(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
20 October 2011 (20.10.2011)

PCT

(10) International Publication Number
WO 2011/129462 A2

(51) International Patent Classification:
C12P 21/02 (2006.01)

(21) International Application Number:
PCT/JP2011/059480

(22) International Filing Date:
12 April 2011 (12.04.2011)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

2010-091719	12 April 2010 (12.04.2010)	JP
2010127403	5 July 2010 (05.07.2010)	RU
2010-201962	9 September 2010 (09.09.2010)	JP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2011/129462 A2

(54) Title: A YEAST EXTRACT CONTAINING γ-Glu-X OR γ-Glu-X-Gly AND A METHOD FOR PRODUCING THE SAME

(57) Abstract: A yeast extract containing a peptide, such as gamma-Glu-X and gamma-Glu-X-Gly, wherein X can represent an amino acid or an amino acid derivative other than Cys and derivatives thereof, is prepared by culturing a yeast in a medium containing a peptide such as gamma-Glu-X, gamma-Glu-X-Gly and X-Gly, and preparing a yeast extract from the obtained cells.

DESCRIPTION

A yeast extract containing γ-Glu-X or γ-Glu-X-Gly and a method for producing the same

BACKGROUND OF THE INVENTION

Technical Field

The present invention relates to a yeast extract containing γ-Glu-X-Gly or γ-Glu-X and a method for producing the same. The yeast extract of the present invention is useful in the field of foodstuffs such as seasonings and health foods.

Background Art

Yeast extracts have a function of imparting atsumi(thickness), umami, etc. to foodstuffs, and have been widely used as seasonings in the field of foodstuffs. Especially, glutathione (henceforth also referred to as "GSH"), which is a tripeptide consisting of glutamic acid, cysteine and glycine, is known to impart kokumi to foodstuffs (Ueda et al., Agric. Biol. Chem., 54, 163-169 (1990), Ueda et al., Biosci. Biotechnolo. Biochem., 61, 1977-1980 (1997)), and seasonings containing GSH have been developed.

Meanwhile, although the calcium sensing receptor (CaSR), which is a G-protein classified into the class C, has been reported to respond to GSH (Wang et al., Journal of Biological Chemistry, 281, 8864-8870 (2006)), the physiological significance thereof has not been clarified. Moreover, this CaSR is present also in the lingual cells, and it was considered to show a certain taste response (Gabriel et al., Biochemical and Biophysical Research Communications, 378, 414-418 (2009)). Then, it has recently been clarified that this CaSR participates in recognition of kokumi in humans (Ohsu et al., Journal of Biological Chemistry, 285, 1016-1022 (2010)). This reference reported that not only GSH has been recognized as a kokumi substance, but also several γ-glutamyl compounds similarly respond to CaSR. Furthermore, it has been reported that peptides represented by the general formula γ-Glu-X or γ-Glu-X-Gly (X can represent an amino acid or amino acid derivative other than Cys), for example, γ-Glu-Met, γ-Glu-Thr, γ-Glu-Val-Gly, etc. have a kokumi-imparting effect (WO2007/055393). Moreover, the group of esters including S- or O-carboxyalkylated γ-glutamyl or β-asparagyl peptides etc., are also reported as kokumi substances (WO2007/042288). Although these peptides impart kokumi to foodstuffs like GSH,

they do not have a reduced SH group unlike GSH. It is known that a substance having the reduced SH group such as GSH is generally unstable, and titer thereof is reduced with formation of disulfide bond (WO2007/042288). However, γ-Glu-X, γ-Glu-X-Gly etc. are considered useful from the viewpoint that the kokumi-imparting peptides not having the reduced SH group are stable.

As for foodstuffs containing a γ-Glu dipeptide, it has been reported that various γ-Glu dipeptides were detected in Gouda cheese ripened over a long period of time, even as long as about 44 weeks (Toelstede, S and Hofmann, T, J. Agric. Food. Chem., 2009). This reference reported that various γ-Glu dipeptides such as γ-Glu-Ala, γ-Glu-Glu and γ-Glu-Gln were detected in a total amount of 3590 μmol/kg of dry materials at most. This value corresponds to 0.088% in terms of weight percentage based on the solid content.

However, a yeast extract containing γ-Glu-X and γ-Glu-X-Gly in an amount enabling impartation of kokumi has not been previously reported.

Furthermore, it is known that the synthesis and decomposition of glutathione, which is one of the γ-glutamyl compounds, is catalyzed by a plurality of enzymes which make up the γ-glutamyl cycle. In particular, γ-glutamyl transpeptidase is known to transfer the glutamate of GSH at the γ–position to another compound having an amino group, resulting in decomposition of GSH to cysteinylglycine (Protein Nucleic acid Enzyme, 1988-7, VOL. 33, NO. 9, ISSN 003909450, Special Issue "Epoch of glutathione research", pp.1432-1433). It is considered that, if the compound having an amino group in this rearrangement reaction is an amino acid, a dipeptide of γ-Glu-X can be generated as a by-product. However, research into making a microorganism effectively produce this by-product has not been positively performed to date, partially because it is a by-product.

Findings about the dipeptide γ-Glu-X that have been reported include an analysis of the fermentation broth of *Micrococcus glutamicus* (Ronald et al., Journal of Biological Chemistry, 240, p2508-2511 (1965)). This reference reported that the fermentation broth was loaded onto various columns to separate peptides etc., and to isolate γ-Glu-Glu, γ-Glu-Val, and γ-Glu-Leu. However, these were found as a result of separation with various columns, and the amounts of these peptides contained in the broth were not determined.

Furthermore, an enzyme responsible for GSH biosynthesis was newly isolated from *Streptococcus agalactiae* and *Clostridium acetobutylicum*, and the substrate specificity thereof was analyzed (Kino et al., JBB research communications, 352, pp.351-359 (2007)). GSH is usually biosynthesized by two different enzymes called

γ-glutamylcysteine synthetase, which combines Glu and Cys to generate γ-Glu-Cys, and glutathione synthetase, which combines the produced γ-Glu-Cys and Gly to generate GSH. However, the aforementioned two kinds of microorganisms have a unique enzyme which is essentially a fusion of γ-glutamylcysteine synthetase and glutathione synthetase. It was reported that, according to an *in vitro* analysis, the substrate recognition of this enzyme was slightly ambiguous, i.e., it also recognized amino acids other than Cys, and as a result, it could generate γ-Glu-X and γ-Glu-X-Gly. However, these are nevertheless *in vitro* results, and it was not described whether or not those microorganisms that produced marked amounts of peptides such as γ-Glu-X and γ-Glu-X-Gly, also contain many compounds having an amino group besides the target X.

Yeast extracts are seasonings which have been widely used in the field of foodstuffs, and are highly accepted by consumers. Therefore, a yeast extract can be used as a carrier of γ-Glu-X-Gly or γ-Glu-X. Yeast strains containing minerals have been studied for this use. It is known that if a metal is added to a medium, yeasts take up the metal into the cells (B. Volesky, H.A., Appl. Microbiol. Biotechnol., 42;797-806 (1995)). In particular, if trace elements such as zinc, iron, copper, manganese, selenium, molybdenum and chromium are added to the medium, yeasts can be used to supply such trace elements via enrichment in foodstuffs (Japanese Patent Laid-open (Kokai) No. 2004-298014). As a result, methods for producing mineral-containing yeast have been developed (Japanese Patent Laid-open No. 54-157890, Japanese Patent Laid-open No. 60-75279, Japanese Patent Publication (Kokoku) No. 6-16702).

Furthermore, mineral-containing yeast also have an advantage concerning taste. For example, yeast containing a high amount of magnesium are described in Japanese Patent Laid-open No. 8-332081. This reference describes that although magnesium-enriched foodstuffs containing inorganic magnesium salt were also marketed, a strong bitterness and astringency was noted due to the mineral salt. As a result, it was quite more difficult to routinely eat the magnesium-enriched foodstuffs containing inorganic magnesium salt as compared to foodstuffs containing naturally occurring magnesium. Japanese Patent Laid-open No. 8-332081 also discloses a technique of producing a natural material by making yeast take up magnesium. As for nutritional merit, the technique disclosed in Japanese Patent Laid-open No. 2008-99578 can be exemplified. According to this reference, although zinc contributes to improving taste and generative function, etc., the reference overlooks that zinc is often not taken in sufficient amounts. If zinc is added during the yeast cultivation process, yeast takes up zinc into cells, but water-soluble zinc binds with a protein or an amino

acid, and accumulates as amorphous zinc at a high concentration. The amorphous zinc is more efficiently absorbed into the body, as compared to crystalline zinc. As a result, improved absorption can be obtained by incorporating zinc into yeast, as compared to simply taking zinc as it is.

As described above, there are various advantages to making yeast take up a target substance and adding either the yeast or a yeast extract to foodstuffs, as compared to simply adding the target substance to foodstuffs. However, unlike minerals, which are essential nutrients, the ability of yeast to take up an amino acid or a peptide is delicately controlled, and it simply applying the technique for incorporating minerals into yeast to the techniques for uptake of amino acid or peptides was considered to be difficult.

There has been much research concerning the generation of GSH or γ-Glu-Cys using yeast Examples of such research include the report that the GSH content was improved by mutagenizing a *Saccharomyces* yeast and selecting a strain having improved zinc resistance (Japanese Patent Laid-open No. 02-295480), the report that suppression of the *MET25* gene expression was derepressed by making a cell contain a mutant *MET30* gene and thereby increase intracellular γ-Glu-Cys content (Japanese Patent Application No. 2002-282743), and so forth. Moreover, the latest scientific findings include uptake of GSH by Hgt1p. Although GSH and the dimer thereof, GSSG, were taken into cells by Hgt1p, uptake of GSH by Hgt1p was not affected even in the presence of excessive amounts of amino acids, various dipeptides, and tripeptides. Therefore, it is considered that Hgt1p is not a nonspecific transporter as once thought, but a transporter specific to GSH (Bourbouloux et al., Journal of Biological Chemistry, 275, pp.13259-13265 (2000)). Furthermore, a search for the active site of Hgt1p has also been performed (Kaur et al., FEMS Yeast Res., 9, 849-866 (2009)).

As described above, although many findings about GSH and the precursor thereof, γ-Glu-Cys, have been reported, there have been no reports about yeast cells containing such a substance as γ-Glu-X and γ-Glu-X-Gly, and a method for producing an extract prepared from the cells.

SUMMARY OF THE INVENTION

Aspects of the present invention include providing a yeast extract containing γ-Glu-X or γ-Glu-X-Gly, and providing a method for producing the yeast.

The above aspects were achieved by finding that a yeast took up γ-Glu-X and γ-Glu-X-Gly (X represents an amino acid or an amino acid derivative other than Cys,

the same shall apply in the following descriptions) into cells, and a yeast extract containing γ-Glu-X or γ-Glu-X-Gly could be produced by preparing the yeast extract from a yeast cultured in a medium containing γ-Glu-X or γ-Glu-X-Gly. Moreover, it was found that if yeast was cultured in a medium containing γ-Glu-X or X-Gly, these compounds were taken up into the cells, and γ-Glu-X-Gly could be generated via an intracellular enzymatic reaction. Furthermore, it was also found that a yeast extract containing γ-Glu-X or γ-Glu-X-Gly could be produced by allowing γ-glutamyl transferase to act on a yeast extract raw material to which an amino acid or a peptide selected from X and X-Gly was added.

It is an aspect of the present invention to provide a yeast extract containing a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof).

It is a further aspect of the present invention to provide the yeast extract as described above, which contains the peptide in a total amount of 0.02% or more.

It is a further aspect of the present invention to provide the yeast extract as described above, wherein X is Val.

It is a further aspect of the present invention to provide the yeast extract as described above, wherein X is nVal.

It is a further aspect of the present invention to provide the yeast extract as described above, wherein the yeast is *Saccharomyces cerevisiae*.

It is a further aspect of the present invention to provide a method for producing a yeast extract containing a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly, which comprises culturing a yeast in a medium containing a peptide selected from the group consisting of γ-Glu-X, γ-Glu-X-Gly and X-Gly, and preparing a yeast extract from the obtained cells, , wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

It is a further aspect of the present invention to provide the method as described above, wherein the medium contains 0.1 ppm or more of the peptide, and the yeast extract contains the peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract.

It is a further aspect of the present invention to provide the method as described above, wherein X is Val.

It is a further aspect of the present invention to provide the method as

described above, wherein the yeast has been modified so that uptake of the peptide into cells is improved.

It is a further aspect of the present invention to provide the method as described above, wherein the activity of Hgt1p of the yeast has been enhanced.

It is a further aspect of the present invention to provide the method as described above, wherein the activity of Ptr2p of the yeast has been enhanced.

It is a further aspect of the present invention to provide the method as described above, wherein the activity of glutathione synthetase of the yeast has been enhanced.

It is a further aspect of the present invention to provide a method for producing a yeast extract containing γ-Glu-nVal, which comprises culturing a yeast in a medium containing nVal.

It is a further aspect of the present invention to provide the method as described above, wherein the activity of γ-glutamylcysteine synthetase of yeast has been enhanced.

It is a further aspect of the present invention to provide the method as described above, wherein the yeast is *Saccharomyces cerevisiae*.

It is a further aspect of the present invention to provide a method for producing a yeast extract containing a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly, which comprises allowing a γ-glutamyl transferase to act on a yeast extract raw material to which an amino acid or a peptide selected from the group consisting of X and X-Gly is added, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

It is a further aspect of the present invention to provide the method as described above, wherein the amino acid or peptide is added in a total amount of 1% or more based on dry weight of the yeast extract raw material, and the yeast extract contains a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract.

It is a further aspect of the present invention to provide the method as described above, wherein X is Val.

It is a further aspect of the present invention to provide the method as described above, wherein X is nVal.

It is a further aspect of the present invention to provide the method as described above, wherein the yeast is *Saccharomyces cerevisiae*.

According to the present invention, a yeast extract containing γ-Glu-X, γ-Glu-X-Gly, or these both can be produced. A yeast extract containing these peptides

is excellent in kokumi.

Moreover, the yeast extract containing γ-Glu-X is also useful as a raw material for producing a yeast extract containing γ-Glu-X-Gly.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows HPLC-MS chromatograms of γ-Glu-Val-Gly, γ-Glu-Val and Val-Gly standard samples.

Fig. 2 shows HPLC-MS chromatograms of γ-Glu-Val-Gly, γ-Glu-Val, Val-Gly, γ-Glu-nVal-Gly, and γ-Glu-nVal standard samples.

Fig. 3 shows the structure of the plasmid pUC19AOX-G418-BRI.

Fig. 4 shows the structure of the plasmid pKS-URA3-13.

Fig. 5 shows construction of the plasmid pKS-URA3-13-kanMX.

Fig. 6 shows construction of the plasmid pLoxP-PADH1-kanR5.

Fig. 7 shows construction of the plasmid pKS-URA3-PADH1-LR.

Fig. 8 shows construction of the plasmid pKS-URA3-P-ADH1.

Fig. 9 shows preparation of a DNA fragment used for promoter substitution.

CDS = coding sequence of target gene

up-CDS = region upstream target gene.

Horizontally stroked boxes designate a 40-base pairs overlapping region, black box designates a 40-base fragment for homologous recombination with 5'-region of target gene.

Fig. 10 shows the map of pUG6-PTDH3.

Fig. 11 shows cloning of the P_{TDH3}-GSH2 cassette into multicopy yeast vector.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The yeast extract in accordance with the presently described subject matter contains a peptide such as γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract, wherein X can represent an amino acid or an amino acid derivative other than Cys and derivatives thereof. The yeast extract can contain the peptide in a total amount of 0.005% or more, 0.02% or more, 0.1% or more, or 0.5% or more, based on dry weight of the yeast extract.

The yeast used as the raw material of the yeast extract is the same as the yeast used for the method described later.

Glu and Gly in the peptide represent glutamic acid and glycine, respectively.

The symbol "-" represents a peptide bond. "γ" of γ-Glu means that another amino acid binds via the carboxy group of the glutamic acid at the γ-position.

