

[DESCRIPTION]

[Title of Invention] METHOD FOR CONSTRUCTING CHIMERIC
PLASMID LIBRARY

[Technical Field]

5 [0001]

The present invention relates to a novel method for constructing a chimeric plasmid library.

[Background Art]

[0002]

10 In association with the progress in synthetic biology, there is an increasing demand for a long-chain DNA in which a plurality of genes are linked. In sequence designing for a long-chain DNA, it is unlikely that a result of interest can be achieved with sequence designing at once because it is necessarily required to learn many expression parameters
15 such as choices of the type of gene to be used or choices of the expression intensity of the gene. Thus, in many cases, the sequence designing is on the premise of performing a DBTL cycle (Design-Build-Test-Learn cycle), in which a long-chain DNA is Designed first, the long-chain
20 DNA is Built, the long-chain DNA is then Tested, the content thereof is Learned, and a new DNA based on the finding is constructed. In order to learn many expression parameters at the same time in this DBTL cycle, a combinatorial library technique for selecting one choice
25 from a plurality of choices for each expression parameter and linking each of them to construct various types of long-chain DNAs is desirable from the efficiency viewpoint. In other words, it is easier to derive the direction of DNA design for each expression parameter in a shorter cycle by
30 simultaneously constructing and comparing a plurality of types of long-chain DNAs with a variety for each expression parameter rather than by constructing and testing only a single long-chain DNA.

35 [0003]

However, synthesis of a long-chain DNA generally incurs costs and takes time, and it is often difficult to construct a plurality of long-chain DNAs. A multiple gene fragment assembling technique for preparing many short DNA fragments with the functional unit of a gene or the like as an index and linking (assembling) them for construction is used for long-chain DNA construction because, for example, the length of a DNA that can be supplied by chemical synthesis is as short as about 200 bases. For this kind of method for assembling DNA fragments, various methods including OGAB method (Patent Literature 1: Japanese Laid-Open Publication No. 2004-129654, Non Patent Literature 1: Tsuge, K., et al., *Nucleic Acids Res.* 31, e133 (2003)), SLIC method (Non Patent Literature 2: Li MZ, Elledge SJ (2007) *Nature Methods* 4:251-256), Golden Gate method (Non Patent Literature 3: Engler, C. et al. *PLoS ONE* (2008)), Gibson Assembly method (Non Patent Literature 4: Gibson, D. G., et al. *Nat. Methods*, 6, 343-345. (2009)), LCR method (Non Patent Literature 5: de Kok, S. et al. *ACS Synth. Biol.* (2014)), gene assembling method of budding yeast (Non Patent Literature 6: Gibson, D. G., et al. *Proc. Nat. Acad. Sci. USA* 6, 105, 20404-20409, 2008), and the like have been developed.

[0004]

In order to easily supply a plurality of types of long-chain DNAs at low cost, it is possible to simultaneously prepare a plurality of short DNA fragments each having a different expression parameter for use in this gene assembling and link them in a combination-manner to create a combinatorial library. A method for constructing a combinatorial library has been developed in the above-described gene assembling method.

[0005]

In addition, it is necessary to make the genotype of a long-chain DNA correspond to the phenotype thereof in

Testing the long-chain DNA combinatorial library supplied by these methods and Learning the direction of the design of expression parameters. There are mainly two methods in conventional construction of a combinatorial library. The first method is constructing one type of long-chain DNA in one gene assembling. In this case, gene assembling with a different material is individually performed as many times as matching to the scale of the combinatorial library. This method has an advantage in that it is possible to quickly grasp the corresponding phenotype even without separately confirming a genotype in Test because it can be grasped beforehand which gene assembling results in which genotype. However, this method has a disadvantage in that it is difficult to enlarge the scale. The second method is mixing all materials to be used in the combinatorial library and constructing a library with one gene assembling. This method has an advantage in that a large-scale combinatorial library can be easily obtained. However, it is necessary to individually confirm the base sequence by sequencing in order to know the genotype of a clone selected from this library, and the longer the chain length of DNA is, the more time it takes to confirm the base sequence. Thus, this method has a problem in that Test is a rate-determining step.

[Citation List]

[Patent Literature]

[0006]

[PL1] Japanese Laid-Open Publication No. 2004-129654 (Japanese Patent No. 4479199)

[Non Patent Literature]

[0007]

[NPL 1] Tsuge, K., et al., Nucleic Acids Res.31, e133, 2003

[NPL 2] Li MZ, Elledge SJ, Nature Methods 4:251-256, 2007

[NPL 3] Engler, C. et al. PLoS ONE, 2008

[NPL 4] Gibson, D. G., et al. Nat. Methods, 6, 343-345.,

2009

[NPL 5] de Kok, S. et al. ACS Synth. Biol., 2014

[NPL 6] Gibson, D. G., et al. Proc. Nat. Acad. Sci. USA 6,
105, 20404-20409, 2008

5 [Summary of Invention]

[Technical Problem]

[0008]

Under this circumstance, the problem to be solved by
the present invention is to provide a novel preparation
10 method of a DNA fragment for microbial cell transformation
by which a long-chain DNA combinatorial library can be
efficiently constructed and by which a long-chain DNA
combinatorial library for the next DBTL cycle can be
quickly prepared even without confirmation of the base
15 sequence, which is a rate-determining step.

[Solution to Problem]

[0009]

It is preferable to use a multiple gene fragment
assembling technique as described above in order to
20 efficiently construct a long-chain DNA combinatorial
library. However, regardless of the type of gene assembling
technique, it is important that gene fragments to be
assembled are even in amount, in other words, it is
important that the molar concentration of each gene
25 fragment is equal. However, it has been difficult to
efficiently construct a combinatorial library comprised of
many choices by utilizing a gene assembling technique
because it has been difficult to adjust the molar
concentration of many gene fragments, particularly over 10,
30 to an equal molar concentration.

[0010]

Gene fragments that are materials for performing gene
assembling generally need to be prepared one by one for
each fragment. In addition, in integrating these fragments
35 to be at an equal molar concentration, the weight

concentration of a DNA is measured and the amount to be added is determined by calculation based on the length of a DNA fragment. However, it was extremely difficult to precisely integrate the fragments to be equimolar due to an error in measurement of a DNA concentration, an error in DNA pipetting, or the like. Meanwhile, when an assembly that is in a plasmid state after being assembled once is cleaved by a restriction enzyme to be reduced to the original material, the resultant material is in an ideal equimolar state. The present inventors found that if this is actually used to perform assembling again, the assembling efficiency is improved by 100-times or greater as compared to the above-described case wherein DNA fragments are manually integrated and assembled (Tsuge, NAR, 2003). Moreover, in the case of an assembly which is cleaved into choice fragments when cleaved by a restriction enzyme, even when the base sequence of the choice fragments is not identified, the presence of actual DNA enables construction of a combinatorial library by mixing the choice fragments with a choice fragment derived from another assembly prepared in the same manner. The present inventors obtained a concept from this result to complete the highly efficient method for constructing a long-chain DNA combinatorial library of the present invention.

[0011]

Specifically, as a result of diligent research to solve the above-described problem to be solved, the present inventors adopted the above-described method so that all the ratios between the molar concentrations of DNA fragments that are used for assembling for a combinatorial library are as close to 1 as possible in a gene assembling method utilizing the plasmid transformation system of *Bacillus subtilis* (OGAB method). Specifically, choice gene fragments to be combinatorialized are linked to be a string to construct a seed plasmid. In addition, other seed

plasmids are constructed for other choice gene fragments to prepare seed plasmids, whose number of types is the same as the maximum number of choices. Cleaving each seed plasmid by a restriction enzyme gives a solution in which the gene fragments are mixed in an equimolar state. Equimolarity of this solution is maintained even when it is mixed with another seed plasmid. Various gene fragments contained in such solution are then linearly linked to give a polymeric DNA in a pseudo-tandem repeat state in which a plasmid vector portion periodically appears, and this DNA is used to transform *Bacillus subtilis*. A combinatorial library is efficiently constructed by circularization in the body of *Bacillus subtilis* utilizing homology to the plasmid vector portion.

[0012]

This method has a feature in that gene fragments at an equal molar concentration necessary for construction of a combinatorial library can be very easily and certainly prepared and the scale of the library construction can be enlarged more than ever before. In addition, with this method, a long-chain DNA combinatorial library of the next cycle can be constructed even without confirming the genotype of the obtained plasmid. Specifically, the present invention is summarized as follows.

