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DESCRIPTION

ENZYME GENE PARTICIPATING IN THE SYNTHESIS OF POLYESTER AND PROCESS FOR PRODUCING POLYESTER USING THE SAME

TECHNICAL FIELD

The present invention relates to genes necessary for the enzymatic synthesis of polyesters, a microorganism fermentatively synthesizing polyesters utilizing the gene, and a method of producing polyesters using the microorganism. More particularly, it relates to genes capable of functioning in a host enzymatically synthesizing plastic-like polymers decomposable under the action of microorganisms in the natural environment (in soil, rivers and seas), a transformant resulting from transformation with the gene and improved in the capacity to fermentatively synthesize plastic-like polymers, and a method of producing polyesters utilizing the transformant.

BACKGROUND ART

At present, a large number of microorganisms are known to store polyester as an energy source substance within cells. A typical example of the polyester is poly-3-hydroxybutyric acid (hereinafter referred to briefly as P(3HB)), which is a homopolymer of 3-hydroxybutyric acid (hereinafter referred to as 3HB for short). It was first discovered in Bacillus megaterium (M. Lemoigne, Ann. Inst. Pasteur, 39, 144 (1925)). P(3BH) is a thermoplastic polymer and is biodegradable in the natural environment and, thus, has recently attracted attention as an ecofriendly plastic. However, P(3HB) is high in crystallinity, and hard and fragile by nature, so that the range of practical application thereof is limited. Therefore, studies have been undertaken to modify the same for bringing about improvements in these properties.

In Japanese Kokai Publication Sho-57-150393 and Japanese

Kokai Publication Sho-59-220192, among others, a technology of producing a copolymer made of 3-hydroxybutyric acid (3HB) and 3-hydroxyvaleric acid (3HV) (hereinafter such copolymer is referred to as P(3HB-co-3HV)) is disclosed. This P(3HB-co-3HV) is rich in flexibility as compared with P(3HB), hence was considered to have a wide application range. In actuality, however, P(3HB-co-3HV) shows only slight changes in physical properties even when the mole fraction of 3HV is increased. In particular, the flexibility, which is required for its use in films and the like, will not be improved. Thus, it has been used only in the field of rigid shaped articles such as shampoo bottles and disposable razor grips.

In recent years, studies have been made concerning copolyesters consisting of two components 3HB and 3-hydroxyhexanoic acid (hereinafter referred to as 3HH for short) (hereinafter such copolyesters are referred to as P(3HB-co-3HH) for short), as described in Japanese Kokai Publication Hei-05-93049 and Japanese Kokai Publication Hei-07-265065, among others. According to these publications, this technology of producing P(3HB-co-3HH) comprises fermentative production thereof from a fatty acid, such as oleic acid, or an oil or fat, such as olive oil, using Aeromonas caviae isolated from soil. Studies concerning the properties of P(3HB-co-3HH) have also been made (Y. Doi, S. Kitamura, H. Abe, Macromolecules, 28, 4822-4823 (1995)). According to this report, when A. caviae is cultured using a fatty acid containing not less than 12 carbon atoms as the only carbon source, P(3HB-co-3HH) with a 3HH content of 11 to 19 mole percent can be fermentatively produced. It has been revealed that the properties of such P(3HB-co-3HH) change from hard and brittle gradually to soft and flexible, to an extent exceeding the flexibility of P(3HB-co-3HV), with the increase in mole fraction of 3HH. However, the above method of production is low in productivity, namely the yield of cells is 4 g/L and the polymer content is 30%. Therefore, methods capable of

attaining higher productivity for practical use have been searched for.

A PHA (polyhydroxyalkanoic acid) synthase gene has been cloned from Aeromonas caviae, which is a producer of P(3HB-co-3HH) (T. Fukui, Y. Doi, J. Bacteriol., vol. 179, No. 15, 4821-4830 (1997); Japanese Kokai Publication Hei-10-108682). This gene was transformed into Ralstonia eutropha (formerly Alcaligenes eutrophus), and cultivation was carried out using the resulting transformant and a vegetable oil as the carbon source, whereby a content in cells of 4 g/L and a polymer content of 80% were attained (T. Hukui et al., Appl. Microbiol. Biotechnol., 49, 333 (1998)). A method of producing P(3HB-co-3HH) using a bacterial species, such as Escherichia coli, or a plant as the host has also been disclosed (WO 00/43525), without describing any productivity data, however.

The above-mentioned polymer P(3HB-co-3HH) can be given a wide range of physical properties, from properties of rigid polymers to properties of flexible polymers, by changing the mole fraction of 3HH and therefore can be expected to be applicable in a wide range, from television boxes and the like, for which rigidity is required, to yarns, films and the like, for which flexibility is required. However, the production methods mentioned above are still poor in the productivity of P(3HB-co-3HH). There is no other way but to say that they are still unsatisfactory as production methods for the practical use of P(3HB-co-3HH).

In a recent study of the production of biodegradable polyesters, Leaf et al. used yeast high in cell productivity as the host (Microbiology, vol. 142, pp. 1169-1180 (1996)). Thus, the polyester synthase gene of Ralstonia eutropha was transformed into Saccharomyces cerevisiae, a kind of yeast, the thus-produced transformant was cultured using glucose as the carbon source, and the accumulation of P(3HB) was confirmed (polymer content 0.5%). However, the polymer produced in this

study was that hard and brittle P(3HB).

Yeast is known to grow fast and be high in cell productivity. Among them, yeasts belonging to the genus Candida attracted attention as single cell proteins in the past and, since then, studies have been made on the production of cells thereof for use as feeds using normal-paraffins as carbon sources. Further, in recent years, vectors functioning in hosts belonging to the genus Candida have been developed, and the production of substances using the recombinant DNA technology has been reported (Kagaku to Seibutsu, vol. 38, No. 9, 614 (2000)). When Candida utilis is used as the host, the α-amylase productivity is as high as about 12.3 g/L. Microorganisms of the genus Candida having such high substance productivity are expected to serves as hosts in polymer production. Furthermore, cells thereof can be separated from the culture fluid with ease as compared with bacteria and, thus, the polymer extraction and purification steps can be facilitated.

Thus, the advent of a method of producing P(3HB-co-3HH) having good physical properties using yeast belonging to the genus Candida has been waited for.

SUMMARY OF THE INVENTION

In view of the above-mentioned state of the art, it is an object of the present invention to provide a gene involved in polyester synthesis which can be expressed in yeast in a functional and efficient manner, a yeast transformant transformed with a gene expression cassette comprising the gene, and a method of producing polyesters having biodegradability and good physical properties which comprises cultivating the transformant obtained in the above manner.

The present inventors made various investigations and, as a result, found that when novel genes, which are capable of being expressed in yeast, are produced by adding a peroxisome-targeting signal-encoding DNA to one or more genes

each coding for an enzyme involved in polyester synthesis, a gene expression cassette is constructed by joining a promoter and terminator capable of substantially functioning in yeast to each of those genes, the resulting gene expression cassette is further transformed into yeast and the resulting transformant is cultivated, a polyester can be produced and recovered from the culture in such a manner that very high productivity can be expected.

Thus, the present invention relates to a gene capable of being expressed in yeast,

which obtainable by addition of a DNA coding for a peroxisome-targeting signal to a gene coding for an enzyme involved in polyester synthesis.

In a preferred embodiment thereof, the invention relates to the gene as mentioned above

herein the enzyme involved in polyester synthesis is a bacterial species-derived enzyme,

more preferably from Aeromonas caviae and

to the gene as mentioned above

wherein at least one CTG codon in the gene coding for the bacterial species-derived enzyme has been modified into TTA, TTG, CTT, CTC or CTA.

In another preferred embodiment, the invention relates to a gene as mentioned above wherein the enzyme involved in polyester synthesis is a polyhydroxyalkanoic acid synthase or R-specific enoyl-CoA hydratase.

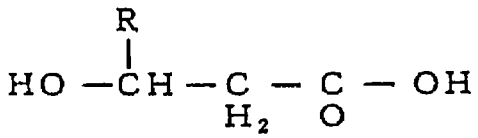
The present invention also relates to an enzyme involved in polyester synthesis,

which is translated from the above gene.