"X" can represent any of 19 kinds of amino acid among the natural amino acids or a derivative thereof, except for Cys and derivatives thereof. Cys represents cysteine, and examples of the derivatives thereof include α-aminobutyric acid, β-aminobutyric acid, and so forth. The aforementioned amino acids except for Cys and derivatives thereof include neutral amino acids such as glycine (Gly), alanine (Ala), valine (Val), leucine (Leu), isoleucine (Ile), serine (Ser), threonine (Thr), methionine (Met), asparagine (Asn), glutamine (Gln) and proline (Pro), acidic amino acids such as aspartic acid (Asp) and glutamic acid (Glu), basic amino acids such as lysine (Lys), arginine (Arg) and histidine (His), and aromatic amino acids such as phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp). In particular, when X is a hydrophobic amino acid, the kokumi effect of the peptide is high, and such a peptide is a particular example. Examples of such hydrophobic amino acids include Val, Ala, Leu, Phe, and so forth.

Examples of the derivatives of amino acid include, for example, norvaline (nVal), norleucine (nLeu), tert-leucine (tLeu), hydroxyproline (Hyp), and so forth.

Among the aforementioned peptides, particular examples are γ-Glu-Val-Gly,γ-Glu-Val, γ-Glu-nVal-Gly, γ-Glu-nVal, and Val-Gly.

In addition, the aforementioned amino acids and amino acid derivatives are all L-isomers.

The form of the yeast extract is not particularly limited, and it may be in the form of powder or solution. The yeast extract can have the same uses as that of conventional yeast extracts, for example, seasonings, food additives, health foods, and so forth. The yeast extract is excellent in its kokumi-imparting effect. Kokumi means a taste that cannot be expressed with the five basic tastes, and means a taste that enhances not only the basic tastes but also marginal tastes of the basic tastes, such as thickness, growth (mouthfulness), continuity, and harmony Since the kokumi-imparting effect is more strongly exerted in the presence of umami or salty taste, an umami substance such as sodium L-glutamate and taste nucleotides, and/or a salty substance such as sodium chloride may be added to the yeast extract. Moreover, an umami substance and/or a salty substance may be added to seasonings, food additives or health foods together with the yeast extract in accordance with the presently described subject matter.

As shown in the examples section, yeast extracts containing γ-Glu-Val-Gly, especially a solution containing a yeast extract, produced by the method described

herein, showed a higher kokumi-enhancing effect as compared to a γ-Glu-Val-Gly solution having the same concentration of γ-Glu-Val-Gly as that of the solution. This indicates the usefulness of the yeast extract.

The yeast extract in accordance with the presently described subject mattercan be produced by, for example, the methods of the present invention described herein.

The first method in accordance with the presently described subject matteris a method for producing a yeast extract containing a peptide selected from γ-Glu-X-Gly and γ-Glu-X, which includes the steps of culturing a yeast in a medium containing a peptide selected from γ-Glu-X-Gly, γ-Glu-X and X-Gly, and preparing a yeast extract from the obtained cells, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

The yeast is not particularly limited, so long as the chosen yeast can take up γ-Glu-X, γ-Glu-X-Gly or X-Gly into the cells thereof. Examples include yeasts belonging to the genus *Saccharomyces* such as *Saccharomyces cerevisiae*, those belonging to the genus *Candida* such as *Candida utilis*, those belonging to the genus *Pichia* such as *Pichia pastoris*, and those belonging to the genus *Schizosaccharomyces* such as *Schizosaccharomyces pombe*. Among these, *Saccharomyces cerevisiae* and *Candida utilis* are particular examples, and are frequently used for production of yeast extracts. The yeast may be a monoploid, or may have diploidy or a further higher polyploidy.

The yeast may be any wild-type strain, or various mutant strains, so long as the chosen yeast can intracellularly take up γ-Glu-X, γ-Glu-X-Gly or X-Gly and accumulate γ-Glu-X and/or γ-Glu-X-Gly in the cells. Examples of mutant strains include a strain with enhanced activities or activity of γ-glutamylcysteine synthetase (GSH1) and/or glutathione synthetase (GSH2). The yeast may also be modified so that the uptake of γ-Glu-X, γ-Glu-X-Gly or X-Gly into the cells is improved. The uptake of γ-Glu-X, γ-Glu-X-Gly or X-Gly can be improved by enhancing an activity of a protein which participates in the uptake of these peptides. Although Hgt1p had been reported to be a transporter specific to GSH, it is shown that, by enhancing the activity of Hgt1p, the uptake of γ-Glu-Val-Gly or γ-Glu-nVal-Gly can be improved (see the examples section). Therefore, it is possible that the uptake of not only γ-Glu-Val-Gly, but also other γ-Glu-X-Gly peptides into the cells can be improved by enhancing the Hgt1p activity. Also, Ptr2p had been reported to be an oligopeptide transporter. It is shown that, by enhancing the activity of Ptr2p, the uptake of Val-Gly seemed to be enhanced (see the examples section). Therefore, it is possible that the uptake of not only Val-Gly, but also other X-Gly peptides into the cells can be improved by

enhancing Ptr2p activity. The nucleotide sequence of the gene coding for Ptr2p of *Saccharomyces cerevisiae* is shown in SEQ ID NO: 58. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 59. The methods for enhancing the activity of the aforementioned enzyme or protein include enhancing expression thereof by replacing the promoter of the gene coding for the enzyme or protein on the chromosome with a stronger promoter, enhancing expression thereof by inserting the target gene into the chromosome to introduce two or more copies thereof, and enhancing expression thereof by incorporating a plasmid containing the target gene into the yeast, or the like.

As the promoter, a highly active conventional promoter may be obtained by using various reporter genes, or a known high expression promoter such as *PGK1*, *PDC1*, *TDH3*, *TEF1* and *HXT7* may be used. Alternatively, a plasmid having the replication origin of CEN4, or a multi-copy plasmid having the replication origin of 2 μm DNA may be used. Furthermore, a transposon may be used in order to introduce a target gene into an arbitrary region of the chromosome, or the target gene may be introduced by using rDNA sequences as a target, which is present in a copy number of 150 in the cell.

Enhancement of the activity of γ-glutamylcysteine synthetase is disclosed in, for example, U.S. Patent No. 7,553,638; Otake Y. et al., Bioscience and Industry, volume 50, No. 10, pp.989-994, 1992, and so forth. Although disruption of the glutathione synthetase gene is disclosed in U.S. Patent No. 7,553,638, the glutathione synthetase activity can be enhanced in the same manner as that for enhancement of the activity of γ-glutamylcysteine synthetase. The activity of Hgt1p can also be enhanced in a similar manner.

The nucleotide sequences of the genes coding for Gsh1p and Gsh2p of *Saccharomyces cerevisiae* are disclosed in *Saccharomyces* Genome Database (http://www.yeastgenome.org/). The nucleotide sequences of the genes coding for Gsh1p and Gsh2p of *Candida utilis* are disclosed in U.S. Patent No. 7,553,638. The nucleotide sequence of the gene coding for Gsh1p of *Saccharomyces cerevisiae* is shown in SEQ ID NO: 60. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 61. The nucleotide sequence of the gene coding for Gsh2p of *Saccharomyces cerevisiae* is shown in SEQ ID NO: 19. The amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 20.

The gene coding for Hgt1p can be obtained from *Saccharomyces* Genome Database (http://www.yeastgenome.org/). Synonyms of Hgt1p include Gsh11p, Opt1p or the like. The sequence disclosed as *GSH11* of *Saccharomyces cerevisiae* is

shown in SEQ ID NO: 21. Moreover, the amino acid sequence encoded by this nucleotide sequence is shown in SEQ ID NO: 22.

The method for producing the yeast extract is explained below.

First, a yeast is cultured in a medium containing the peptide. The medium is not particularly limited, so long as a medium in which the yeast can proliferate is chosen, and is not limited to the SD medium described in the examples. A medium usually used for industrial purposes can be used.

When the yeast is cultured, γ-Glu-X, γ-Glu-X-Gly or X-Gly is added to the aforementioned medium. One type of these peptides may be added, or a arbitrary mixture of two or more kinds of these peptides may be added. These peptides may be present in the medium from the start of the culture, or may be added to the medium at an arbitrary time during the culture. When the peptides are added to the medium during the culture, they can be added at 0 to 50 hours before the end of the culture (0 hour means that the culture is terminated immediately after the addition), 0.1 to 24 hours before the end of the culture, or 0.5 to 6 hours before the end of the culture. Furthermore, when the peptides are added during the culture, they may be continuously added.

Prior to the culture in the medium containing the peptides, a preculture may be performed. The medium used for the preculture may or may not contain the peptides.

The peptides are added to the medium usually in an amount of 0.1 ppm or more, 0.5 ppm or more, 1 ppm or more, 10 ppm or more, or 50 ppm or more, in terms of the final concentration in the culture broth at the time of the addition. Although the upper limit of the amount of the peptides is not particularly limited, it can be exemplified as less than 100,000 or 50,000 ppm from an aspect of production cost, it is usually 10,000 ppm or less, 1,000 ppm or less, or 500 ppm or less.

The peptide amount to be added to the medium is usually not less than 0.1 ppm, 0.5 ppm, 1 ppm, 10 ppm, or 50 ppm in terms of the final concentration in the culture broth at the time of the addition. The upper limit of the peptide amount is not specifically limited. However, the upper limit of the peptide amount can be set, for example, to be not more than 100,000 ppm or not more than 50,000 ppm in view of the production cost. The upper limit of the peptide amount is usually not more than 10,000 ppm, 1,000 ppm, or 500 ppm.

As the culture conditions, the same conditions as those used for usual production of yeast extracts can be used, and they may be suitably changed according to the chosen yeast. Arbitrary methods such as batch culture, fed-batch culture, and continuous culture may be used. When the yeast is *Saccharomyces cerevisiae*, it is

preferably aerobically cultured by shaking or the like at 25 to 35°C, 27 to 33°C, or 28 to 32°C.

If the yeast is cultured as described above, γ-Glu-X, γ-Glu-X-Gly, or both can accumulate in the cells of the yeast. When γ-Glu-X or X-Gly is added to the medium, these peptides accumulate in the cells, and in addition, γ-Glu-X-Gly also accumulates. It is estimated that this is because γ-Glu-X-Gly is produced from γ-Glu-X and X-Gly, which have been taken up into the cells by the action of intracellular γ-glutamyl transferase. As shown in the examples section, the amount of γ-Glu-Val and γ-Glu-Val-Gly in the yeast did not correlate with the amount of GSH in the cells, and therefore it is considered that yeast extracts produced by the conventional methods do not contain γ-Glu-X or γ-Glu-X-Gly at a high concentration, even if they are produced from a yeast containing GSH at a high concentration.

The yeast extract can be prepared from the yeast in the same manner as that used for conventional production of yeast extracts. The yeast extract may be obtained by subjecting the yeast cells to hot water extraction and processing the extract, or by digesting the yeast cells and processing the digestion product. Furthermore, the obtained yeast extract may be concentrated, or may be dried and thereby made into powdered form, if needed.

In such a manner as described above, a yeast extract in which the amount of γ-Glu-X, γ-Glu-X-Gly or both are increased is obtained. The yeast extract can contain γ-Glu-X, γ-Glu-X-Gly, or both in a total amount of 0.005% or more, 0.02% or more, 0.1% or more, or 0.5% or more, based on dry weight of the yeast extract.

By allowing a γ-glutamyl transferase to act on the yeast extract containing γ-Glu-X obtained as described above in the same manner as that of the method described below, a yeast extract containing γ-Glu-X-Gly can be produced.

The second method in accordance with the presently described subject matteris a method for producing a yeast extract containing a peptide selected from γ-Glu-X and γ-Glu-X-Gly, which includes the steps of allowing a γ-glutamyl transferase to act on a yeast extract raw material to which an amino acid or a peptide such as X and X-Gly is added, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

If a γ-glutamyl transferase is allowed to act on X or X-Gly, γ-Glu-X or γ-Glu-X-Gly is generated. Therefore, a yeast extract containing γ-Glu-X or γ-Glu-X-Gly can also be obtained by allowing a γ-glutamyl transferase to act on a yeast extract containing X or X-Gly. The yeast extract containing X and/or X-Gly may be prepared from a yeast cultured in a medium containing X and/or X-Gly, or may be

obtained by adding X and/or X-Gly to a yeast extract raw material.

As the yeast extract raw material, a yeast extract obtained by a conventional method can be used.

One kind of X or X-Gly may be added to the yeast extract raw material, or an arbitrary mixture of two or more kinds of them may be added. X and/or X-Gly is added in a total amount of 1% or more, 5% or more, 10% or more, based on dry weight of the yeast extract raw material.

The reaction catalyzed by the γ-glutamyl transferase is performed in an aqueous solvent such as water or buffers. Specifically, for example, the yeast extract raw material is dissolved in the aqueous solvent, and the γ-glutamyl transferase is added. The reaction conditions can be suitably determined according to the γ-glutamyl transferase to be used. The reaction is usually allowed at pH 3 to 9 and 15 to 70°C for 1 to 300 minutes, or pH 5 to 8 and 30 to 70°C for 5 to 150 minutes.

Concentration of the yeast extract raw material in the aqueous solvent may be determined in view of ease of handling. The concentration is usually 0.1 to 50%, or 0.5 to 20%, in terms of dry weight of the yeast extract raw material.

Examples of the γ-glutamyl transferase include glutaminase, γ-glutamyl transpeptidase (γ-GTP), and so forth. As for the amount of the enzyme, in the case of γ-GTP, it is usually 0.001 to 1000 units/ml, 0.005 to 100 units/ml, y 0.01 to 25 units/ml, or 0.05 to 10 units/ml, wherein 1 unit is defined to be the activity of liberating 1.0 μmole of p-nitroaniline from γ-glutamyl-p-nitroanilide per 1 minute in a solution at pH 8.5 and 25°C (definition described in Sigma General Catalogue, 2008-2009 Edition, p.917). The amount of glutaminase can also be determined in a manner similar to that for γ-GTP.

After the enzymatic reaction, the γ-glutamyl transferase may be inactivated by, for example, a heat treatment at 80 to 100°C, but this is not always necessary.

As a substrate of the γ-glutamyl transferase, a γ-glutamyl compound, for example, GSH, may be added to the reaction mixture. GSH contained in the yeast extract may also be used as a substrate. In this case, a yeast extract prepared from yeast in which the content of GSH is increased, for example, a yeast in which activities or activity of Gsh1p and/or Gsh2p is enhanced can be used. Although a greater GSH content in the yeast extract is preferred, it is usually 1 to 50%, 1 to 30%, or 5 to 20%, based on dry weight of the yeast extract.

In such a manner as described above, a yeast extract in which the amount of γ-Glu-X, γ-Glu-X-Gly, or both is increased is obtained. The yeast extract can contain γ-Glu-X, γ-Glu-X-Gly or both in a total amount of 0.005% or more, 0.02% or more,

0.1% or more, or 0.5% or more, based on dry weight of the yeast extract.

The obtained yeast extract may be concentrated, or may be dried and thereby made into powdered form, if needed.

Examples

Hereinafter, the present invention will be explained more specifically with reference to the following non-limiting examples.

Example 1: Detection of γ-Glu-Val-Gly and γ-Glu-Val in various yeast extracts

γ-Glu-Val-Gly, γ-Glu-Val, and Val-Gly contents in various commercially available yeast extracts were measured by fluorescence derivatization of the peptides with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), and detection by LC-MS/MS according to the method described below. First, to 2.5 μL each of solutions of various yeast extracts diluted to an appropriate concentration, or 2.5 μL each of standard solutions containing γ-Glu-Val-Gly, γ-Glu-Val and Val-Gly, respectively, 2.5 μL of Milli-Q water, 5 μL of a 5 μM internal standard substance solution (L-alanine-3,3,3-d3, Sigma (Ala-D3), DL-valine-2,3,4,4,4,5,5,5-d8, Sigma (Val-d8), both are labeled with stable isotope), and 30 μL of a borate buffer (attached to AccQ-Fluor (registered trademark) Reagent Kit, Nihon Waters) were added. To each mixture, 10 μL of an AQC reagent solution (prepared by dissolving the reagent powder of the aforementioned reagent kit in 1 mL of acetonitrile) was added. This mixture was heated at 55°C for 10 minutes, and then 100 μL of 0.1% formic acid aqueous solution was added to the mixture to prepare a sample for analysis.

Then, the sample for analysis prepared as described above was subjected to separation by the reverse phase liquid chromatography described below, and then introduced into a mass spectrometer. The separation conditions are as follows.