[0013]

[1] A preparation method of a DNA fragment for microbial cell transformation, the DNA fragment having at least one insert DNA unit comprising: a DNA comprising a replication origin effective in a host microorganism; and an insert DNA in which unit DNAs are linked, characterized in that the method comprises:

(A) preparing a plurality of types of plasmids by OGAB method, wherein the plasmids comprise an insert DNA unit in which a plurality of types of unit DNAs capable of being linked in a specific linking order are linked;

(B) processing the plurality of types of plasmids prepared in step (A) with a restriction enzyme suitable for each plasmid to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture solutions; and

(C) re-assembling the unit DNAs by OGAB method using the plurality of types of unit DNA mixture solutions obtained in step (B) to prepare a long-chain DNA fragment.

[2] The preparation method of a DNA fragment for microbial cell transformation of [1], characterized in that all the ratios between molar concentrations for DNA fragments in the unit DNA mixture solutions obtained in step (B) are 0.8 to 1.2.

[3] The preparation method of a DNA fragment for microbial cell transformation of [1] or [2], characterized in that in step (A), the number of types of unit DNAs comprised in one type of insert DNA unit is 3 to 60.

[4] The preparation method of a DNA fragment for microbial cell transformation of any of [1] to [3], wherein the number of types of the restriction enzymes used in step (B) is three or less.

[5] The preparation method of a DNA fragment for microbial cell transformation of any of [1] to [4], wherein the restriction enzyme is a restriction enzyme that produces an overhang end.

[6] A plasmid comprising a DNA fragment for microbial cell transformation obtained by the preparation method of any of [1] to [5].

[7] A preparation method of a DNA fragment for microbial cell transformation, the DNA fragment having at least one insert DNA unit comprising: a DNA comprising a replication origin effective in a host microorganism; and an insert DNA in which unit DNAs are linked, characterized in that the method comprises:

(B') processing the plurality of types of plasmids of

[6] with a restriction enzyme suitable for each plasmid to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture solutions; and

(C) re-assembling the unit DNAs by OGAB method using the unit DNA mixture solutions obtained in step (B') to prepare a long-chain DNA fragment.

[8] The preparation method of a DNA fragment for microbial cell transformation of [7], characterized by selecting a plurality of types of plasmids comprising the obtained long-chain DNA fragment and reusing the plasmids as the plasmids in step (B').

[9] A method for constructing a chimeric plasmid library, using the preparation method of a DNA fragment for microbial cell transformation of any of [1] to [5], [7], and [8].

[Advantageous Effects of Invention]

[0014]

According to the present invention, it is possible to quickly and efficiently construct a long-chain DNA combinatorial library. It is also possible to reuse a plurality of plasmids which are selected from the same library and whose base sequences have not been confirmed for construction of a new chimeric library.

[Brief Description of Drawings]

[0015]

[Figure 1] Figure **1** shows the structure of pGETS302-SfiI-pBR, which is a plasmid vector for assembling.

[Figure 2] Figure **2** shows the detailed structure of a unit DNA constituting an insert unit.

[Figure 3] Figure **3** shows an artificial metabolic pathway of budding yeast which is designed to highly produce isobutanol.

[Figure 4] Figure **4** schematically shows the method for constructing a chimeric plasmid library of the present invention.

[Figure 5] Figure **5** shows the direction of a unitary gene in each plasmid in the first chimeric plasmid library obtained by the method of the present invention and the isobutanol production.

5 [Figure 6] Figure **6** shows the steps for constructing a new combinatorial library, with the plasmid used for transformation as a seed plasmid.

[Figure 7] Figure **7** shows the direction of a unitary gene in each plasmid in the second chimeric plasmid library
10 obtained by the method of the present invention and the isobutanol production.

[Description of Embodiments]

[0016]

The present invention is hereinafter described in
15 detail. As used herein, a molecular biological approach can be performed by a method described in general experiment manuals known to those skilled in the art or a method equivalent thereto, unless specifically and explicitly noted otherwise. Further, the terms used herein are
20 understood in the meaning that is commonly used in the art, unless specifically noted otherwise.

[0017]

<Preparation method of a DNA fragment for microbial cell transformation>

25 The present invention relates to a novel preparation method of a DNA fragment for microbial cell transformation by which a long-chain DNA combinatorial library can be efficiently constructed and by which a new combinatorial library can be easily constructed even without confirmation
30 of the genotype of the resulting clone. Specifically, said preparation method is a preparation method of a DNA fragment for microbial cell transformation having at least one insert DNA unit comprising: a DNA comprising a replication origin effective in a host microorganism; and
35 an insert DNA in which unit DNAs are linked, characterized

in that the method comprises:

(A) preparing a plurality of types of plasmids by OGAB method, wherein the plasmids comprise an insert DNA unit in which a plurality of types of unit DNAs capable of being
5 linked in a specific linking order are linked;

(B) processing the plurality of types of plasmids prepared in step (A) with a restriction enzyme suitable for each plasmid to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture
10 solutions; and

(C) re-assembling the unit DNAs by OGAB method using the plurality of types of unit DNA mixture solutions obtained in step (B) to prepare a long-chain DNA fragment.
[0018]

15 The present invention is a method wherein a DNA (plasmid vector) comprising a replication origin effective in a host microorganism and an insert DNA unit comprising an insert DNA are prepared as a plurality of unit DNAs having a structure in which they can be alternately linked,
20 the unit DNAs are linked to create a DNA fragment that has at least one insert DNA unit and has at least two unit DNAs that are identical, co-culture with the DNA fragment and a competent cell of the host microorganism is then performed, a plasmid DNA is collected from the microorganism to
25 prepare a combinatorial library, and a plasmid DNA selected from the combinatorial library can be utilized as a seed plasmid of a new library.

[0019]

In the present invention, an insert DNA unit refers to
30 a unit that comprises: a DNA comprising a replication origin effective in a host microorganism; and an insert DNA. A DNA fragment for microbial cell transformation comprises one or more insert DNA units. Furthermore, the insert DNA unit can comprise an appropriate base sequence as required
35 in addition to a DNA comprising a replication origin

effective in a host microorganism and an insert DNA. For example, when a plasmid for expressing a gene comprised in an insert DNA is created by the method of the present invention, the insert DNA unit may comprise a base sequence
5 that controls transcription and translation such as promoters, operators, activators, or terminators. Specifically, a promoter used when yeast is a host includes a promoter for a primary metabolite of a glycolysis system and the like.

10 [0020]

In the present invention, a DNA comprising a replication origin effective in a host microorganism can be any DNA as long as the DNA is replicated in a microorganism that can be transformed with a created DNA fragment.
15 Bacteria that belong to the genus *Bacillus* are used as the host microorganism of the present invention. Examples of a specific microorganism and a DNA comprising a replication origin effective in the microorganism include *B. subtilis* (*Bacillus subtilis*) and a DNA having a θ -type replication
20 mechanism, which specifically includes a sequence of a replication origin or the like comprised in a plasmid such as pTB19 (Imanaka, T., et al. *J. Gen. Microbiol.* 130, 1399-1408. (1984)) or pLS32 (Tanaka, T and Ogra, M. *FEBS Lett.* 422, 243-246. (1998), pAM β 1 (Swinfield, T. J., et al. *Gene*
25 87, 79-90. (1990)).

[0021]

In the present invention, an insert DNA refers to a DNA to be cloned, and the type and the size thereof are not particularly limited. The DNA may be any type of DNA which
30 is not only a natural sequence of a prokaryote, a eukaryote, a virus or the like but also an artificially designed sequence. The type of the DNA is not particularly limited. Preferably, the DNA includes a gene group constituting a series of metabolic pathways, an antisense RNA gene group
35 intended to deactivate gene expression present in a host

genome, a mixture of both a metabolic pathway gene group and an antisense RNA group, and the like. The insert DNA of the present invention has a structure in which unit DNAs are linked.

5 [0022]

In the present invention, unit DNAs have a structure in which they can be repeatedly linked to each other while keeping the order. Unit DNAs that are linked in order constitute a DNA fragment that is one insert DNA. The DNA
10 chain length of a unit DNA fragment is not particularly limited. Being linked to each other while keeping the order means that unit DNAs having sequences adjacent to each other on an insert DNA are bound while keeping the order and direction. Further, being repeatedly linked means that
15 the 5' terminal of a unit DNA having the base sequence of the 5' terminal of an insert DNA and the 3' terminal of a unit DNA having the base sequence of the 3' terminal of the insert DNA are bound. Specific examples of such unit DNAs include DNA fragments having terminals that can be
20 repeatedly linked to each other while keeping the order by utilizing complementarity between base sequences in overhang ends of the fragments. The structure of this overhang, including a difference in the shape of overhang between the 5' terminal overhang and the 3' terminal
25 overhang, is not particularly limited as long as it is not a palindromic structure (palindrome). In this regard, it is preferable that an overhang end can be created by digestion with a restriction enzyme in creating a unit DNA. If an enzyme capable of recognizing a specific sequence and
30 creating an overhang end of any sequence near the recognized sequence is used as a restriction enzyme, overhang ends of unit DNA fragments can be different at each linking site, so that the linking order is maintained. Examples of such a restriction enzyme include TALEN and ZNF
35 that are artificial restriction enzymes or CRISPR

technique-related enzymes capable of creating an overhang end such as CRISPR-Cpf1 and the like in addition to general restriction enzymes used for molecular biology. It is preferable to use a Type II restriction enzyme such as AarI, AlwNI, BbsI, BbvI, BcoDI, BfuAI, BglI, BsaI, BsaXI, BsmAI, BsmBI, BsmFI, BspMI, BspQI, BtgZI, DraIII, FokI, PflMI, SfaNI, or SfiI.

