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(54) **CORYNEFORM BACTERIUM TRANSFORMANT AND METHOD FOR PRODUCING PHENOL USING SAME**

MITTEL ZUR TRANSFORMIERUNG CORYNEFORMER BAKTERIEN SOWIE VERFAHREN ZUR PHENOLHERSTELLUNG DAMIT

TRANSFORMÉ DE BACTÉRIE CORYNÉFORME, ET PROCÉDÉ DE FABRICATION DE PHÉNOL

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JP-A- 2007 117 078 JP-A- 2007 514 430

- **N. J. P. WIERCKX ET AL: "Engineering of Solvent-Tolerant Pseudomonas putida S12 for Bioproduction of Phenol from Glucose", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 71, no. 12, 1 December 2005 (2005-12-01), pages 8221-8227, XP055082366, ISSN: 0099-2240, DOI: 10.1128/AEM.71.12.8221-8227.2005**
- **SAKAI SHINSUKE ET AL: "Effect of lignocellulose-derived inhibitors on growth of and ethanol production by growth-arrested Corynebacterium glutamicum R", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 73, no. 7, 2 February 2007 (2007-02-02), pages 2349-2353, XP002536040, ISSN: 0099-2240, DOI: 10.1128/AEM.02880-06**
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Description

TECHNICAL FIELD

5 **[0001]** The present invention relates to a technique for producing phenol. In more detail, the present invention relates to a coryneform bacterium transformant constructed by specific gene recombination and thereby provided with a phenol-producing function, and relates to an efficient phenol-producing process using the transformant.

BACKGROUND ART

10 **[0002]** Against the backdrop of global warming and exhaustion of fossil resources, production of chemical products using renewable resources, along with production of biofuels, is recognized as an emerging industry, biorefinery, which is an important means for realizing a low-carbon society, and has attracted keen attention.

15 **[0003]** However, production of biophenol using renewable resources is less productive as compared to production of lactic acid or ethanol because the metabolic reaction from a raw material saccharide consists of a great many steps. In addition, for the reasons that produced phenol inhibits bacterial proliferation and that phenol is cytotoxic, industrial production of phenol has been considered to be impossible.

20 **[0004]** Important use of phenol is phenol resins. A phenol resin, which is produced by addition condensation of phenol and aldehyde, is one of the oldest plastics, and with its properties including excellent heat resistance and durability, is used for various purposes, such as an alternative automotive material to metal, a semiconductor seal material, and a circuit board even today. Due to extremely high reactivity of phenol and aldehyde as raw materials and to the complicated three-dimensional network structure of resulting phenol resin polymers, precise structural designing and development into nanomaterials thereof had been considered difficult and so had been application to high-value-added use. However, in recent years, the theory of physical-properties of polymers and the simulation thereof have rapidly developed, and therefore it has gradually become possible to create highly functional materials from phenol resins by refining the network structure. Under the circumstances, the phenol resin production in Japan is also increasing year by year.

25 **[0005]** The currently employed industrial production process of phenol (cumene process) is a typical energy-consuming process in the chemical industry using petroleum-derived benzene and propylene as raw materials, and requiring great amounts of solvent and thermal energy. Therefore, in the light of global environment conservation and greenhouse gas reduction, there is an urgent need to develop an environment-conscious, energy saving process that allows production of phenol from renewable resources and can reduce carbon dioxide emissions and waste products, that is, to establish biophenol production technologies.

30 **[0006]** There have not been reported phenol-producing bacteria in nature so far.

35 **[0007]** Also, there have not been known recombinant bacteria-based phenol-producing technologies to achieve a practically sufficient phenol productivity.

[0008] Tyrosine phenol-lyase is an enzyme that catalyzes synthesis of tyrosine from phenol, pyruvic acid, and ammonia and the reverse reaction thereof (for example, PTL 1). PTL 2, for example, teaches synthesis of tyrosine from phenol, pyruvic acid, and ammonia with the use of tyrosine phenol-lyase derived from members of the family Enterobacteriaceae.

40 **[0009]** Also, it is known that efficient tyrosine phenol-lyase production can be achieved by transformation of *Escherichia coli* with tyrosine phenol-lyase genes derived from various living things (PTL 3 to 5).

[0010] Wierckx et al., ("Engineering of Solvent-Tolerant *Pseudomonas putida* S12 for Bioproduction of Phenol from Glucose", Appl. Environ. Microbiol. 2005, 71(12):8221-8227) discloses a transformant for phenol production which was prepared by introducing a tyrosine phenol lyase gene from *Pantoea agglomerans* into *Pseudomonas putida* S12.

45 **[0011]** WO 2009/154122 A1 discloses a coryneform bacterium transformant prepared by transferring an exogenous gene which encodes a protein having a sugar transporter function into a coryneform bacterium capable of utilizing D-xylose.

[0012] Sakai et al., ("Effect of Lignocellulose-Derived Inhibitors on Growth of and Ethanol Production by Growth-Arrested *Corynebacterium glutamicum* R ∇ ", Appl. Environ. Microbiol., 2007, 73 (7) :2349-2353) disclose studies showing the effect of inhibitors on the ethanol production by *Corynebacterium glutamicum*.

50 **[0013]** EP 2 639 306 A1, representing a later published document, discloses a phenol-producing transformant constructed by transferring a gene which encodes an enzyme having 4-hydroxybenzoate decarboxylase activity into *Corynebacterium glutamicum* as a host.

55 **[0014]** EP 2 615 170 A1, also representing a later published document, discloses a phenol-producing *Corynebacterium glutamicum* transformant containing an enzyme having tyrosine phenol-lyase activity, which transformant is used in a process where the transformant is reacted in a reaction mixture containing a saccharide as carbon source under reducing condition.

CITATION LIST

[Patent Literature]

5 [0015]

[PTL 1] JP 2006-320238 A

[PTL 2] JP 08-154675 A

[PTL 3] JP 2005-278453 A

10 [PTL 4] WO 90/04031

[PTL 5] JP 04-218380 A

SUMMARY OF INVENTION

15 TECHNICAL PROBLEM

[0016] An object of the present invention is to provide a microorganism capable of efficiently producing phenol from tyrosine, and a process for efficiently producing phenol from tyrosine.

20 SOLUTION TO PROBLEM

[0017] The present inventors have wholeheartedly carried out investigations in order to achieve the object described above and obtained the following findings.

25 (i) A transformant constructed by transferring a tyrosine phenol-lyase gene into a coryneform bacterium can efficiently produce phenol from tyrosine.

(ii) The transformant can further efficiently produce phenol in the case where the phenol 2-monooxygenase gene (poxF) on the chromosome of the coryneform bacterium as the host has a disruption or deletion.

30 (iii) The transformant has a particularly higher phenol productivity when proliferation is substantially inhibited in a reaction mixture under reducing conditions.

[0018] The present invention, which has been completed based on the above-mentioned findings, provides the following transformant and process for producing phenol.

35 [1] A *Corynebacterium glutamicum* transformant PHE31 (Accession Number: NITE BP-999).

[2] A process for producing phenol, which comprises a step of allowing a phenol-producing transformant of *Corynebacterium glutamicum* comprising a gene which encodes an enzyme having tyrosine phenol-lyase activity to react in a reaction mixture containing tyrosine, a salt thereof, or an ester thereof under reducing conditions, and a step of collecting phenol from the reaction mixture, with the proviso that the case, in which a reaction mixture containing a saccharide as a carbon source is used, is excluded.

[3] The process of the above [2], wherein the gene which encodes an enzyme having tyrosine phenol-lyase activity is a gene of *Pantoea agglomerans*, a gene of *Citrobacter braakii*, a gene of *Desulfitobacterium hafniense*, a gene of *Chloroflexus aurantiacus*, a gene of *Nostoc punctiforme*, or a gene of *Treponema denticola*.

45 [4] The process of the above [2] or [3], wherein the gene which encodes an enzyme having tyrosine phenol-lyase activity is the DNA of the following (a) or (b):

(a) a DNA consisting of the base sequence of SEQ ID NO: 16, a DNA consisting of the base sequence of SEQ ID NO: 23, a DNA consisting of the base sequence of SEQ ID NO: 24, a DNA consisting of the base sequence of SEQ ID NO: 25, a DNA consisting of the base sequence of SEQ ID NO: 26, or a DNA consisting of the base sequence of SEQ ID NO: 27;

(b) a DNA which hybridizes to a DNA consisting of a complementary base sequence of any of the DNAs of (a) under stringent conditions and which encodes a polypeptide having tyrosine phenol-lyase activity.

55 [5] The process of the above [2] to [4], wherein the *Corynebacterium glutamicum* is a *Corynebacterium glutamicum* in which a gene which encodes an enzyme having phenol 2-monooxygenase activity on the chromosome is disrupted or deleted.

[6] The process of the above [2] to [4], wherein the *Corynebacterium glutamicum* is *Corynebacterium glutamicum*

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R (FERM BP-18976), ATCC13032, or ATCC13869.

[7] The process of the above [2] to [4], wherein the *Corynebacterium glutamicum* is a strain of *Corynebacterium glutamicum* R (FERM BP-18976), ATCC13032, or ATCC13869 in which a gene which encodes an enzyme having phenol 2-monooxygenase activity on the chromosome is disrupted or deleted.

[8] The process of the above [2] to [4], wherein the *Corynebacterium glutamicum* is a *Corynebacterium glutamicum* transformant PHE31 having the Accession Number: NITE BP-999.

[9] The process of the above [2], wherein the transformant does not substantially proliferate in the reaction step.

[10] The process of the above [2], wherein the oxidation-reduction potential of the reaction mixture under reducing conditions is -200 mV to -500 mV.

ADVANTAGEOUS EFFECTS OF INVENTION

[0019] With the use of the transformant of the present invention, phenol can be efficiently produced from tyrosine.

[0020] Generally, growth of microorganisms is inhibited by a solvent, such as a phenol, because of its cytotoxicity, and therefore phenol production with the use of microorganisms was difficult. According to the process of the present invention, however, phenol production with the use of microorganisms can be achieved with a practically sufficient efficiency.

BRIEF DESCRIPTION OF DRAWINGS

[0021]

Fig. 1 shows the construct of a plasmid used in Examples.

Fig. 2 shows the influence of phenol on proliferation of various microorganisms under aerobic conditions.

DESCRIPTION OF EMBODIMENTS

[0022] Hereinafter, the present invention will be described in detail.

(I) Phenol-producing transformant

[0023] The transformant of the present invention capable of producing phenol is a transformant constructed by transferring a gene which encodes an enzyme having tyrosine phenol-lyase activity into a coryneform bacterium as a host.