The present invention further relates to a transformant resulting from transformation of at least one gene above-mentioned into yeast. In a preferred embodiment, it relates to a transformant resulting from transformation, into yeast, of a polyester synthesis-associated enzyme gene expression cassette

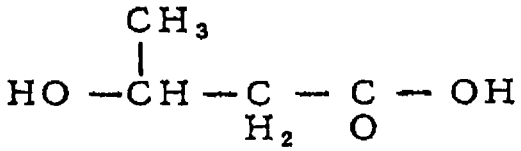
which comprises the above gene, a promoter and a terminator both capable of functioning in the yeast.

In another preferred embodiment, the invention relates to a transformant as defined above wherein the polyester is a homopolymer or copolymer of a 3-hydroxyalkanoic acid(s) represented by the general formula (1):



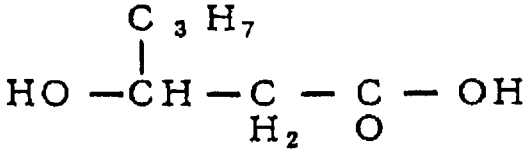
(1)

wherein R represents an alkyl group, and, more preferably to a transformant as defined above wherein the polyester is a copolyester, P(3HB-co-3HH), obtainable by represented by the formula (2):



(2)

and 3-hydroxyhexanoic acid represented by the formula (3):



(3)

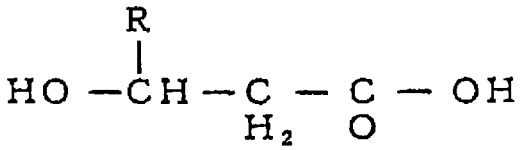
The present invention still further relates to a method of producing polyesters which comprises cultivating the above transformant and recovering a polyester from the culture obtained.

DETAILED DISCLOSURE OF THE INVENTION

In the following, the present invention is described in detail.

(1) Enzyme involved in polyester synthesis and gene coding for same

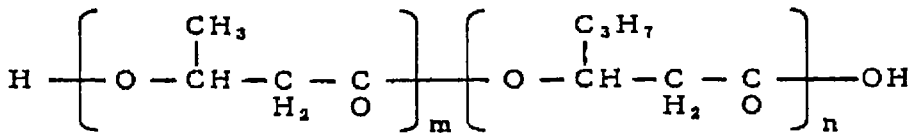
In the practice of the present invention, the polyester is not particularly restricted but preferably is a homopolymer or copolymer of a 3-hydroxyalkanoic acid(s) represented by the general formula (1):



(1)

wherein R represents an alkyl group. Thus, preferred are a homopolymer of one 3-hydroxyalkanoic acid represented by the formula (1) or a copolymer of two or more 3-hydroxyalkanoic acids represented by the formula (1).

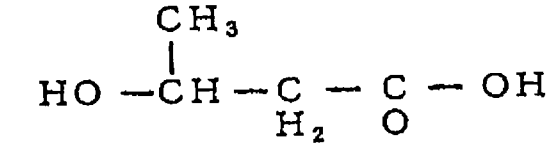
More preferred is a copolymer, P(3HB-co-3HH), represented by the formula (4):



(4)

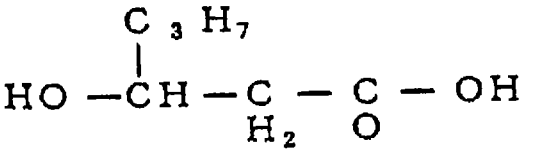
wherein m and n each represents an integer of not less

than 1, as obtainable by copolymerization of 3-hydroxybutyric acid represented by the formula (2):



(2)

and 3-hydroxyhexanoic acid represented by the formula (3):



(3)

In the above general formula (1), R represents an alkyl group and preferably contains 1 to 11 carbon atoms, more preferably 1 to 4 carbon atoms, still more preferably 1 to 3 carbon atoms.

The "enzyme involved in polyester synthesis" so referred to herein includes not only polyester synthase but also polyester intermediate synthase. As the polyester synthase, there may be mentioned, among others, polyhydroxyalkanoic acid (hereinafter referred to as PHA) synthase. As the polyester intermediate synthase, there may be mentioned, among others, R-specific enoyl-CoA hydratase (T. Fukui, et al., FEMS Microbiology Letters, vol. 170, 69-75 (1999)) which converts enoyl-CoA, an intermediate in the β oxidation cycle, to an (R)-3-hydroxyacyl-CoA, which is a monomer, β-ketothiolase and NADPH-dependent acetoacetyl-CoA reductase (Peoples OP, et al., J. Biol. Chem., 264 (26), 15298-15303 (1989)) which dimerize acetyl-CoA and then reduce acetoacetyl-CoA to 3-hydroxybutyryl-CoA, which is a monomer, 3-ketoacyl-CoA reductase (fabG), (Qun Ren, et al., J. Bacteriol., vol. 182, No. 10, pp. 2978-2981), 3-hydroxyacyl-CoA epimerase (fadB),

acyl-CoA dehydrogenase (Qun Ren, et al., Applied and Environmental Microbiology, Vol. 66, No. 4, pp. 1311-1320), etc.

Those enzymes involved in polyester synthesis are not particularly restricted but preferably are enzymes of bacterial origin, more preferably enzymes derived from Aeromonas caviae or Ralstonia eutropha. Thus, for example, enzymes described in Japanese Kokai Publication Hei-10-108682 can be used.

When yeast is transformed with a gene coding for such an enzyme of bacterial origin, the use of the bacterial gene as it is may lead to occurrence, in some host yeasts, of some abnormality in translation of genetic codons in some instances. For example, CTG codon is translated into serine, not into leucine, in the yeast Candida cylindracea (Y. Kawaguchi et al., Nature, 341, 164-166 (1989)) or Candida maltosa (H. Sugiyama et al., Yeast, 11, 43-52 (1995)). When a gene coding for an enzyme involved in polyester synthesis as derived from an organism other than yeasts is expressed in such a yeast, abnormality may occur in translation of a genetic codon, with the result that an enzyme differing in amino acid sequence from the desired enzyme may be produced. Thus, there is the possibility that the enzyme expressed cannot fully carry out the function of the desired enzyme.

Such problem can be solved by using a modified gene derived from the gene in question by converting CTG codon contained in the latter gene to another codon corresponding to leucine (TTA, TTG, CTT, CTC, or CTA) in advance. The leucine codon to be used in substitution for CTG is not restricted but, in view of the efficiency of translation of the gene to be transformed, it is preferably a genetic codon usage of which is high in the host yeast into which the gene is to be transformed. In Candida maltosa, for instance, it is desirable that CTG codon be converted to TTA or TTG, most preferably to TTG.

The codon usage constituting a gene varies from organism to organism. Thus, for amino acids other than tryptophan and

methionine, there is a plurality of genetic codons corresponding to each amino acid but these differ in codon usage in an organism-dependent manner. In Candida maltosa, the genetic codons corresponding to alanine, for instance, are GCT, GCC, GCA and GCG and, among them, GCT is the genetic codon used most frequently in constituting a gene. In this way, which genetic codon is used among a plurality of genetic codons designating one and the same amino acid may differ from organism to organism. It has been pointed out that the efficiency of translation of a gene is high when the gene is composed of genetic codons with high codon usage. The GC contents of Aeromonas caviae-derived PHA synthase gene and R-specific enoyl-CoA hydratase gene, for instance, are 67.16% and 65.77%, respectively, whereas, for the Candida maltosa-derived enzymes so far reported, the GC content of phosphoglycerate kinase is 39.55% and the GC content of P450 (ALK2-A) is 35.67%.

Thus, the GC content in a gene varies as a result of the deviability of codon usage. Therefore, for attaining efficient expression of a gene involved in polyester synthesis in Candida maltosa, for instance, it is preferred that the relevant gene be modified not only by modification of CTG codon into another genetic codon corresponding to leucine, as mentioned above, but also by modification of some or other genetic codon (s) into one(s) with high codon usage in Candida maltosa.