[0023]

Regarding the number of types of restriction enzyme used for creating an overhang end, cleavage by one type of restriction enzyme is preferred for cutting out one unit DNA. Not all unit DNAs necessarily need to be obtained by digestion with the same type of restriction enzyme. However, it is better that the total number of types of restriction enzyme used is smaller, wherein three or less types are preferable, two or less types are more preferable, and one type is even more preferable.

[0024]

One or more unit DNAs among unit DNAs constituting an insert DNA unit need to comprise a replication origin effective in a host cell. The rest of the unit DNAs are elements constituting a contiguous base sequence such as a metabolic pathway cluster, a part or the whole of a contiguous genome sequence of an organism, an artificial gene, or an artificial gene circuit, and there is no limitation that a single unit DNA must match a biologically functional unit.

[0025]

A method for creating a unit DNA may be any method as long as it can create the unit DNA of the present invention. For example, a DNA fragment amplified by a polymerase chain reaction (PCR) using a primer added with a restriction enzyme recognition sequence that produces each overhang end in a base sequence on a template DNA, or a chemically synthesized DNA fragment incorporating a restriction enzyme

recognition sequence to produce any overhang sequence at the end beforehand or the like is cloned into a plasmid vector, and used after the base sequence is confirmed. Each unit DNA is designed to be linked in a specific order to eventually provide a desired DNA fragment for microbial cell transformation. The number (type) of unit DNAs that are linked to constitute an insert DNA of interest is 3 to 60 (types), preferably 5 to 50 (types), more preferably 8 to 25 (types), and even more preferably 10 to 20 (types).

[0026]

Each step of the preparation method of a DNA fragment for microbial cell transformation of the present invention is hereinafter described in detail.

[0027]

[Step (A)]

In step (A) in the preparation method of a DNA fragment for microbial cell transformation of the present invention, a so-called seed plasmid is prepared. The seed plasmid needs to have a structure wherein an appropriate restriction enzyme recognition sequence is introduced at or near a border between unit DNAs in accordance with each design so that the plasmid after construction of an assembly can be divided into unit DNA fragments in consideration of step (B) and step (C). It is preferable to use an enzyme capable of creating an overhang end of any sequence such as AarI, AlwNI, BbsI, BbvI, BcoDI, BfuAI, BglI, BsaI, BsaXI, BsmAI, BsmBI, BsmFI, BspMI, BspQI, BtgZI, DraIII, FokI, PflMI, SfaNI, or SfiI as a restriction enzyme. A plurality of overhang sequences obtained by processing with these restriction enzymes need to be unique sequence in a single seed plasmid. Further, a seed plasmid group needs to share the same overhang sequence in the same chain and in the same order in a recombination unit in a combinatorial library (although a unit DNA often matches said unit, the recombination unit may be comprised of a

plurality of unit DNAs in some seed plasmids).

[0028]

In constructing an OGAB seed plasmid, specifically, it is also possible to create a DNA fragment for microbial cell transformation by linking (ligation) using a DNA ligase or the like in unit DNA mixture solutions in which each of the above-described unit DNAs is adjusted to be almost equimolar. In this regard, the starting material for gene assembling is not limited to only each of the above-described unit DNAs. An assembly prepared by any assembling method can be utilized as long as it eventually has a structure capable of being divided into each unit DNA as described above. In this case, being almost equimolar means that all the ratios between molar concentrations for DNA fragments in the unit DNA mixture solutions are within the range from 0.8 to 1.2, preferably within the range from 0.9 to 1.1, more preferably within the range from 0.95 to 1.05, even more preferably 1.0. All the ratios between molar concentrations for DNA fragments in the unit DNA mixture solutions being within the above-described numerical value range can be also rephrased as the value obtained by dividing the highest value of the concentration of the unit DNAs comprised in the unit DNA mixture solutions by the lowest value being within the range from 1.0 to 1.5, being within the range from 1.0 to 1.2, being 1.0 to 1.1, or being 1.0.

[0029]

The unit DNA of the seed plasmid prepared in this step may be any form such as a gene cluster, a gene, or a gene fragment.

[0030]

Although a method for linking unit DNAs is not particularly limited, it is preferable to perform linking in the presence of a polyethylene glycol and a salt. A salt of a monovalent alkali metal is preferred as the salt.

Specifically, it is more preferable to perform linking in a ligation reaction solution comprising 10% polyethylene glycol 6000 and 250 mM sodium chloride. In addition, although the concentration of each unit DNA in a reaction solution is not particularly limited, it is preferable that each unit DNA has a concentration of 1 fmol/ μ L or greater and is equimolar. Although the enzyme, the reaction temperature, and the time of ligation are not particularly limited, ligation with T4DNA polymerase at 37°C for 30 minutes or more is preferred.

[0031]

A host microorganism for the DNA fragment for microbial cell transformation of the present invention is not particularly limited as long as it has natural transformation ability. Such a microorganism includes a microorganism that has natural transformation ability to process a DNA to a single-stranded DNA to take up the DNA and the like. Specifically, bacteria that belong to the genus *Bacillus*, bacteria that belong to the genus *Streptococcus*, bacteria that belong to the genus *Haemophilus*, bacteria that belong to the genus *Neisseria*, and the like are included. Furthermore, bacteria that belong to the genus *Bacillus* include *B. subtilis* (*Bacillus subtilis*), *B. megaterium* (*Bacillus megaterium*), *B. stearothermophilus* (*Bacillus stearothermophilus*), and the like. The most preferred microorganism among them includes *Bacillus subtilis* having excellent natural transformation ability and recombination ability.

[0032]

A known method suitable for each microorganism can be selected as a method for making a microbial cell competent. Specifically, for example, it is preferable to use a method described in Anagnostopoulou, C. and Spizizen, J. J. *Bacteriol.*, 81, 741-746 (1961) for *Bacillus subtilis*. Further, a known method suitable for each microorganism can

be used as a method for transformation. The liquid quantity of a ligation product given to a competent cell is also not particularly limited. The quantity is preferably from 1/20 to an equal quantity, and more preferably a half quantity relative to a competent cell culture. A known method also can be used as a method for purifying a plasmid from a transformant.

[0033]

It can be confirmed that a plasmid obtained by the above-described method has an insert DNA of interest by a size pattern of a fragment generated by restriction enzyme cleavage, PCR method, or a sequencing method. Further, when the insert DNA has a function such as substance production, confirmation can be made by detecting the function.

[0034]

For adjustment of a seed plasmid used in construction of a combinatorial library, any method can be used as long as it is a common method for purifying a circular plasmid. A method with reduced risk of contamination of a DNA other than a plasmid DNA is desirable. Specifically, a cesium chloride-ethidium bromide density-gradient ultracentrifugation method is preferred.

[0035]

[Step (B)]

This step is processing the plurality of types of plasmids (seed plasmids) prepared in step (A) with a restriction enzyme suitable for each plasmid to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture solutions. The plurality of types of plasmids (seed plasmids) prepared in step (A) are purified to high purity and then cleaved into unit DNAs. For cleavage into unit DNAs, an appropriate restriction enzyme can be selected depending on the design in step (A).

[0036]

Regarding the number of types of restriction enzyme

used for creating an overhang end, cleavage by one type of restriction enzyme is preferred for cutting out one unit DNA. Not all unit DNAs necessarily need to be obtained by digestion with the same type of restriction enzyme. However,
5 it is better that the total number of types of restriction enzyme used is smaller, wherein three or less types are preferable, two or less types are more preferable, and one type is even more preferable.

[0037]

10 The unit DNA mixture solutions obtained in this step are free of DNA fragment other than the plasmid because the seed plasmids are purified to extremely high purity. A prepared long-chain DNA is cleaved with a restriction enzyme and the restriction enzyme is removed, whereby DNA
15 fragment solutions (unit DNA mixture solutions) in which all the ratios between the molar concentrations for DNA fragments are extremely close to 1 can be obtained.