Host

[0024] The coryneform bacterium is a group of microorganisms defined in Bergey's Manual of Determinative Bacteriology, Vol. 8, 599 (1974), and is not particularly limited as long as it proliferates under normal aerobic conditions.

[0025] The specific examples include *Corynebacterium*, *Brevibacterium*, *Arthrobacter*, *Mycobacterium* and *Micrococcus*. Among the coryneform bacteria, *Corynebacterium* is preferred.

[0026] Examples of the *Corynebacterium* include *Corynebacterium glutamicum*, *Corynebacterium efficiens*, *Corynebacterium ammoniagenes*, *Corynebacterium halotolerance*, and *Corynebacterium alkanolyticum*.

[0027] Inter alia, *Corynebacterium glutamicum* is preferred for safety and high phenol production. Examples of preferred strains include *Corynebacterium glutamicum* R (FERM P-18976), ATCC13032, ATCC13869, ATCC13058, ATCC13059, ATCC13060, ATCC13232, ATCC13286, ATCC13287, ATCC13655, ATCC13745, ATCC13746, ATCC13761, ATCC14020, ATCC31831, MJ-233 (FERM BP-1497), and MJ-233AB-41 (FERM BP-1498). Inter alia, strains R (FERM P-18976), ATCC13032, and ATCC13869 are preferred.

[0028] According to molecular biological classification, names of some species of coryneform bacteria, such as *Brevibacterium flavum*, *Brevibacterium lactofermentum*, *Brevibacterium divaricatum*, and *Corynebacterium lilium* are standardized to *Corynebacterium glutamicum* (Liebl, W. et al., Transfer of *Brevibacterium divaricatum* DSM 20297T, "Brevibacterium flavum" DSM 20411, "Brevibacterium lactofermentum" DSM 20412 and DSM 1412, and *Corynebacterium glutamicum* and their distinction by rRNA gene restriction patterns. Int. J. Syst. Bacteriol. 41: 255-260. (1991); and Kazuo Komagata et al., "Classification of the coryneform group of bacteria", Fermentation and industry, 45: 944-963 (1987)).

[0029] *Brevibacterium lactofermentum* ATCC13869, *Brevibacterium flavum* MJ-233 (FERM BP-1497) and MJ-233AB-41 (FERM BP-1498), etc. of the old classification are also suitable as *Corynebacterium glutamicum*.

[0030] Examples of the *Brevibacterium* include *Brevibacterium ammoniagenes* (for example, ATCC6872).

[0031] Examples of the *Arthrobacter* include *Arthrobacter globiformis* (for example, ATCC8010, ATCC4336, ATCC21056, ATCC31250, ATCC31738 and ATCC35698).

[0032] Examples of the *Mycobacterium* include *Mycobacterium bovis* (for example, ATCC19210 and ATCC27289).

[0033] Examples of the *Micrococcus* include *Micrococcus freudenreichii* (for example, NO. 239 (FERM P-13221)), *Micrococcus leuteus* (for example, NO. 240 (FERM P-13222)), *Micrococcus ureae* (for example, IAM1010), and *Micrococcus roseus* (for example, IFO3764).

[0034] The coryneform bacteria may be, let alone a wild strain, a mutant thereof or an artificial recombinant thereof. Examples thereof include disruptants in which a gene of lactate dehydrogenase, phosphoenolpyruvate carboxylase, or malate dehydrogenase is disrupted. Using such a disruptant as a host can improve phenol productivity and reduce production of by-products.

[0035] Inter alia, preferred is a disruptant in which a lactate dehydrogenase gene is disrupted. In the disruptant, the lactate dehydrogenase gene is disrupted and the metabolic pathway from pyruvic acid to lactic acid is blocked. Inter alia, especially preferred is a disruptant of *Corynebacterium glutamicum* R (FERM P-18976) strain in which the lactate dehydrogenase gene is disrupted.

[0036] Such a disruptant can be prepared based on a conventional gene engineering process. Such a lactate dehydrogenase disruptant and the preparation process thereof are described in WO 2005/010182 A1.

[0037] As shown in Example 3, the present inventors found that coryneform bacteria have significantly higher resistance to phenol as compared with other microorganisms. In this regard, coryneform bacteria are preferred in the production of phenol by the process of the present invention.

[0038] In addition, as compared with other aerobic microorganisms, coryneform bacteria can more efficiently produce substances under reducing conditions where the bacteria do not substantially proliferate. In this regard also, coryneform bacteria are preferred in the production of phenol by the process of the present invention.

Tyrosine phenol-lyase gene (tpl)

[0039] Tyrosine phenol-lyase is an enzyme that catalyzes a reaction in which phenol is produced by elimination of pyruvic acid and ammonia from tyrosine and the reverse reaction thereof. Tyrosine phenol-lyase also catalyzes a reaction in which L-DOPA is produced from catechol and pyruvic acid.

[0040] The gene which encodes an enzyme having tyrosine phenol-lyase activity may be of any origin without particular limitation, and preferred are a gene derived from *Pantoea agglomerans*, a gene derived from *Citrobacter braakii*, a gene derived from *Desulfitobacterium hafniense*, a gene derived from *Chloroflexus aurantiacus*, a gene derived from *Nostoc punctiforme*, or a gene derived from *Treponema denticola*. Inter alia, more preferred is a gene derived from *Pantoea agglomerans*.

[0041] Examples of the gene derived from *Pantoea agglomerans* include the DNA consisting of the base sequence of SEQ ID NO: 16, examples of the gene derived from *Citrobacter braakii* include the DNA consisting of the base sequence of SEQ ID NO: 23, examples of the gene derived from *Desulfitobacterium hafniense* include the DNA consisting of the base sequence of SEQ ID NO: 24, examples of the gene derived from *Chloroflexus aurantiacus* include the DNA consisting of the base sequence of SEQ ID NO: 25, examples of the gene derived from *Nostoc punctiforme* include the DNA consisting of the base sequence of SEQ ID NO: 26, and examples of the gene derived from *Treponema denticola* include the DNA consisting of the base sequence of SEQ ID NO: 27.

[0042] In the present invention, a DNA which hybridizes to a DNA consisting of a complementary base sequence of the base sequence of SEQ ID NO: 16, 23, 24, 25, 26, or 27 under stringent conditions and which encodes a polypeptide having tyrosine phenol-lyase activity can also be used.

[0043] The "stringent conditions" as used herein means general conditions, for example, the conditions described in Molecular Cloning, A Laboratory Manual, Second edition, 1989, Vol. 2, p.11. 45. It means, in particular, conditions where hybridization occurs at a temperature 5 to 10°C below the melting temperature (T_m) of a perfect hybrid.

[0044] The tyrosine phenol-lyase activity can be measured by a modified method of the method described in J. Biol. Chem., 245, 1767-1772 (1970) "Materials and Methods". Briefly, by adding the test enzyme to a liquid for testing, a reaction mixture containing 50 mM potassium phosphate buffer at pH 8.0, 2.5 mM L-Tyr, 0.1 mM pyridoxal phosphate, 20% glycerol, and the enzyme was prepared, and the mixture was allowed to react at 30°C for 30 minutes (0, 5, 10, 20, 30 minutes). The reaction was stopped by the addition of 0.6 N hydrochloric acid (final concentration). After the reaction mixture was subjected to centrifugation and filter filtration, the produced phenol was analyzed and quantified by HPLC (Cosmosil C18 ARII, mobile phase: 20% MeOH and 0.07% perchloric acid). The specific activity was calculated based on the initial rate of the reaction and the protein concentration (the amount of the enzyme required to produce 1 μmol of phenol per minute was defined as 1 unit).

[0045] In the present invention, a DNA consisting of a base sequence which has 90% or more, preferably 95% or more, more preferably 98% or more homology with the base sequence of SEQ ID NO: 16, 23, 24, 25, 26, or 27 and which encodes a polypeptide having tyrosine phenol-lyase activity can also be used.

[0046] In the present invention, the base sequence homology was calculated using GENETYX Ver. 8 (made by Genetyx).

[0047] The homolog of the DNA consisting of the base sequence of SEQ ID NO: 16, 23, 24, 25, 26, or 27 can be selected from a DNA library of a different species by, for example, PCR or hybridization using a primer or a probe designed based on these base sequences, according to a conventional method, and as a result, a DNA which encodes a polypeptide having tyrosine phenol-lyase activity can be obtained with a high probability.

Construction of vector for transformation

[0048] The DNA which encodes tyrosine phenol-lyase is amplified by PCR and then cloned into a suitable vector which is replicable in a host.

[0049] The plasmid vector may be any plasmid vector as long as it comprises a gene responsible for autonomously replicating function in a coryneform bacterium. Specific examples of the plasmid vector include pAM330 derived from *Brevibacterium lactofermentum* 2256 (JP 58-67699 A; Miwa, K. et al., Cryptic plasmids in glutamic acid-producing bacteria. Agric. Biol. Chem. 48:2901-2903 (1984); and Yamaguchi, R. et al., Determination of the complete nucleotide sequence of the *Brevibacterium lactofermentum* plasmid pAM330 and the analysis of its genetic information. Nucleic Acids Symp. Ser. 16:265-267 (1985)); pHM1519 derived from *Corynebacterium glutamicum* ATCC13058 (Miwa, K. et al., Cryptic plasmids in glutamic acid-producing bacteria. Agric. Biol. Chem. 48:2901-2903 (1984)) and pCRY30 derived from the same (Kurusu, Y. et al., Identification of plasmid partition function in coryneform bacteria. Appl. Environ. Microbiol. 57:759-764(1991)); pCG4 derived from *Corynebacterium glutamicum* T250 (JP 57-183799 A; and Katsumata, R. et al., Protoplast transformation of glutamate-producing bacteria with plasmid DNA. J. Bacteriol., 159:306-311 (1984)), pAG1, pAG3, pAG14 and pAG50 derived from the same (JP 62-166890 A), and pEK0, pEC5 and pEKEx1 derived from the same (Eikmanns, B.J. et al., A family of *Corynebacterium glutamicum*/Escherichia coli shuttle vectors for cloning, controlled gene expression, and promoter probing. Gene, 102:93-98(1991)); etc.

[0050] Examples of a preferred promoter include promoter PgapA as a promoter of the glyceraldehyde-3-phosphate dehydrogenase A gene (gapA), promoter Pmdh as a promoter of the malate dehydrogenase gene (mdh), and promoter PldhA as a promoter of lactate dehydrogenase A gene (ldhA), all of which are derived from *Corynebacterium glutamicum* R, and inter alia, PgapA is preferred.

[0051] Examples of a preferred terminator include terminator rrnB T1T2 of *Escherichia coli* rRNA operon, terminator trpA of *Escherichia coli*, and terminator trp of *Brevibacterium lactofermentum*, and inter alia, terminator rrnB T1T2 is preferred.

