, potassium hydrogen phosphate, and ammonium hydrogen phosphate; calcium salts such as calcium chloride; iron salts such as ferrous sulfate; manganese salts such as manganese sulfate; zinc salts such as zinc sulfate; magnesium salts such as magnesium sulfate; and EDTA and salts thereof such as disodium EDTA.

5 [0061]

Even in cases where the fermentation feedstock contains pentose, nicotinic acid may be added to the medium as appropriate depending on the growth of the microorganism, as described above. An antifoaming agent may also be added as appropriate.

10 [0062]

Even in cases where the fermentation feedstock contains pentose as a metabolizable carbon source, and a microorganism belonging to the genus *Zymobacter* having a capacity to metabolize pentose is cultured, the culture temperature, the pH during the culturing, the mode of culturing of the microorganism, the aeration conditions, and the conditions for supplying oxygen into the medium, including the preferred conditions therefor, are the same as those described above.

15 [0063]

The culture liquid obtained by the 2,3-butanediol fermentation step contains not only 2,3-butanediol, but also impurities derived from the fermentation feedstock, and ethanol as a by-product of the fermentation. Therefore, 2,3-butanediol contained in the culture liquid needs to be purified in a later process.

20 [0064]

Purification Step

The step of purifying 2,3-butanediol from the 2,3-butanediol culture liquid is not limited, and may be carried out by a method known to those skilled in the art. The present invention preferably comprises a process of distillation of the 2,3-butanediol culture liquid.

25

[0065]

In the distillation of the 2,3-butanediol culture liquid, 2,3-butanediol is recovered from the vapor side. The method of distillation is not limited, and may be any of simple distillation, precision distillation, atmospheric distillation, and
5 distillation under reduced pressure which are commonly applied. The method may be carried out using an apparatus selected from, for example, a thin film distillation apparatus, tray-type distillation apparatus, and packed distillation apparatus. Either a batch method or a continuous method may be applied to the present invention. Distillation under reduced pressure is especially preferred since, in this method, the
10 boiling point can be lowered to suppress generation of impurities. More specifically, the method is preferably carried out at a heating temperature of 60°C to 150°C. In cases where the heating temperature is less than 60°C, the pressure needs to be largely decreased, so that maintenance of the apparatus is very difficult at an industrial level. In cases where the heating temperature is more than 150°C,
15 decomposition of a small amount of sugars and the like remaining in the 2,3-butanediol solution occurs, leading to production of colored substances as by-products, which is not preferred. Thus, the degree of reduction of the pressure is preferably controlled such that distillation of 2,3-butanediol occurs within the heating temperature range described above.

20 [0066]

Although the 2,3-butanediol culture liquid may be subjected as it is to the distillation process, the 2,3-butanediol culture liquid is preferably subjected to a desalting process and/or a concentration process before the distillation process.

[0067]

25 Specific examples of the desalting process include an ion-exchange process. The ion-exchange process is a method for removing ionic components in the 2,3-butanediol culture liquid using an ion exchanger. Examples of ion exchangers

which may be used include ion-exchange resins, ion-exchange membranes, ion-exchange fibers, ion-exchange papers, gel ion exchangers, liquid ion exchangers, zeolite, carbonaceous ion exchangers, and montmorillonite. In the present invention, a process using an ion-exchange resin is preferably employed.

5 [0068]

Ion-exchange resins can be divided into strong anion-exchange resins, weak anion-exchange resins, strong cation-exchange resins, weak cation-exchange resins, chelate-exchange resins, and the like depending on their functional groups. A strong anion-exchange resin is selected from, for example, “Amberlite” IRA410J, IRA411, and IRA910CT, manufactured by Organo Corporation; and “DIAION” SA10A, SA12A, SA11A, NSA100, SA20A, SA21A, UBK510L, UBK530, UBK550, UBK535, and UBK555, manufactured by Mitsubishi Chemical Corporation. Examples of weak anion-exchange resins include “Amberlite” IRA478RF, IRA67, IRA96SB, IRA98, and XE583, manufactured by Organo Corporation; and “DIAION” WA10, WA20, WA21J, and WA30, manufactured by Mitsubishi Chemical Corporation. Examples of strong cation-exchange resins include “Amberlite” IR120B, IR124, 200CT, and 252, manufactured by Organo Corporation; and “DIAION” SK104, SK1B, SK110, SK112, PK208, PK212, PK216, PK218, PK220, and PK228, manufactured by Mitsubishi Chemical Corporation. A weak cation-exchange resins may be selected from, for example, “Amberlite” FPC3500 and IRC76, manufactured by Organo Corporation; and “DIAION” WK10, WK11, WK100, and WK40L, manufactured by Mitsubishi Chemical Corporation.

20 [0069]

In the present invention, an anion-exchange resin(s) and a cation-exchange resin(s) are preferably used in combination as the ion-exchange resin. A strong anionic resin(s) and a strong cationic resin(s) are more preferably used since these can remove various ions. The anion-exchange resin is preferably regenerated with a

dilute aqueous solution of an alkali such as sodium hydroxide, and used as the OH type. The cation-exchange resin is preferably regenerated with a dilute aqueous solution of an acid such as hydrochloric acid, and used as the H type. The method of desalting using the ion-exchange resins may be either a batch method or a column method, and is not limited as long as efficient desalting is possible. In the production process, a column method is preferably employed since the method allows repeated use. The flow rate is usually controlled based on SV (space velocity), and SV is preferably 2 to 50. SV is more preferably 2 to 10 in view of achieving higher purity. Resins are commercially available in the forms of gel types such as a porous type, high porous type, and MR type, and the ion-exchange resins may be in any of these forms. Ion-exchange resins having optimum forms may be selected depending on the quality of the solution.