[0038]

[Step (C)]

20 This step is re-assembling the unit DNAs by OGAB method using the plurality of types of unit DNA mixture solutions obtained in step (B) to prepare a long-chain DNA fragment. It is possible to more efficiently perform gene assembling by performing a gene assembling method (OGAB method) using
25 the DNA fragment solutions (unit DNA mixture solutions) obtained in step (B) in which all the ratios between the molar concentrations for DNA fragments are extremely close to 1 as a starting material. The explanation in step (A) can be applied to a method for re-assembling the unit DNAs
30 by OGAB method using the unit DNA mixture solutions in this step.

[0039]

Furthermore, the present invention also includes a preparation method of a DNA fragment for microbial cell
35 transformation, the DNA fragment having at least one insert

DNA unit comprising: a DNA comprising a replication origin effective in a host microorganism; and an insert DNA in which unit DNAs are linked, characterized in that the method comprises:

- 5 (B') processing the plurality of types of plasmids prepared by the above-described method of the present invention with a restriction enzyme suitable for each plasmid to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture solutions; and
- 10 (C) re-assembling the unit DNAs by OGAB method using the unit DNA mixture solutions obtained in step (B') to prepare a long-chain DNA fragment.

[0040]

- A plurality of types of plasmids comprising the long-chain DNA fragment obtained by the above-described method of the present invention can be selected and reused as the plasmids in step (B').

[0041]

- The present invention also includes a plasmid
- 20 comprising the DNA fragment for microbial cell transformation obtained by the above-described preparation method of the present invention. The present invention also includes a method for constructing a chimeric plasmid library using the preparation method of the present
- 25 invention.

[Examples]

[0042]

- The present invention is specifically described in the following Examples. However, the present invention is not
- 30 limited by these Examples.

[0043]

The common test method or the like such as reagents used in the Examples is as follows.

- RM125 strain (Uozumi, T., et al. *Mol. Gen. Genet.*, 152, 65-69 (1977)) and its derivative strain, BUSY9797 strain,
- 35

were used as a host of *Bacillus subtilis*. As a plasmid vector capable of being replicated in *Bacillus subtilis*, pGET118 (Kaneko, S., et al. *Nucleic Acids Res.* 31, e112 (2003)) was used. Carbenicillin, which is an antibiotic, was purchased from Wako Pure Chemical Industries. Tetracycline, which is an antibiotic, was purchased from Sigma. SfiI and BspQI, which are restriction enzymes, were purchased from NEB. T4 DNA Ligase was purchased from Takara Bio. Takara Ligation Kit (Mighty) (Takara Bio) was used for common ligation for constructing a plasmid of *Escherichia coli*. KOD plus polymerase of TOYOBO was used for a PCR reaction for preparation of a unit DNA. Meanwhile, Ex-Taq HS manufactured by Takara Bio was used for colony PCR for sequencing a DNA cloned into a plasmid. pMD-19 (simple) was purchased from Takara Bio. For Plasmid Safe, which is an enzyme for purifying a circular plasmid, a product manufactured by EPICENTER was used. 2-Hydroxyethyl agarose, which is a low melting point agarose gel for DNA electrophoresis, was purchased from Sigma. UltraPure Agarose of Invitrogen was used for other common agarose gels for electrophoresis. Phenol:chloroform:isoamyl alcohol 25:24:1 and TE saturated phenol (containing 8-quinolinol) manufactured by Nacalai Tesque were used. Lysozyme was purchased from Wako Pure Chemical Industries. A medium component of an LB medium and agar manufactured by Becton Dickinson were used. IPTG (isopropyl β -D-thiogalactopyranoside) manufactured by Wako Pure Chemical Industries was used. For all other medium components and biochemical reagents, products manufactured by Wako Pure Chemical Industries were used.

[0044]

Either of *Escherichia coli* DH5 α strain, JM109 strain, or TOP10 strain was used for constructing a plasmid which is not specifically noted. QIAprep Spin Miniprep Kit of Qiagen was used for purification of a small amount of a

constructed plasmid from *Escherichia coli* while QIAfilter Midi Kit of Qiagen was used for purification of a large amount. MinElute Reaction Cleanup Kit of Qiagen or QIAquick PCR purification Kit of Qiagen was used for DNA cleanup from an enzyme reaction solution. MinElute Gel Extraction Kit of Qiagen was used for purification from a gel block obtained after separation on ordinary agarose gel electrophoresis. As an ultratrace spectrophotometer, nanodrop 2000 of Thermo was used. 3130xl genetic analyzer, which is a fluorescent automatic sequencer manufactured by Applied Biosystems, was used for sequencing.

[0045]

Other common DNA manipulations were performed according to a standard protocol (Sambrook, J., et al., Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989)). *Bacillus subtilis* transformation and plasmid extraction by OGAB method or the like were performed according to a known method (Tsuge, K., et al., Nucleic Acids Res. 31, e133. (2003)).

[0046]

1. Preparation of an insert DNA unit

(1) Construction of a plasmid vector for assembling

pGETS302-SfiI-pBR, a plasmid vector for assembling, is an *Escherichia coli*-*Bacillus subtilis*-yeast shuttle plasmid vector that has a replication origin of pBR322 of *Escherichia coli*, repA, which is a replication origin functional in *Bacillus subtilis*, and ARS4 and CEN6 capable of being replicated in budding yeast. This is a plasmid constructed through multi-stage processes based on pGETS109 (Tsuge, et. al., Nucleic Acids Res., 31, e133. (2003)). Figure 1 shows the structure thereof, and SEQ ID NO: 1 shows the base sequence thereof. The cloning site for the gene to be assembled is between two SfiI cleavage sites, and the largest 15 kb SfiI fragment is used in assembling.

Ampicillin was used for selection in *Escherichia coli*. Sterile water was added to 5 µg of this plasmid so that the total volume was 40 µl, followed by adding 5 µl of 10 × NEB2.1 Buffer attached to the restriction enzyme and 5 µl of SfiI (NEB), restriction enzyme, and causing the mixture thereof to react at 50°C for 2 hours. The resultant liquid was subjected to separation by low melting point agarose gel electrophoresis, an about 15 kb fragment of the vector body was then cut out from the gel, a DNA fragment of interest was purified, the DNA fragment was then dissolved into 20 µl TE, 1 µl of the solution was collected and the concentration thereof was measured by an ultratrace spectrophotometer.

[0047]

(2) Method for designing a unit DNA overhang sequence

As unit DNAs constituting one insert unit, there are 14 fragments in total including pGETS302, which is a vector for assembling, as shown in Figure 2. There are 12 genes in total constituting a group involved in an isobutanol metabolic pathway in budding yeast. These genes are defined as the 1st to the 12th unit DNA in order. kanMX4, which acts as a selection marker for transformation in budding yeast, is defined as the 13th unit DNA, and an assembling vector is defined as the 14th unit DNA. The 1st to 14th unit DNAs are contiguous in the same order as the number and form a structure in which the 14th unit DNA and the 1st unit DNA are linked, thereby forming one insert unit. The terminal of each unit DNA has a unique 3-base 3' terminal overhang bases which are designated for each number of unit DNA on each of the left and right of the fragment. With this complementarity, a linking partner is designated. The specific configuration is as follows. (14th unit DNA)-GTT-(1st unit DNA)-TGA-(2nd unit DNA)-CGA-(3rd unit DNA)-TGT-(4th unit DNA)-GAT-(5th unit DNA)-TTG-(6th unit DNA)-GTC-(7th unit DNA)-ATG-(8th unit DNA)-TGG-(9th unit DNA)-TAG-

(10th unit DNA)-ACT-(11th unit DNA)-GTA-(12th unit DNA)-CTT-(13th unit DNA)-TCT-(14th unit DNA).

[0048]

2. Regulation of a gene expression level of budding yeast
5 isobutanol-producing gene group

(1) Budding yeast

Budding yeast (*Saccharomyces cerevisiae*) is a eukaryotic microorganism. Research on budding yeast has been advanced as a eukaryotic model microorganism, its
10 genome sequence has been completely revealed, and various information has been accumulated. Budding yeast performs alcohol fermentation as anaerobic respiration. Budding yeast has been utilized for fermentation of beer, wine, Japanese sake or the like for a long time, and has been
15 widely used as a host for bioethanol production because of its high ethanol production ability. Currently, budding yeast is also widely used for industrial purposes as a host for production of a useful substance other than ethanol, and is also utilized for production of an added value
20 product such as dyes, perfumes, or supplements in addition to higher alcohols having three or more carbons chain or various organic acids. Unlike a bacterium, which is a prokaryote, budding yeast, which is a eukaryote, has an organelle such as a mitochondrion or a nucleus. Further,
25 since budding yeast is generally in a monocistronic expression format instead of a polycistronic expression format, one promoter is required for one gene. For example, 12 promoters are required to express 12 genes.