Codon usage in Candida maltosa is described in Klaus Wolf (ed.): Nonconventional Yeasts in Biotechnology (published by Springer). Thus, GCT is preferred as the genetic codon for alanine, AGA for arginine, AAC or AAT for asparagine, GAT for aspartic acid, TGT for cysteine, GGT for glycine, CAA for glutamine, GAA for glutamic acid, CAC or CAT for histidine, ATT for isoleucine, TTG or TTA for leucine, AAA for lysine, TTC or TTT for phenylalanine, CCA for proline, TCT for serine, ACT for threonine, TAT or TAC for tyrosine, and GTT for valine. The genetic codons to be employed are not particularly restricted

to these, however.

For example, when Candida maltosa is used as a host, the polyhydroxyalkanoic acid (PHA) synthase gene shown under SEQ ID NO:5, and the R-specific enoyl-CoA hydratase gene shown under SEQ ID NO:6 can be utilized as the genes for enzymes involved in polyester synthesis in the practice of the present invention.

As for the nucleotide sequences of these genes represented by SEQ ID NO:5 or 6, one or a plurality of nucleotides may have undergone mutation, such as deletion, substitution and/or addition, in their nucleotide sequences provided that they can provide substantial enzyme activities.

In this description, the expression "the nucleotide sequences in which one or a plurality of nucleotides may have undergone mutation, such as deletion, substitution and/or addition" means the nucleotide sequences with deletion, substitution, and/or addion, wherein the number of the nucleotide is in the extent of capable of being deleted, substituted, and/or added by the known method in the prior art, such as "Protein-Nucleic acid- Enzyme, a special issue, gene-amplifying PCR method TAKKAJ 35(17), 2951-3178(1990)", or "PCR technology(1990), edited by Henry A. Erlich, translated by Ikunoshin Kato", etc.

The DNA comprising the above nucleotide sequence may be the DNA capable of hybridizing with DNA, which comprises the nucleotide sequence of SEQ ID NO:5 or 6, in a stringent condition, provided that they can provide substantial enzyme activities.

"The DNA capable of hybridizing with DNA, which comprises the nucleotide sequence of SEQ ID NO:5 or 6, in a stringent condition" referred to herein means the DNA obtainable by colony hybridization method, plaque hybridization method, Southern hybridization method, among others, using a DNA comprising the nucleotide sequence of SEQ ID NO:5 or 6 as a probe. Those skilled in the art may easily obtain the desired DNA by carrying out said hybridization according to the method described in Molecular Cloning 2nd Edt. (Cold Spring Harbor Laboratory Press,

1989).

(2) Genes capable of being expressed in yeast, added a DNA coding for a peroxisome targeting signal to a gene coding for an enzyme involved in polyester synthesis

A characteristic feature of the present invention is that genes capable of being expressed in yeast, added a DNA coding for a peroxisome targeting signal to such a gene coding for an enzyme involved in polyester synthesis as mentioned above is utilized.

In fermentative production of a polyester using yeast, carbohydrates, fats and oils, fatty acids, n-paraffins and the like, which can be utilized by the yeast, can be used as the carbon sources, without any particular restriction. In fermentative polyester production using fats and oils, fatty acids, n-paraffins or the like as carbon sources, these carbon sources are metabolized via the β oxidation cycle, and the metabolic intermediates in the β oxidation cycle are utilized as substrates for polyester synthesis with good efficiency (T. Fukui, Y. Doi, J. Bacteriol., 179, No. 15, 4821-4830 (1997); Q. Ren et al., J. Bacteriol., 182, No. 10, 2978-2981 (2000)). Since the β oxidation in yeast is carried out in peroxisomes, which are intracellular microbodies, localization, in peroxisomes, of the enzyme(s) involved in polyester synthesis is favorable for efficient polyester synthesis.

The proteins to be transferred to peroxisomes are synthesized on free ribosomes and, owing to the function of a peroxisome-targeting signal occurring in the protein sequences, they are transferred to peroxisomes (S. Subramani, J. Membrane Biol., 125, 99-106 (1992); Y. Itai, Kagaku to Seibutsu 35, No. 10, 687-695 (1997); E. H. Hettema, Biochim. Biophys. Acta, 1451, 17-34 (1999)). Therefore, by adding a DNA coding for such peroxisome-targeting signal to a gene coding for an enzyme involved in polyester synthesis, it becomes possible to localize the enzyme involved in polyester synthesis in peroxisomes for efficient polyester synthesis.

In plants, targeting of the PHA synthase gene toward peroxisomes results is reported (WO 99/35278).

Known as peroxisome-targeting signals occurring at the carboxyl terminus are sequences comprising three amino acid residues, namely "(serine/alanine/cysteine)-(lysine/arginine/histidine)-leucine". The expression (serine/alanine/cysteine), for instance, as used herein means any one of serine, alanine and cysteine. For the targeting of an enzyme involved in polyester synthesis toward peroxisomes, the addition of the above three-amino-acid sequence to the carboxyl terminus of the enzyme is sufficient. Among the peroxisome-targeting carboxyl-terminal signals, the most commonly known one, namely "serine-lysine-leucine" (hereinafter referred to as SKL for short) (SEQ ID NO:1) or the sequence "alanine-lysine-isoleucine" (hereinafter referred to as AKI for short) (SEQ ID NO:2), which is known as the peroxisome-targeting carboxyl-terminal signal in Candida tropicalis (J. D. Aitchison et al., J. Biol. Chem., 266, 23197-23203 (1991)), is preferably added to the carboxyl terminus.

The nucleotide sequence corresponding to such amino acid sequence is not particularly restricted. In the case of SKL, the nucleotide sequence shown under SEQ ID NO:3 can be utilized and, in the case of AKI, that shown under SEQ ID NO:4 can be used.

Further, sequences occurring in the vicinity of the N terminus and comprising 9 amino acid residues, namely "(arginine/lysine)-(leucine/valine/isoleucine)-(5 amino acid residues)-(histidine/glutamine)-(leucine/alanine)", are also known as peroxisome-targeting signals. By inserting and adding these sequences into an enzyme involved in polyester synthesis, it is possible to cause localization of the enzyme in peroxisomes.

The novel gene resulting from addition of a DNA coding

for such a peroxisome-targeting signal as mentioned above to a gene coding for an enzyme involved in polyester synthesis can be constructed by chemical synthesis or by the PCR method, for instance. In the following, a method of adding SKL to the carboxyl terminus of the PHA synthase gene shown under SEQ ID NO:5 or the R-specific enoyl-CoA hydratase gene shown under SEQ ID NO:6, each as a gene for an enzyme involved in polyester synthesis, by PCR is described as an example.

A novel gene (hereinafter referred to as ORF2S for short) (SEQ ID NO:7) resulting from addition of a DNA coding for a peroxisome-targeting signal to the PHA synthase gene can be constructed by carrying out PCR using, as primers for PCR, oligonucleotides shown under SEQ ID NO:11 and SEQ ID NO:12 and, as a template, the PHA synthase gene shown under SEQ ID NO:5. In the same manner, a novel gene (hereinafter referred to as ORF3S for short) (SEQ ID NO:9) resulting from addition of a DNA coding for a peroxisome-targeting signal to the R-specific enoyl-CoA hydratase gene can be constructed by carrying out PCR using, as primers for PCR, oligonucleotides shown under SEQ ID NO:14 and SEQ ID NO:15 and, as a template, the R-specific enoyl-CoA hydratase gene shown under SEQ ID NO:6. The PCR conditions to be used here may be arbitrary provided that the target gene fragments can be amplified.

In the same manner as mentioned above, a DNA coding for AKI can be added to the carboxyl terminus of the PHA synthase gene or R-specific enoyl-CoA hydratase gene. By carrying out PCR using primers shown under SEQ ID NO:11 and SEQ ID NO:13 and the PHA synthase gene as a template, it is possible to construct a novel gene (hereinafter referred to as ORF2A) (SEQ ID NO:8). In the same way, by carrying PCR using primers shown under SEQ ID NO:14 and SEQ ID NO:16 and the R-specific enoyl-CoA hydratase gene as a template, it is possible to construct a novel gene (hereinafter referred to as ORF3A) (SEQ ID NO:10).