[0070]

Specific examples of the desalting process also include nanofiltration membrane treatment. For example, in the nanofiltration membrane treatment of the 2,3-butanediol culture liquid, as disclosed in JP 2010-150248 A, filtration of the 2,3-butanediol culture liquid through a nanofiltration membrane allows efficient separation of 2,3-butanediol into the permeate side, and inorganic salts, sugars, and colored components into the feed side.

[0071]

Examples of the material of the nanofiltration membrane include polymer materials such as piperazine polyamide, polyamide, cellulose acetate, polyvinyl alcohol, polyimide, and polyester; and inorganic materials such as ceramics. A nanofiltration membrane is generally used as a spiral-wound membrane element, or as a flat membrane or a hollow fiber membrane. The nanofiltration membrane used in the present invention is preferably a spiral-wound membrane element.

[0072]

Specific examples of the nanofiltration membrane element preferably used in the present invention include “GEsepa”, which is a cellulose acetate nanofiltration membrane manufactured by GE Osmonics; NF99 and NF99HF, which are nanofiltration membranes having a functional layer composed of a polyamide, manufactured by Alfa-Laval; NF-45, NF-90, NF-200, and NF-400, which are nanofiltration membranes having a functional layer composed of a cross-linked piperazine polyamide, manufactured by Filmtec Corporation; and SU-210, SU-220, SU-600, and SU-610, which are nanofiltration membrane elements manufactured by Toray Industries, Inc., containing UTC60 manufactured by the same manufacturer. Among these, the nanofiltration membrane element is more preferably NF99 or NF99HF, which are nanofiltration membranes having a functional layer composed of a polyamide, manufactured by Alfa-Laval; NF-45, NF-90, NF-200, or NF-400, which are nanofiltration membranes having a functional layer composed of a cross-linked piperazine polyamide, manufactured by Filmtec Corporation; or SU-210, SU-220, SU-600, or SU-610, which are nanofiltration membrane modules manufactured by Toray Industries, Inc., containing UTC60 manufactured by the same manufacturer. The nanofiltration membrane element is still more preferably SU-210, SU-220, SU-600, or SU-610, which are nanofiltration membrane elements manufactured by Toray Industries, Inc., containing UTC60 manufactured by the same manufacturer, whose major component is a cross-linked piperazine polyamide.

[0073]

The filtration through a nanofiltration membrane may be carried out under pressure, and the filtration pressure is preferably within the range of 0.1 MPa to 8 MPa. In cases where the filtration pressure is less than 0.1 MPa, the membrane permeation rate may be low, while in cases where the filtration pressure is more than 8 MPa, the membrane may be damaged. In cases where the membrane is used at a filtration pressure of 0.5 MPa to 7 MPa, the membrane permeation flux is high, so

that the 2,3-butanediol fermentation liquid can be efficiently allowed to permeate, and the possibility of damaging the membrane is small, which is more preferred. The membrane is especially preferably used at a filtration pressure of 1 MPa to 6 MPa.

5 [0074]

The concentration of 2,3-butanediol in the 2,3-butanediol solution that is to be filtered through the nanofiltration membrane is not limited. In cases where the concentration of 2,3-butanediol in the 2,3-butanediol solution is high, the concentration of 2,3-butanediol contained in the permeate is also high, so that the energy required for concentrating 2,3-butanediol can be saved, and the cost can therefore be reduced, which is preferred.

[0075]

In the desalting process, either the ion-exchange treatment or the nanofiltration membrane treatment may be carried out, or both of these may be employed in combination. It is preferred to carry out at least the ion-exchange treatment. In cases where the nanofiltration membrane treatment and the ion-exchange treatment are employed in combination, the order of these treatments is not limited. Preferably, the 2,3-butanediol culture liquid is subjected to the nanofiltration membrane treatment, and then the 2,3-butanediol culture liquid containing reduced inorganic salts obtained from the permeate side is subjected to the ion-exchange treatment. By this, inorganic salts and organic acids that partially pass through the nanofiltration membrane can be removed with the ion-exchange resin, so that the removal rate of inorganic salts can be increased.

[0076]

25 The method of concentrating the 2,3-butanediol culture liquid may be a general method known to those skilled in the art, and examples of the method include methods using a reverse osmosis membrane, and methods by concentration under

heat using an evaporator. A method using a reverse osmosis membrane is preferably applied.

[0077]

In the method using a reverse osmosis membrane, a 2,3-butanediol solution is
5 filtered through a reverse osmosis membrane to allow water to permeate into the
permeate side, while 2,3-butanediol is retained and concentrated in the feed side of
the membrane. Examples of reverse osmosis membranes preferably used in the
present invention include composite membranes having a cellulose acetate polymer
as a functional layer (hereinafter also referred to as cellulose acetate reverse osmosis
10 membranes) and composite membranes having a polyamide functional layer
(hereinafter also referred to as polyamide reverse osmosis membranes). Here,
examples of the cellulose acetate polymer include polymers prepared with organic
acid esters of cellulose such as cellulose acetate, cellulose diacetate, cellulose
triacetate, cellulose propionate, and cellulose butyrate, which may be used
15 individually, or as a mixture or mixed ester of two or more of these. Examples of
the polyamide include linear polymers and cross-linked polymers constituted by
aliphatic and/or aromatic diamine monomers. Examples of the form of the
membrane that may be used as appropriate include a flat membrane, spiral-wound
membrane, and hollow fiber membrane.