(1) HPLC: Agilent 1200 Series
(2) Separation column: Unison UK-Phenyl, internal diameter: 2.0 mm, length: 100 mm, particle size: 3 μm (Imtakt)
(3) Column temperature: 40°C
(4) Mobile phase A: 25 mM Ammonium formate (pH 6.0 adjusted by aqueous ammonia)
(5) Mobile phase B: methanol
(6) Flow rate: 0.25 mL/min
(7) Elution conditions: elution was performed by using mixtures of the mobile phase A

and the mobile phase B. The ratios of the mobile phase B to the mixtures are as follows: 0 minute (5%), 0 to 17 minutes (5 to 40%), 17 to 17.1 minutes (40 to 80%), 17.1 to 19 minutes (80%), 19 to 19.1 minutes (80 to 5%), 19.1 to 27 minutes (5%).

Then, derivatized compounds of γ-Glu-Val-Gly, γ-Glu-Val, and Val-Gly eluted under the aforementioned separation conditions were introduced into a mass analyzer, and quantified by HPLC-MS chromatography. The analysis conditions are as follows.

(1) Mass analyzer: AB Sciex API3200 QTRAP
(2) Detection mode: Selected Ion Monitoring (positive ion mode)
(3) Selected ion: Table 1

Table 1

Derivatized compound	First mass analyzer (Q1)	Second mass analyzer (Q3)
γ -Glu-Val-Gly	474.2	171.2
γ -Glu-Val	417.4	171.1
Val-Gly	345.4	171.1
Ala-d3	263.0	171.1
Val-d8	296.0	171.1

The derivatized compounds of γ-Glu-Val-Gly, γ-Glu-Val, and Val-Gly were quantified by using analysis software, Analyst ver. 1.4.2 (AB Sciex). As the internal standard substance for performing the quantification, derivatized compounds of Ala-d3 were used in the case of γ-Glu-Val-Gly and γ-Glu-Val, and a derivatized compound of Val-d8 was used in the case of Val-Gly, respectively. The results are shown in Table 2. In the table, "ND" means that the amount was below quantitation limit (the same shall apply to the following). The analysis results (HPLC-MS chromatograms) of derivatized internal standard amino acids, γ-Glu-Val-Gly, γ-Glu-Val, and Val-Gly standard samples are shown in Fig. 1.

Table 2

	γ -Glu-Val-Gly	γ -Glu-Val	Val-Gly
Brand A	ND	0.3 ppm	0.5 ppm
Brand B	ND	ND	ND
Brand C	2.2 ppm	19.2 ppm	11.1 ppm
Brand D	ND	ND	0.4 ppm
Brand E	3.1 ppm	25.9 ppm	33.1 ppm
Brand F	ND	ND	0.4 ppm
Brand G	4.6 ppm	38.3 ppm	0.4 ppm

As shown in Table 1, the the amount of γ-Glu-Val-Gly in the various yeast extracts was several ppm at most. The amount of each of γ-Glu-Val and Val-Gly was on the order of several tens of ppm at most.

Example 2: Detection of γ-Glu-Val-Gly and γ-Glu-Val in cells of various yeasts

Then, the intracellular content of γ-Glu-Val-Gly and γ-Glu-Val in various yeasts were measured. The *Saccharomyces cerevisiae* S288C strain was used as a standard, and the *Saccharomyces cerevisiae* AJ14819 strain (deposited as an international deposit as FERM BP-08502 strain) was used for it's high GSH content. The S288C strain is stored at the independent administrative agency, National Institute of Technology and Evaluation, Biological Resource Center (NBRC, NITE Biological Resource Center, 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba-ken, 292-0818, Japan) with the number of NBRC1136, and can be provided therefrom. This strain is also stored at the American Type Culture Collection (12301 Parklawn Drive, Rockville, Maryland 20852, United States of America) with the number of ATCC 26108, and can be provided therefrom. The AJ14819 strain was obtained by mutagenizing a monoploid yeast strain obtained from a commercially available *Saccharomyces cerevisiae* strain with EMS, and then selecting a mutant strain in which expression of the *MET25* gene is not suppressed by methionine. The resulting strain was deposited at the independent administrative agency, Agency of Industrial Science and Technology, International Patent Organism Depository (Tsukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on September 11, 2002, and assigned an accession number of FERM P-19007. Then, the deposit was converted to an international deposit under the provisions of the Budapest Treaty on October 1, 2003, and assigned an accession number of FERM BP-08502 (Japanese Patent No. 4352877).

One loop of each of the aforementioned strains was inoculated into SD medium (50 ml in 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm.

Composition of SD medium:

Glucose	2%
Nitrogen Base	1-fold concentration

Nitrogen Base of 10-fold concentration was obtained by dissolving a mixture of 1.7 g of Bacto Yeast Nitrogen Base w/o Amino Acids and Ammonium Sulfate (Difco) and 5 g of ammonium sulfate in 100 ml of sterilized water, adjusting the solution to about pH 5.2, and sterilizing the solution by filter filtration.

Absorbance of the obtained culture broth was measured, the culture broth was inoculated into SD medium (400 ml in a 2 L-volume conical flask with baffle fins, a plurality of flasks) so that OD660 was 0.01 at the start of the culture (absorbance was measured by using DU640 SPECTROPHTOMETER, BECKMAN COULTER), and culture was performed at 30°C for 19 hours with shaking by rotation at a velocity of 120 rpm. From the obtained culture broth, 400 OD units of the cells (1 OD unit is defined as cells contained in 1 ml of culture broth of which OD660 is 1) were collected by centrifugal separation. The supernatant was removed as much as possible, and the residual cells were suspended in 45 ml of Milli-Q water. The cells were collected again by centrifugal separation, and resuspended in 45 ml of Milli-Q water. By repeating this operation 3 times in total, the medium was completely removed from the cells. The washed cells were suspended in about 1.5 ml of Milli-Q water, and the suspension was heated at 70°C for 10 minutes. By this step, the extractable components contained in the cells were extracted. Then, the extract and the cell residue were separated by centrifugation.

Cell debris were removed from the extract using a centrifugal filtration membrane of 10 kDa (Amicon Ultra - 0.5 mL 10K, MILLIPORE, Catalogue Number UFC501096)), the obtained fraction was derivatized with the AQC reagent in the same manner as that used in Example 1, and γ-Glu-Val-Gly and γ-Glu-Val were measured by LC-MS/MS. Separately, the amount of GSH in the cells was measured in a conventional manner (GSH in the extract was derivatized by using a fluorescence reagent ABD-F that binds to thiol group, separated and quantified by HPLC). Furthermore, dry cell weight was measured after the washed cells were dried at 104°C for 4 hours. From the amounts of γ-Glu-Val-Gly and γ-Glu-Val in the extract prepared

from the cells that was contained in a certain volume of culture broth and dry cell weight measured as described above, the amount of these peptides based on weight of dried cells was calculated. The results are shown in Table 3.

Table 3

	γ -Glu-Val-Gly (ppm)	γ -Glu-Val (ppm)	GSH (%)
S288C	1	3	0.43
AJ14819	1	2	1.06

As a result, particular correlation between the γ-Glu-Val-Gly or γ-Glu-Val content and the GSH content was not observed.

Example 3: Addition of γ-Glu-Val-Gly to S288C strain (1)

One loop of the S288C strain was inoculated into the SD medium (50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the obtained culture broth was measured, the culture broth was inoculated into SD medium (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 16 hours with shaking by rotation at a velocity of 120 rpm. To the SD medium, γ-Glu-Val-Gly (purchased from Kokusan Chemistry) was added in advance at a final concentration of 100 ppm.

Using the above culture broth, content of γ-Glu-Val-Gly in dry cells was calculated in the same manner as in Example 2. As a result, the γ-Glu-Val-Gly content was about 20,000 ppm (= 2%).

Furthermore, when the content of γ-Glu-Val-Gly in the washing solution was measured after various washing steps, the content was 0.01 ppm, i.e., below the quantitation limit, after centrifugation three times. The cells were washed once, and therefore it was confirmed that γ-Glu-Val-Gly in the medium was sufficiently removed in the washing process by four separation steps, and did not remain in the cell extract.

Example 4: Addition of γ-Glu-Val-Gly to S288C strain (2)

One loop of the S288C strain was inoculated into SD medium (50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the obtained culture broth was measured, the

culture broth was inoculated into SD medium (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 19 hours with shaking by rotation at a velocity of 120 rpm. After 19 hours, γ-Glu-Val-Gly aqueous solutions of various concentrations were added to obtain predetermined concentrations, and the culture was continued for 1 hour. Using these culture broths, the γ-Glu-Val-Gly content in dry cells was calculated in the same manner as in Example 2. Furthermore, the solid content of the extract was calculated, and the amount of γ-Glu-Val-Gly in the solid content was also calculated. The results are shown in Table 4.

Table 4

Amount of γ-Glu-Val-Gly added to medium (ppm)	0.01	0.1	1	5	10	100
γ-Glu-Val-Gly content in dry cells (ppm)	5	28	363	2647	5341	10191
γ-Glu-Val-Gly content in solid content of extract (ppm)	21	96	1110	10119	17408	32183

As a result, it was seen that as the amount of γ-Glu-Val-Gly increased, the γ-Glu-Val-Gly content in the dried cells and solid content of the extract increased.

Example 5: Production of yeast extract containing γ-Glu-Val-Gly

A yeast extract was produced from yeast cells cultured with the addition of γ-Glu-Val-Gly. One loop of the S288C strain was inoculated into SD medium (50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the obtained culture broth was measured, the culture broth was inoculated into SD medium (400 ml in a 2 L-volume conical flask with baffle fins, 12 flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 19 hours with shaking by rotation at a velocity of 120 rpm. After 19 hours, a γ-Glu-Val-Gly aqueous solution was added at a final concentration of 50 ppm, and the culture was continued for 1 hour.

The cells were collected from the total culture broth, and washed in the same manner as that of Example 2. The washed cells in an amount of 400 OD units were suspended in 1.5 ml of Milli-Q water. This suspension was maintained at 70°C for 10 minutes to produce an extract from the yeast cells. Furthermore, the suspension was

centrifuged to remove the cell residues and thereby collect only the extract. The γ-Glu-Val-Gly concentration of this extract (extract of addition experiment) was measured to be about 300 ppm, and solid content was about 1%. The γ-Glu-Val-Gly concentration in an extract prepared in the same way but without adding γ-Glu-Val-Gly to the culture broth (extract of no addition experiment) was below the quantitation limit, and solid content was about 1%.

The extracts prepared as described above were evaluated for kokumi in the presence of MSG (sodium glutamate) as follows. As a control, an aqueous solution containing 0.2% MSG and 0.5% NaCl was used, and the organoleptic score thereof was defined to be 0.0. Then, an aqueous solution containing 0.2% MSG, 0.5% NaCl and 10 ppm of γ-Glu-Val-Gly was used as a standard solution for kokumi, and the organoleptic score thereof was defined to be 3.0. By using these two kinds of solutions as standards of kokumi intensity, the kokumi intensity of the extract was evaluated using an aqueous solution containing the extract, 0.2% MSG, and 0.5% NaCl as a test sample. The amount of the extract of the addition experiment in the test sample was adjusted so that the γ-Glu-Val-Gly concentration became 10 ppm. The amount of the extract of the no addition experiment was adjusted so that the solid content in the test sample was the same as that in the test sample of the extract of the addition experiment. That is, the γ-Glu-Val-Gly concentrations in the standard solution of kokumi and the test sample containing the extract of the addition experiment were equivalent, and the solid contents in the test sample containing the extract of the addition experiment and the test sample containing the extract of the no addition experiment were equivalent.

The test samples prepared as described above were evaluated for kokumi by four special panelists. As a result, all four of the panelists evaluated that kokumi obtained with the extract of the addition experiment was stronger than that obtained with the same concentration of γ-Glu-Val-Gly. Furthermore, kokumi was evaluated at different stages of tasting, that is, it was evaluated for initial and middle tastes and also for aftertaste. The averages of the scores of the four panelists for each type of kokumi were used as the evaluation scores. The results are shown in Table 5. The initial and middle tastes means the taste sensed at 0 to 4 seconds after eating, and the aftertaste means the taste sensed 5 seconds after eating and thereafter. As seen from these results, it is estimated that kokumi for initial and middle tastes and kokumi for aftertaste of the extract prepared by adding γ-Glu-Val-Gly during the culture process was enhanced, because γ-Glu-Val-Gly was metabolized in a certain manner, or because of synergism of γ-Glu-Val-Gly and a component in the yeast extract.

Table 5

	Evaluation score for kokumi for initial and middle tastes	Evaluation score for kokumi for aftertaste
Control	0.0	0.0
Standard solution (10 mM γ -Glu-Val-Gly, 0.2% MSG, 0.5% NaCl)	3.0	3.0
Extract of addition experiment	4.0 ± 0.1	3.9 ± 0.3
Extract of no addition experiment	2.5 ± 0.9	1.8 ± 0.6

Example 6: Addition of γ-Glu-Val to the S288C strain (1)

One loop of the S288C strain was inoculated into SD medium (50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the resulting culture broth was measured, the culture broth was inoculated into SD medium (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 24 hours with shaking by rotation at a velocity of 120 rpm. To the SD medium, γ-Glu-Val (purchased from Bachem) was added in advance to a final concentration of 100 ppm. Using the culture broth, the amounts of γ-Glu-Val and γ-Glu-Val-Gly present in dry cells were calculated in the same manner as that of Example 2. The results are shown in Table 6.

Table 6

	γ-Glu-Val (ppm)	γ-Glu-Val-Gly (ppm)
No addition	3	0
Addition of γ-Glu-Val	7171	20

As a result, a part of γ-Glu-Val taken up into the yeast cells was converted into γ-Glu-Val-Gly.

Example 7: Addition of γ-Glu-Val to the S288C strain (2)

One loop of the S288C strain was inoculated into SD medium (50 ml in a 500

ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the resulting culture broth was measured, the culture broth was inoculated into SD medium (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 19 hours with shaking by rotation at a velocity of 120 rpm. After 19 hours, γ-Glu-Val aqueous solutions of various concentrations were added to obtain predetermined concentrations, and the culture was continued for 1 hour. Using these culture broths, the γ-Glu-Val content in the dry cells was calculated in the same manner as that of Example 2. Furthermore, the solid content of the extracted extract was calculated, and the amount of γ-Glu-Val in the solid content of the extract was also calculated. The results are shown in Table 7.

Table 7

Amount of γ-Glu-Val added to medium (ppm)	0	25	100	200
γ-Glu-Val content in dry cells (ppm)	2	1468	6369	10930
γ-Glu-Val content in solid content of extract (ppm)	9	8491	27599	49474

As a result, as the addition amount of γ-Glu-Val increased, the γ-Glu-Val content in the dry cells and solid content of the extract increased.

Example 8: Addition of Val-Gly to the S288C strain

One loop of the S288C strain was inoculated into SDP medium (ammonium sulfate used for the preparation of the SD medium was replaced with proline, final concentration of proline was 0.1 g/L, 50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the resulting culture broth was measured, the culture broth was inoculated into SDP medium (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 24 hours with shaking by rotation at a velocity of 120 rpm. To the SDP medium, Val-Gly (purchased from Bachem) was added in advance to a final concentration of 100 ppm. Using the culture broth, the amount of γ-Glu-Val-Gly in dry cells was calculated in the same manner as that of Example 2. The results are shown in Table 8.

Table 8

	γ-Glu-Val-Gly (ppm)
Val-Gly no addition experiment	0
Val-Gly addition experiment	2

When Val-Gly was added to the medium, γ-Glu-Val-Gly accumulated in the yeast cells.

Example 9: Yeast extract obtained with addition of Val-Gly on which γ-glutamyl transferase was made to act

A 1% aqueous solution of the yeast extract containing about 8% of GSH based on the solid content (yeast extract sample was prepared by adding reagent grade GSH into the commercially available yeast extract) was prepared, and adjusted to pH 7.0 with NaOH. To this solution, powdered Val-Gly was added to final concentrations in the aqueous solutions of 400 ppm, 800 ppm and 4000 ppm to prepare test samples. An aqueous solution of the yeast extract without addition of Val-Gly was also used as a control. To these test samples, a glutaminase (Glutaminase Daiwa SD-C100S, Daiwa kasei) was added at 1 mg/ml, and the enzymatic reaction was allowed at 37°C for 120 minutes. Furthermore, γ-GTP (γ-Glutamyltranspeptidase from equine kidney, Sigma, Code G9270-100UN) was added at 0.05 mg/ml instead of the glutaminase, and the enzymatic reaction was similarly allowed at 37°C for 120 minutes. The reaction mixtures were immediately cooled on ice, and the amounts of γ-Glu-Val-Gly, GSH and Cys-Gly were measured.