[0049]

30 (2) Isobutanol metabolic pathway design

The main application of isobutanol is as an organic synthesis solvent, a paint remover, and a raw material of i-butyl methacrylate. In addition, isobutanol can be converted to isobutylene by dehydration, can be utilized as
35 a raw material of a fuel mixture agent or a bio-jet fuel

such as ethyl tert-butyl ether (ETBE), and can be further utilized as a raw material of various polymers by conversion of isobutylene to isooctene (diisobutylene). Although budding yeast originally produces ethanol as a main product, budding yeast slightly produces isobutanol as fusel alcohol. Figure 3 shows an artificial metabolic pathway of budding yeast which is designed to highly produce isobutanol. If two genes (e.g., *kivd* derived from *Lactococcus lactis* and *ADH6* derived from budding yeast) encoding a keto-acid decarboxylase (KDC) and an alcohol dehydrogenase (ADH) are introduced to 2-keto-isovalerate in the L-valine metabolic pathway in budding yeast, isobutanol production is increased. Thus, these genes were added to the subject of assembling. However, isobutanol cannot be efficiently produced due to a plurality of causes such as 2-keto-isovalerate that is a substrate being originally produced in the mitochondrion or NADPH required by a ketol-acid reductoisomerase (*ILV5*) and *ADH6* being deficient. Thus, three genes encoding an acetolactate synthase (*ILV2*), a ketol-acid reductoisomerase (*ILV5*), and a dihydroxy acid dehydratase (*ILV3*) constituting the pathway from pyruvic acid to 2-keto-isovalerate (the metabolic pathway indicated by a double line in Figure 3) which is performed in the mitochondrion, and a malic enzyme (*MAE1*) for adjusting NADPH in the mitochondrion, i.e., four genes in total (the genes indicated by a double underline in Figure 3), were added to the subject of assembling for the purpose of enhancing the expression of those genes. Furthermore, three genes of *ilv2CEc*, *ilvDL1* and *alsLp* were added to the subject of assembling so that the metabolic pathway of the above genes is also constructed on the cytoplasm side, and three genes of a carbonic acid fixing enzyme (*PYC2*), a malic dehydrogenase (*MDH2*), and *sMAE1* from which a mitochondrial localization signal of the N terminal of a malic enzyme (*MAE1*) is removed were added to the subject of

assembling in order to solve the deficiency in NADPH in the cytoplasm, in other words, six genes in total (the genes indicated by a single underline in Figure 3) were added to the subject of assembling. The above-described 12 genes

5 were each introduced with a promoter and a terminator of a primary metabolic pathway capable of being strongly expressed in yeast.

[0050]

(3) Seed plasmid 1: design of an overexpressing gene group

10 set

Expression cassettes using 12 types of promoters and terminators were designed so that 12 genes can be expressed in budding yeast. Specifically, promoters and terminators of ADH1, FBA1, HXT7, PDC1, PGK1, SED1, TDH1, TDH2, TDH3,

15 TEF1, TEF2, and TPI1 were used to design 12 types of expression cassettes (the arrows on the gene ORF in Figure 4 indicate the promoter sequence while the pins indicate the terminator sequence). A sequence (...atgAGAAGAGCTCTTCataa...) in which two BspQI sites are

20 reversely placed was added between the promoter and the terminator of each expression cassette so that the portion from the start codon (ATG) to the stop codon (TAA) of a gene to be inserted can be subcloned. For PDC1 promoter and TDH2 promoter, a sequence in which G in position -492 was

25 mutated to C and a sequence in which C in position -462 was mutated to G were used for PDC1 promoter and TDH2 promoter, respectively, in order to delete the BspQI sites contained in the sequence. A restriction enzyme site (SfiI site) designed such that a unique 3-base 3' terminal overhang

30 designated for each number of a unit DNA appears after cleavage with SfiI was added to the left and right terminal sequences of the 12 types of expression cassettes comprising a promoter and a terminator, and the sequence was designed such that a linking partner is designated by

35 complementarity. These expression cassettes comprising 12

types of promoters and terminators were designed to be cloned into a pMA or pMK vector. Next, *ilvEc*, *ilvDL1*, *alsLp*, *kivd*, *ILV3*, *ILV5*, *ADH6*, *PYC2*, *ILV2*, *MDH2*, *maeBEc*, and *sMAE1* were selected as 12 genes constituting a group involved in an isobutanol metabolic pathway in budding yeast, and the sequence was modified so that the start codon and the stop codon of each gene were unified to ATG and TAA, respectively. These genes were also designed to be added with a sequence (TAGGCTCTTCAatg...taaAGAAGAGCCTA) in which a BspQI site is placed at both terminals so that these genes can be subcloned to any of the 12 types of expression cassettes (Figure 2). These genes having a BspQI site at both terminals were designed so as to be cloned into a pCR-BluntII-TOPO vector. Finally, 12 types of overexpression cassettes in total (*ilvCEc*-1st, *ilvDL1*-2nd, *alsLp*-3rd, *kindEc*-4th, *ILV3*-5th, *ILV5*-6th, *ADH6*-7th, *PYC2*-8th, *ILV2*-9th, *MDH2*-10th, *maeBEc*-11th, and *sMAE1*-12th) were designed (SEQ ID NOs: 2 to 13) so that *ilvEc*, *ilvDL1*, *alsLp*, *kivd*, *ILV3*, *ILV5*, *ADH6*, *PYC2*, *ILV2*, *MDH2*, *maeBEc*, or *sMAE1* would be inserted to each of the BspQI sites of the 12 types of expression cassettes having the promoters and terminators of *ADH1*, *FBA1*, *HXT7*, *PDC1*, *PGK1*, *SED1*, *TDH1*, *TDH2*, *TDH3*, *TEF1*, *TEF2*, and *TPI1* cloned into pMA or pMK. In addition, a KanMX fragment (*kanMX4*-13th) was introduced as the 13th fragment (SEQ ID NO: 14) to enable selection with an agent in budding yeast.

[0051]

(4) Seed plasmid 2: design of an expression suppressing gene group set

Although the same promoter and terminator sequence was used while following (3) Seed plasmid 1: design of an overexpressing gene group set, an ORF fragment of each gene to be inserted was designed to be in an opposite direction relative to the overexpression gene group set. Specifically, the plasmid configured to be able to subclone the portion

from the start codon (ATG) to the stop codon (TAA) of a gene to be inserted between the promoter and the terminator of each expression cassette which was designed in (3) was cleaved by BspQI and a sequence in which two BspQI sites
 5 are reversely placed is newly linked thereto, whereby a plasmid with a changed overhang sequence (the underlined sequence of ...atgttaAGAAAGAGCTCTTCattaa....) was prepared. The base sequences of the expression suppressing cassettes (ilvCEc-as-1st, ilvDL1-as-2nd, alsLp-as-3rd, kindEc-as-4th,
 10 ILV3-as-5th, ILV5-as-6th, ADH6-as-7th, PYC2-as-8th, ILV2-as-9th, MDH2-as-10th, maeBEc-as-11th, and sMAE1-as-12th) created through these processes are shown in SEQ ID NOs: 15 to 26. Regarding a unit DNA, the unit DNAs of seed plasmid 2 are consequently longer than the unit DNAs of seed
 15 plasmid 1 by six bases of the overhang sequence that were newly introduced. The same marker as seed plasmid 1 was used for KanMX, which is an agent selection marker.

[0052]

(5) Construction of a seed plasmid

20 A gene (ORF region) was amplified from budding yeast (YPH499 strain) using the PCR method. First, primers in which the restriction enzyme recognition site determined above was added to the 5' terminal of primers for amplifying a DNA sequence between the overhang combinations
 25 determined above in a position to cut out a desired overhang and in which the sequence of TAG was further added to the 5' terminal were used. A DNA fragment of a designated region was amplified from the budding yeast genome by using a pair of these primers. The reaction
 30 condition of PCR was set by adding, for each reaction (50 μ l), 5 μ l of KOD Plus 10 $\times$ buffer Ver. 2, 3 μ l of 25 mM MgSO₄, 5 μ l of dNTP (2 mM each), 1 μ l of KOD Plus (1 unit/ μ l), 48 pg of lambda phage DNA (TOYOBO), 15 pmol of primers (F primer and R primer, respectively), and sterile
 35 water, and the reaction was performed with GeneAmp PCR

System 9700, Applied Biosystems) according to the following program. After incubation at 94°C for 2 minutes, 30 cycles each comprised of 98°C for 10 seconds, 55°C for 30 seconds, and 68°C for 1 minute were performed, and final incubation was performed at 68°C for 7 minutes.