Transformation

[0052] As a method of transformation, any publicly known method can be used without limitation. Examples of such a known method include the calcium chloride/rubidium chloride method, the calcium phosphate method, DEAE-dextran transfection, and electroporation. Inter alia, preferred for a coryneform bacterium is electroporation, which can be performed by a known method (Kurusu, Y. et al., Electroporation-transformation system for Coryneform bacteria by auxotrophic complementation. , Agric. Biol. Chem. 54:443-447 (1990) ; and Vertes A.A. et al., Presence of mrr- and mcr-like restriction systems in Coryneform bacteria. Res. Microbiol. 144:181-185 (1993)).

[0053] The transformant is cultured using a culture medium usually used for culture of microorganisms. The culture medium may be a natural or synthetic medium containing a carbon source, a nitrogen source, inorganic salts, other nutritional substances, etc.

[0054] Examples of the carbon source include carbohydrates and sugar alcohols such as glucose, fructose, sucrose, mannose, maltose, mannitol, xylose, arabinose, galactose, starch, molasses, sorbitol and glycerol; organic acids such as acetic acid, citric acid, lactic acid, fumaric acid, maleic acid and gluconic acid; and alcohols such as ethanol and propanol. Hydrocarbons, such as normal paraffin, etc. may also be used as desired. These carbon sources may be used alone or as a mixture of two or more thereof. The concentration of these carbon sources in the culture medium is usually about 0.1 to 10 w/v%.

[0055] Examples of the nitrogen source include inorganic or organic ammonium compounds, such as ammonium chloride, ammonium sulfate, ammonium nitrate, and ammonium acetate; urea; aqueous ammonia; sodium nitrate; and potassium nitrate. Nitrogen-containing organic compounds, such as corn steep liquor, meat extract, peptone, N-Z-amine, protein hydrolysate, amino acid, etc. may also be used. These nitrogen sources may be used alone or as a mixture of two or more thereof. The concentration of these nitrogen sources in the culture medium varies depending on the kind of the nitrogen compound, but is usually about 0.1 to 10 w/v%.

[0056] Examples of the inorganic salts include potassium dihydrogen phosphate, dipotassium hydrogenphosphate, magnesium sulfate, sodium chloride, iron(II) nitrate, manganese sulfate, zinc sulfate, cobalt sulfate, and calcium carbonate. These inorganic salts may be used alone or as a mixture of two or more thereof. The concentration of the inorganic salts in the culture medium varies depending on the kind of the inorganic salts, but is usually about 0.01 to 1 w/v%.

[0057] Examples of the nutritional substances include meat extract, peptone, polypeptide, yeast extract, dry yeast,

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corn steep liquor, skim milk powder, defatted soybean hydrochloric acid hydrolysate, and extract from animals, plants or microorganisms, and degradation products thereof. The concentration of the nutritional substances in the culture medium varies depending on the kind of the nutritional substances, but is usually about 0.1 to 10 w/v%. Further, vitamins may be added as needed. Examples of the vitamins include biotin, thiamine (vitamin B1), pyridoxine (vitamin B6), pantothenic acid, inositol, nicotinic acid, etc.

[0058] The pH of the culture medium is preferably about 5 to 8.

[0059] Examples of the preferable microbial culture medium include A medium (Inui, M. et al., Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J. Mol. Microbiol. Biotechnol.* 7:182-196 (2004)), BT medium (Omumasaba, C. A. et al., *Corynebacterium glutamicum* glyceraldehyde-3-phosphate dehydrogenase isoforms with opposite, ATP-dependent regulation. *J. Mol. Microbiol. Biotechnol.* 8:91-103 (2004)), etc.

[0060] The culture temperature is about 15 to 45°C, and the culture period is about 1 to 7 days.

Disruption or deletion in host chromosomal gene

[0061] In the coryneform bacterium as a host, the gene which encodes an enzyme having phenol 2-monooxygenase activity (poxF) on the chromosome preferably has a disruption or deletion for further efficient phenol production.

[0062] Replacement of a gene on the chromosome with the corresponding gene having a disruption or deletion can be achieved by creating a gene with deletion mutation for not allowing production of a normally functioning enzyme protein, and transforming a bacterium with a DNA comprising the mutated gene for recombination in which the gene on the chromosome and the mutated gene are exchanged. An enzyme protein encoded by a gene having a disruption or deletion, even when produced, has a conformation different from that of the wild type, and has no or reduced function. The gene deletion or gene disruption by way of such gene replacement through homologous recombination has already been established, and examples thereof include a method using a plasmid containing a temperature sensitive replication origin or a plasmid capable of conjugal transfer, and a method using a suicide vector not having a replication origin that works in a host (U.S. Pat. No. 6303383 and JP 05-007491 A).

[0063] Specifically, by the method described in Example 1, a coryneform bacterium in which the phenol 2-monooxygenase gene (poxF) is disrupted or deleted can be obtained.

(II) Process for producing phenol

[0064] Phenol can be produced by a process comprising a step of allowing the above-described transformant of the present invention to react in a reaction mixture containing tyrosine, and a step of collecting phenol from the reaction mixture.

Proliferation of microorganism

[0065] Before the reaction, the transformant is preferably cultured and proliferated under aerobic conditions at about 25 to 38°C for about 12 to 48 hours.

Culture medium

[0066] The culture medium used for aerobic culture of the transformant before the reaction may be a natural or synthetic medium containing a carbon source, a nitrogen source, inorganic salts, other nutritional substances, etc.

[0067] Examples of the carbon source that can be used include saccharides (monosaccharides such as glucose, fructose, mannose, xylose, arabinose, and galactose; disaccharides such as sucrose, maltose, lactose, cellobiose, xylobiose, and trehalose; polysaccharides such as starch; and molasses); sugar alcohols such as mannitol, sorbitol, xylitol, and glycerol; organic acids such as acetic acid, citric acid, lactic acid, fumaric acid, maleic acid and gluconic acid; alcohols such as ethanol and propanol; and hydrocarbons such as normal paraffin.

[0068] These carbon sources may be used alone or as a mixture of two or more thereof.

[0069] Examples of the nitrogen source that can be used include inorganic or organic ammonium compounds, such as ammonium chloride, ammonium sulfate, ammonium nitrate, and ammonium acetate; urea; aqueous ammonia; sodium nitrate; and potassium nitrate. Nitrogen-containing organic compounds, such as corn steep liquor, meat extract, peptone, N-Z-amine, protein hydrolysate, amino acid, etc. may also be used. These nitrogen sources may be used alone or as a mixture of two or more thereof. The concentration of these nitrogen sources in the culture medium varies depending on the kind of the nitrogen compound, but is usually about 0.1 to 10 w/v%.

[0070] Examples of the inorganic salts include potassium dihydrogen phosphate, dipotassium hydrogenphosphate, magnesium sulfate, sodium chloride, iron(II) nitrate, manganese sulfate, zinc sulfate, cobalt sulfate, and calcium car-

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bonate. These inorganic salts may be used alone or as a mixture of two or more thereof. The concentration of the inorganic salts in the culture medium varies depending on the kind of the inorganic salts, but is usually about 0.01 to 1 w/v%.

[0071] Examples of the nutritional substances include meat extract, peptone, polypeptone, yeast extract, dry yeast, corn steep liquor, skim milk powder, defatted soybean hydrochloric acid hydrolysate, and extract from animals, plants or microorganisms, and degradation products thereof. The concentration of the nutritional substances in the culture medium varies depending on the kind of the nutritional substances, but is usually about 0.1 to 10 w/v%.

[0072] Further, vitamins may be added as needed. Examples of the vitamins include biotin, thiamine (vitamin B1), pyridoxine (vitamin B6), pantothenic acid, inositol, nicotinic acid, etc.

[0073] The pH of the culture medium is preferably about 6 to 8.

[0074] Specific examples of the preferable culture medium for coryneform bacteria include A medium (Inui, M. et al., Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *J. Mol. Microbiol. Biotechnol.* 7:182-196 (2004)), BT medium (Omumasaba, C. A. et al., *Corynebacterium glutamicum* glyceraldehyde-3-phosphate dehydrogenase isoforms with opposite, ATP-dependent regulation. *J. Mol. Microbiol. Biotechnol.* 8:91-103 (2004)), etc. Such a culture medium can be used after prepared so as to contain a saccharide at a concentration in the above-mentioned range.

Reaction mixture

[0075] As the reaction mixture, water, a buffer solution, an inorganic salt medium, or the like, containing a phenol precursor (raw material for phenol) can be used.

[0076] As the precursor, tyrosine (L-tyrosine, D-tyrosine, or a mixture thereof), a salt thereof, or an ester thereof may be used. Inter alia, preferred is L-tyrosine, a salt thereof, or an ester thereof. Examples of the salt include a sodium salt, a potassium salt, and a hydrochloride. Examples of the ester include esters with alcohols having 1 to 4 carbon atoms. Since tyrosine is poorly soluble in water, preferably used is a tyrosine salt, and more preferred is a sodium salt. These precursors may be used alone or a mixture of two or more kinds.

[0077] The concentration of tyrosine, a salt thereof, or an ester thereof in the reaction mixture is preferably about 0.5 to 10 w/v%, more preferably about 1 to 7 w/v%, and still more preferably about 2 to 5 w/v%. When the concentration is in the above range, phenol can be efficiently produced.

[0078] Examples of the buffer solution include a phosphate buffer, a Tris buffer, a carbonate buffer, etc. The concentration of the buffer solution is preferably about 10 to 150 mM.

[0079] Examples of the inorganic salt medium include a medium containing one or more kinds of inorganic salts including potassium dihydrogen phosphate, dipotassium hydrogenphosphate, magnesium sulfate, sodium chloride, iron(II) nitrate, manganese sulfate, zinc sulfate, cobalt sulfate, and calcium carbonate. Inter alia, preferred is a medium containing magnesium sulfate. Specific example of the inorganic salt medium include BT medium (Omumasaba, C. A. et al., *Corynebacterium glutamicum* glyceraldehyde-3-phosphate dehydrogenase isoforms with opposite, ATP-dependent regulation. *J. Mol. Microbiol. Biotechnol.* 8:91-103 (2004)) etc. The concentration of the inorganic salts in the culture medium varies depending on the kind of the inorganic salts, but is usually about 0.01 to 1 w/v%.

[0080] The pH of the reaction mixture is preferably about 6 to 8. During the reaction, the pH of the reaction mixture is preferably kept nearly neutral, in particular at around 7 with the use of aqueous ammonia, aqueous sodium hydroxide, or the like, under the control of a pH controller (for example, Type: DT-1023 made by Able).