As a result, as shown in the following tables, very little γ-Glu-Val-Gly was generated in the Val-Gly no addition experiment, but as the addition amount of Val-Gly was increased, more γ-Glu-Val-Gly was generated. When γ-GTP was used, in particular, production of γ-Glu-Val-Gly was remarkable.

Table 9: Concentrations of components in reaction mixture observed with glutaminase

		γ-Glu-Val-Gly (ppm)	GSH (ppm)	Cys-Gly (ppm)
No addition	Before reaction	0.0	835.6	6.3
	After reaction	0.0	3.1	701.6
400 ppm	Before reaction	0.0	862.4	9.9
	After reaction	1.4	3.5	715.0
800 ppm	Before reaction	0.0	851.5	6.7
	After reaction	3.8	3.6	705.7
4000 ppm	Before reaction	0.0	826.2	6.1
	After reaction	16.0	3.4	683.3

Table 10: Concentrations of components in reaction mixture observed with γ-GTP

		γ-Glu-Val-Gly (ppm)	GSH (ppm)	Cys-Gly (ppm)
No addition	Before reaction	0.0	813.6	5.2
	After reaction	0.8	432.4	335.1
400 ppm	Before reaction	0.0	824.6	4.5
	After reaction	150.9	333.2	388.2
800 ppm	Before reaction	0.0	836.9	6.1
	After reaction	157.9	316.3	417.3
4000 ppm	Before reaction	0.0	857.2	6.7
	After reaction	340.5	180.8	462.6

If Val or nVal is added instead of Val-Gly, and the enzyme reaction is allowed to proceed in a similar manner, γ-Glu-Val or γ-Glu-nVal is also produced.

Indeed, the same experiments were executed by adding 4000ppm Val, 800ppm nVal, or 4000ppm nVal to a final concentration instead of Val-Gly. Also in this experiment, the yeast extract prepared from *Candida utilis* was used as commercially available yeast extract. Moreover, dry matter of the reaction mixture was measured to calculate the amount of γ-Glu-Val or γ-Glu-nVal in the dry matter base. As a result, 0.239% γ-Glu-Val on the dry matter base of yeast extract was produced when 4000ppm Val was added as a final concentration, 0.709% γ-Glu-nVal on the dry matter base of yeast extract was produced when 800ppm nVal was added as a final concentration, and 1.029% γ-Glu-nVal on the dry matter base of yeast extract was produced when

4000ppm nVal was added as a final concentration. The γ-Glu-nVal content was measured in the same manner described in the experimental example 1 by using 1μM γ-Glu-nVal as a standard solution. The analysis result (HPLC-MS chromatogram) of derivatized γ-Glu-nVal standard sample is shown in Fig. 2.

Example 10: Addition of γ-Glu-Val to GSH2-enhanced strain

A *GSH2* expression-enhanced strain can be bred by the following procedure.

1) Acquisition of uracil auxotrophic strain (*ura3* mutant) from the S288C strain

A uracil auxotrophic strain can be obtained by spreading a *Saccharomyces cerevisiae* strain treated with a mutagen in a conventional manner on an agar medium containing 5-FOA, and selecting an *ura3* mutant strain from the strains that grow (see, for example, METHODS IN YEAST GENETICS, 2000 EDITION, p.179). A uracil auxotrophic strain can also be obtained by introducing an *URA3*-neighboring DNA except for the *URA3* gene into the S288C strain, and disrupting the *URA3* gene, as shown below.

First, a 500-bp upstream region of *URA3* was amplified by PCR using the primers shown in SEQ ID NOS: 1 and 2, and the chromosomal DNA of the S288C strain as the template. Furthermore, a 500-bp downstream region of *URA3* was also amplified using the primers shown in SEQ ID NOS: 3 and 4. As for PCR conditions, a cycle consisting of thermal denaturation at 94°C for 10 seconds, annealing at 55°C for 10 seconds, and extension at 72°C for 1 minute was repeated 25 times. Then, overlap PCR was performed using the above two kinds of DNA fragments purified by ethanol precipitation as templates and the primers shown in SEQ ID NOS: 5 and 6 to obtain a 1-kb DNA fragment consisting of the 500-bp upstream region and 500-bp downstream region of the *URA3* gene ligated together. The S288C strain was transformed with this DNA fragment, and then cultured overnight in the SD medium to which uracil was added, and the cells were applied to 5-FOA plate medium. The ura3Δ0 strain was obtained from the resulting transformants.

2) Preparation of a template plasmid for promoter substitution

First, the *URA3* locus was amplified by PCR using the primers of SEQ ID NOS: 7 and 8 and the chromosomal DNA of a *Saccharomyces cerevisiae* wild-type strain as the template (thermal denaturation: 94°C for 10 seconds, annealing: 50°C for 10 seconds, extension: 72°C for 1 minute, 25 cycles). The resulting DNA fragment was purified by ethanol precipitation, and then digested with *Sph*I and *Eco*RI, and the

product was inserted into the plasmid pUC19 at the *Sph*I-*Eco*RI sites to obtain pUC19-URA3. Then, the *PGK1* promoter region was amplified from the chromosomal DNA of the *Saccharomyces cerevisiae* wild-type strain using the primers shown in SEQ ID NOS: 9 and 10. This DNA fragment was digested with *Pst*I, and inserted into pUC19-URA3 digested with *Pst*I and treated with CIAP at the *Pst*I site to obtain pUC19-PGK1p-URA3. In a similar manner, the *PGK1* promoter amplified using the primers shown in SEQ ID NOS: 11 and 12 was digested with *Aat*II, and inserted into pUC19-PGK1p-URA digested with *Aat*II and treated with CIAP at the *Aat*II site to obtain pUC19-PGK1p-URA3-PGK1p. When the nucleotide sequence of the prepared template plasmid was confirmed by sequencing, it was equivalent to the nucleotide sequence expected when using the chromosomal DNA of the S288C strain as the template. Therefore, even if the chromosomal DNA of the S288C strain is used as the template instead of chromosomal DNA of a wild-type strain, a similar plasmid can be prepared.

3) Introduction of *PGK1* promoter into *GSH2* gene on chromosome

PCR is performed using the primer of SEQ ID NO: 13, which has a *GSH2* upstream sequence at the 5' end, the primer of SEQ ID NO: 14, which has a part of a sequence in ORF starting from the start codon of the *GSH2* gene, and pUC19-PGK1p-URA3-PGK1p as the template (thermal denaturation: 94°C for 10 seconds, annealing: 60°C for 10 seconds, extension: 72°C for 4 minutes, 25 cycles) to prepare a DNA fragment having *URA3* between *PGK1* promoters. The ura3Δ0 strain can be transformed with this DNA fragment, and plated on an SD plate medium to obtain transformants, and a strain in which the *GSH2* promoter is replaced with the *PGK1* promoter-URA3-PGK1 promoter can be obtained from the transformants.

4) Elimination of *URA3* selective marker and substitution of promoter for *GSH2* gene

The strain in which the *PGK1* promoter-URA3-PGK1 promoter substitutes for the *GSH2* promoter is cultured overnight in a uracil-supplemented SD medium, and an appropriate volume of the culture is applied to 5-FOA plate medium. From colonies that appeared, a strain in which *URA3* is removed by homologous recombination between the introduced *PGK1* promoters, and the *GSH2* promoter is replaced with the *PGK1* promoter, can be obtained. Furthermore, by introducing a DNA amplified using a wild-type genome as the template and the primers of SEQ ID NOS: 15 and 16 into the above strain, a strain with a genotype that is the same as that of the ura3Δ0 strain except that *URA3* is returned to wild-type, and the *GSH2* promoter is replaced

with the *PGK1* promoter, can be obtained.

A yeast extract is produced from the strain obtained as described above, and evaluated in the same manner as described in Examples 3, 4, and 6 to 8.

Example 11: Addition of a γ-Glu-Val-Gly to *HGT1*-enhanced strain

An *HGT1* expression-enhanced strain can be obtained in the same manner as described in Example 10. Specifically, by performing PCR using pUC19-PGK1p-URA3-PGK1p as a template, the primer of SEQ ID NO: 17, which has a *GSH1* upstream sequence at the 5' end, and the primer of SEQ ID NO: 18, which has a part of a sequence in ORF starting from the start codon of the *GSH11* gene, and introducing the obtained DNA fragment into sporulating yeast, cells in which the promoter of *GSH11* is replaced with the *PGK1* promoter are obtained.

A yeast extract is produced from the strain obtained as described above, and evaluated in the same manner as described in Examples 3, 4, and 6 to 8.

Example 12: Evaluation of γ-Glu-Val-Gly uptake activity of *HGT1* or *PRT2* modified strain

The influence of Hgt1p, which has been considered to be a GSH specific transporter, and Ptr2p, which is a peptide transporter, on γ-Glu-Val-Gly accumulation was evaluated as follows.

1)The acquisition of uracil auxotrophic mutant (*ura3* mutant) from S288C

The geneticin (G418)-resistance cassette (P_{ADH1}-kanR) was amplified by PCR using pUC19AOX-G418-BRI (Fig. 3, SEQ ID NO: 23, Olga A. et al., Mol Biotechnol., Pubslished online December 19, 2010)) as the template and primers URA3Km-L1 (SEQ ID NO: 24) and URA3Km-R2 (SEQ ID NO: 25). The plasmid pUC19AOX-G418-BRI consists of the kanamycin resistant gene (*neo*) from *E. coli* transposon Tn5 under control of *S. cerevisiae ADH1* (*ADC1*) promoter cloned in pUC19 vector. BR1 means that in this plasmid the *Bam*HI and *Eco*RI sites, which are present in parental pUC19AOX-G418 between P_{ADH1} and *kaznR*, were deleted. The P_{ADH1}-kanR cassette or its equivalent can also be obtained by PCR using the plasmid pKat7 (Lang-Hinrichs, C. et al., 1989. Applied Microbiology and Biotechnology. 30:388-394) or pUM2 (Merckelbach A, et al., 1993, Appl Microbiol Biotechnol. Nov;40:361-364) instead of pUC19AOX-G418-BRI as a template and the above described primers URA3Km-L1 and URA3Km-R2.

40 nucleotides at 5'-end of primer URA3Km-L1 are homologous to the *URA3*

upstream region, and 40 nucleotides at 5'-end of primer URA3Km-R2 are complementary to the *URA3* downstream region.

The resulting DNA fragment was used for transformation of *S. cerevisiae* S288C. Transformants were selected on YPD plates containing 200 μg/ml of G418. Deletion of the *URA3* gene was verified by PCR with primers ura3up2 (SEQ ID NO: 26) and ura3dn2 (SEQ ID NO: 27).

The resulting strain was S288C ura3Δ0::P_{ADH1}-kanR. It may also be referred to as S288C ura3Δ0. This strain was used to construct a *HGT1* or *PTR2* overxpressing strain.

On the other hand, another uracil auxotrophic mutant was acquired as follows in order to use it to construct a strain in which *HGT1* or *PTR2* is deleted . A fragment which contains *URA3* with promoter and terminator regions was amplified by PCR using chrojmosomal DNA of S288C as the template and the aforementioned primers ura3up2 and ura3dn2. This fragment was cloned into the *Sma*I site of pBluescript II KS (+), and its structure was confirmed by sequencing analysis. The resulting plasmid was referred to as pKS-URA3-13 (Fig. 4, SEQ ID NO: 28).

Plasmid pKS-URA3-13 was digested with *Stu*I and *Nco*I, and then treated with Klenow fragment to blunt-end the *Nco*I site. The resulting 4.38-kb fragment was ligated with the 1.64-kb *Hin*cII – *Hin*cII fragment from pUG6 [Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., and Hegemann, J.H. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Research 24, 2519-2524], containing a loxP-kanMX-loxP module. The resulting plasmid was referred to as pKS-URA3-13-kanMX. Its map and scheme of construction is shown in Fig. 5, SEQ ID NO: 29.

Then, the kanMX gene flanked by URA3 fragments was amplified by PCR using the aforementioned primers ura3up2 and ura3dn2, and pKS-URA3-13-kanMX as the template. The resulting fragment was used for transformation of S. cerevisiae S288C strain, and then transformants were selected on YPD medium containing 200 μg/ml of G418. As a result, a S288C derivative strain with deletion of 227 nucleotides of coding region of *URA3* was obtained. This deletion was referred to as ura3Δ227::loxP-kanMX-loxP. To delete the kanMX marker, the resulting strain was transformed with plasmid pSH47 [Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., and Hegemann, J.H. 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Research 24, 2519-2524], containing a gene encoding Cre-recombinase under the control of the *GAL1* promoter and *URA3* as a selective marker. Marker excision occurred after induction of Cre-recombinase synthesis by shifting cells from the YPD medium to YPG (containing galactose). The resulting strain

from which the plasmid pSH47 had been deleted was designated S288C ura3Δ227::loxP.

2) Acquisition of *HGT1* deletion mutant

DNA fragment containing an Agleu2-CaURA3-Agleu2 cassette was amplified by PCR using plasmid pAG61 [Goldstein, A.L., Pan, X., and McCusker, J.H. 1999. Heterologous URA3MX cassettes for gene replacement in Saccharomyces cerevisiae. Yeast 15, 507-511] as the template and primers hgt1-pUG6u (SEQ ID NO: 30) and hgt1-pUG6d (SEQ ID NO: 31). The Agleu2-CaURA3-Agleu2 cassette contains *Candida albicans URA3* gene under control of *Ashbya gossypii TEF* promoter and terminator, surrounded with two identical fragments of *Ashbya gossypii LEU2* gene. Each of the *LEU2* gene fragments is a 470bp sequence spanning from C-terminal region to 3' UTR of the gene. The Agleu2-CaURA3-Agleu2 cassette is excisable from the plasmid pAG61.

47 nucleotides at the 5'-end of primer hgt1-pUG6u are homologous to the *HGT1* 5'- region, and 44 nucleotides at the 5'-end of the primer hgt1-pUG6d are complementary to the *HGT1* 3'- region.

The resulting fragment was used for transformation of the S288C ura3Δ227::loxP strain. Transformants were selected on plates containing SD medium. The resulting strain S288C ura3Δ227::loxP hgt1Δ1981::Agleu2-CaURA3-Agleu2, in which nucleotides at position 1981 to 2400 of the coding region of the *HGT1* gene was deleted, was referred to as S288C hgt1Δ.

The deletion of the *HGT1* gene was verified by PCR using the primers hgt1-51 (SEQ ID NO: 32) and hgt1-32 (SEQ ID NO: 33).

3) Acquisition of *PTR2* deletion mutant

The P_{ADH1}-kanR module from plasmid pUC19AOX-G418-BRI was excised with *Xba*I and *Sac*I and subcloned into the same sites of plasmid pUG6. The resulting plasmid was referred to as pLoxP-PADH1-kanR5 (Fig. 6).

A DNA fragment containing the loxP-P_{ADH1}-kanR-loxP module was amplified by PCR using the pLoxP-PADH1-kanR5 plasmid as the template and primers ptr2-pUG6u (SEQ ID NO: 34) and ptr2-pUG6d (SEQ ID NO: 35).

47 nucleotides at the 5'-end of primer hgt1-pUG6u are homologous to the *PTR2* 5'- region, and 44 nucleotides at the 5'-end of primer hgt1-pUG6d are complementary to the *PTR2* 3'- region.

The resulting fragment was used for transformation of S288C ura3Δ227::loxP

strain. Transformants were selected on plates containing YPD medium with 200 μg/ml of G418. The resulting strain S288C ura3Δ227::loxP ptr2Δ967::loxP-P_{ADH1}-kanR-loxP, in which nucleotides at postions 422 to 1388 of the coding region of the *PTR2* gene are deleted, is referred to as S288C ptr2Δ.

The deletion of the *PTR2* gene was verified by PCR using primers ptr2-51 (SEQ ID NO: 36) and ptr2-31 (SEQ ID NO: 37).

4) Acquisition of the *HGT1* and *PTR2* deletion mutant from the S288C strain

The strain S288C hgt1Δ ptr2Δ was constructed in a manner similar to the S288C ptr2Δ strain, but the strain S288C hgt1Δ was used for transformation.

5) Acquisition of an Hgt1p overproducing strain

A DNA fragment containing the promoter of *ADH1* was amplified by PCR using chromosomal DNA of *S. cerevisiae* strain S288C as the template and primers adh1L1 (SEQ ID NO: 38) and adh1R1 (SEQ ID NO: 39). The resulting fragment was cloned into the *Sma*I site of plasmid pUC19 and its structure was confirmed by sequence analysis. The resulting plasmid was referred to as pUC19-PADH1-r (Fig. 7).