The nucleotide sequences of the above-mentioned novel genes shown under SEQ ID NO: 7 to SEQ ID NO:10 may have undergone

deletion, substitution and/or addition of one or a plurality of nucleotides respectively provided that the corresponding enzymes have substantial polyester synthesizing activity. The DNAs comprising the above nucleotide sequences may respectively be the DNAs capable of hybridizing with DNA, which comprises the sequences illustrated above as SEQ ID, in a stringent condition, provided that they can provide substantial enzyme activities.

The gene resulting from addition of a DNA coding a peroxisome-targeting signal to the genes coding for the above enzyme involved in polyester synthesis is a gene capable of being expressed in yeast. The "yeast" so referred to herein is not particularly restricted. Thus, usable are kinds of yeast deposited with organism depositories (e.g. IFO, ATCC, etc.) and belonging to such genera as Aciculoconidium, Ambrosiozyma, Arthroascus, Arxiozyma, Ashbya, Babjevia, Bensingtonia, Botryoascus, Botryozyma, Brettanomyces, Bullera, Bulleromyces, Candida, Citeromyces, Clavispora, Cryptococcus, Cystofilobasidium, Debaryomyces, Dekkera, Dipodascopsis, Dipodascus, Eeniella, Endomycopsella, Eremascus, Eremothecium, Erythrobasidium, Fellomyces, Filobasidium, Galactomyces, Geotrichum, Guilliermondella, Hanseniaspora, Hansenula, Hasegawaea, Holtermannia, Hormoascus, Hyphopichia, Issatchenkia, Kloeckera, Kloeckeraspora, Kluyveromyces, Kondoa, Kuraishia, Kurtzmanomyces, Leucosporidium, Lipomyces, Lodderomyces, Malassezia, Metschnikowia, Mrakia, Myxozyma, Nadsonia, Nakazawaea, Nematospora, Ogataea, Oosporidium, Pachysolen, Phachytichospora, Phaffia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Saitoella, Sakaguchia, Saturnospora, Schizoblastosporion, Schizosaccharomyces, Schwanniomyces, Sporidiobolus, Sporobolomyces, Sporopachydermia, Stephanoascus, Sterigmatomyces, Sterigmatosporidium, Symbiotaphrina, Sympodiomyces, Sympodiomycopsis, Torulaspora, Trichosporiella, Trichosporon, Trigonopsis, Tsuchiyaea,

Udeniomyces, Waltomyces, Wickerhamia, Wickerhamiella, Williopsis, Yamadazyma, Yarrowia, Zygoascus, Zygosaccharomyces, Zygowilliopsis, and Zygozyma.

Among the yeast described above, in view of high proliferation potency when fat and oils are used as carbon sources, high safety of strains, and the fact that separation of cells from culture fluid is relatively easy, preferably is one belonging to the genus Candida or Yarrowia, more preferably Candida maltosa or Yarrowia lipolytica, still more preferably Candida maltosa.

From the gene resulting from addition of a DNA coding for a peroxisome-targeting signal to the genes coding for an enzyme involved in polyester synthesis, there is translated the enzyme involved in polyester synthesis.

(3) Gene expression cassette construction

For the above gene resulting from addition of a DNA coding for a peroxisome-targeting signal to a gene coding for an enzyme involved in polyester synthesis to be expressed in yeast, it is necessary that such DNA sequences as a promoter, UAS, occur on the 5′ upstream of the gene and such DNA sequences as poly(A) additional signal, terminator, etc. on the 3′ downstream of the gene. When an appropriate site satisfying the above conditions occurs on a yeast chromosome, the gene in question may be directly inserted therein. Alternatively, the gene may be inserted into a plasmid having an appropriate promoter and terminator so that yeast may be transformed with the resulting plasmid. In the practice of the present invention, a gene expression cassette is preferably constructed by joining a promoter to the gene on the 5′ upstream thereof and a terminator on the 3′ downstream thereof so that the cassette may be used in transforming yeast.

Any promoter and terminator sequences may be used provided that they can function in yeast. While, among the promoters, there are ones causing constitutive expression and ones causing inducible expression, either type of promoter may

be used. In the practice of the present invention, it is desirable that the promoter and terminator can function in Candida maltosa, hence the promoter and terminator be derived from Candida maltosa. More preferably, the Candida maltosa ALK1, ALK5 or POX2 gene-derived promoter and the ALK1 gene-derived terminator are utilized.

Thus, for example, the promoter ALK1p (SEQ ID NO:17) of the Candida maltosa ALK1 gene (GenBank D00481), the promoter ALK5p (SEQ ID NO:18) of the ALK5 gene, and the promoter POX2p (SEQ ID NO:19) of the POX2 gene (GenBank D21228), among others, can be used as the promoter. The terminator ALK1t (SEQ ID NO:20) of the Candida maltosa ALK1 gene and the like can be used as the terminator. The nucleotide sequences of the above promoters and terminators each may have undergone deletion, substitution and/or addition of one or more nucleotides provided that they can function in Candida maltosa. The DNA comprising the above nucleotide sequences may respectively be the DNA capable of hybridizing in a stringent condition with DNA containing the sequences illustrated above as SEQ ID, provided that they can function in Candida maltosa.

The promoter is ligated to the 5′ upstream of the gene coding for an enzyme involved in polyester synthesis with an added DNA coding for a peroxisome-targeting signal, and the terminator is ligated to the 3′ downstream of the gene coding for the enzyme involved in polyester synthesis with the added peroxisome-targeting signal-encoding DNA.

The vector to be used in constructing the gene expression cassette may be any of those capable of autonomous replication in Escherichia coli. It may further have a region capable of autonomous replication in yeast. The vector capable of autonomous replication in yeast is maintained in microbial cells. It is also possible to integrate the gene expression cassette into a chromosome. As an example, pUTU1 capable of autonomous replication in Candida maltosa can be used (M. Ohkuma, et al., J. Biol. Chem., vol. 273, 3948-3953 (1998)).

Appropriate restriction enzyme sites for joining the promoter and terminator to the structural gene can be formed by utilizing the PCR method. The primer sequences to be used in PCR are not particularly restricted but, for example, the sequences shown under SEQ ID NO:21 to SEQ ID NO:28 can be used. The PCR conditions are arbitrary provided that the desired gene fragments can be amplified.

Although the method of constructing the gene expression cassette according to the present invention is not particularly restricted, a construction method in which the above-mentioned two genes, namely ORF2S and ORF3S, are used as the genes resulting from addition of a DNA sequence coding for a peroxisome-targeting signal to a gene coding for an enzyme involved in polyester synthesis is described below as an example.

(a) The case of using ALK1p, ALK5p and ALK1t

As for the promoter region, ALK1p whose 5′ terminus is PvuII and 3′ terminus is NdeI can be prepared using the ALK1 gene as a template, together with primers shown under SEQ ID NO:21 and SEQ ID NO:22. As for the terminator region, ALK1t whose 5′ end is HindIII and 3′ end is EcoRV can be prepared using the ALK1 gene as a template, together with primers shown under SEQ ID NO:27 and SEQ ID NO:28. Usable as the vectors are the vector pUTA1 (Fig. 1) constructed by using pUTU1 and the Candida maltosa ADE1 gene (SEQ ID NO:29) (GenBank D00855) (S. Kawai, et al., Agric. Biol. Chem., vol. 55, 59-65 (1991)) while changing the marker gene from uracil to adenine, and pUCNT (WO 94/03613).

ALK1p is joined to pUCNT at the PvuII-NdeI site to construct pUCNT-ALK1p, and ALK1t is ligated to pUCNT at the HindIII-EcoRV site to construct pUCNT-ALK1t. Then, ALK1p is excised from pUCNT-ALK1p with PvuII and NdeI, and joined to pUCNT-ALK1t at the PvuII-NdeI site. Thus, pUAL1 (Fig. 2) can be constructed.

Then, ORF2S is joined to pUAL1 at the NdeI-PstI site.