20 [0078]

Specific examples of the reverse osmosis membrane used in the present
invention include polyamide reverse osmosis membrane modules manufactured by
Toray Industries, Inc., such as low-pressure type modules SU-710, SU-720, SU-720F,
SU-710L, SU-720L, SU-720LF, SU-720R, SU-710P, and SU-720P, as well as high-
25 pressure type modules SU-810, SU-820, SU-820L, and SU-820FA; cellulose acetate
reverse osmosis membranes manufactured by the same manufacturer, SC-L100R,
SC-L200R, SC-1100, SC-1200, SC-2100, SC-2200, SC-3100, SC-3200, SC-8100,

and SC-8200; NTR-759HR, NTR-729HF, NTR-70SWC, ES10-D, ES20-D, ES20-U, ES15-D, ES15-U, and LF10-D, manufactured by Nitto Denko Corporation; RO98pHt, RO99, HR98PP, and CE4040C-30D, manufactured by Alfa-Laval; “GE Sepa” manufactured by GE; and BW30-4040, TW30-4040, XLE-4040, LP-4040, LE-4040, SW30-4040, and SW30HRLE-4040, manufactured by FilmTec Corporation.

[0079]

In the present invention, the concentration through a reverse osmosis membrane is carried out under pressure. The filtration pressure is preferably within the range of 1 MPa to 8 MPa since, in cases where the filtration pressure is less than 1 MPa, the membrane permeation rate may be low, while in cases where the filtration pressure is more than 8 MPa, the membrane may be damaged. In cases where the filtration pressure is within the range of 1 MPa to 7 MPa, the membrane permeation flux is high, so that the 2,3-butanediol solution can be efficiently concentrated. The filtration pressure is most preferably within the range of 2 MPa to 6 MPa in view of reducing the possibility of damaging the membrane. In cases of a 2,3-butanediol solution at a low concentration, a method using a reverse osmosis membrane is preferred in view of the cost.

[0080]

Prior to the above process, a process of separating bacterial cells contained in the 2,3-butanediol culture liquid is preferably included. Examples of methods which can be used for the process of separating bacterial cells include centrifugation and filtration separation. One of, or the combination of, these methods may be employed.

[0081]

The 2,3-butanediol produced by the present invention is characterized in that it has high purity. Examples of methods for measuring impurities contained in the

2,3-butanediol include combinations of purity measurement by gas chromatography (GC), purity measurement by UV detection using high performance liquid chromatography (HPLC), and/or the like. Since these are based on evaluation of the ratio of the area of 2,3-butanediol in the total area of detected peaks, a higher ratio means a higher purity of 2,3-butanediol.

EXAMPLES

[0082]

The present invention is described below concretely by way of Examples. However, the present invention is not limited to these Examples.

[0083]

Reference Example 1 Measurement of kLa

A dissolved-oxygen electrode (manufactured by ABLE Corporation) was inserted into a culture apparatus (manufactured by ABLE Corporation; jar capacity, 1.5 L) to measure the dissolved-oxygen level under various aeration/stirring conditions, and kLa was determined by the dynamic method using nitrogen gas. First, in the culture vessel, 1 L of water was placed, and nitrogen gas was sufficiently blown into the water while the water temperature was controlled at 30°C and the water was stirred at a constant rate. When the electrode value became minimum, zero calibration of the dissolved-oxygen electrode was carried out. Thereafter, the aeration gas was switched from nitrogen gas to air or nitrogen gas at a predetermined aeration rate, and changes in the dissolved-oxygen level with time thereafter were measured to determine kLa. Table 1 shows kLa obtained for various aeration/stirring conditions.

[0084]

[Table 1]

Aeration rate (vvm)	Stirring rate (rpm)	kLa(h ⁻¹)
0.1 (Nitrogen gas)	400	0
0.025	400	9
0.2	400	17
0.6	400	28
0.6	500	57

[0085]

Examples 1 to 6: kLa

<2,3-Butanediol Fermentation>

5 The *Zymobacter palmae* (*Z. palmae*) ATCC51623 strain was subjected to static culture in a test tube containing 5 mL of T medium (20 g/L Glucose, 10 g/L Yeast extract, 10 g/L KH₂PO₄, 2 g/L (NH₄)₂SO₄, 0.5 g/L MgSO₄·7H₂O, pH 6.0) at 30°C for 24 hours, to perform pre-preculture. The whole pre-preculture liquid was then added to an Erlenmeyer flask containing 50 mL of T medium, and static culture

10 was carried out at 30°C for 16 hours, to perform preculture. The preculture liquid was added to 1 L of the 2,3-butanediol fermentation medium having the composition shown in Table 2, and culturing was performed at a temperature of 30°C at each kLa described in Table 1 while the pH was maintained at 5.5 by neutralization with potassium hydroxide. Collection of the culture liquid was carried out using an

15 autosampler (manufactured by ABLE Corporation) at 2-hour intervals after the beginning of the culturing for up to 40 hours. The glucose concentration and the 2,3-butanediol concentration in the culture liquid collected were measured.

[0086]

[Table 2]

20

Glucose	40	g
Corn steep liquor (solid content, 50%)	30	g
(NH ₄) ₂ SO ₄	5.8	g
KH ₂ PO ₄	1.75	g
K ₂ HPO ₄	9.2	g
(NH ₄) ₂ HPO ₄	2.9	g
CaCl ₂ ·2H ₂ O	8.8	mg
FeSO ₄ ·7H ₂ O	44	mg
MnSO ₄ ·5H ₂ O	1.28	mg
ZnSO ₄ ·7H ₂ O	0.9	mg
MgSO ₄ ·7H ₂ O	219	mg
EDTA·2Na	44	mg
Unit (1/Liter)		

[0087]

The following are conditions for measuring each concentration by high performance liquid chromatography (HPLC, manufactured by Shimadzu Corporation). The 2,3-butanediol concentration and the glucose concentration after the fermentation, and the fermentation yield, were determined by the following measurement methods.