The *Hin*dIII-*Hin*dIII fragment (1,16 kb) from the plasmid pKS-URA3-13 was subcloned into the *Hin*dIII site of the plasmid pBluescript II KS(+) yielding pKS-URA3H-d. The *Kpn*I-*Sal*I fragment from the plasmid pUC19-PADH1-r was cloned into the SalI - KpnI sites of pKS-URA3H-d. The resulting plasmid was referred to as pKS-URA3-P-ADH1 (Fig. 8).

Then, plasmid pKS-URA3-PADH1 was digested with *Cla*I and *Bsr*GI. The digested plasmid was blunt-ended with Klenow fragment and self-ligated. The resulting plasmid, referred to as pKS-URA3-PADH1-5'd, was digested with *Eco*RI, treated with Klenow fragment, and then digested with *Xba*I. The resulting fragment was ligated with a fragment of P_{ADH1}, and excised from plasmid pUC19-PADH1-r with *Xba*I and *Bst*Z17I. The resulting plasmid was referred to as pKS-URA3-PADH1-LR (Fig. 7).

A DNA fragment containing a P_{ADH1}-URA3-P_{ADH1} cassette was amplified by PCR using the plasmid pKS-URA3-PADH1-LR as the template and primers PADH1-HGT1 (SEQ ID NO: 40) and HGT1-PADH1 (SEQ ID NO: 41).

40 nucleotides at the 5'-end of primer PADH1-HGT1 are complementary to the first 40 nucleotides of the *HGT1* coding region, and 40 nucleotides at the 5'-end of primer HGT1-PADH1 are homologous to the *HGT1* upstream region.

A DNA fragment containing the *HGT1* upstream region was amplified by PCR using chromosomal DNA of S. cerevisiae strain S288C as the template and primers

hgt53 (SEQ ID NO: 42) and hgt33 (SEQ ID NO: 43).

Then, fragments obtained as described above were fused by PCR using primers hgt53 and PADH1-HGT1 as it was described in Shevchuk, N.A., Bryksin, A.V., Nusinovich, Y.A., Cabello, F.C., Sutherland M., Ladisch S. 2004. Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. Nucleic Acids Res.32(2):e19. The resulting fragment was used for transformation of strain S288C ura3Δ0::P_{ADH1}-kanR. Transformants were selected on plates containing SD medium. As a result, the strain S288C ura3Δ0::P_{ADH1}-kanR P_{ADH1}-URA3-P_{ADH1}-HGT1 was obtained. It was referred to as S288C P_{ADH1}-HGT1.

The scheme for the promoter replacement experiment is provided in Fig. 9. Primer hgt53 corresponds to Primer1, primer hgt33 corresponds to Primer2, primer HGT1-PADH1 corresponds to Primer3, primer PADH1-HGT1 corresponds to Primer4.

6) Acquisition of Ptr2p overproducing strain

Replacement of the native promoter of *PTR2* was carried out in a manner similar to the replacement of the promoter of *HGT1* (Fig. 9).

Primers used:

ptr52 (SEQ ID NO: 44), correspond to Primer1;

ptr32 (SEQ ID NO: 45), correspond to Primer2;

PTR2-PADH1 (SEQ ID NO: 46), correspond to Primer4;

PADH1-PTR2 (SEQ ID NO: 47), correspond to Primer3;

40 nucleotides at the 5'-end of primer PADH1-PTR2 are complementary to the first 40 nucleotides of the *PTR2* coding region, and 30 nucleotides at the 5'-end of the primer PTR2-PADH1 are homologous to the *PTR2* upstream region.

7) Evaluation of mutants

Each strain was cultivated in 50-ml tubes containing 5 ml of SD medium with 100ppm γ-Glu-Val-Gly as a final concentration. Tubes were inoculated by overnight culture of the corresponding strain at initial OD_{600}=0.5, and placed in a rotation shaker at 240 rpm, 30°C. The cultivation time was 7 hours. Then, cells from 2 ml of broth culture were harvested by centrifugation, and were washed with 1ml of milliQ water three times. Then, washed cells were suspended in 1ml of milliQ and were incubated at 70°C for 10 min with a water bath to extract intracellular materials. After cell debris and the extract were separated by centrifugation, the aqueous phase was transferred to a clean vial and the vial was then vacuum-dried. Thus, a dried extract was prepared. Then, the amount of γ-Glu-Val-Gly in the dried extract was measured with LC-MS/MS

analysis. The detailed procedures are described below.

Equipment:

Mass spectrometer: Triple Quadrupole Agilent 6410

HPLC: Agilent 1200

Columns: Thermo Hypersil-Keystone C18 100mm*2.1mm*5μm

1. Sample was dissolved in 100 μl of water, and then 100 μl of acetonitrile(MeCN) was added, and then centrifuged.
2. 190 μl of supernatant was transferred to a new tube and dried.
3. Pellet was dissolved in 40 μl of water.
4. For derivatization, 20 μl of the obtained solution was mixed with 60 μl of buffer and 80 μl of derivatization reagent, and incubated at 55°C for 10 min.
5. After incubation, 200 μl of 0.1% solution of HCOOH in water was added to stop AQC derivatization reaction.

40 μl of the resultingsolution was taken for HPLC, and γ-Glu-Val-Gly in the elutants was analyzed with MS/MS detector.

HPLC conditions:

mobile phase A – 0.1% HCOOH in H_2O

mobile phase B – 0.1% HCOOH in MeCN.

Flow rate – 0.250 ml/min

Table 11: Gradient conditions:

Time, min	mobile phase A	mobile phase B
0	95	5
0,5	95	5
30	75	25
35	20	80
40	20	80
41	95	5

Table 12: Detecting conditions in Mass spectrometer

Compound	Precursor ion (m/z)	Product ion (m/z)
γ-Glu-Val-Gly	474.2	304.2 300.1 229.0

With these data, the amount of intracellular γ-Glu-Val-Gly was caluculated. The results are shown in Tables 13 and 14. It was shown that the *HGT1* gene is essential to transport γ-Glu-Val-Gly from the medium, but *PTR2* is not.

Table 13: evaluation of *HGT1* and/or *PTR2* deleting strains

Strain	OD_{600}	γ-Glu-Val-Gly ($mg\ l^{-1}\ OD_{600}^{-1}$)
S288C	3.3	1.93
S288C hgt1Δ	3.3	0.01
S288C ptr2Δ	3.8	1.84
S288C hgt1Δ ptr2Δ	3.4	0.01

Table 14: evaluation of Hgt1p or Ptr2p overproducing strains

Strain	OD_{600}	γ-Glu-Val-Gly ($mg\ l^{-1}\ OD_{600}^{-1}$)
S288C	3.40	3.3
S288C P_{ADH1}-HGT1	1.64	28.6
S288C P_{ADH1}-PTR2	2.64	2.6

Example 13: Effect of the addition of γ-Glu-Val-Gly to a Hgt1p overproducing strain

The S288C P_{ADH1}-HGT1, Hgt1p overproducing strain obtained in Example 12, and its parental strain S288C, were evaluated for their ability to take up γ-Glu-Val-Gly in the same manners as described in Example 4. The amount of γ-Glu-Val-Gly added into the medium was 100ppm as a final concentration. As a result, intracellular content of γ-Glu-Val-Gly in S288C on the dry weight cell was about 0.9%, but intracellular content of γ-Glu-Val-Gly in S288C P_{ADH1}-HGT1 on the dry weight cell was about 4.9%. This result confirms the effect of Hgt1p overproduction.

Example 14: Effect of the addition of γ-Glu-nVal-Gly to S288C strain

γ-Glu-nVal-Gly uptake ability of S288C was evaluated in the same manner as described in example 4. γ-Glu-nVal-Gly was added into the medium in advance at a final concentration of 10ppm or 100ppm. The γ-Glu-nVal-Gly content was measured in the same manner described in experimental example 1 by using 1μM γ-Glu-nVal-Gly as a standard solution instead of γ-Glu-Val-Gly. As a result, the γ-Glu-nVal-Gly content in the dry cell was under the quantitative limit when γ-Glu-nVal-Gly was not added into the medium, but γ-Glu-nVal-Gly content in the dry cell was about 0.07% when γ-Glu-nVal-Gly was added into the medium at a final concentration of 10ppm, and γ-Glu-nVal-Gly content in the dry cell was about 0.09% when γ-Glu-nVal-Gly was added into the medium at a final concentration of 100ppm. The analysis result (HPLC-MS chromatogram) of derivatized γ-Glu-nVal-Gly standard sample is shown in Fig. 2.

Example 15: Effect of the addition of γ-Glu-nVal to S288C strain

γ-Glu-nVal uptake ability of S288C was evaluated in the same manner as described in example 7. To the SD medium, γ-Glu-nVal was added in advance at a final concentration of 10ppm or 100ppm. And the γ-Glu-nVal content was measured in the same manner described in the experimental example 1 by using 1μM γ-Glu-nVal as a standard solution instead of γ-Glu-Val. As a result, the γ-Glu-nVal content in the dry cell was under the quantitative limit when γ-Glu-nVal was not added into the medium, but γ-Glu-nVal content in the dry cell was about 0.03% when γ-Glu-nVal was added into the medium at a final concentration of 10ppm, and γ-Glu-nVal content in the dry cell was about 0.13% when γ-Glu-nVal was added into the medium at a final concentration of 100ppm.

Example 16: Production of yeast extract containing γ-Glu-nVal -Gly

A yeast extract was produced from yeast cells cultured with addition of γ-Glu-nVal –Gly in the same manner as described in example 5. One loop of the S288C strain was inoculated into SD medium (50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the resulting culture broth was measured, the culture broth was inoculated into SD medium (400ml in a 2 L-volume conical flask with baffle fins, 12 flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 19 hours with shaking by rotation at a velocity of 120 rpm. After 19 hours, a γ-Glu-nVal -Gly solution was added to a final concentration of 50 ppm, and the culture was continued for 1 hour.

The cells were collected from the obtained total culture broth, and washed in the same manner as described in Example 2. The washed cells in an amount of 400 OD

units were suspended in 1.5 ml of Milli-Q water. This suspension was maintained at 70°C for 10 minutes to produce an extract from the yeast cells. Furthermore, the suspension was centrifuged to remove the cell residues and thereby collect only the extract. The γ-Glu-nVal -Gly concentration of this extract (extract of addition experiment) was measured to be about 100 ppm, and the solid content was about 1%. The γ-Glu-nVal -Gly concentration of an extract prepared by the same operation without adding γ-Glu-nVal -Gly to the culture broth (extract of no addition experiment) was below the detection limit, and the solid content was about 1%.

The extracts prepared as described above were evaluated for kokumi in the presence of MSG (sodium glutamate) as follows. As a control, an aqueous solution containing 0.2% MSG and 0.5% NaCl was used, and the organoleptic score thereof was defined to be 0.0. Then, an aqueous solution containing 0.2% MSG, 0.5% NaCl and 1 ppm of γ-Glu-nVal-Gly was used as a standard solution for kokumi, and the organoleptic score thereof was defined to be 3.0. By using these two kinds of solutions as standards of kokumi intensity, kokumi intensity of the extract was evaluated. The kokumi intensity of the extracts was evaluated using an aqueous solution containing the extract, 0.2% MSG and 0.5% NaCl as a test sample. The amount of the extract of the addition experiment in the test sample was adjusted so that the γ-Glu-nVal-Gly concentration became 1 ppm. The amount of the extract of the no addition experiment was adjusted so that the solid content in the test sample was the same as that in the test sample of the extract of the addition experiment. That is, the γ-Glu-nVal-Gly concentrations in the standard solution of kokumi and the test sample containing the extract of the addition experiment were equivalent, and the solid contents in the test sample containing the extract of the addition experiment and the test sample containing the extract of the no addition experiment, were equivalent. Moreover, a model sample was prepared by adding a γ-Glu-nVal -Gly solution into the extract of no addition experiment so as to be at an equivalent concentration of γ-Glu-nVal -Gly as compared to the extract of addition experiment. Therefore the γ-Glu-nVal -Gly concentration contained in the model sample was about 100 ppm, and the solid content was about 1%.

The test samples prepared as described above were evaluated for kokumi by four special panelists. As a result, all four of the panelists evaluated that kokumi obtained with the extract of the addition experiment was stronger than that obtained with the same concentration of γ-Glu-nVal-Gly. Furthermore, kokumi was evaluated for initial and middle tastes, and for aftertaste. Averages of the scores of the four panelists for each taste point of kokumi were used as the evaluation scores. The

results are shown in Table 15. The initial and middle tastes means the taste at 0 to 4 seconds after eating, and the aftertaste means the taste 5 seconds after eating and thereafter. As seen from these results, it is estimated that kokumi for initial and middle tastes and kokumi for aftertaste of the extract prepared by adding γ-Glu-nVal-Gly during the culture process was enhanced, because γ-Glu-nVal-Gly was metabolized in a certain manner, or because of synergism of γ-Glu-nVal-Gly and a component in the yeast extract.

Table 15

	Evaluation score for kokumi for initial and middle tastes	Evaluation score for kokumi for aftertaste
Control	0.0	0.0
Standard solution (1 mM γ -Glu-nVal-Gly, 0.2% MSG, 0.5% NaCl)	3.0	3.0
Extract of addition experiment	4.2 ± 0.7	3.6 ± 0.5
Extract of no addition experiment	1.1 ± 0.9	1.1 ± 0.9
Model sample	3.3 ± 0.2	3.3 ± 0.2

Example 17: Effect of addition of γ-Glu-nVal-Gly to an Hgt1p overproducing strain

S288C P_{ADH1}-HGT1, Hgt1p overproducing strain obtained in Example12, and its parental strain S288C were evaluated for their γ-Glu-nVal-Gly uptake abilities in the same manners as described in Example 4 and Example 13. The amount of γ-Glu-nVal-Gly added to the medium was 100 ppm as a final concentration. As a result, intracellular content of γ-Glu-Val-Gly in S288C on the dry weight cell was about 0.09%, but intracellular content of γ-Glu-nVal-Gly in S288C P_{ADH1}-HGT1 on the dry weight cell was about 2.84%.

Example 18: Effect of addition of Val-Gly to a Ptr2p overproducing strain

S288C P_{ADH1}-HGT1, Hgt1p overproducing strain obtained in Example12, S288C P_{ADH1}-PTR2, Ptr2p overproducing strain obtained in Example12, and their parental strain S288C were evaluated for their γ-Glu-Val-Gly producing abilities from Val-Gly. Each strain was cultivated in 50-ml tubes containing 10 ml of SD medium with 100 ppm Val-Gly as a final concentration. Tubes were inoculated by overnight

culture of corresponding strain at initial OD_{600}=0.01, and placed in rotation shaker at 240 rpm, 30°C. Cultivation time was for 20 hours. Then, cells from 2 ml of broth culture were harvested by centrifugation, and washed with 1ml of milliQ water three times. Then, washed cells were suspended in 1ml of milliQ and incubated at 70°C for 10 min with a water bath to extract intracellular materials. After cell debris and the extract were separated by centrifugation, the aqueous phase was transferred to a clean vial and then the vial was vacuum-dried. Thus, the dried extract was prepared. Then γ-Glu-Val-Gly in the dried extract was measured with LC-MS/MS analysis as described in Example 12. As seen from the results, *PTR2* seems to be a transporter of Val-Gly from the medium, and was effective to increase cellular γ-Glu-Val-Gly content from Val-Gly.

Table 16: evaluation of Hgt1p or Ptr2p overproducing strains

Strain	OD_{600}	γ-Glu-Val-Gly ($\mu g\ l^{-1}\ OD_{600}^{-1}$)
S288C	3.38	0.04
S288C P_{ADH1}-HGT1	3.96	0.03
S288C P_{ADH1}-PTR2	1.86	8.89

Example 19: Effect of addition of nVal to a *GSH1* overexpressing strain

S288C strain was cultivated in SDP medium in the same manner as the Val-Gly no addition experiment described in Example 8. Then, cellular γ-Glu-nVal-Gly and γ-Glu-nVal were measured but were below the detection limit. Therefore, the effect of addition of nVal to the *GSH1* overexpressing strain was evaluated.

First of all, *GSH1* overexpressing strain was constructed as described below.