Reaction conditions

[0081] The reaction temperature, that is, the temperature for keeping the transformant alive during the reaction is preferably about 20 to 50°C, and more preferably about 25 to 47°C. When the temperature is in the above range, phenol can be efficiently produced.

[0082] The reaction period is preferably about 1 to 7 days, and more preferably about 1 to 3 days.

[0083] The culture may be a batch process, a fed-batch process, or a continuous process. Inter alia, a batch process is preferred.

<Reducing conditions>

[0084] The reaction may be performed under aerobic conditions or reducing conditions, but preferably is performed under reducing conditions. Under reducing conditions, coryneform bacteria do not substantially proliferate and can further efficiently produce phenol.

[0085] The "reducing conditions" is defined based on the oxidation-reduction potential of the reaction mixture. The oxidation-reduction potential of the reaction mixture is preferably about -200 mV to -500 mV, and more preferably about -250 mV to -500 mV.

[0086] The reducing conditions of the reaction mixture can be simply estimated with the use of resazurin indicator (in reducing conditions, decolorization from blue to colorless is observed). However, for precise measurement, a redox-potential meter (for example, ORP Electrodes made by BROADLEY JAMES) is used.

[0087] As a method of preparing a reaction mixture under reducing conditions, any publicly known method can be used without limitation. For example, as a liquid medium for preparation of the reaction mixture, an aqueous solution for a reaction mixture may be used instead of distilled water or the like. As reference for preparation of the aqueous solution for a reaction mixture, for example, the method for preparing a culture medium for strictly anaerobic microorganisms, such as sulfate-reducing microorganisms (Pfennig, N. et al.: The dissimilatory sulfate-reducing bacteria, In The Prokaryotes, A Handbook on Habitats, Isolation and Identification of Bacteria, Ed. by Starr, M. P. et al. Berlin, Springer Verlag, 926-940, 1981, or Nogeikagaku Jikken-sho, Ed. by Kyoto Daigaku Nogakubu Nogeikagaku Kyoshitsu, Vol.3, Sangyo Tosho, 1990, Issue 26) may be used, and such a method provides an aqueous solution under desired reducing conditions.

[0088] Specifically, by treating distilled water or the like with heat or under reduced pressure for removal of dissolved gases, an aqueous solution for a reaction mixture under reducing conditions can be obtained. In this case, for removal of dissolved gases, especially dissolved oxygen, distilled water or the like may be treated under reduced pressure of about 10 mmHg or less, preferably about 5 mmHg or less, more preferably about 3 mmHg or less, for about 1 to 60 minutes, preferably for about 5 to 40 minutes.

[0089] Alternatively, by adding a suitable reducing agent (for example, thioglycolic acid, ascorbic acid, cysteine hydrochloride, mercaptoacetic acid, thiol acetic acid, glutathione, sodium sulfide, etc.), an aqueous solution for a reaction mixture under reducing conditions can be prepared.

[0090] These methods may be suitably combined to prepare an effective aqueous solution for a reaction mixture under reducing conditions.

[0091] It is preferred to maintain the reducing conditions of the reaction mixture during the reaction. For maintenance of reducing conditions, it is preferred that oxygen from the outside of the reaction system is prevented to the utmost extent from entering the system. Specific examples of the method employed for this purpose include a method comprising encapsulating the reaction system with inert gas, such as nitrogen gas, carbon dioxide gas, etc. In some cases, for allowing the metabolic functions in the cells of the aerobic bacterium of the present invention to work effectively during the reaction, addition of a solution of various nutrients or a reagent solution for adjusting and maintaining the pH of the reaction system may be needed. In such a case, for more effective prevention of oxygen incorporation, it is effective to remove oxygen in the solutions to be added, in advance.

Collection of phenol

[0092] Through the culture performed in the above manner, phenol is produced in the reaction mixture. Phenol can be collected by collecting the reaction mixture, and it is also feasible to isolate phenol from the reaction mixture by a known method. Examples of such a known method include distillation, the membrane permeation method, and the organic solvent extraction method.

EXAMPLES

[0093] Hereinafter, the present invention will be described in more detail by way of Examples, but the present invention is not limited thereto.

Example 1

Cloning and expression of phenol-producing gene

(1) Extraction of chromosomal DNA from microorganisms

[0094] To extract chromosomal DNA from *Corynebacterium glutamicum* R (FERM P-18976), the bacterium was inoculated, with the use of a platinum loop, into A medium (2 g of $(\text{NH}_2)_2\text{CO}$, 7 g of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g of KH_2PO_4 , 0.5 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL of 0.06% (w/v) $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ + 0.042% (w/v) $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 1 mL of 0.02% (w/v) biotin solution, 2 mL of 0.01% (w/v) thiamin solution, 2 g of yeast extract, and 7 g of vitamin assay casamino acid were dissolved in 1 L of distilled water), which was supplemented with 50% (w/v) glucose as a carbon source to a final concentration of 4%, and cultured with shaking at 33°C until the logarithmic growth phase. After the bacterial cells were collected, chromosomal DNA was recovered from the collected cells with the use of a DNA extraction kit (trade name: GenomicPrep Cells and Tissue DNA Isolation Kit, made by Amersham) according to the instruction manual.

[0095] To extract chromosomal DNA from *Pantoea agglomerans* NBRC12686, the bacterium was inoculated into

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NBRC Medium No. 802 (10 g of polypeptone, 2 g of yeast extract, and 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ were dissolved in 1 L of distilled water) with the use of a platinum loop, and cultured with shaking at 30°C until the logarithmic growth phase. After the bacterial cells were collected, chromosomal DNA was recovered from the collected cells with the use of a DNA extraction kit (trade name: GenomicPrep Cells and Tissue DNA Isolation Kit, made by Amersham) according to the instruction manual.

(2) Construction of cloning vectors

Construction of cloning vector pCRB22

[0096] A DNA fragment comprising a DNA replication origin sequence of pCASE1, a plasmid derived from *Corynebacterium casei* JCM12072 (hereinafter abbreviated as pCASE1-ori) and a DNA fragment comprising a cloning vector pHSG298 (made by Takara Bio, Inc.) were amplified by the following PCR method.

[0097] In the PCR, the following sets of primers were synthesized based on SEQ ID NO: 1 (pCASE1-ori sequence) and SEQ ID NO: 2 (cloning vector pHSG298) for cloning of the pCASE1-ori sequence and the cloning vector pHSG298, and were used.

Primers for pCASE1-ori sequence amplification

[0098]

(a-1); 5'-AT AGATCT AGAACGTCCGTAGGAGC-3' (SEQ ID NO: 3)

(b-1); 5'-AT AGATCT GACTTGGTTACGATGGAC-3' (SEQ ID NO: 4)

[0099] Primers (a-1) and (b-1) each have a BglII restriction enzyme site added thereto.

Primers for cloning vector pHSG298 amplification

[0100]

(a-2); 5'-AT AGATCT AGGTTTCCCGACTGGAAAG-3' (SEQ ID NO: 5)

(b-2); 5'-AT AGATCT CGTGCCAGCTGCATTAATGA-3' (SEQ ID NO: 6)

[0101] Primers (a-2) and (b-2) each have a BglII restriction enzyme site added thereto.

[0102] As the template DNA, total DNA extracted from *Corynebacterium casei* JCM12072 obtained from Japan Collection of Microorganisms (JCM) and cloning vector pHSG298 (made by Takara Bio, Inc.) were used.

[0103] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara Bio, Inc.) as a reaction reagent under the conditions described below.

Reaction mixture:

[0104]

TaKaRa LA Taq™ (5 units/ μL)	0.5 μL
10 \times LA PCR™ Buffer II (Mg^{2+} free)	5 μL
25 mM MgCl_2	5 μL
dNTP Mixture (2.5 mM each)	8 μL
Template DNA	5 μL (DNA content: 1 μg or less)
The above 2 primers*)	0.5 μL each (final conc. : 1 μM)
Sterile distilled water	25.5 μL

The above ingredients were mixed, and 50 μL of the reaction mixture was subjected to PCR.

*) For amplification of the pCASE1-ori sequence, a combination of primers (a-1) and (b-1), and for amplification of the cloning vector pHSG298, a combination of primers (a-2) and (b-2) were used.

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PCR cycle:

[0105]

5	Denaturation step:	94°C, 60 seconds
	Annealing step:	52°C, 60 seconds
	Extension	step: 72°C
	pCASE1-ori sequence:	150 seconds
10	Cloning vector pHSG298:	180 seconds

[0106] A cycle consisting of the above 3 steps was repeated 30 times.

[0107] Using 10 μ L of the above-produced reaction mixture, 0.8% agarose gel electrophoresis was performed. In the case of the pCASE1-ori sequence, an about 1.4-kb DNA fragment was detected. In the case of the cloning vector pHSG298, an about 2.7-kb DNA fragment was detected.

[0108] 10 μ L of the about 1.4-kb DNA fragment comprising the pCASE1-ori sequence derived from *Corynebacterium casei*, and 10 μ L of the about 2.7-kb DNA fragment comprising the cloning vector pHSG298, both amplified by the above PCR, were each cut with the use of restriction enzyme BglII and processed at 70°C for 10 minutes for deactivation of the restriction enzyme. Both were mixed, and 1 μ L of T4 DNA ligase 10 \times buffer solution and 1 unit of T4 DNA ligase (made by Takara Bio, Inc.) were added thereto. Sterile distilled water was added thereto so that the total volume was 10 μ L, and the mixture was allowed to react at 15°C for 3 hours for ligation. This was named Ligation Liquid A.

[0109] With the use of the Ligation Liquid A, *Escherichia coli* JM109 was transformed by the calcium chloride method (Journal of Molecular Biology, 53, 159 (1970)) and was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) containing 50 μ g/mL of kanamycin.

[0110] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of restriction enzyme BglII to confirm the inserted fragment. As a result, in addition to an about 2.7-kb DNA fragment of the cloning vector pHSG298, an about 1.4-kb DNA fragment of the pCASE-ori sequence was confirmed.

[0111] The cloning vector comprising the pCASE1-ori sequence was named pCRB22.

30

Construction of cloning vector pCRB207

[0112] A DNA fragment comprising a promoter sequence of the gapA gene encoding the glyceraldehyde-3-phosphate dehydrogenase (hereinafter abbreviated as PgapA) derived from *Corynebacterium glutamicum* R, and a DNA fragment comprising an rrnBT1T2 bidirectional terminator sequence (hereinafter abbreviated as terminator sequence) derived from a cloning vector pKK223-3 (made by Pharmacia) were amplified by the following method.

[0113] In the PCR, the following sets of primers were synthesized based on SEQ ID NO: 7 (PgapA sequence) and SEQ ID NO: 8 (terminator sequence) for cloning of the PgapA sequence and the terminator sequence, and were used.