[0053]

The amplified DNA fragment was applied with a voltage of 100 V (about 8 V/cm) by a general-purpose agarose gel electrophoresis device (i-MyRun.N, electrophoresis system for nucleic acids, Cosmo Bio) and subjected to electrophoresis for 1 hour in the presence of 1 × TAE (Tris-Acetate-EDTA Buffer) buffer in 0.7% low melting point agarose gel (2-Hydroxyethyl Agarose TypeVII, Sigma), whereby the plasmid vector and the unit DNAs were separated.

This electrophoresis gel was stained with 100 ml of 1 × TAE buffer comprising 1 µg/ml of ethidium bromide (Sigma) for 30 minutes and illuminated with an ultraviolet ray with a long wavelength (366 nm) to be visualized, whereby a PCR product having a size of interest was cut out with a razor and collected in a 1.5 ml tube. 1 × TAE buffer was added to the collected low melting point agarose gel (about 300 mg) so that the total volume was about 700 µl, which was then kept at a constant temperature of 65°C for 10 minutes to thereby dissolve the gel. An equal quantity of TE saturated phenol (Nacalai Tesque) was then added and mixed well to deactivate the restriction enzyme. The mixture was separated into a phenol phase and an aqueous phase by centrifugation (20,000 × g, 10 minutes), and the aqueous phase (about 900 µl) was collected in a new 1.5 ml tube. 500 µl of 1-butanol (Wako Pure Chemical Industries) was added thereto and mixed well, followed by separation by centrifugation (20,000 × g, 1 minute) and removal of water-saturated 1-butanol. This operation was repeated until the volume of the aqueous phase was 450 µl or less, thereby decreasing the volume of the aqueous phase. 50 µl of 3M

potassium acetate-acetic acid buffer (pH 5.2) and 900 μ l of ethanol were added thereto and centrifugation ($20,000 \times g$, 10 minutes) was performed to precipitate the DNA, which was then rinsed with 70% ethanol and dissolved into 20 μ l of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This collected DNA was preserved at -20°C until it was used.

[0054]

The obtained DNA fragment was cloned into an Escherichia coli plasmid vector by the TA cloning method by the method shown below. 1 μ l of 10 $\times$ Ex-Taq Buffer attached to Ex-Taq, which is an enzyme for PCR reaction of TAKARA, 0.5 μ l of 100 mM dATP, and 0.5 μ l of Ex-Taq were added to 8 μ l of the DNA fragment, and the mixture thereof was kept at a constant temperature of 65°C for 10 minutes to thereby add an overhang of A to the 3' terminal of the DNA fragment. 1 μ l of pMD19-Simple of TAKARA and 3 μ l of sterile water were mixed to 1 μ l of this DNA fragment solution, 5 μ l of TAKATA Ligation (Mighty) Mix was then added thereto, and the mixture thereof was kept at a constant temperature of 16°C for 30 minutes. 5 μ l of this ligation solution was added to 50 μ l of chemical competent cells of Escherichia coli DH5 α , and the mixture thereof was kept at a constant temperature on ice for 15 minutes, was then given a heat shock at 42°C for 30 seconds, was left on ice for 2 minutes, and was then added with 200 μ l of LB medium. The mixture thereof was kept at a constant temperature of 37°C for 1 hour, was then spread on an LB plate comprising 1.5% agar and comprising carbenicillin at a concentration of 100 $\mu\text{g/ml}$, and was cultured overnight at 37°C , whereby a transformant of the plasmid was obtained.

[0055]

The obtained colony was prepared using a reagent for preparing a template DNA for PCR (Cica geneus $^{\circledR}$ DNA preparation reagent, Kanto Kagaku). Specifically, 2.5 μ l of solution in which reagent a and reagent b in the reagent

kit were mixed at a ratio of 1:10 was prepared, and a small quantity of the colony on the plate collected by a toothpick was suspended in the solution, followed by processing the suspension at 72°C for 6 minutes and then
5 processing it at 94°C for 3 minutes. 2.5 µl of 10× enzyme for TAKARA Ex-Taq, 2 µl of 2.5 mM dNTP solution, 0.25 µl of 10 pmol/µl of M13F primer, 0.25 µl of 10 pmol/µl of M13R primer, 17 µl of sterile water, and 0.5µl of Ex-TaqHS were added to the obtained liquid, and the mixture thereof was
10 incubated at 94°C for 5 minutes, followed by performing 30 cycles each consisting of 98°C for 20 seconds, 55°C for 30 seconds, and 72°C for 1 minute to amplify the DNA. The base sequence of this PCR product was analyzed to study whether it completely matches a desired sequence. Finally, a
15 correct sequence was obtained from all clones.
[0056]

Escherichia coli transformants having a plasmid into which a DNA fragment having a desired sequence was cloned were each cultured in 2 ml of LB medium containing 100
20 µg/ml of carbenicillin at 37°C and 120 spm overnight. The obtained microbial body was purified by using QIAfilter Plasmid miniKit (Qiagen) according to the manual. The obtained plasmid was cleaved by BspQI and an ORF region was collected by size fractionation by electrophoresis.
25 [0057]

Thermo Fisher was asked to synthesize a DNA (for overexpression of Figure 2) designed such that DNA fragments in which a promoter and a terminator of yeast are linked at a BspQI site can be cut out at a SfiI site. These
30 DNA fragments were delivered while being cloned into a plasmid vector pAM or pMK. This plasmid was cleaved by BspQI and a BspQI fragment of the above-described ORF was linked thereto to make a construct, which was prepared as a plasmid having the unit DNAs of seed plasmid 1. The
35 sequences of these unit DNA fragments are as shown in SEQ

ID NOs: 2 to 13. For seed plasmid 2, the above-described DNA fragments cloned into pMA or pMK in which a promoter and a terminator are linked at a BspQI site were cleaved by BspQI and introduced with a linker DNA, thereby making a new construct as in the construct for expression suppression of Figure 2. This plasmid was cleaved by BspQI and a BspQI fragment of the above-described ORF was introduced to make a construct. *Escherichia coli* transformants each having these plasmids into which a DNA fragment having a desired sequence was cloned were each cultured in 2 ml of LB medium containing 100 µg/ml of carbenicillin at 37°C and 120 rpm overnight. The obtained microbial body was purified by using QIAfilter Plasmid miniKit (Qiagen) according to the manual. 10 µl of the obtained plasmid was fractionated, 30 µl of sterile water, 5 µl of 10 × NEB buffer#2, and 5 µl of SfiI restriction enzyme (NEB) were added thereto, and the mixture thereof was caused to react at 50°C for 2 hours, thereby separating the unit DNA fragments from the plasmid vector. The resulting product was applied with voltage of 50 V (about 4 V/cm) by a general-purpose agarose gel electrophoresis device and subjected to electrophoresis for 1 hour in the presence of 1 × TAE buffer in 0.7% low melting point agarose gel, whereby the plasmid vector and the unit DNAs were separated. This electrophoresis gel was stained with 100 ml of 1 × TAE buffer comprising 1 µg/ml of ethidium bromide (Sigma) for 30 minutes and illuminated with an ultraviolet ray with a long wavelength (366 nm) to be visualized, whereby a portion around 3 kb was cut out with a razor and collected in a 1.5 ml tube. The collected low melting point agarose gel (about 300 mg) was purified in the above-described manner and dissolved into 20 µl TE. The unit DNA plasmid prepared in this manner was quantified by a fluorescence plate reader for SYBR GreenII, which is a nucleic acid fluorescent dye, using a calibration curve

created based on a dilution series of commercially available Lambda phage genome DNA (TOYOBO).

[0058]

(6) Gene assembling

5 11 μ l of 2 $\times$ ligation buffer was added to 10 μ l of mixture solution comprising 0.1 fmol or greater of the unit DNAs of SEQ ID NOs: 2 to 14 for assembling of seed plasmid 1 or the unit DNAs of SEQ ID NOs: 14 to 26 for assembling of seed plasmid 2 and pGETS302-SfiI (SEQ ID NO: 1) that is
10 a vector for gene assembling in equimolar amounts. The whole mixture was kept at a constant temperature of 37°C for 5 minutes, followed by adding 1 μ l of T4 DNA ligase (Takara) and keeping the mixture at a constant temperature of 37°C for 4 hours. 10 μ l thereof was collected and
15 subjected to electrophoresis to confirm ligation. Subsequently, 10 μ l thereof was collected in a new tube, 100 μ l of *Bacillus subtilis* competent cells were added thereto, and the mixture thereof was rotary-cultured by a duck rotor at 37°C for 30 minutes. Subsequently, 300 μ l of
20 LB medium was added, and the mixture thereof was rotary-cultured by a duck rotor at 37°C for 1 hour, followed by spreading the culture solution on an LB plate containing 10 μ g/ml of tetracycline and culturing overnight at 37°C. From the colonies, 100 transformants were obtained for both the
25 overexpression construct and the gene expression suppressing construct. The plasmid was extracted and the pattern of restriction enzyme cleavage was analyzed to select one transformant having a structure of interest (the seed plasmids of step (A) of Figure 4) for each of the
30 constructs.