[0088]

Measurement of 2,3-Butanediol Concentration

10 Column: Shodex Sugar SH1011 (manufactured by Showa Denko K. K.)

Column temperature: 65°C

Mobile phase: 0.05 M aqueous sulfuric acid solution, 0.6 mL/min.

Detection: RI

[0089]

15 Measurement of Glucose Concentration

Column: Asahipak NH2P50 4E (manufactured by Showa Denko K. K.)

Column temperature: 30°C

Mobile phase: water:acetonitrile = 1:3, 0.6 mL/min.

Detection: RI

[0090]

Fermentation Yield

Based on the 2,3-butanediol concentration and the glucose concentration
5 measured by the above HPLC analysis, the fermentation yield was calculated using
the following equation.

Fermentation yield (%) = $100 \times \{ (2,3\text{-butanediol concentration in culture liquid}) - (2,3\text{-butanediol concentration in medium}) \} / \{ (\text{glucose concentration in medium}) - (\text{glucose concentration in culture liquid}) \}$

10 [0091]

The concentrations of 2,3-butanediol and glucose in the culture liquid and the
fermentation yield at the time point when the glucose concentration in the culture
liquid became closest to 0 are shown in Table 3. The 2,3-butanediol culture liquid
obtained was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The
15 supernatant was then filtered through a microfiltration membrane (manufactured by
Toray Industries, Inc.) to remove the bacterial cells. This process was carried out
twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result.

[0092]

<Ion-exchange Purification of 2,3-Butanediol Culture Liquid>

20 The culture liquid obtained by the fermentation test described above was
subjected to removal of residual ions by ion-exchange treatment. A strong anion-
exchange resin IRA120J (manufactured by Organo Corporation) and a strong cation-
exchange resin IR410 (manufactured by Organo Corporation) that were regenerated
with 1 N sodium hydroxide or 1 N hydrochloric acid into the OH type or H type,
25 respectively, were used. The amount of resin was calculated such that the total
amount of inorganic salts and organic acids was the same as the exchange capacity of
the ion-exchange resin. Columns were filled with the ion-exchange resins, and the

culture liquid was passed through the anion-exchange resin and then through the cation-exchange resin at a flow rate SV of 5.

[0093]

<Distillation Purification of 2,3-Butanediol>

5 The 2,3-butanediol solution after the ion-exchange treatment was subjected to removal of water with a film evaporator MF-10 (manufactured by Tokyo Rikakikai). At this time, water was allowed to evaporate at a degree of reduction of pressure of 30 hPa and a heating temperature of 60°C. The concentrated 2,3-butanediol solution was distilled under reduced pressure (5 mmHg) to obtain purified 2,3-
10 butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the following measurement methods.

[0094]

GC Purity

 The 2,3-butanediol after the distillation was analyzed by gas chromatography
15 (GC; manufactured by Shimadzu Corporation), and the GC purity was calculated according to the following equation based on the ratio of the peak area of 2,3-butanediol in the total detected peak area.

$$\text{GC purity (\%)} = 100 \times (\text{2,3-butanediol peak area}) / (\text{total detected peak area})$$

[0095]

20 The analysis conditions for the gas chromatography were as follows.

Column: RT-BDEXM (0.25 mm × 30 m, manufactured by Restek)

Column temperature: 75°C

Vaporizing chamber, detector temperature: 230°C

Carrier gas: He

25 Linear velocity: 35 cm/sec.

Detection: flame ionization detector (FID)

[0096]

Distillation Yield

The distillation yield was calculated according to the following equation based on

the amount of 2,3-butanediol fed before the distillation calculated from the
 5 2,3-butanediol concentration measured by HPLC analysis and the amount of liquid fed, and

the recovery of 2,3-butanediol calculated from the amount of distillate after the distillation and the above-described GC purity.

Distillation yield (%) = $100 \times \{(\text{amount of distillate after distillation}) \times (\text{GC purity})\}$
 10 $/ \{(\text{2,3-butanediol concentration before distillation}) \times (\text{amount of liquid fed before distillation})\}$

[0097]

Total 2,3-Butanediol Yield

The total 2,3-butanediol yield was calculated according to the following
 15 equation based on the fermentation yield calculated from the results of the fermentation step, and the distillation yield calculated from the results of the purification step. The results on the total 2,3-butanediol yield are shown in Table 3.

Total 2,3-butanediol yield (g/g sugar) = $(\text{fermentation yield}) / 100 \times (\text{distillation yield}) / 100$

20 [0098]

[Table 3]

		Example 1	Example 2	Example 3	Example 4	Example 5	Example 6
kLa (h ⁻¹)		0	3	9	17	28	57
Before culturing	Glucose concentration (g/L)	42.0	42.1	42.0	40.8	39.5	41.1
	2,3-BDO concentration (g/L)	0.0	0.0	0.0	0.0	0.0	0.0
After culturing	Glucose concentration (g/L)	26.1	11.6	2.1	0.5	0.2	3.4
	2,3-BDO concentration (g/L)	1.1	7.8	16.0	18.9	18.2	17.5
	Fermentation yield (%)	6.8	25.5	40.0	46.8	46.4	46.5
After distillation	Distillation yield (%)	78.6	79.3	82.4	82.6	83.2	82.7
	GC purity (%)	>99.9	>99.9	>99.9	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.053448	0.202215	0.3296	0.386568	0.386048	0.384555

[0099]

Examples 7 to 11: pH

The *Z. palmae* ATCC51623 strain was cultured by the pre-preculture method and the preculture method described in Examples 1 to 6. The preculture liquid was added to 1 L of the 2,3-butanediol fermentation medium having the composition shown in Table 2, and culturing was carried out at an aeration rate of 0.2 vvm, stirring rate of 400 rpm, and temperature of 30°C while the pH was maintained at one of 4.5, 4.8, 5.2, 5.5, 6.1, and 6.5 by neutralization with potassium hydroxide.