40 Primers for PgapA sequence amplification

[0114]

(a-3); 5'-CTCT GTCGAC CCGAAGATCTGAAGATTCCTG-3' (SEQ ID NO: 9)

45 (b-3) ; 5'-CTCT GTCGAC GGATCC CCATGG

TGTGTCTCCTCTAAAGATTGTAGG-3' (SEQ ID NO: 10)

50 [0115] Primer (a-3) has a Sall restriction enzyme site added thereto, and primer (b-3) has Sall, BamHI, and NcoI restriction enzyme sites added thereto.

Primers for terminator sequence amplification

[0116]

55 (a-4); 5'-CTCT GCATGC CCATGG CTGTTTTGGCGGATGAGAGA-3' (SEQ ID NO: 11)

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(b-4); 5'-CTCT GCATGC TCATGA AAGAGTTTGTAGAAACGCAAAAAGG-3' (SEQ ID NO: 12)

[0117] Primer (a-4) has SphI and NcoI restriction enzyme sites added thereto, and primer (b-4) has SphI and BspHI restriction enzyme sites added thereto.

[0118] As the template DNA, the chromosomal DNA extracted from *Corynebacterium glutamicum* R (FERM P-18976) and the plasmid pKK223-3 (made by Pharmacia) were used.

[0119] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara Bio, Inc.) as a reaction reagent under the conditions described below.

Reaction mixture:

[0120]

TaKaRa LA Taq™ (5 units/μL)	0.5 μL
10× LA PCR™ Buffer II (Mg ²⁺ free)	5 μL
25 mM MgCl ₂	5 μL
dNTP Mixture (2.5 mM each)	8 μL
Template DNA	5 μL (DNA content: 1 μg or less)
The above 2 primers ^{*)}	0.5 μL each (final conc.: 1 μM)
Sterile distilled water	25.5 μL

The above ingredients were mixed, and 50 μL of the reaction mixture was subjected to PCR.

*) For amplification of the PgapA sequence, a combination of primers (a-3) and (b-3), and for amplification of the terminator sequence, a combination of primers (a-4) and (b-4) were used.

PCR cycle:

[0121]

Denaturation step:	94°C, 60 seconds
Annealing step:	52°C, 60 seconds
Extension step:	72°C
PgapA sequence:	45 seconds
Terminator sequence:	30 seconds

[0122] A cycle consisting of the above 3 steps was repeated 30 times.

[0123] Using 10 μL of the above-produced reaction mixture, 0.8% agarose gel electrophoresis was performed. In the case of the PgapA sequence, an about 0.6-kb DNA fragment was detected. In the case of the terminator sequence, an about 0.4-kb DNA fragment was detected.

[0124] 10 μL of the about 0.6-kb DNA fragment comprising the PgapA sequence derived from *Corynebacterium glutamicum* R, which was amplified by the above PCR, and the about 4.1-kb cloning vector pCRB22 were each cut with the use of restriction enzyme Sall and processed at 70°C for 10 minutes for deactivation of the restriction enzyme. Both were mixed, and 1 μL of T4 DNA ligase 10× buffer solution and 1 unit of T4 DNA ligase (made by Takara Bio, Inc.) were added thereto. Sterile distilled water was added thereto so that the total volume was 10 μL, and the mixture was allowed to react at 15°C for 3 hours for ligation. This was named Ligation Liquid B.

[0125] With the use of the Ligation Liquid B, *Escherichia coli* JM109 was transformed by the calcium chloride method (Journal of Molecular Biology, 53, 159 (1970)) and was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) containing 50 μg/mL of kanamycin.

[0126] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of restriction enzyme Sall to confirm the inserted fragment. As a result, in addition to an about 4.1-kb DNA fragment of the cloning vector pCRB22, an about 0.6-kb DNA fragment of the PgapA sequence was confirmed.

[0127] The cloning vector comprising the PgapA sequence was named pCRB206.

[0128] 10 μL of the about 0.4-kb DNA fragment comprising the terminator sequence derived from the plasmid pKK223-3, which was amplified by the above PCR, was cut with the use of restriction enzymes NcoI and BspHI, 2 μL of the above cloning vector pCRB206 was cut with the use of restriction enzyme NcoI, and both were processed at 70°C for 10 minutes

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for deactivation of the restriction enzymes. Both were mixed, and 1 μL of T4 DNA ligase 10 \times buffer solution and 1 unit of T4 DNA ligase (made by Takara Bio, Inc.) were added thereto. Sterile distilled water was added thereto so that the total volume was 10 μL , and the mixture was allowed to react at 15°C for 3 hours for ligation. This was named Ligation Liquid C.

5 [0129] With the use of the Ligation Liquid C, *Escherichia coli* JM109 was transformed by the calcium chloride method (Journal of Molecular Biology, 53, 159 (1970)) and was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) containing 50 $\mu\text{g}/\text{mL}$ of kanamycin.

[0130] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of the restriction enzyme to confirm the inserted fragment. As a result, 10 in addition to an about 4.7-kb DNA fragment of the cloning vector pCRB206, an about 0.4-kb DNA fragment of the terminator sequence was confirmed.

[0131] The cloning vector comprising the rrnBT1T2 terminator sequence was named pCRB207.

Construction of cloning vector pCRB209

15 [0132] A DNA fragment comprising a promoter sequence of the gapA (glyceraldehyde 3-phosphate dehydrogenase A) gene (hereinafter abbreviated as PgapA) derived from *Corynebacterium glutamicum* R was amplified by the following method.

[0133] In the PCR, the following set of primers was synthesized based on SEQ ID NO: 13 (pCRB207) for cloning of 20 the pCRB207 sequence, and was used.

Primers for pCRB207 sequence amplification

[0134]

25

(a-5); 5'-CTCT CATATG CTGTTTTGGCGGATGAGAG-3' (SEQ ID NO: 14)

(b-5); 5'-CTCT CATATG GTGTCTCCTCTAAAGATTGTAGG-3' (SEQ ID NO: 15)

[0135] Primers (a-5) and (b-5) each have an NdeI restriction enzyme site added thereto.

30 [0136] As the template DNA, the cloning vector pCRB207 comprising a gapA promoter and a rrnBT1T2 terminator sequence was used.

[0137] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara SHUZO) as a reaction reagent under the conditions described below.

35 Reaction mixture:

[0138]

40 TaKaRa LA Taq™ (5 units/ μL)	0.5 μL
10 \times LA PCR™ Buffer II (Mg ²⁺ free)	5 μL
25 mM MgCl ₂	5 μL
dNTP Mixture (2.5 mM each)	8 μL
Template DNA	5 μL (DNA content: 1 μg or less)
45 The above 2 primers ^{*)}	0.5 μL each (final conc.: 1 μM)
Sterile distilled water	25.5 μL

The above ingredients were mixed, and 50 μL of the reaction mixture was subjected to PCR.

*) For amplification of the pCRB207 sequence, a combination of primers (a-5) and (b-5) was used.

50 PCR cycle:

[0139]

55 Denaturation step:	94°C, 60 seconds
Annealing step:	52°C, 60 seconds
Extension step:	72°C, 307 seconds

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[0140] A cycle consisting of the above 3 steps was repeated 30 times.

[0141] Using 10 μ L of the above-produced reaction mixture, 0.8% agarose gel electrophoresis was performed, and an about 5.1-kb DNA fragment comprising the cloning vector pCRB207 was detected.

[0142] 10 μ L of the about 5.1-kb DNA fragment comprising the gene derived from pCRB207, which was amplified by the above PCR, was cut with the use of restriction enzyme NdeI and processed at 70°C for 10 minutes for deactivation of the restriction enzyme. To this, 1 μ L of T4 DNA ligase 10 \times buffer solution and 1 unit of T4 DNA ligase (made by Takara SHUZO) were added. Sterile distilled water was added thereto so that the total volume was 10 μ L, and the mixture was allowed to react at 15°C for 3 hours for ligation. This was named Ligation Liquid D.

[0143] With the use of the Ligation Liquid D, *Escherichia coli* JM109 was transformed by the calcium chloride method (Journal of Molecular Biology, 53, 159 (1970)) and was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) containing 50 μ g/mL of kanamycin.

[0144] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of restriction enzyme NdeI to confirm the inserted restriction enzyme site.

[0145] The cloning vector comprising the PgapA sequence and the rrnBT1T2 terminator sequence was named pCRB209.

(3) Cloning of phenol-producing gene

Cloning of phenol-producing gene derived from *Pantoea agglomerans*

[0146] A DNA fragment comprising the *tpl* gene which is derived from *Pantoea agglomerans* and which encodes a gene having tyrosine phenol-lyase activity was amplified by the PCR method as described below.

[0147] In the PCR, the following set of primers was synthesized based on SEQ ID NO: 16 (the *tpl* gene of *Pantoea agglomerans*) with the use of "394 DNA/RNA Synthesizer" made by Applied Biosystems for cloning of the *tpl* gene, and was used. Primers for *tpl* gene amplification

(a-6); 5'-CTCT CATATG AACTATCCTGCCGAGC-3' (SEQ ID NO: 17)

(b-6); 5'-CTCT CATATG TTAAATAAAGTCAAAACGCGCAGTAAAG-3' (SEQ ID NO: 18)

[0148] Primers (a-6) and (b-6) each have an NdeI restriction enzyme site added thereto.

[0149] As the template DNA for *Pantoea agglomerans*, the chromosomal DNA extracted from *Pantoea agglomerans* NBRC12686 obtained from NITE Biological Resource Center (NBRC) was used.

[0150] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara Bio, Inc.) as a reaction reagent under the conditions described below.

Reaction mixture:

[0151]

TaKaRa LA Taq™ (5 units/ μ L)	0.5 μ L
10 \times LA PCR™ Buffer II (Mg ²⁺ free)	5 μ L
25 mM MgCl ₂	5 μ L
dNTP Mixture (2.5 mM each)	8 μ L
Template DNA	5 μ L (DNA content: 1 μ g or less)
The above 2 primers*)	0.5 μ L each (final conc. : 1 μ M)
Sterile distilled water	25.5 μ L

The above ingredients were mixed, and 50 μ L of the reaction mixture was subjected to PCR.

*) For amplification of the *tpl* gene of *Pantoea agglomerans*, a combination of primers (a-6) and (b-6) was used.

PCR cycle:

[0152]

Denaturation step:	94°C, 60 seconds
Annealing step:	52°C, 60 seconds

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(continued)

Extension step: 72°C
Pantoea agglomerans tpl gene 82 seconds

5

[0153] A cycle consisting of the above 3 steps was repeated 30 times.

[0154] With the use of 10 µL of the reaction mixture produced above, 0.8% agarose gel electrophoresis was performed. As a result, detected was an about 1.4-kb DNA fragment of the *Pantoea agglomerans* tpl gene.