[0059]

(7) High purity-purification of a seed plasmid

A plasmid DNA with high purity was supplied by a cesium chloride-ethidium bromide density-gradient
35 ultracentrifugation method. Specifically, 200 ml of an LB

medium supplemented with an antibiotic (tetracycline) was prepared, 100 ml of each thereof was placed in a 500 ml conical flask and cultured overnight at 37°C. After sufficient proliferation, 100 µl of 1M IPTG was added to
5 each flask to increase the copy number of the plasmid and the mixture thereof was further cultured for about 3 to 12 hours. After culture was complete, 50 ml of each resulting product was dispensed into four 50 ml tubes (Falcon 2070) and centrifuged at 5,000 rpm for 10 minutes. The
10 supernatant was disposed of, and the bacterial pellet was completely loosened by vortexing. 10 mg/ml of Sol.I solution containing lysozyme (composition: 50 mM glucose, 25 mM Tris-Cl (pH 8.0), and 10 mM EDTA) was prepared, and 2.5 ml of each solution was added to the four tubes
15 containing bacteria and mixed well. The resulting mixture was incubated at 37°C for 30 minutes. Centrifugation was performed at 5,000 rpm for 10 minutes, the supernatant was removed by decanting, 2.5 ml of Sol.I free of lysozyme was newly added to each of the four tubes, and the pellet was
20 uniformly suspended. Fresh Sol.II (composition: 0.2 N NaOH and 1% (w/v) sodium dodecyl sulfate) was prepared, 5 ml of each solution was added to the four tubes, and the mixture thereof was slowly mixed to make it transparent. 3.75 ml of Sol.III (composition: 60 ml 5M potassium acetate, 11.5 ml
25 glacial acetic acid, and 28.5 ml water) was added to each tube and mixed with strong force to a certain extent so that the white turbid substance could be uniformly dispersed. Centrifugation was performed at 5,000 rpm for 10 minutes, and the supernatant was aspirated with a pipette
30 and transferred to four new 50 ml tubes (Falcon 2070) with a screw cap. 5 ml of phenol/chloroform was added to each tube and mixed hard. Centrifugation was performed at 5,000 rpm for 10 minutes, and the supernatant was aspirated with a pipette and transferred to four new 50 ml tubes (Falcon
35 2070) with a screw cap. 25 ml of 100% ethanol was added to

each of the tubes and mixed, followed by centrifugation at 5,000 rpm for 10 minutes. The supernatant was removed. 2.5 ml of each solution (final concentration of 10 µg/ml) in which 10 µl of 10 mg/ml of RNaseA solution was added to 10
5 ml of TE was added to each tube and the precipitate was dissolved. The liquid in the four tubes was collected in one tube and incubated for 30 minutes with an incubator with a gas phase at 37°C. After incubation was complete, 5 ml of phenol/chloroform was added and mixed well, followed
10 by centrifugation at 5,000 rpm for 10 minutes. The supernatant was transferred to a new 50 ml tube, 1 ml of Sol.III was added thereto, and 25 ml of 100% ethanol was then added to the mixture and mixed. Subsequently, centrifugation was performed at 5,000 rpm for 10 minutes to
15 remove the supernatant. 5.4 ml of TE was added to the precipitate and completely dissolved. Next, precisely weighed 6.40 g of cesium chloride was placed therein and completely dissolved. Furthermore, 2.6 ml of 1.3 g/ml of cesium chloride solution (solution made by mixing 1.3 g
20 cesium chloride and 1 ml water, in which volumetric adjustment was not performed) was added. Finally, 600 µl of 10 mg/ml of ethidium bromide solution was added and mixed well. One ultracentrifugation tube (Beckman 362181) was prepared and the above mixture was transferred to the
25 ultracentrifugation tube. Water or 1.3 g/ml of cesium chloride solution (with a specific gravity of about 1.5 g/ml) was added to finely adjust the weight so that a difference in weight from the balance would be 20 mg or less. Centrifugation was performed with an
30 ultracentrifugation instrument (Beckman Coulter) under the following condition. Centrifugation was performed at a temperature of 18°C, a rate of 50,000 rpm, an acceleration of Max, and a deceleration of Max for 15 hours or more. After centrifugation was complete, a 1 ml syringe set with
35 a needle (21G × 5/8") was prepared and inserted into the

ccc-form plasmid band to collect the plasmid solution and transfer it to a 15 ml tube under observation with a ultraviolet ray (365 nm). 500 μ l of Sol.III was added thereto, followed by adding water so that the total volume was 3 ml. Furthermore, 9 ml of 100% ethanol was added. Centrifugation was performed at 5,000 rpm for 10 minutes to remove the supernatant. 700 μ l of TE was added to the obtained precipitate in the 15 ml tube and the DNA was dissolved. The resulting product was transferred to a 1.5 ml tube, 600 μ l of 1-butanol was added thereto and mixed, the mixture thereof was centrifuged at 15,000 rpm for about 10 seconds to separate the mixture into two layers, and the upper butanol layer was disposed of. 600 μ l of 1-butanol was newly added and mixed, the mixture thereof was centrifuged at 15,000 rpm for about 10 seconds to separate the mixture into two layers, and the upper butanol layer was disposed of. This operation was continued until the water layer was 450 μ l or less. 50 μ l of Sol.III was added and 900 μ l of 100% ethanol was further added. Centrifugation was performed at 15,000 rpm for 10 minutes. The supernatant was disposed of and the precipitate was rinsed with 70% ethanol. The precipitate was dissolved into 22 μ l of TE.

[0060]

(8) Production of a unit DNA from a seed plasmid

Preparation of a unit DNA of step (B) in Figure 4 was performed in the following manner. 300 ng of the seed plasmid purified to high purity by an ultracentrifugation method was fractionated and diluted to 40 μ l with sterile water, 5 μ l of 10 $\times$ NEBuffer#2 and 5 μ l of restriction enzyme SfiI (NEB) were then added, and the mixture thereof was caused to react at 37°C for 2 hours. 1 μ l of the reaction solution was subjected to electrophoresis to confirm cleavage. Subsequently, the reaction solutions of two seed plasmids were integrated, and 450 μ l of

phenol/chloroform/isoamyl alcohol (25:24:1) (Nacalai Tesque) was added thereto and mixed, followed by separating the mixture thereof into a phenol phase and an aqueous phase by centrifugation (20,000 × g, 10 minutes) and
5 collecting the aqueous phase (about 900 µl) in a new 1.5 ml tube. 500 µl of 1-butanol (Wako Pure Chemical Industries) was added thereto and mixed well, followed by separation by centrifugation (20,000× g, 1 minute) and removal of water-saturated 1-butanol. This operation was repeated until the
10 volume of the aqueous phase was 450 µl or less, thereby decreasing the volume of the aqueous phase. 50 µl of 3M potassium acetate-acetic acid buffer (pH 5.2) and 900 µl of ethanol were added thereto and centrifugation (20,000 × g, 10 minutes) was performed to precipitate the DNA, which was
15 then rinsed with 70% ethanol and dissolved into 20 µl of TE. [0061]

(9) Construction of a combinatorial library

Construction of a combinatorial library in step (C) of Figure 4 was performed in the following manner. The DNA
20 mixture solution obtained in (8) was assembled by the gene assembling method shown in (6) to obtain about 400 transformants. Colonies of 96 strains were randomly selected from the obtained transformants and cultured overnight in an LB medium containing 2 ml of 10 µg/ml of
25 tetracycline. IPTG was added to amplify the copy number of the inside plasmid so that the final concentration was 1 mM, and the mixture thereof was further cultured at 37°C for 3 hours. Plasmids were extracted from the obtained microbial
body. The direction of the gene of each of these extracted
30 plasmids was determined by the PCR method using a primer set shown in SEQ ID NOs: 27 to 62 (Figure 5). As a result, there were 75 clones having all elements of 12 genes, and a partial loss or an overlap of a unit DNA was found in 21 clones. There were 71 types of different combinations in 75
35 clones, and an overlap in types was found in 4 clones.