1) Acquisition of uracil auxotrophic strain (*ura3* mutant) of a *Saccharomyces cerevisiae* wild type strain

Uracil auxotrophic strain (*ura3* mutant) was obtained in the same manner described in Example 10, but a *Saccharomyces cerevisiae* wild type strain (MAT α type haploid strain) was used instead of S288C strain. The resulting strain was given a private number AJ14956, and was deposited at the independent administrative agency, Agency of Industrial Science and Technology, International Patent Organism Depository (Tukuba Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) on August 18, 2010, and assigned an accession number of FERM

P-22000. Then, the deposit was converted to an international deposit under the provisions of the Budapest Treaty on November 17, 2010, and assigned an accession number of FERM BP-11299.

2) Acquisition of *GSH1* overexpressing strain

Template plasmid pAUA (Olga, A.S. et al, 2010, Mol Biotechnol, published on line; 19 December 2010, DOI 10.1007/s12033-010-9362-6) was used to substitute the native promoter of GSH1 with the promoter of *ADH1*. Then, the native promoter of *GSH1* in AJ14956 was replaced with the promoter of *ADH1* as we reported previously (Olga, A.S. et al, 2010). Then, the deleted *URA3* gene was returned to the wild-type strain in the same manner as described in Example 10. Thus, a *GSH1* overexpressing strain AG1 was obtained, which was, of course, non-auxotrophic to uracil.

Then, this AG1 strain was cultivated in SD medium supplemented with nVal. More specifically, one loop of the AG1 strain was inoculated into SD medium (50 ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the obtained culture broth was measured, and the culture broth was inoculated into SDP medium (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C for 23 hours with shaking by rotation at a velocity of 120 rpm. After 23 hours, nVal solution was added to a final concentration of 10 ppm or 100ppm, and the cultures were continued for 1 hour. Then, the amount of γ-Glu-nVal was measured in the same manner described in the experimental example 15. The γ-Glu-nVal content was 0.07% when 10 ppm of nVal was added to the medium and the γ-Glu-nVal content was 0.37% when 100 ppm of nVal was added to the medium. Then, yeast extract containing γ-Glu-nVal was prepared from the broth culture cultivated with 100 ppm nVal basically in the same manner as described in Example 16. This yeast extract contained 1.44% γ-Glu-nVal at the dry matter base. This yeast extract would give strong kokumi for initial and middle taste from its character.

Example 20: Effect of addition of γ-Glu-Val to a *GSH2* overexpressing strain (1)

GSH2 overexpressing strain PG2 was constructed basically in the same manner as described in Example 10 except that AJ14956 described in Example 19 was used instead of ura3Δ0 from S288C. As a control, WT strain was obtained by returning the deleted *URA3* gene into wild type gene from AJ14956 in the same manner described in Example 10 and Example 19.

One loop of the WT strain or PG2 strain was inoculated into SD medium (50

ml in a 500 ml-volume Sakaguchi flask), and cultured at 30°C for 24 hours with shaking at a velocity of 120 rpm. Absorbance of the obtained culture broth was measured, and the culture broth was inoculated into the SD medium supplemented with various concentrations of γ-Glu-Val (400 ml in a 2 L-volume conical flask with baffle fins, two or more flasks) so that OD660 was 0.01 at the start of the culture, and culture was performed at 30°C with shaking by rotation at a velocity of 120 rpm until OD660 reached to the predetermined value. Then, the amount of γ-Glu-Val-Gly in dry cells and the solid content of the extracted extract were calculated in the same manner described previously. As shown in the results, PG2, a *GSH2* overexpressing strain, took up γ-Glu-Val from the medium and converted it into γ-Glu-Val-Gly in the cell.

Table 17: evaluation of *GSH2* overexpressing strain

Strain	γ-Glu-Val in the medium	CT (hr)	OD_{660}	γ-Glu-Val-Gly content in dry cells (ppm)	γ-Glu-Val-Gly content in solid content of extract (ppm)
WT	0 ppm	19	1.67	0	0
WT	100 ppm	19	1.79	7	29
WT	500 ppm	19	1.69	7	29
PG2	0 ppm	21	1.68	0	0
PG2	100 ppm	21	1.74	81	384
PG2	500 ppm	21	1.65	123	585

Example 21: Effect of addition of γ-Glu-Val to *GSH2* overexpressing strain (2)

1) Preparation of template plasmid with excisable kanMX marker and *TDH3* promoter

The DNA fragment containing promoter of the *TDH3* gene, encoding the glyceraldehyde 3-phosphate dehydrogenase, was amplified by PCR, using the chromosomal DNA of S288C strain as a template, and tdh3-EcoR1 (SEQ ID NO: 48) and tdh3-Pci1 (SEQ ID NO: 49) primers (thermal denaturation: 94°C for 20 seconds, annealing: 50°C for 20 seconds, extension: 72°C for 30 seconds, 30 cycles). Resulting fragment was cloned into *Sma*I site of pUC57 vector. The plasmid where promoter of TDH3 gene was orientated in direction from *Hin*dIII site to *Eco*RI site was selected and referred to as pUC57-PTDH3. The structure of cloned DNA fragment was confirmed by sequence analysis.

The *Nde*I-*Sal*I DNA fragment of pUC57-PTDH3 was cloned into *Nde*I-*Sal*I sites of pUG6 (Güldener, U., Heck, S., Fiedler, T., Beinhauer, J., and Hegemann, J.H.

1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Research 24, 2519-2524). Resulting plasmid was referred to as pUG6-PTDH3 (Fig. 10).

2) Substitution of *GSH1* and *GSH2* native promoters with promoter of *TDH3*

The loxP-kanMX-loxP-P_{TDH3} cassette was amplified by PCR using the pUG6-PTDH3 as a template and GSH1-pUG (SEQ ID NO: 50) and PTDH3-GSH1 (SEQ ID NO: 51) primers (thermal denaturation: 94°C for 20 seconds, annealing: 48°C for 60 seconds, extension: 72°C for 120 seconds, 2 cycles; 94°C for 20 seconds, annealing: 65°C for 20 seconds, extension: 72°C for 120 seconds, 20 cycles). Resulting DNA fragment was used for transformation of S288C strain. Transformants were selected on YPD medium containing 200 μg/ml of G418. As a result, the S288C derivative strain with promoter of *GSH1* gene replaced with promoter of *TDH3* was obtained (S288C loxP-kanMX-loxP-P_{TDH3}-GSH1 strain). The *ura3* derivative of this strain (S288C ura3 loxP-kanMX-loxP-P_{TDH3}-GSH1 strain) was obtained then, by selection on 5-FOA containing medium according conventional method (see, for example, METHODS IN YEAST GENETICS, 2000 EDITION, p.179). The kanMX marker was deleted from resulting strain by means of transformation by pSH47 plasmid, as it is described in Example 12. As a result the S288C ura3 loxP-P_{TDH3}-GSH1 strain was obtained.

The native promoter of *GSH2* gene was then replaced with promoter of *TDH3*. For this purpose, the loxP-kanMX-loxP-P_{TDH3} cassette was amplified by PCR using the pUG6-PTDH3 as a template and PTDH3-GSH2 (SEQ ID NO: 52) and GSH2-pUG (SEQ ID NO: 53) primers (thermal denaturation: 94°C for 20 seconds, annealing: 48°C for 60 seconds, extension: 72°C for 120 seconds, 2 cycles; 94°C for 20 seconds, annealing: 65°C for 20 seconds, extension: 72°C for 120 seconds, 20 cycles). The GSH2 upstream region was amplified by PCR using chromosomal DNA of S288C strain as a template and GSH2up2 (SEQ ID NO: 54) and GSH2d_tail (SEQ ID NO: 55) primers (thermal denaturation: 94°C for 20 seconds, annealing: 48°C for 20 seconds, 72°C for 30 seconds, 30 cycles). The resulting fragments were fused by means of overlap extension PCR (Shevchuk, N.A. at al. 2004. Construction of long DNA molecules using long PCR-based fusion of several fragments simultaneously. Nucleic Acids Res.32(2):e19) using GSH2up2 (SEQ ID NO: 54) and PTDH3-GSH2 (SEQ ID NO: 52) primers (thermal denaturation: 94°C for 20 seconds, annealing: 55°C for 20 seconds, 72°C for 120 seconds, 30 cycles). Resulting DNA fragment was used for transformation of S288C ura3 loxP-P_{TDH3}-GSH1 strain. Transformants were selected on

YPD medium containing 200 μg/ml of G418. As a result, S288C ura3 loxP-P_{TDH3}-GSH1 loxP-kanMX-loxP-P_{TDH3}-GSH2 strain was obtained.
Then, the kanMX marker was deleted from this strain as was described above, and the S288C ura3 loxP-P_{TDH3}-GSH1 loxP-P_{TDH3}-GSH2 strain was obtained. It was referred to as Y3560.

3) Construction of *GSH2* overexpressed plasmid

The P_{TDH3}-GSH2 construction was amplified using Y3560 chromosomal DNA as a template and yepgsh2-F (SEQ ID NO: 56) and yepgsh2-R (SEQ ID NO: 57) primers. The 45 nucleotides of 5' sequences of these primers were complementary to regions flanking the *Sma*I – *Bam*HI fragment in YEp24 plasmid (Fig. 11). Then the YEp24 plasmid was digested with *Sma*I and *Bam*HI and the larger of the resulting fragments were isolated by means of agarose gel electrophoresis. The resulting P_{TDH3}-GSH2 and YEp24 fragments were mixed and used for transformation of S288C ura3Δ227::loxP and Y3560 strains. Transformants were selected on SD medium. Resulting plasmid was referred to as YEp24-PTDH3-GSH2, and resulting strains were referred to as S288C ura3Δ227/YEp24-PTDH3-GSH2 and Y3560/YEp24-PTDH3-GSH2, respectively.

4) Evaluation of mutants

Each of the S288C ura3Δ227/YEp24-PTDH3-GSH2, Y3560/YEp24-PTDH3-GSH2, S288C and Y3560 strains, was cultivated in 50-ml tubes containing 10 ml of SD medium with 100ppm γ-Glu-Val as a final concentration. For Y3560 strain medium was also supplemented with uracil (20 mg/L). Tubes were inoculated by overnight culture of corresponding strain at initial OD_{600}=0.01, and placed in rotation shaker at 240 rpm, 30°C for 18 hours. Then cells from 2 ml of broth culture were harvested by centrifugation, and were washed by 1ml of milliQ water three times. Then washed cells were suspended in 1ml of milliQ and were incubated at 70°C for 10 min with a water bath to extract intracellular materials. After cell debris and the extract were separated by centrifugation, aqueous phase was transferred to clean vial and then the vial was vacuum-dried. Thus the dried extract was prepared. Then γ-Glu-Val-Gly in the dried extract was measured with LC-MS/MS analysis as it is described in example 12. The results are shown in Table 18.

As shown in the results, *GSH2* overexpressing strains converted γ-Glu-Val into γ-Glu-Val-Gly in the cell.

Table 18. The influence of YEp24-PTDH3-GSH2 on γ-EV to γ-EVG conversion

Strain	OD_{600}	γ-EVG, μg l^{-1} OD_{600}^{-1}
S288C	3.1	6
S288C ura3Δ227/YEp24-PTDH3-GSH2	3.0	705
Y3560	2.7	110
Y3560/YEp24-PTDH3-GSH2	2.5	2039

CLAIMS

1. A yeast extract containing a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

2. The yeast extract according to claim 1, which contains the peptide in a total amount of 0.02% or more.

3. The yeast extract according to claim 1 or 2, wherein X is Val.

4. The yeast extract according to claim 1 or 2, wherein X is nVal.

5. The yeast extract according to any one of claims 1 to 4, wherein the yeast is *Saccharomyces cerevisiae*.

6. A method for producing a yeast extract containing a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly, which comprises culturing a yeast in a medium containing a peptide selected from the group consisting of γ-Glu-X, γ-Glu-X-Gly and X-Gly, and preparing a yeast extract from the obtained cells, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

7. The method according to claim 6 wherein the medium contains 0.1 ppm or more of the peptide, and the yeast extract contains the peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract.

8. The method according to claim 6 or 7, wherein X is Val.

9. The method according to claim 6 or 7, wherein X is nVal.

10. The method according to any one of claims 6 to 9, wherein the yeast has been modified so that uptake of the peptide into cells is improved.

11. The method according to claim 10, wherein the activity of Hgt1p of the yeast has been enhanced.

12. The method according to claim 10, wherein the activity of Ptr2p of the yeast has been enhanced.

13. The method according to any one of claims 10 to 12, wherein the activity of glutathione synthetase of the yeast has been enhanced.

14. A method for producing a yeast extract containing γ-Glu-nVal, which comprises culturing a yeast in a medium containing nVal.

15. The method according to claim 14, wherein the activity of γ-glutamylcysteine synthetase of yeast has been enhanced.

16. The method according to any one of claims 6 to 15, wherein the yeast is *Saccharomyces cerevisiae*.

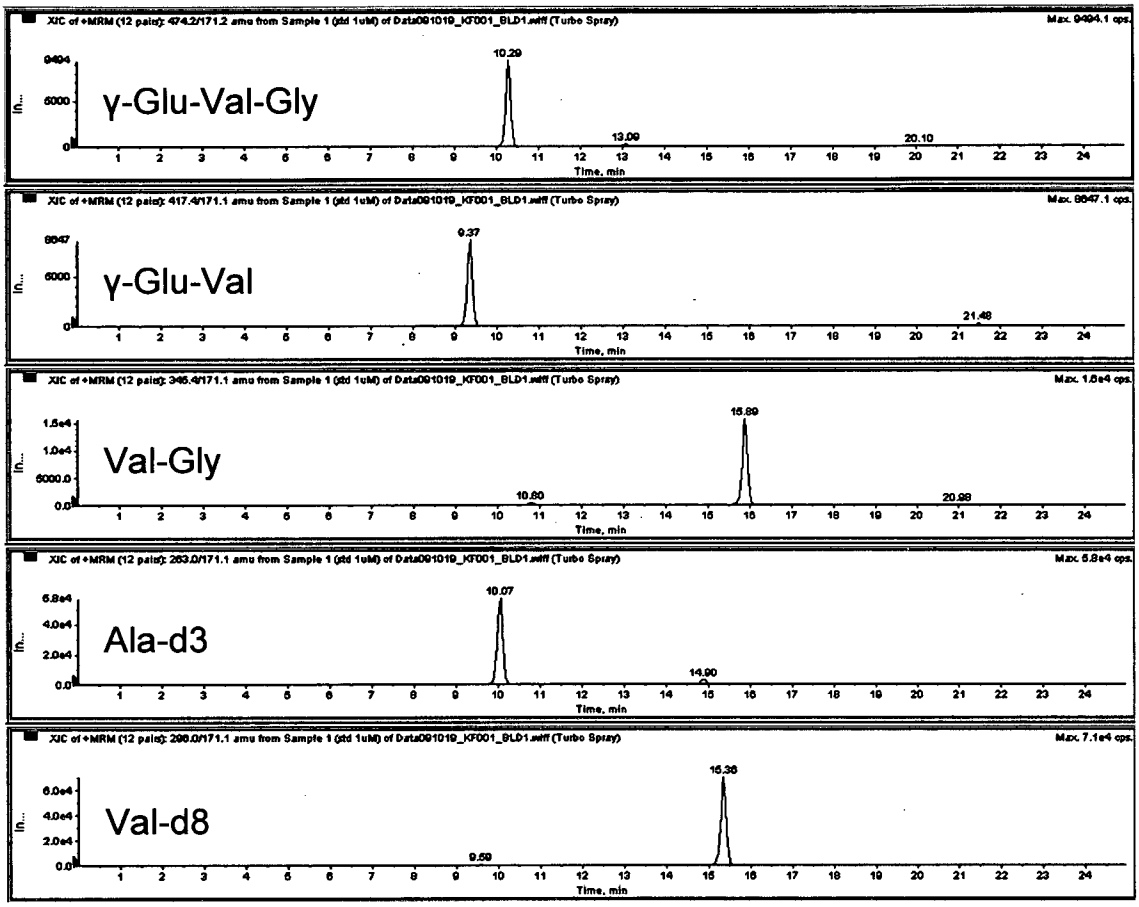
17. A method for producing a yeast extract containing a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly, which comprises allowing a γ-glutamyl transferase to act on a yeast extract raw material to which an amino acid or a peptide selected from the group consisting of X and X-Gly is added, wherein X represents an amino acid or an amino acid derivative other than Cys and derivatives thereof.