10 (4) Construction of phenol-producing gene expression plasmid Cloning of phenol-producing gene to pCRB209

[0155] 10 µL of the about 1.4-kb DNA fragment comprising the tpl gene derived from *Pantoea agglomerans* amplified by the PCR in the above (3), and 2 µL of the cloning vector pCRB209 comprising promoter PgapA were each cut with the use of restriction enzyme NdeI, and were processed at 70°C for 10 minutes for deactivation of the restriction enzyme. Both were mixed, and 1 µL of T4 DNA ligase 10× buffer solution and 1 unit of T4 DNA ligase (made by Takara Bio, Inc.) were added thereto. Sterile distilled water was added thereto so that the total volume was 10 µL, and the mixture was allowed to react at 15°C for 3 hours for ligation. The resulting liquid was named Ligation Liquid E.

[0156] With the use of the obtained Ligation Liquid E, *Escherichia coli* JM109 was transformed by the calcium chloride method (Journal of Molecular Biology, 53, 159 (1970)) and was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) containing 50 µg/mL of kanamycin.

[0157] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of restriction enzyme to confirm the inserted fragment. As a result, in addition to an about 5.1-kb DNA fragment of the plasmid pCRB209, confirmed was an about 1.4-kb inserted fragment of the tpl gene derived from *Pantoea agglomerans* (Ligation Liquid E).

[0158] The plasmid comprising the tpl gene derived from *Pantoea agglomerans* was named pCRB209-tpl/PA (Fig. 1).

25 (5) Construction of plasmid for *Corynebacterium glutamicum* chromosomal gene disruption

30 Construction of plasmid for *Corynebacterium glutamicum* poxF gene disruption

[0159] A DNA fragment required for constructing a plasmid for markerless disruption of the poxF gene on the chromosome of *Corynebacterium glutamicum* was amplified by the PCR method as described below.

[0160] In the PCR, the following sets of primers were synthesized based on the sequence of *Corynebacterium glutamicum* R with the use of "394 DNA/RNA Synthesizer" made by Applied Biosystems, and were used.

35

Primers for amplification of poxF-1 region

[0161]

40 (a-7); 5'-CTCT TCTAGA TACGTCCTAAACACCCGAC-3' (SEQ ID NO: 19)

(b-7); 5'-GACCAACCATTGCTGACTTGCGTATCCATAGTCAGGCTTC-3' (SEQ ID NO: 20)

[0162] Primer (a-7) has an XbaI restriction enzyme site added thereto.

45 Primers for amplification of poxF-2 region

[0163]

50 (a-8); 5'-CAAGTCAGCAATGGTTGGTC-3' (SEQ ID NO: 21)

(b-8); 5'-CTCT TCTAGA TGATCAGTACCAAGGGTGAG-3' (SEQ ID NO: 22)

[0164] Primer (b-8) has an XbaI restriction enzyme site added thereto.

[0165] As the template DNA, the chromosomal DNA extracted from *Corynebacterium glutamicum* R was used.

[0166] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara Bio, Inc.) as a reaction reagent under the conditions described below.

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Reaction mixture:

[0167]

5	TaKaRa LA Taq™ (5 units/μL)	0.5 μL
	10× LA PCR™ Buffer II (Mg ²⁺ free)	5 μL
	25 mM MgCl ₂	5 μL
	dNTP Mixture (2.5 mM each)	8 μL
10	Template DNA	5 μL (DNA content: 1 μg or less)
	The above 2 primers ^{*)}	0.5 μL each (final conc. : 1 μM)
	Sterile distilled water	25.5 μL

The above ingredients were mixed, and 50 μL of the reaction mixture was subjected to PCR.

15 *) For amplification of the poxF-1 region, a combination of primers (a-7) and (b-7), and for amplification of the poxF-2 region, a combination of primers (a-8) and (b-8) were used.

PCR cycle:

[0168]

20	Denaturation step:	94°C, 60 seconds
	Annealing step:	52°C, 60 seconds
	Extension step:	72°C
25	poxF-1 region:	50 seconds
	poxF-2 region:	50 seconds

[0169] A cycle consisting of the above 3 steps was repeated 30 times.

30 [0170] Using 10 μL of the above-produced reaction mixture, 0.8% agarose gel electrophoresis was performed. An about 0.8-kb DNA fragment in the case of the *Corynebacterium glutamicum* poxF-1 region, and an about 0.8-kb DNA fragment in the case of the poxF-2 region were detected.

[0171] Subsequently, 1 μL each of the poxF-1 region fragment and the poxF-2 region fragment, which were amplified by the above PCR, were mixed and subjected to PCR for ligation.

35 [0172] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara Bio, Inc.) as a reaction reagent under the conditions described below.

Reaction mixture:

[0173]

40	TaKaRa LA Taq™ (5 units/μL)	0.5 μL
	10× LA PCR™ Buffer II (Mg ²⁺ free)	5 μL
	25 mM MgCl ₂	5 μL
	dNTP Mixture (2.5 mM each)	8 μL
45	The above 2 fragments ^{*)}	1 μL each
	Sterile distilled water	29.5 μL

The above ingredients were mixed, and 50 μL of the reaction mixture was subjected to PCR.

50 *) poxF-1 region fragment and poxF-2 region fragment were used.

PCR cycle:

[0174]

55	Denaturation step:	95°C, 20 seconds
	Annealing step:	52°C, 5 seconds
	Extension step:	72°C, 50 seconds

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[0175] A cycle consisting of the above 3 steps was repeated 30 times.

[0176] Further, using, as the template DNA, the obtained fragment in which *poxF*-1 and *poxF*-2 were ligated, a *poxF* deletion fragment was amplified by PCR.

[0177] Actual PCR was performed with the use of a thermal cycler, GeneAmp PCR System 9700 (made by Applied Biosystems) and TaKaRa LA Taq (made by Takara Bio, Inc.) as a reaction reagent under the conditions described below.

Reaction mixture:

[0178]

TaKaRa LA Taq™ (5 units/μL)	0.5 μL
10× LA PCR™ Buffer II (Mg ²⁺ free)	5 μL
25 mM MgCl ₂	5 μL
dNTP Mixture (2.5 mM each)	8 μL
Template DNA	5 μL (DNA content: 1 μg or less)
The above 2 primers ^{*)}	0.5 μL each (final conc. : 1 μM)
Sterile distilled water	25.5 μL

The above ingredients were mixed, and 50 μL of the reaction mixture was subjected to PCR.

*) For amplification of the *poxF* deletion fragment, a combination of primers (a-7) and (b-8) was used.

PCR cycle:

[0179]

Denaturation step:	95°C, 20 seconds
Annealing step:	52°C, 5 seconds
Extension step:	72°C, 50 seconds

[0180] A cycle consisting of the above 3 steps was repeated 30 times.

[0181] Using 10 μL of the above-produced reaction mixture, 0.8% agarose gel electrophoresis was performed, and an about 1.6-kb fragment of the *poxF* deletion fragment was detected.

[0182] 10 μL of the about 1.7-kb DNA fragment of the *poxF* deletion fragment derived from *Corynebacterium glutamicum* R, which was amplified by the above PCR, and 2 μL of an about 4.4-kb plasmid pCRA725 for markerless chromosomal gene transfection (J. Mol. Microbiol. Biotechnol., Vol. 8, 243-254, 2004 (JP 2006-124440 A) were each cut with the use of restriction enzyme XbaI, and processed at 70°C for 10 minutes for deactivation of the restriction enzyme. Both were mixed, and 1 μL of T4 DNA ligase 10× buffer solution and 1 unit of T4 DNA ligase (made by Takara Bio, Inc.) were added thereto. Sterile distilled water was added thereto so that the total volume was 10 μL, and the mixture was allowed to react at 15°C for 3 hours for ligation. This was named Ligation Liquid F.

[0183] With the use of the Ligation Liquid F, *Escherichia coli* JM109 was transformed by the calcium chloride method (Journal of Molecular Biology, 53, 159 (1970)) and was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% sodium chloride, and 1.5% agar) containing 50 μg/mL of kanamycin.

[0184] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of restriction enzyme XbaI to confirm the inserted fragment. As a result, in addition to an about 4.4-kb DNA fragment of the plasmid pCRA725, an about 1.7-kb inserted fragment of the *poxF* deletion gene derived from *Corynebacterium glutamicum* (Ligation Liquid F) was confirmed.

[0185] The plasmid comprising the *poxF* deletion gene derived from *Corynebacterium glutamicum* was named pCRA725-*poxF*/CG.

(6) Construction of strain in which a gene associated with degradation of phenol is disrupted

[0186] Vector pCRA725 for markerless chromosomal gene transfection is a plasmid that cannot be replicated within *Corynebacterium glutamicum* R. With the use of the plasmid pCRA725-*poxF*/CG, transformation of *Corynebacterium glutamicum* R was performed by electroporation (Agric. Biol. Chem., Vol. 54, 443-447 (1990) and Res. Microbiol., Vol. 144, 181-185 (1993)), and the strain was applied to A agar medium (A liquid medium and 1.5% agar) containing 50 μg/mL of kanamycin. The single crossover strain obtained on the above medium was applied to BT agar medium (2 g of (NH₂)₂CO, 7 g of (NH₄)₂SO₄, 0.5 g of KH₂PO₄, 0.5 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 1 mL of 0.06% (w/v)

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$\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ + 0.042% (w/v) $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 1 mL of 0.02% (w/v) biotin solution, 2 mL of 0.01% (w/v) thiamin solution dissolved in 1 L of distilled water, and 1.5% agar) containing 10% (w/v) sucrose.

[0187] In the case of a strain having a single crossover of the plasmid pCRA725-poxF/CG with the homologous region on the chromosome, the strain shows kanamycin resistance resulting from the expression of the kanamycin resistance gene on the pCRA725-poxF/CG and mortality on a culture medium containing sucrose resulting from the expression of the *Bacillus subtilis* sacR-sacB gene. In the case of a strain having a double crossover of the plasmid pCRA725-poxF/CG, the strain shows kanamycin sensitivity resulting from the loss of the kanamycin resistance gene on the pCRA725-poxF/CG and growing ability on a culture medium containing sucrose resulting from the loss of the sacR-sacB gene. The markerless chromosomal gene disruptant shows kanamycin sensitivity and growing ability on a culture medium containing sucrose. Therefore, a strain that showed kanamycin sensitivity and growing ability on a culture medium containing sucrose was selected.

[0188] The obtained markerless poxF gene disruptant of *Corynebacterium glutamicum* R was named *Corynebacterium glutamicum* Δ poxF.