Collection of the culture liquid was carried out using an autosampler (manufactured by ABLE Corporation) at 2-hour intervals after the beginning of the culturing for up to 40 hours. The glucose concentration and the 2,3-butanediol concentration in the culture liquid collected were measured according to the methods described in Examples 1 to 6. The concentrations of 2,3-butanediol and glucose in the culture liquid and the fermentation yield at the time point when the glucose concentration in the culture liquid became closest to 0 are shown in Table 4. The 2,3-butanediol culture liquid after the fermentation test was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The supernatant was then filtered through a

microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result. The culture liquid obtained was subjected to desalting and distillation treatment by the methods described in Examples 1 to 6 to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the methods described in Examples 1 to 6. The results are shown in Table 4.

[0100]

[Table 4]

		Example 7	Example 8	Example 9	Example 10	Example 11
pH		4.5	4.8	5.2	6.1	6.5
Before culturing	Glucose concentration (g/L)	41.5	41.6	42.0	40.4	38.8
	2,3-BDO concentration (g/L)	0.0	0.0	0.0	0.0	0.0
After culturing	Glucose concentration (g/L)	11.5	0.0	2.3	1.6	2.6
	2,3-BDO concentration (g/L)	6.5	12.4	18.1	18.5	13.8
	Fermentation yield (%)	21.5	29.8	45.7	47.7	38.0
After distillation	Distillation yield (%)	79.1	81.5	82.9	82.6	83.2
	GC purity (%)	>99.9	>99.9	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.170065	0.24287	0.378853	0.394002	0.31616

10

[0101]

Examples 12 to 16: Sugar Concentration

The *Z. palmae* ATCC51623 strain was cultured by the pre-preculture method and the preculture method described in Examples 1 to 6. The preculture liquid was added to 1 L of the 2,3-butanediol fermentation medium having the same composition as shown in Table 2 except that the glucose concentration was adjusted to one of 12, 20, 100, 180, and 250 g/L. Culturing was then carried out at an

15

aeration rate of 0.2 vvm, stirring rate of 400 rpm, and temperature of 30°C while the pH was maintained at 5.5 by neutralization with potassium hydroxide. Collection of the culture liquid was carried out using an autosampler (manufactured by ABLE Corporation) at 2- to 8-hour intervals after the beginning of the culturing. The glucose concentration and the 2,3-butanediol concentration in the culture liquid collected were measured according to the methods described in Examples 1 to 6. The concentrations of 2,3-butanediol and glucose in the culture liquid and the fermentation yield at the time point when the glucose concentration in the culture liquid became closest to 0 are shown in Table 5. The 2,3-butanediol culture liquid after the fermentation test was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The supernatant was then filtered through a microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result. The culture liquid obtained was subjected to desalting and distillation treatment by the methods described in Examples 1 to 6 to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the methods described in Examples 1 to 6. The results are shown in Table 5.

[0102]

[Table 5]

		Example 12	Example 13	Example 14	Example 15	Example 16
Before culturing	Glucose concentration (g/L)	12.5	20.4	100.8	187.7	253.9
	2,3-BDO concentration (g/L)	0.0	0.0	0.0	0.0	0.0
After culturing	Glucose concentration (g/L)	0.0	0.4	3.1	0.3	5.6
	2,3-BDO concentration (g/L)	5.3	8.8	44.2	82.6	115.5
	Fermentation yield (%)	42.4	44.2	45.2	44.1	46.5
After distillation	Distillation yield (%)	78.8	79.5	84.6	84.3	86.4
	GC purity (%)	>99.9	>99.9	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.334112	0.35139	0.382392	0.371763	0.40176

[0103]

Examples 17 to 31

Expression of four kinds of enzyme genes in cells of the transformant described in Patent Document 1 and Patent Document 2 was confirmed. This transformant has been deposited with International Patent Organism Depository, National Institute of Advanced Industrial Science and Technology, under accession No. FERM BP-10048 as of June 30, 2004, under the Budapest Treaty. Since Patent Document 2 is US 7,569,379 B, this transformant is already available.

[0104]

Examples 17 to 21: kLa

<2,3-Butanediol Fermentation>

As a microorganism belonging to the genus *Zymobacter* given a capacity to metabolize xylose, *Zymobacter palmae* [pMFY31-xt] disclosed in Patent Documents 1 and 2 was used. *Zymobacter palmae* [pMFY31-xt] is a known transformant prepared by transformation of *Zymobacter palmae* with a recombinant wide host-range vector plasmid to which exogenous genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase are introduced. As described above, *Zymobacter palmae* [pMFY31-xt] has been deposited under accession No. FERM BP-10048 under the Budapest Treaty, and is available. *Z. palmae* [pMFY31-xt]

was subjected to static culture in a test tube containing 5 mL of TX medium (40 g/L Xylose, 10 g/L Yeast extract, 10 g/L KH_2PO_4 , 2 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.5 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, pH 6.0) supplemented with 100 $\mu\text{g/mL}$ ampicillin, at 30°C for 24 hours. The whole culture liquid was then added to an Erlenmeyer flask containing 50 mL of TX medium, and static culture was carried out at 30°C for 24 hours. The whole culture liquid was then added to 1 L of the 2,3-butanediol fermentation medium having the composition shown in Table 6, and culturing was performed at a temperature of 30°C under the aeration/stirring conditions described in Table 1 while the pH was maintained at 5.5 by neutralization with potassium hydroxide. The culture liquid was collected as appropriate during the culturing, and the xylose concentration and the 2,3-butanediol concentration were measured.