Thus, a plasmid, pUAL-ORF2S (Fig. 3), can be constructed.

Further, ORF2S is excised, together with the upstream promoter and downstream terminator, from the plasmid pUAL-ORF2S using EcoT22I, followed by joining to pUTA1 at the PstI site, whereby pHA2S (Fig. 4) can be constructed.

The above construction scheme is summarized in Fig. 8 and Fig. 9. The symbol "*1" in Fig. 8 or 9 represents that this is where Fig. 8 or 9 are connected.

On the other hand, ALK5p (SEQ ID NO:18) whose 5' end is PvuII and 3' end is NdeI can be prepared using the Candida maltosa ALK5 gene (GenBank D12717) as a template, together with primers shown under SEQ ID NO:23 and SEQ ID NO:24. This DNA fragment is substituted for ALK1p in pUAL1, whereby pUAL5 can be constructed. The ALK5 promoter and ALK1 terminator are excised from this plasmid using PvuI and PvuII and joined to the commercial vector pSTV28 at the SmaI-PvuI site, whereby pSTAL5 can be constructed. ORF3S is joined to this plasmid at the NdeI-PstI site. Thus, pSTAL5-ORF3S (Fig. 5) can be constructed.

Further, ORF3S is excised, together with the upstream promoter and downstream terminator, from pSTAL5-ORF3S using SalI, followed by ligating to pHA2S at the SalI site, whereby a plasmid, pHA23S2 (Fig. 6), can be constructed.

The above construction scheme is summarized in Fig. 10 and Fig. 11. The symbol "*2" in Fig. 10 or 11 represents that this is where Fig. 10 or 11 are connected.

(b) The case of using ALK1p, POX2p and ALK1t

POX2p (SEQ ID NO:19) whose 5' end is PvuII and 3' end is NdeI can be prepared using the Candida maltosa POX2 gene (GenBank D21228) as a template, together with primers shown under SEQ ID NO:25 and SEQ ID NO:26. This DNA fragment is substituted for the lac promoter in pUCNT-ORF3St, whereby pPOX2-ORF3S can be constructed. ORF3S is excised, together with the upstream promoter and downstream terminator, from pPOX2-ORF3S using SalI, whereby plasmid pHA23S1 (Fig. 7), which

is joined to pHA2S at the SalI site, can be constructed. The above construction scheme is summarized in Fig. 12.

In the above manner, the gene expression cassette for the production of a polyester resulting from homopolymerization or copolymerization of a 3-hydroxyalkanoic acid(s) represented by the general formula (1) in the yeast Candida maltosa can be constructed.

(4) Host

The "yeast" so referred to herein is not particularly restricted. Thus, usable are yeasts deposited with organism depositories (e.g. IFO, ATCC, etc.) and belonging to such genera as Aciculoconidium, Ambrosiozyma, Arthroascus, Arxiozyma, Ashbya, Babjevia, Bensingtonia, Botryoascus, Botryozyma, Brettanomyces, Bullera, Bulleromyces, Candida, Citeromyces, Clavispora, Cryptococcus, Cystofilobasidium, Debaryomyces, Dekkera, Dipodascopsis, Dipodascus, Eeniella, Endomycopsella, Eremascus, Eremothecium, Erythrobasidium, Fellomyces, Filobasidium, Galactomyces, Geotrichum, Guilliermondella, Hanseniaspora, Hansenula, Hasegawaea, Holtermannia, Hormoascus, Hyphopichia, Issatchenkia, Kloeckera, Kloeckeraspora, Kluyveromyces, Kondoa, Kuraishia, Kurtzmanomyces, Leucosporidium, Lipomyces, Lodderomyces, Malassezia, Metschnikowia, Mrakia, Myxozyma, Nadsonia, Nakazawaea, Nematospora, Ogataea, Oosporidium, Pachysolen, Phachytichospora, Phaffia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Saitoella, Sakaguchia, Saturnospora, Schizoblastosporion, Schizosaccharomyces, Schwanniomyces, Sporidiobolus, Sporobolomyces, Sporopachydermia, Stephanoascus, Sterigmatomyces, Sterigmatosporidium, Symbiotaphrina, Sympodiomyces, Sympodiomycopsis, Torulaspora, Trichosporiella, Trichosporon, Trigonopsis, Tsuchiyaea, Udeniomyces, Waltomyces, Wickerhamia, Wickerhamiella, Williopsis, Yamadazyma, Yarrowia, Zygoascus, Zygosaccharomyces, Zygowilliopsis, and Zygozyma.

The yeast to be used as the transformant host in the practice of the present invention is not particularly restricted but, in view of high proliferation potency when fat and oils are used as carbon sources, high safety of strains, and the fact that separation of cells from culture fluid is relatively easy, preferably is one belonging to the genus Candida or Yarrowia, more preferably Candida maltosa or Yarrowia lipolytica, still more preferably Candida maltosa. Among the yeasts usable as hosts, the Candida maltosa AC16 strain has been internationally deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary, AIST Tsukuba Central 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan, as of November 15, 2000 under the accession number FERM BP-7366.

(5) Transformant preparation

The transformant according to the present invention is yeast with one or more kind of genes, each resulting from addition of a DNA coding for a peroxisome-targeting signal to the gene coding for the above-mentioned enzyme involved in polyester synthesis, as introduced therein. It is preferred that one or more kind of enzyme gene expression cassettes for the production of enzyme(s) involved in polyester synthesis, which cassettes each comprises the above gene, a promoter and a terminator both capable of functioning in the yeast, are introduced in the yeast.

The gene expression cassette recombinant vector involved in polymer synthesis can be introduced into yeast in the conventional manner, using, for example, the calcium phosphate method (Lederberg, E. M. et al., J. Bacteriol., 119, 1072 (1974)) or the electroporation method (Current Protocols in Molecular Biology, vol. 1, 1.8, p. 4 (1994)). Commercially available transformation kits such as Fast Track TM-Yeast Transformation Kit SM (Geno Technology) can also be utilized.

As an example, the Candida maltosa CHA1 strain (S. Kawai, et al., Agric. Biol. Chem., vol. 55, 59-65 (1991)) can be used

as the host. By transforming this strain using the gene expression cassette involved in polymer synthesis by the transformation method mentioned above, it is possible to produce Candida maltosa transformants having the above-mentioned plasmid pHA2S or pHA23S2, for instance.

Among such transformants, the Candida maltosa AC16(pHA23S1) strain obtainable by transformation of the plasmid pHA23S1 has been internationally deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary, AIST Tsukuba Central No. 6, 1-1-1 Higashi, Tsukuba, Ibaraki, Japan, as of October 3, 2001 under the accession number FERM BP-7762, and the Candida maltosa AC16(pHA23S2) obtainable by introduction of the plasmid pHA23S2 as of the same date under the accession number FERM BP-7763.

(6) Polyester production

In accordance with the polyester production method of the present invention, a polyester is recovered from the culture obtainable by cultivating the above-mentioned transformant of the present invention.

Thus, the polyester production according to the present invention can be carried out by adding the above transformant to a culture medium, and cultivating the same, followed by recovering the product polyester from the cultured cells or culture obtained. The cultivation temperature is within a temperature range in which the organism can grow, preferably 15°C to 40°C, more preferably 20°C to 40°C, still more preferably 28°C to 34°C. The cultivation time is not particularly restricted but, for example in the case of batch cultivation, preferably is about 1 to 7 days, and continuous cultivation also can be carried out.

The culture medium is not particularly restricted provided that the yeast can utilize the medium. Thus, for example, media containing a carbon source(s), a nitrogen source(s), inorganic salts, and other organic nutrient sources

can be used.

The carbon source is not particularly restricted provided that the yeast can assimilate it. Thus, for example, carbohydrates, fats and oils, fatty acids, n-paraffins and the like can be used. As the carbohydrates, there may be mentioned, for example, glucose, sucrose, glycerol, etc. As the fats and oils, there may be mentioned, for example, rapeseed oil, coconut oil, palm oil, palm kernel oil, etc. As the fatty acids, there may be mentioned, for example, hexanoic acid, octanoic acid, decanoic acid, lauric acid, oleic acid, palmitic acid, linolic acid, linolenic acid, myristic acid, and like saturated and unsaturated acids, as well as esters and salts of these fatty acids and other fatty acid derivatives. As the n-paraffins, there may be mentioned, for example, dodecane, tetradecane, etc.