(7) Construction of transgenic strain for phenol-producing gene

[0189] With the use of the above-described plasmid pCRB209-tpl/PA, transformation of *Corynebacterium glutamicum* Δ poxF was performed by electroporation (Agric. Biol. Chem., Vol. 54, 443-447 (1990) and Res. Microbiol., Vol. 144, 181-185 (1993)), and the strain was applied to A agar medium containing 50 $\mu\text{g}/\text{mL}$ of kanamycin.

[0190] A growing strain on the culture medium was subjected to liquid culture in the usual manner. Plasmid DNA was extracted from the culture and cut with the use of restriction enzyme to confirm the inserted plasmid. As a result, transfection of the above-constructed plasmid pCRB209-tpl/PA was confirmed.

[0191] The obtained strain was named *Corynebacterium glutamicum* PHE31.

[0192] *Corynebacterium glutamicum* PHE31 was deposited in Incorporated Administrative Agency National Institute of Technology and Evaluation, NITE Patent Microorganisms Depository (2-5-8 Kazusakamatari, Kisarazu-shi, Chiba 292-0818 Japan) under Accession Number NITE BP-999 on November 2, 2010.

Example 2

Experiment of phenol production using *Corynebacterium glutamicum* phenol-producing gene transgenic strain and *Corynebacterium glutamicum* by-product formation pathway disruptant

[0193] The *Corynebacterium glutamicum* PHE31 (the markerless chromosomal poxF gene disruptant transfected with phenol-producing gene expression plasmid pCRB209-tpl/PA) prepared in Example 1 was applied to A agar medium (2 g of $(\text{NH}_2)_2\text{CO}$, 7 g of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g of KH_2PO_4 , 0.5 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL of 0.06% (w/v) $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ + 0.042% (w/v) $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 1 mL of 0.02% (w/v) biotin solution, 2 mL of 0.01% (w/v) thiamin solution, 2 g of yeast extract, 7 g of vitamin assay casamino acid, 40 g of glucose, and 15 g of agar were suspended in 1 L of distilled water) containing 50 $\mu\text{g}/\text{mL}$ of kanamycin, and left stand in the dark at 28°C for 20 hours.

[0194] An inoculation loop of the *Corynebacterium glutamicum* PHE31 grown on a plate as above was inoculated into a test tube containing 10 mL of A liquid medium containing 50 $\mu\text{g}/\text{mL}$ of kanamycin, and aerobically cultured with shaking at 28°C for 15 hours.

[0195] The *Corynebacterium glutamicum* PHE31 grown in the above conditions was inoculated into a 2 L-conical flask containing 500 mL of A liquid medium containing 50 $\mu\text{g}/\text{mL}$ of kanamycin, and aerobically cultured with shaking at 28°C for 15 hours.

[0196] The bacterial cells cultured and proliferated as above were collected by centrifugation (5,000 \times g at 4°C for 15 minutes). The obtained bacterial cells were suspended in 50 mL of BT (-urea) liquid medium (0.7% ammonium sulfate, 0.05% potassium dihydrogen phosphate, 0.05% dipotassium hydrogen phosphate, 0.05% magnesium sulfate heptahydrate, 0.0006% iron sulfate heptahydrate, 0.00042% manganese sulfate hydrate, 0.00002% biotin and 0.00002% thiamine hydrochloride) so that the final concentration of the bacterial cell was 10%. To a 100-mL medium bottle, the cell suspension was transferred, L-tyrosine disodium salt was added as a substrate, and the reaction was allowed to proceed under reducing conditions (the ORP of the reaction mixture: -450 mV) in a water bath kept at 33°C with stirring. As for the addition of L-tyrosine disodium salt, 40 mM L-tyrosine disodium salt was added at 0, 1, 3, and 10 hours after the start of the reaction.

[0197] A sample of the reaction mixture was centrifuged (15,000 \times g at 4°C for 10 minutes), and the obtained supernatant was used for quantitative determination of phenol.

[0198] As a result, in the reaction under reducing conditions, *Corynebacterium glutamicum* PHE31 had produced 34 mM of phenol in 24 hours.

Example 3

Test for suitability as a host for phenol production Influence of phenol on aerobic proliferation

- 5 **[0199]** A growth inhibition test in aerobic culture was performed to examine the influence of phenol on *Corynebacterium glutamicum*, *Escherichia coli*, and *Pseudomonas putida*. *Pseudomonas putida* S12, which was used for the test, is reported to be a solvent-resistant strain. In the report, disclosed is an unparalleled technology using the strain as a host in phenol production.
- 10 **[0200]** *Corynebacterium glutamicum* R was applied to A agar medium (2 g of $(\text{NH}_2)_2\text{CO}$, 7 g of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g of KH_2PO_4 , 0.5 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL of 0.06% (w/v) $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ + 0.042% (w/v) $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 1 mL of 0.02% (w/v) biotin solution, 2 mL of 0.01% (w/v) thiamin solution, 2 g of yeast extract, 7 g of vitamin assay casamino acid, 40 g of glucose, and 15 g of agar were suspended in 1 L of distilled water) and was left stand in the dark at 33°C for 15 hours.
- 15 **[0201]** An inoculation loop of the *Corynebacterium glutamicum* R grown on a plate as above was inoculated into a test tube containing 10 mL of A liquid medium (2 g of $(\text{NH}_2)_2\text{CO}$, 7 g of $(\text{NH}_4)_2\text{SO}_4$, 0.5 g of KH_2PO_4 , 0.5 g of K_2HPO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mL of 0.06% (w/v) $\text{Fe}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ + 0.042% (w/v) $\text{MnSO}_4 \cdot 2\text{H}_2\text{O}$, 1 mL of 0.02% (w/v) biotin solution, 2 mL of 0.01% (w/v) thiamin solution, 2 g of yeast extract, 7 g of vitamin assay casamino acid, and 40 g of glucose were suspended in 1 L of distilled water) and was aerobically cultured with shaking at 33°C for 13 hours.
- 20 **[0202]** The *Corynebacterium glutamicum* R grown in the above conditions was inoculated into 100 mL of A liquid medium in such a way that the initial bacterial cell concentration would be $\text{OD}_{610}=0.05$, phenol was added at the same time in such a way that the final concentration would be 0, 0.16, 0.2, 0.24, or 0.32 mM, and aerobic culture was performed with shaking at 33°C. The growth of bacterial cells was determined by absorbance measurement at OD_{610} .
- 25 **[0203]** *Escherichia coli* JM109 was applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar) and was left stand in the dark at 37°C for 15 hours.
- 30 **[0204]** An inoculation loop of the *Escherichia coli* JM109 grown on a plate as above was inoculated into a test tube containing 10 mL of LB liquid medium (1% polypeptone, 0.5% yeast extract, and 0.5% NaCl), and aerobic culture was performed with shaking at 37°C for 13 hours.
- 35 **[0205]** The *Escherichia coli* JM109 grown in the above conditions was inoculated into 100 mL of LB liquid medium in such a way that the initial bacterial cell concentration would be $\text{OD}_{610}=0.05$, phenol was added at the same time in such a way that the final concentration would be 0, 0.16, or 0.20 mM, and aerobic culture was performed with shaking at 37°C. The growth of bacterial cells was determined by absorbance measurement at OD_{610} .
- 40 **[0206]** *Pseudomonas putida* F1 and S12 were applied to LB agar medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 1.5% agar) and were left stand in the dark at 30°C for 15 hours.
- 45 **[0207]** An inoculation loop of each of the *Pseudomonas putida* F1 and S12 grown on a plate as above was inoculated into a test tube containing 10 mL of LB (+glucose) liquid medium (1% polypeptone, 0.5% yeast extract, 0.5% NaCl and 0.4% glucose), and aerobic culture was performed with shaking at 30°C for 13 hours.
- 50 **[0208]** The *Pseudomonas putida* F1 and S12 grown in the above conditions were each inoculated into 100 mL of LB (+glucose) liquid medium in such a way that the initial bacterial cell concentration would be $\text{OD}_{610}=0.05$, phenol was added at the same time in such a way that the final concentration would be 0, 0.10, or 0.20 mM, and aerobic culture was performed with shaking at 30°C. The growth of bacterial cells was determined by absorbance measurement at OD_{610} . Fig. 2 shows analysis results of the influence of phenol addition on aerobic proliferation.
- [0209]** The proliferation of *Escherichia coli* was significantly affected by 0.16% phenol and completely inhibited by 0.20% phenol.
- [0210]** *Pseudomonas putida* F1, and *Pseudomonas putida* S12, which was reported as a solvent-resistant strain, showed a similar tendency, and the proliferation thereof was significantly affected by 0.10% phenol and completely inhibited by 0.20% phenol.
- [0211]** In contrast, the proliferation of *Corynebacterium glutamicum* was hardly affected by 0.16% phenol, which significantly affected the proliferation of *Escherichia coli*. Even in the presence of 0.20% phenol, which completely inhibited the proliferation of *Escherichia coli* and *Pseudomonas putida*, *Corynebacterium glutamicum* showed favorable growth. Further, *Corynebacterium glutamicum* was able to proliferate in the presence of 0.24% phenol.
- [0212]** Thus, it was shown that *Corynebacterium glutamicum* has a higher resistance to phenol as compared with *Escherichia coli* and *Pseudomonas putida*, and is highly suitable as a host in phenol production.

INDUSTRIAL APPLICABILITY

- 55 **[0213]** According to the process of the present invention, phenol can be produced from tyrosine with a practical efficiency using microorganisms.

SEQUENCE LISTING

[0214]

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 <120> Transformant of coryneform group of bacteria and method of producing
 phenol using thereof

10 <130> C01F3971

 <150> JP2010-258089
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 45

Claims

- 50 1. A process for producing phenol, which comprises a step of allowing a phenol-producing transformant of *Corynebacterium glutamicum* comprising a gene which encodes an enzyme having tyrosine phenol-lyase activity to react in a reaction mixture containing tyrosine, a salt thereof, or an ester thereof under reducing conditions, and a step of collecting phenol from the reaction mixture, with the proviso that the case, in which a reaction mixture containing a saccharide as a carbon source is used, is excluded.
- 55 2. The process of claim 1, wherein the gene which encodes an enzyme having tyrosine phenol-lyase activity is a gene of *Pantoea agglomerans*, a gene of *Citrobacter braakii*, a gene of *Desulfitobacterium hafniense*, a gene of *Chloroflexus aurantiacus*, a gene of *Nostoc punctiforme*, or a gene of *Treponema denticola*.