[0105]

[Table 6]

Xylose	40	g
Corn steep liquor	30	g
$(\text{NH}_4)_2\text{SO}_4$	5.8	g
KH_2PO_4	1.75	g
K_2HPO_4	9.2	g
$(\text{NH}_4)_2\text{HPO}_4$	2.9	g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	8.8	mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	44	mg
$\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$	1.28	mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.9	mg
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	219	mg
$\text{EDTA} \cdot 2\text{Na}$	44	mg
Unit (1/Liter)		

15

[0106]

The following are conditions for measuring each concentration by high performance liquid chromatography (HPLC, manufactured by Shimadzu Corporation). The 2,3-butanediol concentration and the sugar concentration after

the fermentation, and the fermentation yield, were determined by the following measurement methods.

[0107]

Measurement of 2,3-Butanediol Concentration

5 Column: Shodex Sugar SH1011 (manufactured by Showa Denko K. K.)

Column temperature: 65°C

Mobile phase: 0.05 M aqueous sulfuric acid solution, 0.6 mL/min.

Detection: RI

[0108]

10 Measurement of Sugar Concentration

Column: Asahipak NH2P50 4E (manufactured by Showa Denko K. K.)

Column temperature: 30°C

Mobile phase: water:acetonitrile = 1:3, 0.6 mL/min.

Detection: RI

15 [0109]

Fermentation Yield

Based on the 2,3-butanediol concentration and the sugar concentration measured by the above HPLC analysis, the fermentation yield was calculated using the following equation.

20 Fermentation yield (%) = $100 \times \{ (2,3\text{-butanediol concentration in culture liquid}) - (2,3\text{-butanediol concentration in medium}) \} / \{ (\text{sugar concentration in medium}) - (\text{sugar concentration in culture liquid}) \}$

[0110]

25 The concentrations of 2,3-butanediol and xylose in the culture liquid and the fermentation yield at the time point when the xylose concentration in the culture liquid became closest to 0 are shown in Table 7. The 2,3-butanediol culture liquid obtained was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The

supernatant was then filtered through a microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result.

[0111]

5 <Ion-exchange Purification of 2,3-Butanediol Culture Liquid>

The culture liquid obtained by the fermentation test described above was subjected to removal of residual ions by ion-exchange treatment. A strong anion-exchange resin IRA120J (manufactured by Organo Corporation) and a strong cation-exchange resin IR410 (manufactured by Organo Corporation) that were regenerated with 1 N sodium hydroxide or 1 N hydrochloric acid into the OH type or H type, respectively, were used. The amount of resin was calculated such that the total amount of inorganic salts and organic acids was the same as the exchange capacity of the ion-exchange resin. Columns were filled with the ion-exchange resins, and the culture liquid was passed through the anion-exchange resin and then through the cation-exchange resin at a flow rate SV of 5.

[0112]

<Distillation Purification of 2,3-Butanediol>

The 2,3-butanediol solution after the ion-exchange treatment was subjected to removal of water with a film evaporator MF-10 (manufactured by Tokyo Rikakikai). At this time, water was allowed to evaporate at a degree of reduction of pressure of 30 hPa and a heating temperature of 60°C. The concentrated 2,3-butanediol solution was distilled under reduced pressure (5 mmHg) to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the following measurement methods.

25 [0113]

GC Purity

The 2,3-butanediol after the distillation was analyzed by gas chromatography

(GC; manufactured by Shimadzu Corporation), and the GC purity was calculated according to the following equation based on the ratio of the peak area of 2,3-butanediol in the total detected peak area.

$$\text{GC purity (\%)} = 100 \times (\text{2,3-butanediol peak area}) / (\text{total detected peak area})$$

5 The analysis conditions for the gas chromatography were as follows.

Column: RT-BDEXM (0.25 mm × 30 m, manufactured by Restek)

Column temperature: 75°C

Vaporizing chamber, detector temperature: 230°C

Carrier gas: He

10 Linear velocity: 35 cm/sec.

Detection: flame ionization detector (FID)

[0114]

Distillation Yield

15 The distillation yield was calculated according to the following equation based on

the amount of 2,3-butanediol fed before the distillation calculated from the 2,3-butanediol concentration measured by HPLC analysis and the amount of liquid fed, and

20 the recovery of 2,3-butanediol calculated from the amount of distillate after the distillation and the above-described GC purity.

$$\text{Distillation yield (\%)} = 100 \times \{(\text{amount of distillate after distillation}) \times (\text{GC purity})\} / \{(2,3\text{-butanediol concentration before distillation}) \times (\text{amount of liquid fed before distillation})\}$$

[0115]

25 Total 2,3-Butanediol Yield

The total 2,3-butanediol yield was calculated according to the following equation based on the fermentation yield calculated from the results of the

fermentation step, and the distillation yield calculated from the results of the purification step. The results on the total 2,3-butanediol yield are shown in Table 7.