When the promoter expression is of the inducible type, an appropriate inducer (e.g. alcohol) is to be added. In some instances, the inducer may serve as the main carbon source.

As an example, Candida maltosa can be cultivated using fats or oils as carbon sources. In the case of yeast, which cannot assimilate fats or oils efficiently or at all, improvements can be achieved by adding lipase to the medium. Furthermore, yeast can be provided with the ability to assimilate fats and oils by transformation with a lipase gene.

Further, the proportion of a component(s) having a odd number of carbon atoms in the polyester carbon chain resulting from homopolymerization or copolymerization of 3-hydroxyalkalnoic acids represented by the general formula (1) can be increased by using, as carbon sources, fatty acids or n-paraffins whose carbon chain has an odd number of carbon atoms.

As the nitrogen source, there may be mentioned, for example, ammonia, ammonium chloride, di-ammonium sulfate, di-ammonium hydrogenphosphate, and other ammonium salts, as well as peptone, meat extract, yeast extract, and the like.

As the inorganic salts, there may be mentioned, among others, potassium dihydrogenphosphate, di-potassium hydrogenphosphate, magnesium hydrogenphosphate, magnesium sulfate, and sodium chloride.

The other organic nutrient sources include, among others, amino acids such as glycine, alanine, serine, threonine and proline; and vitamins such as vitamin B1, vitamin B12, biotin, nicotinamide, pantothenic acid, and vitamin C.

In the practice of the present invention, the polyester is recovered from yeast cells using the following method, for instance. After completion of cultivation, cells are separated from the culture fluid using a centrifuge, for instance, and the cells are washed with distilled water and methanol or the like, and then dried. The polyester is extracted from these dried cells using an organic solvent such as chloroform. The cell fraction is removed from the organic solvent solution containing the polyester by filtration, for instance. A poor solvent, such as methanol or hexane, is added to the filtrate to cause the polyester to precipitate out. The precipitate polyester is separated from the supernatant by filtration or centrifugation, and dried. The polyester can be thus recovered.

Since yeast cells are used as polyester producer cells in accordance with the present invention, such simple and easy methods of separation and recovery as mentioned above can be utilized.

The polyester obtained is analyzed by gas chromatography and/or nuclear magnetic resonance spectrometry, for instance.

The polyester production method according to the present invention has the constitution mentioned above and, therefore, can produce polyesters, which are homopolymers or copolymers of a 3-hydroxyalklanoic acid(s) represented by the general formula (1), with good productivity.

Further, by producing, using the above-mentioned plasmid pHA2S or the like, a transformant of Candida maltosa which has

a gene resulting from addition of a DNA coding for a peroxisome-targeting signal to a gene coding for an enzyme involved in polyester synthesis, together with a promoter and terminator both capable of functioning in yeast, and cultivating the same, the copolyester P(3HB-co-3HH), which results from copolymerization of 3-hydroxybutyric acid represented by the formula (2) given hereinabove and 3-hydroxyhexanoic acid represented by the formula (3) given above, can be produced.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic diagram of the plasmid pUTA1 used as a vector as described in the example section.

Fig. 2 is a schematic diagram of the plasmid pUAL1 constructed as described in the example section.

Fig. 3 is a schematic diagram of the plasmids pUAL-ORF2S and pUAL-ORF2A constructed as described in the example section.

Fig. 4 is a schematic diagram of the plasmids pHA2S and pHA2A constructed as described in the example section.

Fig. 5 is a schematic diagram of the plasmid pSTAL5-ORF3S constructed as described in the example section.

Fig. 6 is a schematic diagram of the plasmid pHA23S2 constructed as described in the example section.

Fig. 7 is a schematic diagram of the plasmid pHA23S1 constructed as described in the example section.

Fig. 8 and Fig. 9 show a plasmid construction scheme illustrating the methods of construction of the plasmids pHA2S and pHA2A for use in producing transformants of the present invention.

Fig. 10 and Fig. 11 show a plasmid construction scheme illustrating the method of construction of the plasmid pHA23S2 for use in producing transformants of the present invention.

Fig. 12 shows a plasmid construction scheme illustrating the method of construction of the plasmid pHA23S1 for use in producing transformants of the present invention.

BEST MODE FOR CARRYING OUT THE INVENTION

The following examples illustrate the present invention more specifically. These examples are, however, by no means limitative of the technical scope of the present invention.

(Example 1) Designing and synthesis of genes involved in polyester synthesis

Based on the amino acid sequences of Aeromonas caviae-derived PHA synthase and R-specific enoyl-CoA hydratase (T. Fukui, et al., FEMS Microbiology Letters, vol. 170, 69-75 (1999)), which converts the β oxidation cycle intermediate enoyl-CoA to the monomer (R)-3-hydroxyacyl-CoA, the corresponding enzyme genes involved in polyester synthesis were synthesized.

Since Candida maltosa is yeast translating the CTG codon into serine, not leucine, CTG was not assigned to the leucine codon. That codon with high codon usage in Candida maltosa was preferentially selected as the codon corresponding to each amino acid. For codon usage, Klaus Wolf: Nonconventional Yeasts in Biotechnology (published by Springer) was referred to.

The gene designing was carried out in the following manner. Based on the DNA sequences and amino acid sequences of Aeromonas caviae-derived PHA synthase and R-specific enoyl-CoA hydratase, which converts the β oxidation cycle intermediate enoyl-CoA to the monomer (R)-3-hydroxyacyl-CoA, optimal codons were assigned to the respective amino acids.

Further, for creating two KpnI sites in the DNA sequence for Aeromonas caviae-derived PHA synthase, the 969th T was modified into C, and the 1449th T into C.

The above substitutions in the above gene do not change the corresponding amino acid sequence.

In this way, the PHA synthase gene (ORF2) (SEQ ID NO:5) and R-specific enoyl-CoA hydratase gene (ORF3) (SEQ ID NO:6)

were designed and chemically synthesized.

(Example 2) Construction of an enzyme gene expression cassette involved in polyester synthesis

(a) The case of using ALK1p, ALK5p and ALK1t

For causing expression of ORF2 and ORF3 in Candida maltosa, it was decided that a Candida maltosa-derived promoter be ligated to the 5′ upstream of each gene, and a Candida maltosa-derived terminator to the 3′ downstream of each gene. Thus, the ALK1 gene (GenBank D00481) promoter ALK1p (SEQ ID NO:17) was selected for joining to ORF2, and the ALK5 gene (GenBank D12717) promoter ALK5p (SEQ ID NO:18) for joining to ORF3. The Candida maltosa ALK1 gene terminator ALK1t (SEQ ID NO:20) was selected for joining to the 3′ downstream of each. For producing restriction enzyme recognition sites for joining the promoter and terminator to the structural gene, the PCR method was utilized. The primer sequences used for PCR are shown under SEQ ID NO:21 to SEQ ID NO:28. As for the PCR conditions, 25 cycles each comprising 1 minute at 94°C, 2 minutes at 55°C and 3 minutes at 72°C were repeated to amplify each gene fragment. The polymerase used was Takara Shuzo's ExTaq.

As for ALK1p, ALK1p whose 5′ terminus is PvuII and 3′ terminus is NdeI was prepared using the ALK1 gene as a template and using SEQ ID NO:21 and SEQ ID NO:22. As for ALK5p, ALK5p whose 5′ terminus is PvuII and 3′ terminus is NdeI was prepared using the ALK5 gene as a template and using SEQ ID NO:23 and SEQ ID NO:22. As for ALK1t, ALK1t whose 5′ terminus is HindIII and 3′ terminus is EcoRV was prepared using the ALK1 gene as a template and using SEQ ID NO:27 and SEQ ID NO:28.