[0062]

(10) Introduction of a combinatorial library to yeast

96 combinatorial libraries obtained in (9) were introduced to yeast by using a lithium acetate (LiAc) method. Specifically, *S. cerevisiae* YPH499 strain that is a parent strain was inoculated onto 5 mL of YPDA medium (10 g/L of dried yeast extract [manufactured by Nacalai Tesque], 20 g/L of peptone [manufactured by Becton Dickinson (BD Difco)], 20g/L of glucose, and 40 mg/L of adenine sulfate) and cultured at 30°C and at 150 rpm overnight. The culture was centrifuged at 3,000 rpm for 5 minutes and the medium was disposed of, followed by suspending the microbial body pellet with 5 mL of sterile distilled water. Furthermore, centrifugation was performed at 3,000 rpm for 5 minutes, the supernatant was then disposed of, and the microbial body pellet was suspended in 1.5 mL of TE/LiAc solution (150 µL of 10 × TE, 150 µL of 10 × LiAc, and 1,200 µL of sterile distilled water). 100 µL of the microbial body suspension was transferred to a 1.5 mL Eppendorf tube, 1 to 5 µL of plasmid DNA (combinatorial library) and 2 µL of Carrier DNA [manufactured by Takara Bio (Clontech)] were added, 600 µL of TE/LiAc/PEG solution (60 µL of 10 × TE, 60 µL of 10 × LiAc, and 480 µL of 50% PEG3350 solution) was then added, and the mixture thereof was mixed by vortexing for 10 seconds. After the mixture solution was incubated at 30°C for 30 minutes, 70 µL of dimethyl sulfoxide (DMSO) was added and inverted and mixed, followed by further incubation at 42°C for 15 minutes. After centrifugation was performed at 14,000 rpm for 5 seconds, the supernatant was completely removed, 250 µL of 100 × amino acid stock solution free of L-leucine (4 g/L of adenine sulfate, 2 g/L of L-histidine, 4 g/L of L-tryptophan, 2 g/L of uracil, and 3 g/L of L-lysine) was added, the microbial body pellet was suspended, and 550 µL of sterile distilled water was added, followed by spreading the entire amount of the suspension

on an agar plate of an SD medium (6.7 g/L of yeast nitrogen base without amino acids (YNB) [manufactured by Becton Dickinson (BD Difco)] and 20 g/L of glucose) (20 g/L of agar powder was added to the medium) and dried, which was
5 then incubated at 30°C for 3 days to obtain a transformant. [0063]

(11) Evaluation of isobutanol productivity in budding yeast

The colony of the obtained yeast transformant was inoculated onto 5 mL of SD selective medium (SD medium
10 added with 100 × amino acid stock solution free of L-leucine) and cultured at 30°C and at 150 opm for 3 days. After centrifugation was performed at 3,000 rpm for 5 minutes and the medium was disposed of, the microbial body pellet was suspended in 5 mL of sterile distilled water.
15 After further centrifugation was performed at 3,000 rpm for 5 minutes and the supernatant was disposed of, the microbial body pellet was suspended in 5 mL of a new SD selective medium and cultured at 30°C and at 150 opm for 48 hours. After the culture was centrifuged at 3,000 rpm for 5
20 minutes, the supernatant was collected. 5100 µL of the collected medium supernatant was added to 45900 µL of acetone, the mixture thereof was mixed by vortexing and centrifuged at 12,000 rpm for 5 minutes, and the supernatant was then collected. The collected supernatant
25 was transferred to a glass vial, and the concentration of isobutanol contained in the medium was quantified by using a DB-FFAP column [manufactured by Agilent Technologies] with a gas chromatography mass spectrometer (GCMS QP2010 Ultra [manufactured by Shimadzu]). As a result, strains
30 showing various isobutanol productions represented by 146 mg/L of clone 96 were obtained as shown in Figure 5. Among them, strains with higher isobutanol production (29 mg/L and 15 mg/L, respectively) than the production of the yeast strains into which a seed plasmid for overexpression and a
35 seed plasmid for gene expression suppression were

introduced were obtained.

[0064]

(12) Selection of an excellent plasmid from a library and re-construction of a library

5 A new combinatorial library was constructed, with a plasmid used in transformation for clone 8, 42, 68, or 96 whose isobutanol production was 120 mg/ml or greater as a seed plasmid (Figure 6). First, a large quantity of *Bacillus subtilis* having the above-described plasmid was
10 cultured, the plasmid was extracted by the ultracentrifugation method shown in (7), and unit DNAs mixed in an equimolar state were prepared by the method shown in (8). Subsequently, the gene assembling shown in (6) was performed to construct a combinatorial library of
15 the 2nd cycle consisting of about 200 transformants. 24 colonies randomly selected from this library were subjected to extraction of the plasmid from *Bacillus subtilis*, the obtained plasmid was individually introduced to yeast by the method shown in (10), and the isobutanol production was
20 measured by the method shown in (11). The direction of the gene in each unit DNA was separately identified by the method described in (9) for the plasmid extracted from *Bacillus subtilis*. Figure 7 shows these results. Regarding the library, the 6th, 9th, 11th, and 12th unit DNAs, which
25 are common in the four seed plasmids, were common in 22 clones excluding two clones, i.e., clones 3 and 12 in which assembling was incomplete, and the rest of the unit DNAs generally reflected the composition ratio of the seed plasmids as expected. There were 19 types of different
30 combinations among 22 clones, and 2 types among them were identical to clones 68 and 96 of the first library that were seed plasmids. Clones 8, 4, 23, and 13 had isobutanol production of 173, 171, 169, 164 mg/l, respectively, wherein many clones showing higher productivity than the
35 highest value of 146 mg/L of the first library were

obtained.

[Industrial Applicability]

[0065]

According to the present invention, it is possible to
5 quickly and efficiently construct a long-chain DNA
combinatorial library. In particular, since the present
invention is able to reuse a plurality of plasmids selected
from the same library for construction of a new chimeric
library even without confirming the genotype of the
10 obtained plasmid, the present invention has a feature in
that it is possible to quickly construct the second and
subsequent libraries.

[Claims]

[Claim 1]

A preparation method of a DNA fragment for microbial cell transformation, the DNA fragment having at least one
5 insert DNA unit comprising: a DNA comprising a replication origin effective in a host microorganism; and an insert DNA in which unit DNAs are linked, characterized in that the method comprises:

(A) preparing a plurality of types of plasmids by OGAB
10 method, wherein the plasmids comprise an insert DNA unit in which a plurality of types of unit DNAs capable of being linked in a specific linking order are linked;

(B) processing the plurality of types of plasmids prepared in step (A) with a restriction enzyme suitable for
15 each plasmid to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture solutions; and

(C) re-assembling the unit DNAs by OGAB method using the plurality of types of unit DNA mixture solutions
20 obtained in step (B) to prepare a long-chain DNA fragment.

[Claim 2]

The preparation method of a DNA fragment for microbial cell transformation of claim 1, characterized in that all the ratios between molar concentrations for DNA fragments
25 in the unit DNA mixture solutions obtained in step (B) are 0.8 to 1.2.

[Claim 3]

The preparation method of a DNA fragment for microbial cell transformation of claim 1 or 2, characterized in that
30 in step (A), the number of types of unit DNAs comprised in one type of insert DNA unit is 3 to 60.

[Claim 4]

The preparation method of a DNA fragment for microbial cell transformation of any one of claims 1 to 3, wherein
35 the number of types of the restriction enzymes used in step

(B) is three or less.

[Claim 5]

The preparation method of a DNA fragment for microbial cell transformation of any one of claims 1 to 4, wherein
5 the restriction enzyme is a restriction enzyme that produces an overhang end.

[Claim 6]

A plasmid comprising a DNA fragment for microbial cell transformation obtained by the preparation method of any
10 one of claims 1 to 5.

[Claim 7]

A preparation method of a DNA fragment for microbial cell transformation, the DNA fragment having at least one insert DNA unit comprising: a DNA comprising a replication
15 origin effective in a host microorganism; and an insert DNA in which unit DNAs are linked, characterized in that the method comprises:

(B') processing the plurality of types of plasmids of claim 6 with a restriction enzyme suitable for each plasmid
20 to cleave the plasmids into unit DNAs and preparing a plurality of types of unit DNA mixture solutions; and

(C) re-assembling the unit DNAs by OGAB method using the unit DNA mixture solutions obtained in step (B') to prepare a long-chain DNA fragment.

25 [Claim 8]

The preparation method of a DNA fragment for microbial cell transformation of claim 7, characterized by selecting a plurality of types of plasmids comprising the obtained long-chain DNA fragment and reusing the plasmids as the
30 plasmids in step (B').

[Claim 9]

A method for constructing a chimeric plasmid library, using the preparation method of a DNA fragment for microbial cell transformation of any one of claims 1 to 5,
35 7, and 8.