Total 2,3-butanediol yield (g/g sugar) = (fermentation yield) / 100 × (distillation yield) / 100

5 [0116]

[Table 7]

		Example 17	Example 18	Example 19	Example 20	Example 21
kLa (h ⁻¹)		0	9	17	28	57
Before culturing	Xylose concentration (g/L)	41.8	41.7	41.2	39.9	41.3
	2,3-BDO concentration (g/L)	0.0	0.0	0.0	0.0	0.0
After culturing	Xylose concentration (g/L)	5.8	20.8	0.0	1.1	0.0
	2,3-BDO concentration (g/L)	6.1	4.6	10.3	10.3	11.1
	Fermentation yield (%)	17.0	22.0	25.0	26.6	26.8
After distillation	Distillation yield (%)	80.3	81.9	82.5	82.4	82.3
	GC purity (%)	>99.9	>99.9	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.13651	0.18018	0.20625	0.219184	0.220564

[0117]

Examples 22 to 25: pH

10 *Z. palmae* [pMFY31-xt] was cultured by the method described in Examples 17 to 21. The resulting culture liquid, prepared by culturing in a flask, was added to 1 L of the 2,3-butanediol fermentation medium having the composition shown in Table 6, and culturing was carried out at an aeration rate of 0.2 vvm, stirring rate of 400 rpm, and temperature of 30°C while the pH was maintained at one of 4.5, 4.8, 15 6.1, and 6.5 by neutralization with potassium hydroxide. The culture liquid was collected as appropriate during the culturing, and the xylose concentration and the 2,3-butanediol concentration were measured by the methods described in Examples 17 to 21. The concentrations of 2,3-butanediol and xylose in the culture liquid and the fermentation yield at the time point when the xylose concentration in the culture

liquid became closest to 0 are shown in Table 8. The 2,3-butanediol culture liquid after the fermentation test was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The supernatant was then filtered through a microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result. The culture liquid obtained was subjected to desalting and distillation treatment by the methods described in Examples 17 to 21 to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the methods described in Examples 17 to 21. The results are shown in Table 8.

[0118]

[Table 8]

		Example 22	Example 23	Example 24	Example 25
pH		4.5	4.8	6.1	6.5
Before culturing	Xylose concentration (g/L)	41.6	39.8	41.0	40.6
	2,3-BDO concentration (g/L)	0.0	0.0	0.0	0.0
After culturing	Xylose concentration (g/L)	13.6	0.8	0.0	0.3
	2,3-BDO concentration (g/L)	3.7	7.7	9.3	6.1
	Fermentation yield (%)	13.1	19.7	22.8	15.2
After distillation	Distillation yield (%)	80.7	82.2	83.0	82.8
	GC purity (%)	>99.9	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.105717	0.161934	0.18924	0.125856

[0119]

Examples 26 to 28: Sugar Concentration

Z. palmae [pMFY31-xt] was cultured by the method described in Examples 17 to 21. The resulting culture liquid, prepared by culturing in a flask, was added to 1 L of the 2,3-butanediol fermentation medium having the same composition as shown in Table 6 except that the xylose concentration was adjusted to one of 5.0, 15, and 100 g/L. Culturing was then carried out at an aeration rate of 0.2 vvm, stirring rate of 400 rpm, and temperature of 30°C while the pH was maintained at 5.5 by

neutralization with potassium hydroxide. The culture liquid was collected as appropriate during the culturing, and the xylose concentration and the 2,3-butanediol concentration were measured by the methods described in Examples 17 to 21. The concentrations of 2,3-butanediol and xylose in the culture liquid and the fermentation yield at the time point when the xylose concentration in the culture liquid became closest to 0 are shown in Table 9. The 2,3-butanediol culture liquid after the fermentation test was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The supernatant was then filtered through a microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result. The culture liquid obtained was subjected to desalting and distillation treatment by the methods described in Examples 17 to 21 to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the methods described in Examples 17 to 21. The results are shown in Table 9.

[0120]

[Table 9]

		Example 26	Example 27	Example 28
Before culturing	Xylose concentration (g/L)	5.3	12.8	95.8
	2,3-BDO concentration (g/L)	0.0	0.0	0.0
After culturing	Xylose concentration (g/L)	0.0	0.0	18.6
	2,3-BDO concentration (g/L)	1.5	3.5	17.1
	Fermentation yield (%)	28.2	27.4	22.2
After distillation	Distillation yield (%)	78.9	79.2	85.9
	GC purity (%)	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.222498	0.217008	0.190698

[0121]

Examples 29 to 31: Pentose Ratio

Z. palmae [pMFY31-xt] was cultured by the method described in Examples 17 to 21. The resulting culture liquid, prepared by culturing in a flask, was added to

1 L of the 2,3-butanediol fermentation medium having the same composition as shown in Table 6 except that the xylose and glucose concentrations (g/L) were adjusted to one of (40:20), (20:40), and (2.5:40), respectively. Culturing was then carried out at an aeration rate of 0.2 vvm, stirring rate of 400 rpm, and temperature of 30°C while the pH was maintained at 5.5 by neutralization with potassium hydroxide. The culture liquid was collected as appropriate during the culturing, and the sugar concentration and the 2,3-butanediol concentration were measured by the methods described in Examples 17 to 21. The 2,3-butanediol concentration and the sugar concentration in the culture liquid and the fermentation yield at the time point when the sugar concentration in the culture liquid became closest to 0 are shown in Table 10. The 2,3-butanediol culture liquid after the fermentation test was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The supernatant was then filtered through a microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained as a result. The culture liquid obtained was subjected to desalting and distillation treatment by the methods described in Examples 17 to 21 to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the methods described in Examples 17 to 21. The results are shown in Table 10.