Used as the vector for finally ligating with ORF2 or ORF3 was the vector pUTA1 (Fig. 1), wherein the marker gene is changed from uracil to adenine, constructed by using pUTU1 (M. Ohkuma, et al., J. Biol. Chem., vol. 273, 3948-3953 (1998)) produced by joining the Candida maltosa-derived autonomously

replicating sequence (ARS) (GenBank D29758) and the URA3 gene (GenBank D12720) to pUC19 (Takara Shuzo), and the Candida maltosa ADE1 gene (SEQ ID NO:29) (GenBank D00855) (S. Kawai, et al., Agric. Biol. Chem., vol. 55, 59-65 (1991)).

Then, pUCNT-ALK1p was constructed by joining ALK1p to pUCNT (WO 94/03613) at the PvuII-NdeI site. pUCNT-ALK1t was also constructed by joining ALK1t to pUCNT at the HindIII-SspI site. ALk1p was excised from pUCNT-ALK1p with PvuII and NdeI and joined to pUCNT-ALK1t at the PvuII-NdeI site to thereby construct pUAL1 (Fig. 2).

It was decided that a peroxisome-targeting signal be added to the carboxy terminus of each of the above-mentioned ORF2 and ORF3 so that each of them may be expressed in Candida maltosa and targeted to peroxisomes. Used as the peroxisome-targeting signal to be added to the carboxy terminus was the amino acid sequence Ser-Lys-Leu (SKL) or Ala-Lys-Ile (AKI). Sequences of the genes (ORF2S, ORF2A, ORF3S, and ORF3A) obtained by adding these amino acids to the ORF2 or ORF3 gene are shown under SEQ ID NO:7 to SEQ ID NO:10. With the ORF2 DNA as a template, ORF2S was constructed using the primers shown under SEQ ID NO:11 and SEQ ID NO:12, and ORF2A was constructed using the primers shown under SEQ ID NO:11 and SEQ ID NO:13. Similarly, with the ORF3 DNA as a template, ORF3S was constructed using the primers shown under SEQ ID NO:14 and SEQ ID NO:15, and ORF3A using the primers shown under SEQ ID NO:14 and SEQ ID NO:16. The PCR conditions were the same as mentioned above.

The DNAs amplified were treated with NdeI and PstI, and ORF2S was joined to pUAL1 at the NdeI-PstI site to construct the plasmid pUAL-ORF2S, and pUAL-ORF2A (Fig. 3) was constructed in the same manner. ORF3S was joined to pUCNT-ALK1t at the NdeI-PstI site to construct pUCNT-ORF3St. ORF3A was constructed in the same manner as ORF3S.

ORF2S or ORF2A was excised, together with the upstream promoter and downstream terminator, from the plasmid pUAL-ORF2S

or pUAL-ORF2A using EcoT22I, and joined to pUTA1 at the PstI site. pHA2S and pHA2A were thus constructed (Fig. 4).

ALK5p (SEQ ID NO:18) whose 5′ terminus is PvuII and 3′ terminus is NdeI was constructed using SEQ ID NO; 23 and SEQ ID NO:24 with the Candida maltosa ALK5 gene (GenBank D12717) as a template. This DNA fragment was substituted for ALK1p in pUAL1, whereby pUAL5 was constructed. The ALK5 promoter and ALK1 terminator were excised from this plasmid using PvuI and PvuII and joined to pSTV28 (product of Takara Shuzo) at the SmaI-PvuI site to construct pSTAL5. ORF3S was joined to this plasmid at the NdeI-PstI site, whereby pSTAL5-ORF3S (Fig. 5) was constructed.

ORF3S was excised, together with the upstream promoter and downstream terminator, from pSTAL5-ORF3S using SalI and joined to pHA2S at the SalI site to construct the plasmid pHA23S2 (Fig. 6).

(b) The case of using ALK1p, POX2p and ALK1t

Based on the Candida maltosa POX2 gene (GenBank S21228), the primers shown under SEQ ID NO:25 and SEQ ID NO:26 were designed. Using these, with the Candida maltosa IAM 12247 genomic DNA as a template, POX2p (SEQ ID NO:19) whose 5′ terminus is PvuII and 3′ terminus is NdeI was prepared. This DNA fragment was substituted for the lac promoter in pUCNT-ORF3St to construct pPOX2-ORF3S. ORF3S was excised, together with the upstream promoter and downstream terminator, from pPOX2-ORF3S using SalI and joined to pHA2S at the SalI site, whereby the plasmid pHA23S1 (Fig. 7) could be constructed.

The preparation schemes for all the above plasmids are shown in Fig. 8 to Fig. 12.

(Example 3) Transformant construction

Unless otherwise specified, the reagents used in yeast cultivation were commercial products available from Wako Pure Chemical Industries. In the practice of the present invention, a number of commercial kits were used. Unless otherwise

specified, they were used according to the manuals attached thereto.

The host used was the Candida maltosa AC16 strain, which is a strain with the ADE1 gene disrupted and has been internationally deposited with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary (accession number FERM BP-7366), and the above-mentioned gene expression cassettes of the present invention, namely the plasmids pHA2S, pHA2A, pHA23S1, and pHA23S2, were respectively introduced into the host. Each plasmid constructed was introduced into the host by the electroporation technique. The gene transfer apparatus used was ELECTRO CELL MANIPULATOR 600 (product of BTX). The cuvettes used were BM 6200 cuvettes produced by BIO MEDICAL CORPORATION CO. LTD. Each plasmid (1 µl) was added to 100 µl of competent cells. 100 µl of the thus-prepared competent cell/plasmid solution was taken and poured into a cuvette, which was set on the pulse generator. Then, electric pulses were applied under the following conditions: electrostatic capacity 40 µF, resistance value 246 ohm, and voltage 1.9 kV. After pulse application, 1 ml of 1 M sorbitol was added to each cuvette and, after gentle mixing, the cuvette was allowed to stand at room temperature for 1 hour. After plasmid introduction, the cells were cultured on a selective plate (0.67 w/v % Yeast Nitrogen base without amino acid (product of Difco), 2 w/v % glucose, 2 w/v % agar), to give transformants. Among the transformants obtained, the one harboring the plasmid pHA23S1 has been internationally deposited as Candida maltosa AC16(pHA23S1) with the National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary under the accession number FERM BP-7762, and the one harboring the plasmid pHA23S2 as Candida maltosa AC16(pHA23S2) under the accession number FERM BP-7763.

(Example 4) Polymer production using the transformants

(a) Polymer production using the transformants resulting from introduction of the plasmid pHA2S or pHA2A

The Candida maltosa transformants resulting from introduction of the gene necessary for polymer production were cultivated in the following manner. YNB medium (0.67 w/v % Yeast Nitrogen base without amino acid) supplemented with 1 w/v % of casamino acids and 1 v/v % of tetradecane was used as the medium.

A 500-ml Sakaguchi flask containing 50 ml of the above medium was inoculated with 500 µl of a glycerol stock of each transformant and, after 20 hours of cultivation, the culture was inoculated into a 2-L Sakaguchi flask containing 300 mL of the production medium at an inoculum size of 10 v/v %. This was cultivated at an incubation temperature of 30°C and a shaking speed of 90 rpm for 4 days. Cells were recovered from the culture fluid by centrifugation, suspended in 80 ml of distilled water, and disrupted using an ultrahigh pressure homogenizer (APV's Rannie 2000, at 15,000 Psi for 15 minutes), followed by centrifugation. The precipitate obtained was washed with methanol and then lyophilized.

The lyophilized cells were ground, 100 ml of chloroform was added thereto, and the mixture was stirred overnight for effecting extraction. The cells were removed by filtration, the filtrate was concentrated to 1 to 2 ml using an evaporator, and about 10 ml of hexane was added to the concentrate to cause the polymer P(3HB-co-3HH) to precipitate out. The cultivation results thus obtained are shown in Table 1.