18. The method according to claim 17, wherein the amino acid or peptide is added in a total amount of 1% or more based on dry weight of the yeast extract raw material, and the yeast extract contains a peptide selected from the group consisting of γ-Glu-X and γ-Glu-X-Gly in a total amount of 0.005% or more based on dry weight of the yeast extract.

19 The method according to claim 17 or 18, wherein X is Val.

20. The method according to claim 17 or 18, wherein X is nVal.

21. The method according to any one of claims 17 to 20, wherein the yeast is *Saccharomyces cerevisiae*.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Val-d8
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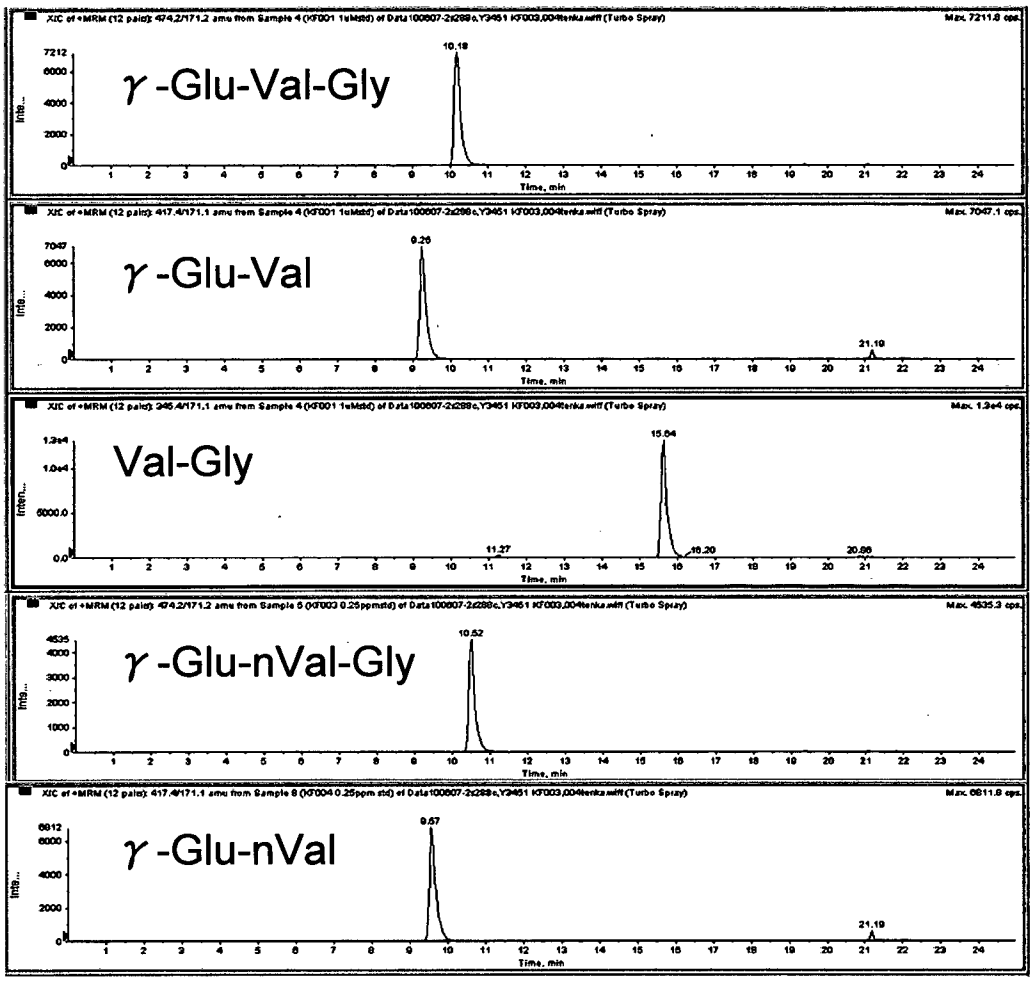
9.59
Time, min

Fig. 1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γ-Glu-nVal
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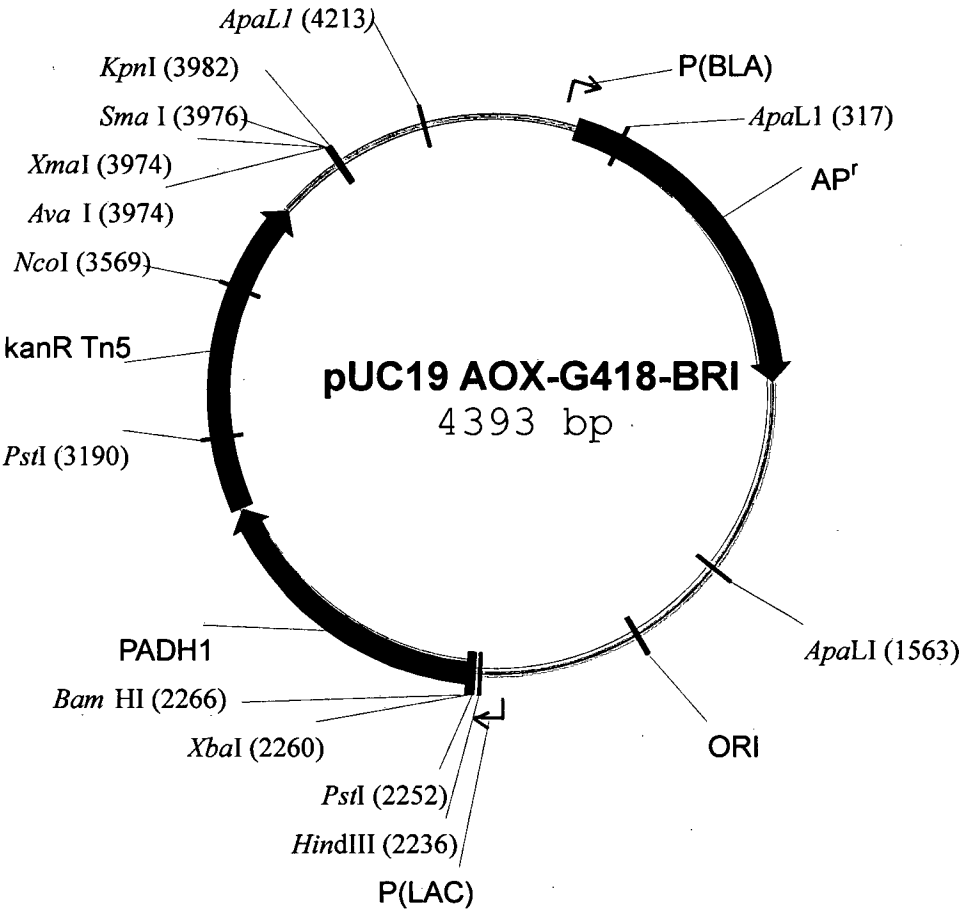
Time, min

Fig. 2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Sma I (3976)
XmaI (3974)
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NcoI (3569)
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PstI (3190)
PADH1
Bam HI (2266)
XbaI (2260)
PstI (2252)
HindIII (2236)
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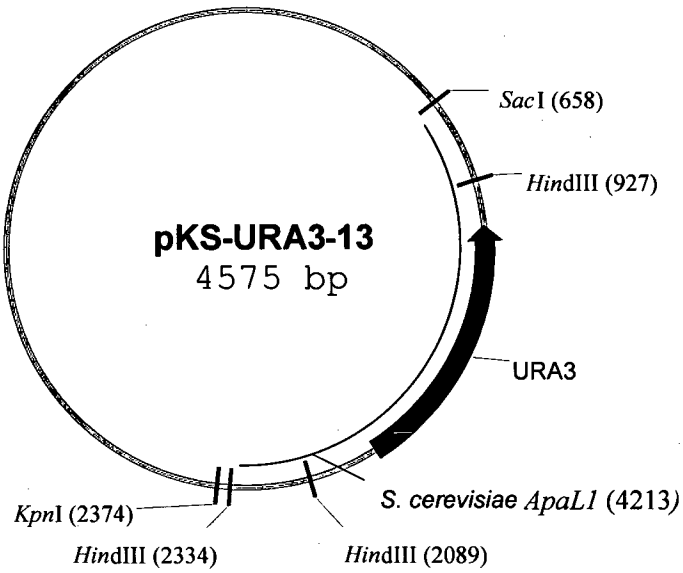
Fig. 3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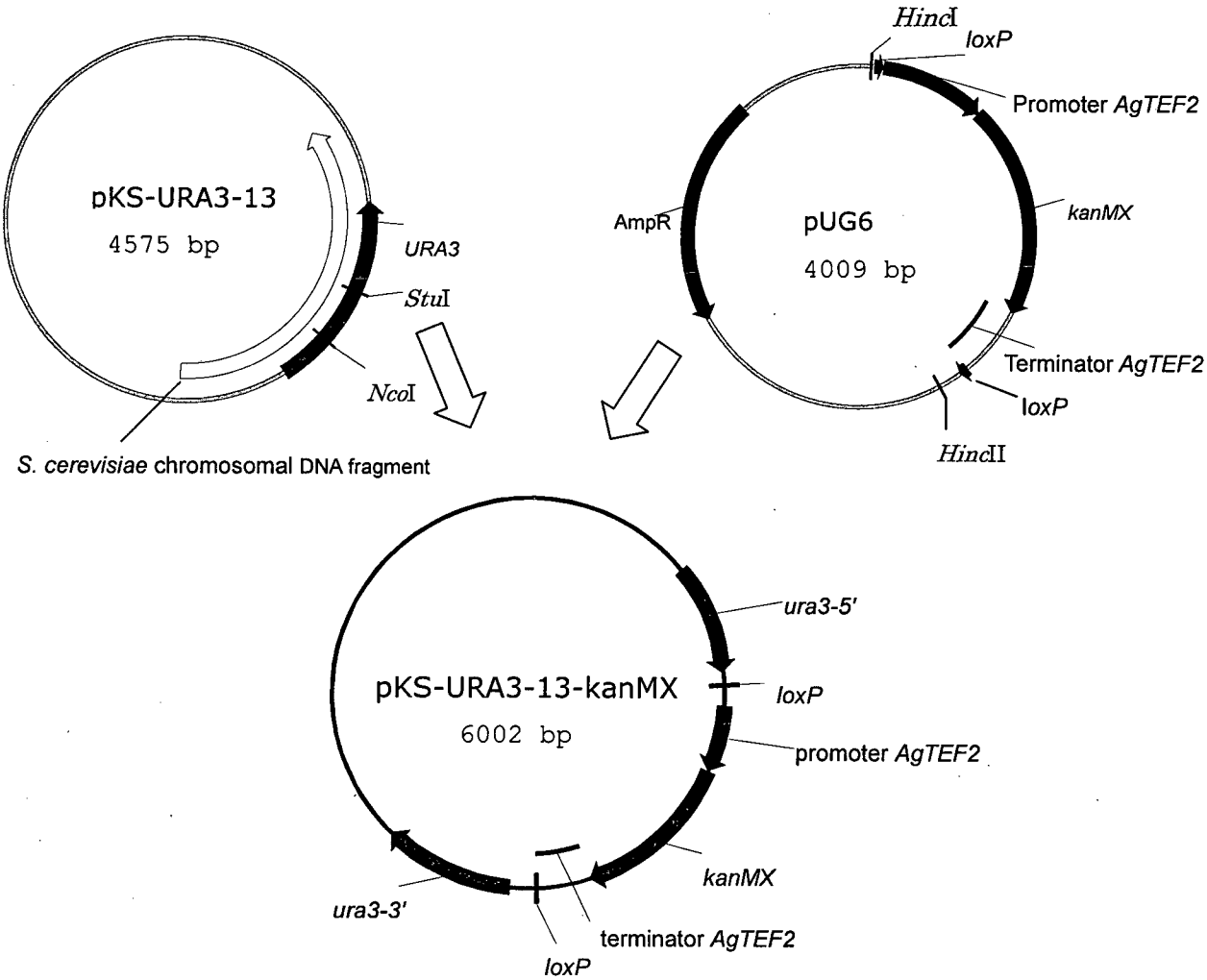
HindIII (927)
URA3
S. cerevisiae ApaL1 (4213)
KpnI (2374)
HindIII (2334)
HindIII (2089)

Fig. 4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Terminator AgTEF2
loxP
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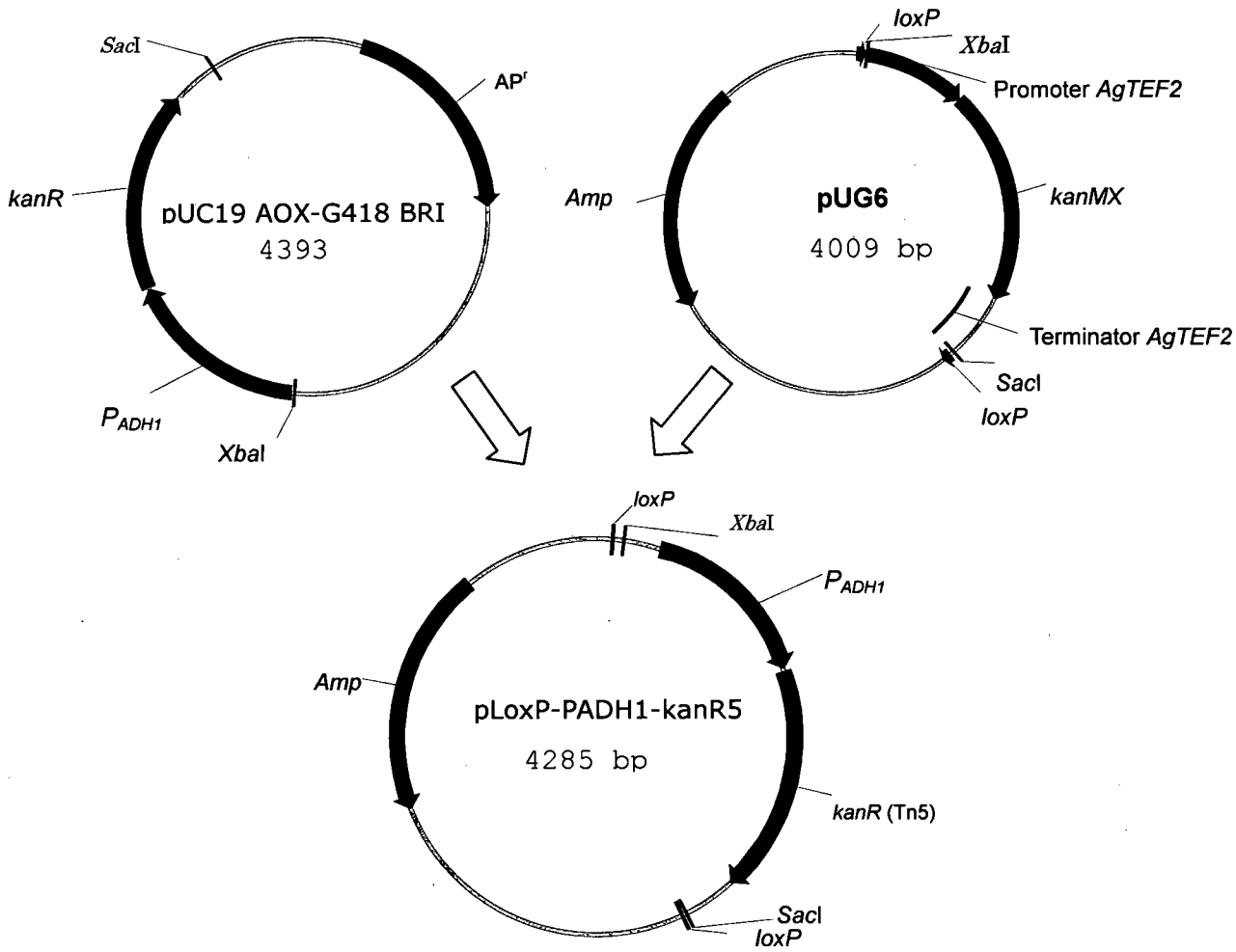
Fig. 5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pUG6
4009 bp
kanMX
Terminator AgTEF2
SacI
loxP
loxP
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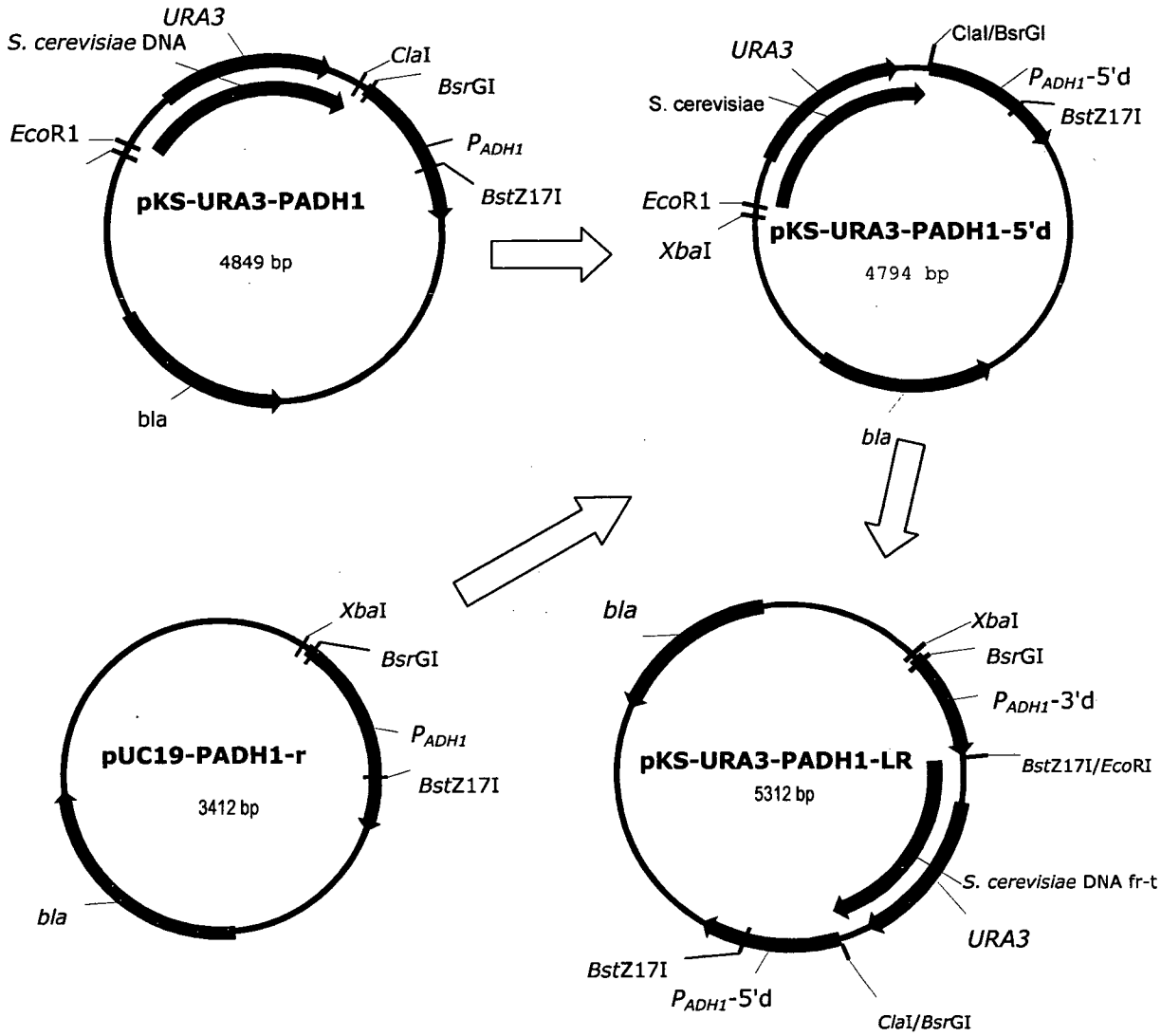
kanR (Tn5)
SacI
loxP