[0122]

[Table 10]

		Example 29	Example 30	Example 31
Before culturing	Xylose concentration (g/L)	2.6	23.9	40.5
	Glucose concentration (g/L)	40.3	45.9	19.8
	2,3-BDO concentration (g/L)	0.0	0.0	0.0
After culturing	Xylose concentration (g/L)	0.0	2.0	4.3
	Glucose concentration (g/L)	0.0	0.0	0.0
	2,3-BDO concentration (g/L)	18.7	21.0	15.3
	Fermentation yield (%)	43.6	30.9	27.4
After distillation	Distillation yield (%)	83.2	84.3	84.1
	GC purity (%)	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.362752	0.260487	0.230434

[0123]

Examples 32 to 35: Organic Nitrogen Source

The *Z. palmarum* ATCC51623 strain was cultured by the pre-preculture method and the preculture method described in Examples 1 to 6. The preculture liquid was added to 1 L of the 2,3-butanediol fermentation medium having the same composition as shown in Table 2 except that the corn steep liquor was replaced with various organic nitrogen sources shown in Table 11. Culturing was then carried out at an aeration rate of 0.2 vvm, stirring rate of 400 rpm, and temperature of 30°C while the pH was maintained at 5.5 by neutralization with potassium hydroxide. Collection of the culture liquid was carried out using an autosampler (manufactured by ABLE Corporation) at 2- to 4-hour intervals after the beginning of the culturing. The glucose concentration and the 2,3-butanediol concentration in the culture liquid collected were measured by the methods described in Examples 1 to 6. The concentrations of 2,3-butanediol and glucose in the culture liquid and the fermentation yield at the time point when the glucose concentration in the culture liquid became closest to 0 are shown in Table 12. The 2,3-butanediol culture liquid after the fermentation test was recovered, and centrifuged at 4°C at 8000 rpm for 15 minutes. The supernatant was then filtered through a microfiltration membrane (manufactured by Toray Industries, Inc.) to remove the bacterial cells. This process was carried out twice, and a total of 2 L of 2,3-butanediol culture liquid was obtained

as a result. The culture liquid obtained was subjected to desalting and distillation treatment by the methods described in Examples 1 to 6 to obtain purified 2,3-butanediol. The GC purity and the distillation yield of the 2,3-butanediol after the distillation were determined by the methods described in Examples 1 to 6. The results are shown in Table 12.

[0124]

A good 2,3-BDO yield could be obtained with any of the organic nitrogen sources. The highest 2,3-BDO yield could be obtained in the case where corn steep liquor was used as shown in Example 4.

[0125]

[Table 11]

Organic nitrogen source	Concentration (g/L, in terms of solid content)
Soybean hydrolysate	15
Animal tissue hydrolysate	15
Casein hydrolysate	15
Yeast extract	15

[0126]

[Table 12]

		Example 32	Example 33	Example 34	Example 35
Organic nitrogen source		Soybean hydrolysate	Animal tissue hydrolysate	Casein hydrolysate	Yeast extract
Before culturing	Glucose concentration (g/L)	39.8	41.1	40.5	39.7
	2,3-BDO concentration (g/L)	0.0	0.0	0.0	0.0
After culturing	Glucose concentration (g/L)	0.3	3.1	1.1	0.0
	2,3-BDO concentration (g/L)	16.3	11.7	10.8	12.3
	Fermentation yield (%)	41.2	30.9	27.4	30.9
After distillation	Distillation yield (%)	83.1	81.9	82.3	79.6
	GC purity (%)	>99.9	>99.9	>99.9	>99.9
Total 2,3-BDO yield (g/g sugar)		0.342372	0.253071	0.225502	0.245964

INDUSTRIAL APPLICABILITY

[0127]

2,3-Butanediol (2,3-BDO) obtained by the method of the present invention is
5 a useful compound as an intermediate material for pharmaceuticals and cosmetics,
and as a material for inks, perfumes, liquid crystals, insecticides, softening agents,
explosives, plasticizers, and the like.

CLAIMS

1. A method for producing 2,3-butanediol, comprising the steps of:
culturing a microorganism belonging to the genus *Zymobacter* in a
fermentation feedstock containing a carbon source (Step A); and
5 purifying 2,3-butanediol from culture liquid obtained in said step (Step B).
2. The method according to claim 1, wherein said microorganism is *Zymobacter palmae*.
3. The method according to claim 1 or 2, wherein said Step A is culturing under aerated conditions.
- 10 4. The method according to any one of claims 1 to 3, wherein said Step A is culturing at a volumetric oxygen transfer coefficient (kLa) of not less than 9 h^{-1} .
5. The method for producing 2,3-butanediol according to any one of claims 1 to 4, wherein said Step A is culturing at a pH of 5 to 7.
6. The method according to any one of claims 1 to 5, wherein said Step A is
15 culturing in a medium whose total sugar concentration is not less than 20 g/L.
7. The method according to any one of claims 1 to 6, wherein said carbon source contains a pentose, and said microorganism belonging to the genus *Zymobacter* has a capacity to metabolize said pentose, and wherein said capacity to metabolize said pentose is given by the introduction of a gene involved in pentose metabolism.
- 20 8. The method according to any one of claims 1 to 7, wherein said microorganism is a transformed microorganism belonging to the genus *Zymobacter* in which an exogenous gene(s) encoding at least one enzyme selected from the group consisting of xylose isomerase, xylulokinase, transaldolase, and transketolase is/are introduced.
- 25 9. The method according to claim 8, wherein said microorganism is a transformed microorganism belonging to the genus *Zymobacter* in which exogenous genes encoding xylose isomerase, xylulokinase, transaldolase, and transketolase are introduced.

10. The method according to any one of claims 7 to 9, wherein said pentose contained in said fermentation feedstock in said Step A is xylose.
11. The method according to any one of claims 7 to 10, wherein the abundance of xylose with respect to total sugar in said fermentation feedstock is 5 to 100%.
- 5 12. The method according to any one of claims 7 to 11, wherein said fermentation feedstock contains a sugar liquid derived from a biomass.
13. The method according to any one of claims 1 to 12, wherein said fermentation feedstock in said Step A contains corn steep liquor.
14. The method according to any one of claims 1 to 13, wherein said Step B
10 comprises a distillation process.
15. The method according to claim 14, comprising a desalting process before said distillation process.
16. The method according to claim 15, comprising an ion-exchange process as said desalting process.