Table 1 Cultivation results with pHA2S and pHA2A

Plasmid	Cell amount (g/L)	Polymer content (wt %)	3HH composition (mol %)
pHA2S	6.97	3.2	12
pHA2A	7.53	0.49	8.6

(b) Polymer production using the transformants resulting from introduction of the plasmid pHA2S, pHA23S1 or pHA23S2

YNB medium (0.67 w/v % Yeast Nitrogen base without amino acid) supplemented with 2 v/v % of dodecane or 2 v/v % of coconut oil was used as the medium.

The cultivation conditions were the same as described above under (a) except that the cultivation period was 2 days. The polymer P(3HB-co-3HH) was extracted in the same manner as described above under (a). The cultivation results are shown in Table 2.

Table 2 Cultivation results with pHA2S, pHA23S1 and pHA23S2

Plasmid	Carbon source	Cell amount (g/L)	Polymer content (wt %)
pHA2S	Dodecane	4.89	5.36
	Coconut oil	5.14	0.95
pHA23S1	Dodecane	4.48	9.38
	Coconut oil	5.89	1.47
pHA23S2	Dodecane	5.85	6.15
	Coconut oil	5.58	1.50

(Comparative Example 1)

Cultivation was carried out in the same manner as described in Example 4(a) except using the transformant (cell amount: 10.3 g/L) in which a plasmid pUTA-ORF23(WO01/88144), constructed by a polyester synthase gene incapable of adding a DNA coding for a peroxisome-targeting signal, was transformed. As a result, the content of the polymer P(3HB-co-3HH) was 0.1% by weight.

As described above, by constructing polyester synthase gene involved in polyester synthesis by adding a DNA coding a peroxisome-targeting signal, transforming a enzyme gene expression cassette containing that gene, promoter and terminator into yeast, and cultivating the thus-obtained

transformant, it becomes possible to produce greater amount of polyester compared with the case which a polyester synthase gene incapable of adding a DNA coding for a peroxisome-targeting signal is used.

INDUSTRIAL APPLICABILITY

The present invention makes it possible to produce polyesters resulting from homopolymerization or copolymerization of a 3-hydroxyalkanoic acid(s) represented by the general formula (1) and having biodegradability and good physical properties by using yeast with high productivity.

CLAIMS

1. A gene capable of being expressed in yeast, which obtainable by addition of a DNA coding for a peroxisome-targeting signal to a gene coding for an enzyme involved in polyester synthesis.

2. The gene according to Claim 1, wherein the peroxisome-targeting signal comprises the amino acid sequence shown under SEQ ID NO: 1 or SEQ ID NO:2.

3. The gene according to Claim 1 or 2, wherein the peroxisome-targeting signal-encoding DNA has the nucleotide sequence shown under SEQ ID NO:3 or SEQ ID NO:4.

4. The gene according to any of Claims 1 to 3, wherein the enzyme involved in polyester synthesis is a bacterial species-derived enzyme.

5. The gene according to Claim 4, wherein the bacterial species is Aeromonas caviae.

6. The gene according to Claim 4 or 5, wherein at least one CTG codon in the gene coding for the bacterial species-derived enzyme has been modified into TTA, TTG, CTT, CTC or CTA.

7. The gene according to any of Claims 1 to 6, wherein the enzyme involved in polyester synthesis is a polyhydroxyalkanoic acid synthase or R-specific enoyl-CoA hydratase.

8. The gene according to Claim 7, wherein the polyhydroxyalkanoic acid synthase is encoded by a gene comprising the nucleotide sequence shown under SEQ

ID NO:5.

9. The gene according to Claim 7,
wherein the R-specific enoyl-CoA hydratase is encoded by a gene comprising the nucleotide sequence shown under SEQ ID NO:6.

10. The gene according to Claim 8,
which comprises the nucleotide sequence shown under SEQ ID NO:7 or SEQ ID NO:8.

11. The gene according to Claim 9,
which comprises the nucleotide sequence shown under SEQ ID NO:9 or SEQ ID NO:10.

12. An enzyme involved in polyester synthesis,
which is translated from the gene according to any of Claims 1 to 11.

13. A transformant resulting from transformation of at least one gene according to any of Claims 1 to 11 into yeast.

14. A transformant resulting from transformation, into yeast, of a polyester synthesis-associated enzyme gene expression cassette
which comprises the gene according to any of Claims 1 to 11, a promoter and a terminator both capable of functioning in the yeast.

15. The transformant according to Claim 14,
wherein the promoter and terminator are derived from Candida maltosa.

16. The transformant according to Claim 14 or 15,
wherein the promoter is derived from Candida maltosa ALK1,

ALK5 or POX2.

17. The transformant according to any of Claims 14 to 16, wherein the terminator is derived from Candida maltosa ALK1.

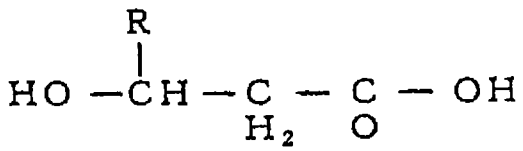
18. The transformant according to any of Claims 13 to 17, which is derived from yeast species belonging to the genus Aciculoconidium, Ambrosiozyma, Arthroascus, Arxiozyma, Ashbya, Babjevia, Bensingtonia, Botryoascus, Botryozyma, Brettanomyces, Bullera, Bulleromyces, Candida, Citeromyces, Clavispora, Cryptococcus, Cystofilobasidium, Debaryomyces, Dekkera, Dipodascopsis, Dipodascus, Eeniella, Endomycopsella, Eremascus, Eremothecium, Erythrobasidium, Fellomyces, Filobasidium, Galactomyces, Geotrichum, Guilliermondella, Hanseniaspora, Hansenula, Hasegawaea, Holtermannia, Hormoascus, Hyphopichia, Issatchenkia, Kloeckera, Kloeckeraspora, Kluyveromyces, Kondoa, Kuraishia, Kurtzmanomyces, Leucosporidium, Lipomyces, Lodderomyces, Malassezia, Metschnikowia, Mrakia, Myxozyma, Nadsonia, Nakazawaea, Nematospora, Ogataea, Oosporidium, Pachysolen, Phachytichospora, Phaffia, Pichia, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycodes, Saccharomycopsis, Saitoella, Sakaguchia, Saturnospora, Schizoblastosporion, Schizosaccharomyces, Schwanniomyces, Sporidiobolus, Sporobolomyces, Sporopachydermia, Stephanoascus, Sterigmatomyces, Sterigmatosporidium, Symbiotaphrina, Sympodiomyces, Sympodiomycopsis, Torulaspora, Trichosporiella, Trichosporon, Trigonopsis, Tsuchiyaea, Udeniomyces, Waltomyces, Wickerhamia, Wickerhamiella, Williopsis, Yamadazyma, Yarrowia, Zygoascus, Zygosaccharomyces, Zygowilliopsis, or Zygozyma.

19. The transformant according to Claim 18, wherein the yeast belongs to the genus Candida or

Yarrowia.

20. The transformant according to Claim 19, wherein the yeast is Candida maltosa.

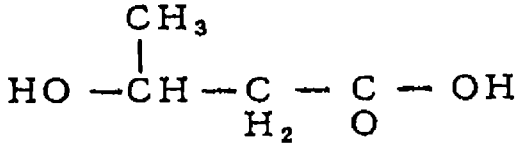
21. The transformant according to any of Claims 13 to 20, wherein the polyester is a homopolymer or copolymer of a 3-hydroxyalkanoic acid(s) represented by the general formula (1):



(1)

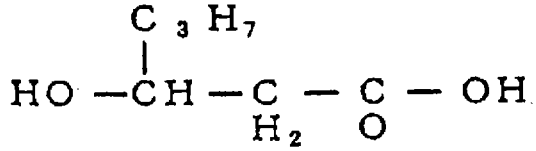
wherein R represents an alkyl group.

22. The transformant according to any of Claims 13 to 21, wherein the polyester is a copolyester, P(3HB-co-3HH), obtainable by copolymerization of 3-hydroxybutyric acid represented by the formula (2):



(2)

and 3-hydroxyhexanoic acid represented by the formula (3):



(3)

23. The method of producing polyesters using the transformant according to any of Claims 13 to 22,

which method comprises cultivating the transformant and recovering the product polyester from the culture obtained.