3. The process of claim 1 or 2, wherein the gene which encodes an enzyme having tyrosine phenol-lyase activity is the DNA of the following (a) or (b):

(a) a DNA consisting of the base sequence of SEQ ID NO: 16, a DNA consisting of the base sequence of SEQ ID NO: 23, a DNA consisting of the base sequence of SEQ ID NO: 24, a DNA consisting of the base sequence of SEQ ID NO: 25, a DNA consisting of the base sequence of SEQ ID NO: 26, or a DNA consisting of the base sequence of SEQ ID NO: 27;

(b) a DNA which hybridizes to a DNA consisting of a complementary base sequence of any of the DNAs of (a) under stringent conditions and which encodes a polypeptide having tyrosine phenol-lyase activity.

4. The process of any one of claims 1 to 3, wherein the *Corynebacterium glutamicum* is a *Corynebacterium glutamicum* in which a gene which encodes an enzyme having phenol 2-monooxygenase activity on the chromosome is disrupted or deleted.

5. The process of any one of claims 1 to 3, wherein the *Corynebacterium glutamicum* is ATCC13032, or ATCC13869.

6. The process of any one of claims 1 to 3, wherein the *Corynebacterium glutamicum* is a strain of ATCC13032, or ATCC13869 in which a gene which encodes an enzyme having phenol 2-monooxygenase activity on the chromosome is disrupted or deleted.

7. The process of any of claims 1 to 6, wherein the transformant does not substantially proliferate in the reaction step.

8. The process of any one of claims 1 to 7, wherein the oxidation-reduction potential of the reaction mixture under reducing conditions is -200 mV to -500 mV.

Patentansprüche

1. Verfahren zur Herstellung von Phenol, umfassend einen Schritt, bei dem eine phenolproduzierende *Corynebacterium glutamicum* Transformante, die ein Gen aufweist, das für ein Enzym mit Tyrosin-Phenol-Lyase-Aktivität kodiert, in einer Reaktionsmischung unter reduzierenden Bedingungen reagiert, welche Reaktionsmischung Tyrosin, ein Salz davon oder einen Ester davon beinhaltet, sowie einen Schritt, Phenol aus der Reaktionsmischung zu sammeln, mit der Maßgabe, dass der Fall, in dem eine Reaktionsmischung, die ein Saccharid als Kohlenstoffquelle enthält, verwendet wird, ausgeschlossen ist.

2. Verfahren nach Anspruch 1, wobei das Gen, das für ein Enzym mit Tyrosin-Phenol-Lyase-Aktivität kodiert, ein Gen von *Pantoea agglomerans*, ein Gen von *Citrobacter braakii*, ein Gen von *Desulfitobacterium hafniense*, ein Gen von *Chloroflexus aurantiacus*, ein Gen von *Nostoc punctiforme* oder ein Gen von *Treponema denticola* ist.

3. Verfahren nach Anspruch 1 oder 2, worin das Gen, das für ein Enzym mit Tyrosin-phenol-Lyase-Aktivität kodiert, die DNA der folgenden (a) oder (b) ist:

(a) eine DNA, bestehend aus der Basensequenz von SEQ ID Nr.: 16, eine DNA, bestehend aus der Basensequenz von SEQ ID Nr.: 23, eine DNA, bestehend aus der Basensequenz von SEQ ID Nr.: 24, eine DNA, bestehend aus der Basensequenz von SEQ ID Nr.: 25, eine DNA, bestehend aus der Basensequenz von SEQ ID Nr.: 26, oder eine DNA, bestehend aus der Basensequenz von SEQ ID Nr.: 27;

(b) eine DNA, die unter stringenten Bedingungen mit einer DNA hybridisiert, die aus einer komplementären Basensequenz einer der DNAs aus (a) besteht, und die für ein Polypeptid mit Tyrosin-Phenol-Lyase-Aktivität kodiert.

4. Verfahren nach einem der Ansprüche 1 bis 3, wobei die *Corynebacterium glutamicum* Transformante eine *Corynebacterium glutamicum* Transformante ist, bei der ein Gen, das für ein Enzym mit Phenol-2-Monooxygenase-Aktivität kodiert, auf dem Chromosom ausgeschaltet oder deletiert ist.

5. Verfahren nach einem der Ansprüche 1 bis 3, wobei die *Corynebacterium glutamicum* Transformante ATCC13032 oder ATCC13869 ist.

6. Verfahren nach einem der Ansprüche 1 bis 3, wobei die *Corynebacterium glutamicum* Transformante ein Stamm

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von ATCC13032 oder ATCC13869 ist, in der ein Gen, das für ein Enzym mit Phenol-2-Monooxygenase-Aktivität kodiert, auf dem Chromosom ausgeschaltet oder deletiert ist.

- 5
7. Verfahren nach einem der Ansprüche 1 bis 6, wobei die Transformante im Reaktionsschritt nicht wesentlich proliferiert.
8. Verfahren nach einem der Ansprüche 1 bis 7, worin das Oxidations-ReduktionsPotential der Reaktionsmischung unter reduzierenden Bedingungen -200 mV bis -500 mV beträgt.

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Revendications

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1. Procédé de production de phénol, qui comprend une étape de réaction d'un transformant producteur de phénol de *Corynebacterium glutamicum* comprenant un gène qui code pour une enzyme ayant une activité tyrosine phénol-lyase dans un mélange de réaction contenant de la tyrosine, un sel de celle-ci, ou un ester de celle-ci dans des conditions réductrices, et une étape de collecte de phénol à partir du mélange de réaction, à condition que le cas dans lequel un mélange de réaction contenant un saccharide en tant que source de carbone est utilisé, soit exclu.

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2. Procédé selon la revendication 1, dans lequel le gène qui code pour une enzyme ayant une activité tyrosine phénol-lyase est un gène de *Pantoea agglomerans*, un gène de *Citrobacter braakii*, un gène de *Desulfitobacterium hafniense*, un gène de *Chloroflexus aurantiacus*, un gène de *Nostoc punctiforme* ou un gène de *Treponema denticola*.

25

3. Procédé selon la revendication 1 ou 2, dans lequel le gène qui code pour une enzyme ayant une activité tyrosine phénol-lyase est l'ADN des (a) ou (b) suivants :

(a) un ADN constitué de la séquence de bases de SEQ ID NO: 16, un ADN constitué de la séquence de bases de SEQ ID NO: 23, un ADN constitué de la séquence de bases de SEQ ID NO: 24, un ADN constitué de la séquence de bases de SEQ ID NO: 25, un ADN constitué de la séquence de bases de SEQ ID NO: 26 ou un ADN constitué de la séquence de bases de SEQ ID NO: 27 ;

30

(b) un ADN qui s'hybride à un ADN constitué d'une séquence de bases complémentaire de l'un quelconque des ADN de (a) dans des conditions stringentes et qui code pour un polypeptide ayant une activité tyrosine phénol-lyase.

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4. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le *Corynebacterium glutamicum* est un *Corynebacterium glutamicum* dans lequel un gène qui code pour une enzyme ayant une activité phénol 2-monooxygénase sur le chromosome est interrompu ou délété.

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5. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le *Corynebacterium glutamicum* est ATCC13032, ou ATCC13869.

45

6. Procédé selon l'une quelconque des revendications 1 à 3, dans lequel le *Corynebacterium glutamicum* est une souche d'ATCC13032, ou ATCC13869 dans laquelle un gène qui code pour une enzyme ayant une activité phénol 2-monooxygénase sur le chromosome est interrompu ou délété.

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7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel le transformant ne prolifère sensiblement pas dans l'étape de réaction.

8. Procédé selon l'une quelconque des revendications 1 à 7, dans lequel le potentiel d'oxydoréduction du mélange de réaction dans des conditions réductrices est de -200 mV à -500 mV.

55

Fig. 1

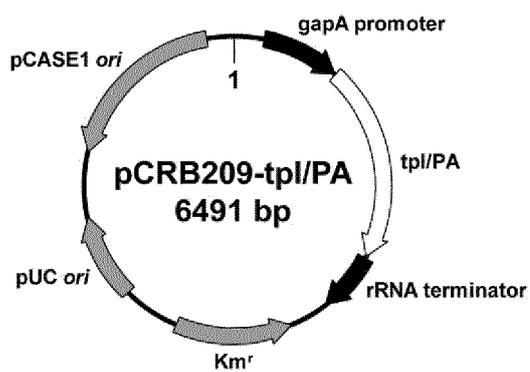
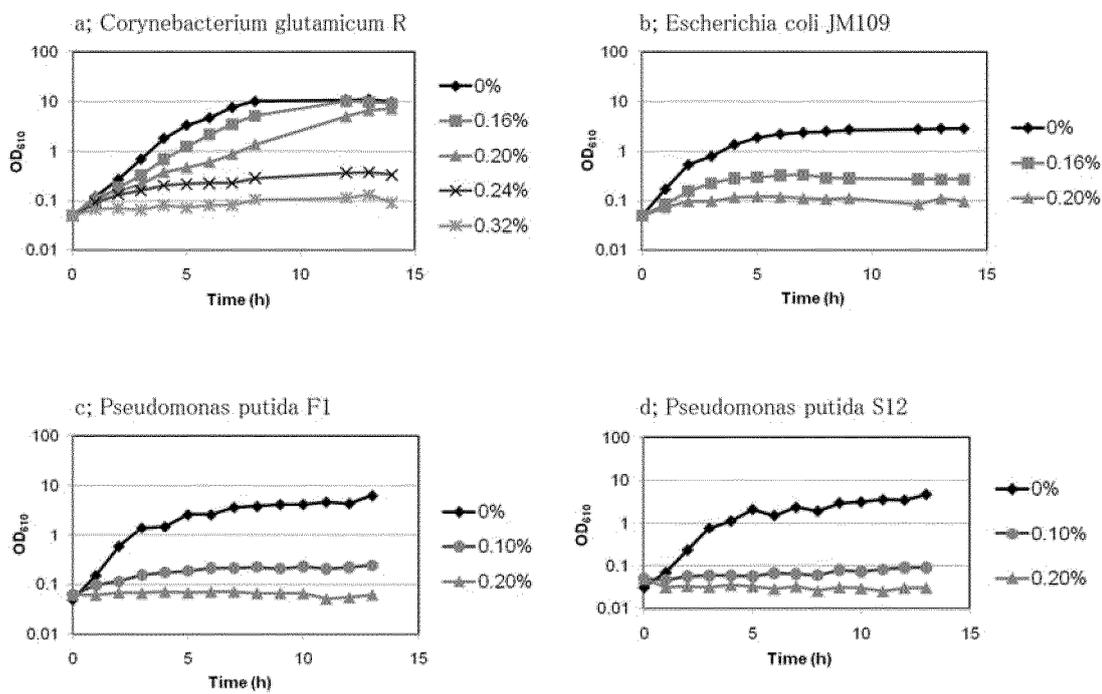


Fig. 2



REFERENCES CITED IN THE DESCRIPTION

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