Fig. 6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BstZ17I
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URA3
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S. cerevisiae
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EcoR1
XbaI
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bla
XbaI
BsrGI
PADH1
pUC19-PADH1-r
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bla
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BsrGI
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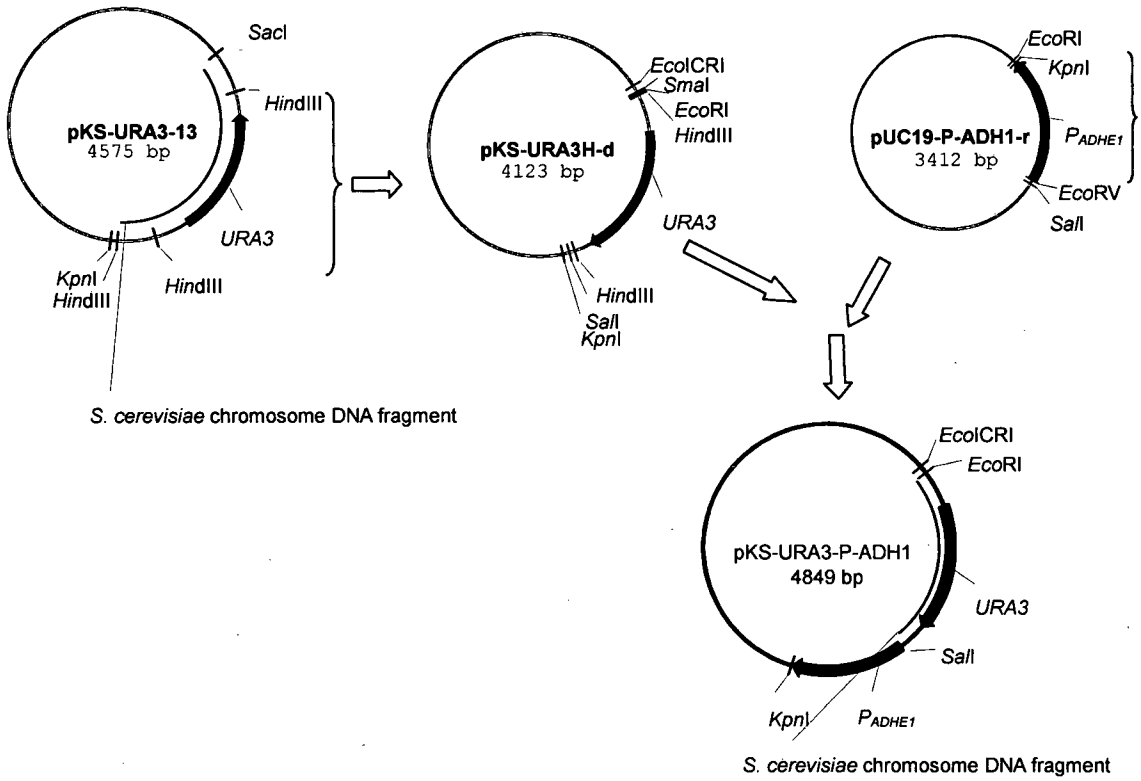
ClaI/BsrGI

Fig. 7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EcoRI
HindIII
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URA3
HindIII
SalI
KpnI
EcoRI
KpnI
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SalI
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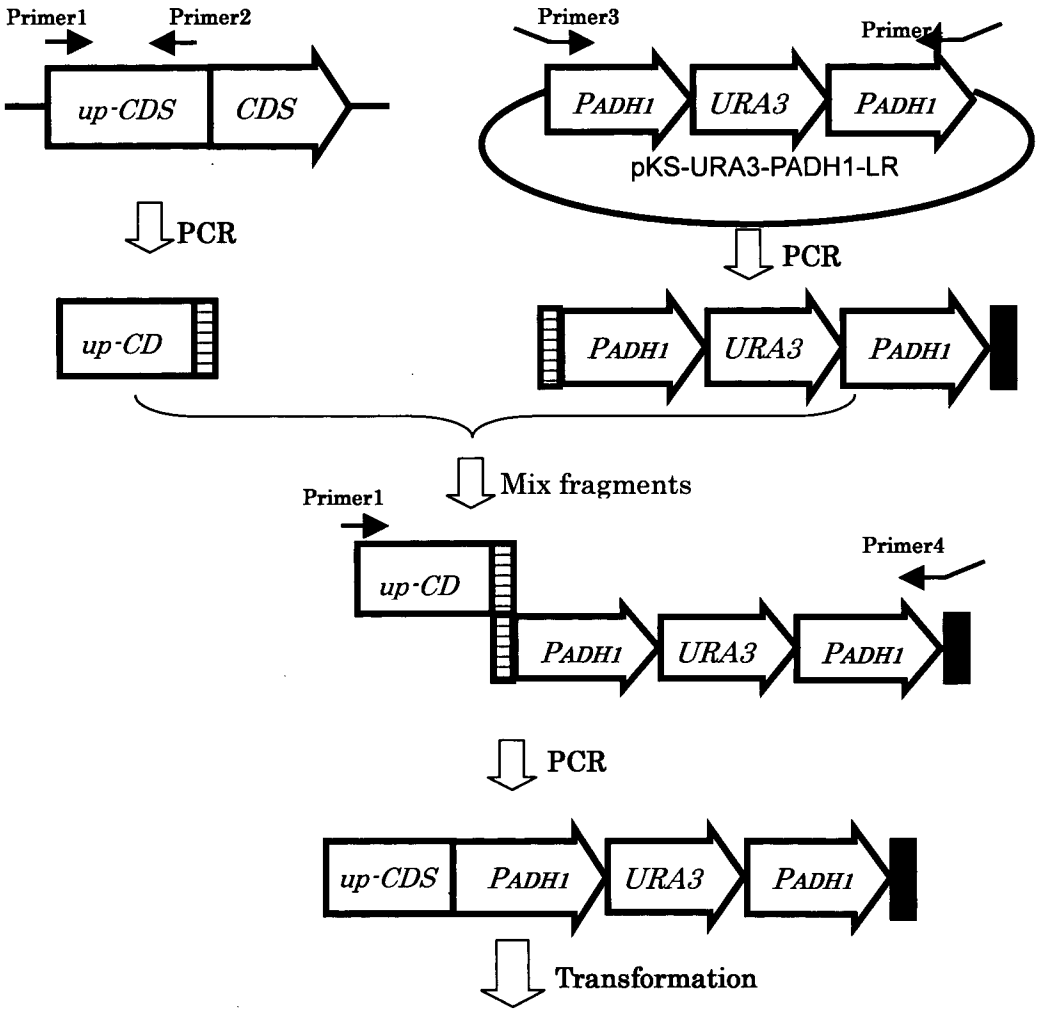
S. cerevisiae chromosome DNA fragment

Fig. 8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Primer2
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CDS
PCR
up-CD
Primer3
Primer4
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URA3
PADH1
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PCR
PADH1
URA3
PADH1
Mix fragments
Primer1
up-CD
Primer4
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URA3
PADH1
PCR
up-CDS
PADH1
URA3
PADH1
Transformation

Fig. 9

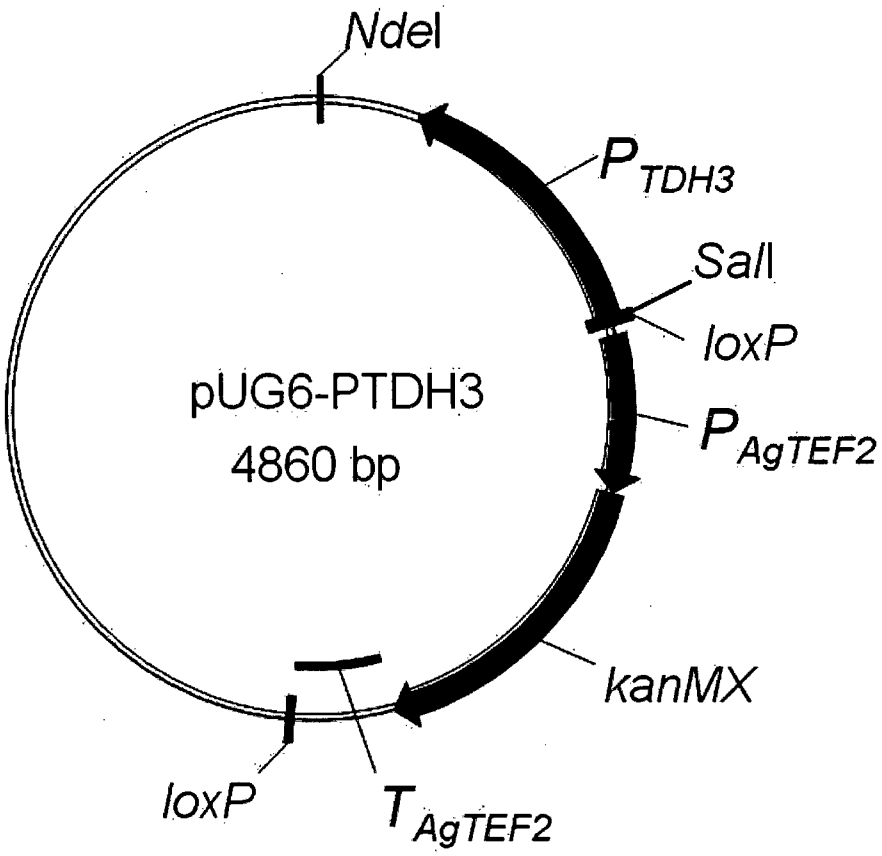
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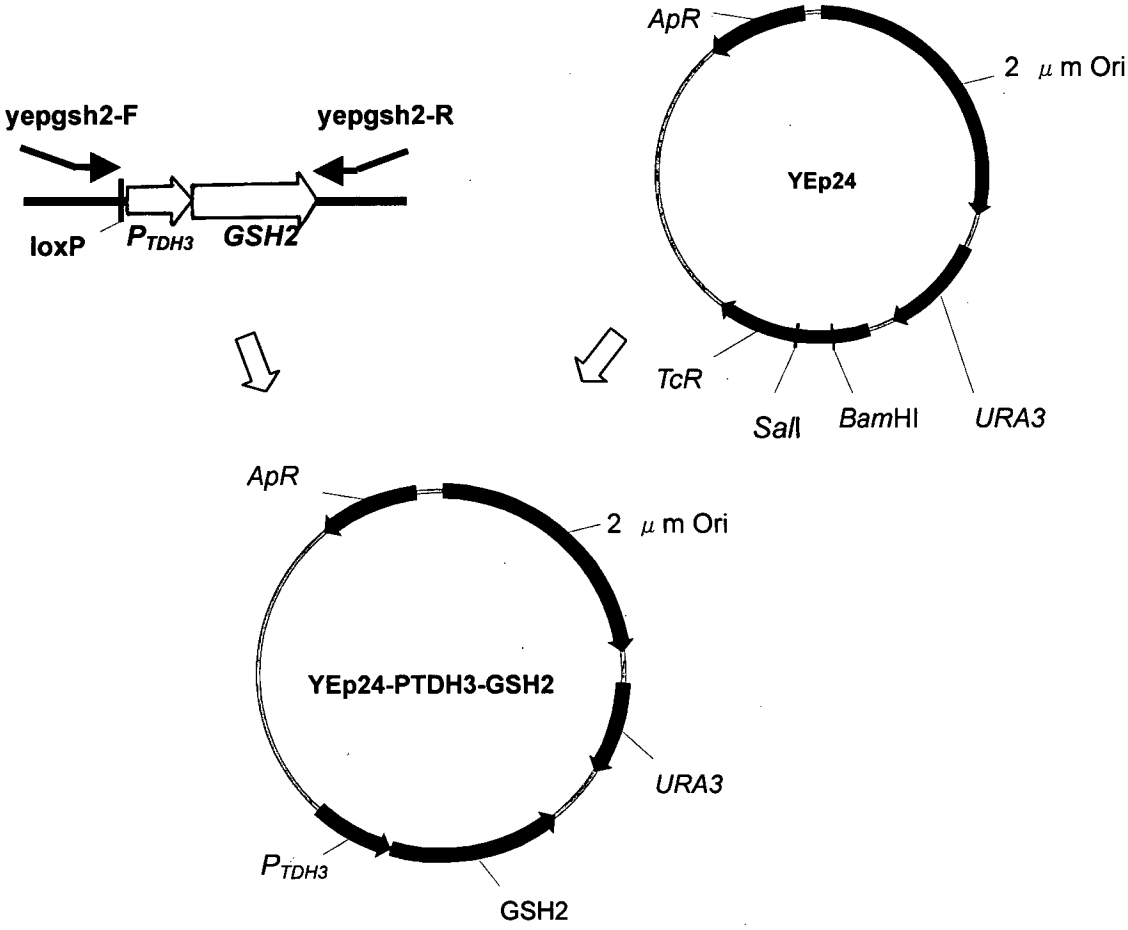
Fig. 10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ApR
2 μm Ori
YEp24-PTDH3-GSH2
URA3
PTDH3
GSH2

Fig. 11