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(54) Title: GENE ENCODING PROTEIN RESPONSIBLE FOR FLOCCULATION PROPERTY OF YEAST AND USE THEREOF

(57) Abstract: The present invention relates to a gene encoding a protein responsible for flocculation property of yeast and use thereof, in particular, a brewery yeast with appropriate flocculation property for producing desired alcoholic beverages, alcoholic beverages produced with said yeast, and a method for producing said beverages. More particularly, the present invention relates to a yeast to which suitable flocculation property for brewing desired alcoholic beverages was imparted by controlling expression level of a gene encoding a protein responsible for flocculation property of brewery yeast, especially a gene specific to a lager brewing yeast, to alcoholic beverages produced with said yeast and to a method for producing said beverages.

DESCRIPTION

GENE ENCODING PROTEIN RESPONSIBLE FOR FLOCCULATION PROPERTY OF YEAST AND USE THEREOF

TECHNICAL FIELD

The present invention relates to a gene encoding a protein responsible for flocculation property of yeast and use thereof, in particular, a brewery yeast for producing alcoholic beverages with appropriate flocculation property, alcoholic beverages produced with said yeast, and a method for producing said beverages. More particularly, the present invention relates to a yeast, which shows appropriate flocculation property by controlling expression level of ECM25, YJL083W, G0N7, YUR1, MNN11, TIR1, TIR2, TIR3, SAC1, HYM1, YKL061W, DCW1, ZRT3, LHS1 or MNN4 gene encoding a protein responsible for flocculation property of brewery yeast, especially non-ScECM25, non-ScYJL083W, non-ScG0N7, non-ScYUR1, non-ScMNN11, non-ScTIR1, non-ScTIR2, non-ScTIR3, non-ScSAC1, non-ScHYM1, non-ScYKL061W, non-ScDCW1, non-ScZRT3, non-ScLHS1 or non-ScMNN4 gene specific to a lager brewing yeast, to a method for selecting said yeast, to a method for breeding said yeast and to a method for producing alcoholic beverages with said yeast.

BACKGROUND ART

Flocculation property of yeast used for fermentation in alcoholic beverages is very important. Flocculation property of yeast means property of forming aggregation as a result of interaction among each individual yeast cells, thus sedimenting to the bottom of the liquid in a liquid culture medium.

For example, yeasts used for fermentation of lager-type beer, which is popularly drunk at the present day, are also called bottom fermenting yeast, because the yeast have a character of flocculating and sedimenting to the bottom of fermentation broth near the end of fermentation. In beer brewing, yeast is recovered after fermentation and the recovered yeast is used at the subsequent fermentation. Thus, flocculation property of yeast is very important property from the standpoint of working efficiency of brewing process. That is, yeast having poor flocculation property does not

sediment at the end of fermentation, and there is a problem that extraneous steps such as centrifugation are required for recover it. On the other hand, yeast having undesirably high flocculation property may sediment during fermentation, which can result in immature termination of fermentation. In that case, flavor and taste of resultant product are also seriously influenced. Accordingly, it is very important to use yeast having suitable flocculation property for production of desired alcoholic beverages.

Further, flocculation of yeast is actively studied, because efficiency is required especially in the field of industrial alcohol production (Novel agglutinative alcohol fermenting yeast, Japanese Examined Patent Publication (Kokoku) No. H6-36734) and wastewater treatment (Japanese Patent No. 3044284), as well as in the field of alcoholic beverage production.

FLO gene family (FLO1, FLO5, FLO8, FLO9, FLO10, FLO11) and SFL1, etc. have ever been recognized as genes responsible for flocculation property of yeast as a result of enormous quantity of investigations on flocculation property, which is of importance for industry, of yeast as stated above.

In the investigation, for example, property of FLO1 gene was analyzed at molecular level (Bidard et al., YEAST, 11(9), 809, 1995). Method for controlling flocculation property of beer yeast was investigated as well using Flo1p (Japanese Patent No. 3643404).

Method of controlling flocculation property of yeast by regulating expression of FLO8 gene (Japanese Examined Patent Publication (Kokoku) No. H8-270), and method of judging flocculating property using nucleotide sequence of FLO5 gene (International Publication No. WO01/040514), etc. are also reported.

On the other hand, some of yeast cell surface proteins specific to flocculating yeast are known as well. However, knowledge of each proteins is not sufficient for research to control flocculation property of beer yeast.

DISCLOSURE OF INVENTION

Under the above situations, there has been a need for breeding yeasts having flocculation property suitable for production of desired alcoholic beverages to make high-efficiency production of alcoholic beverages possible by using a gene encoding a protein responsible for flocculation

property of brewery yeast and said protein.

The present inventors made extensive studies to solve the above problems and as a result, succeeded in identifying and isolating a gene encoding a protein responsible for flocculation property of yeast from beer yeast. Moreover, the present inventors produced transformed yeast in which expression of the obtained gene was controlled to verify that flocculation property can be actually controlled, thereby completing the present invention.

Thus, the present invention relates to a gene encoding a protein responsible for flocculation property of brewery yeast, to a protein encoded by said gene, to a transformed yeast in which the expression of said gene is controlled, to a method for controlling flocculation property using a yeast in which the expression of said gene is controlled, or the like. More specifically, the present invention provides the following polynucleotides, a vector or a DNA fragment comprising said polynucleotide, a transformed yeast introduced with said vector or DNA fragment, a method for producing alcoholic beverages by using said transformed yeast, and the like.

(1) A polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising a polynucleotide consisting of the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 (SEQ ID NO:1; SEQ ID NO:2; SEQ ID NO:3; SEQ ID NO:4; SEQ ID NO:5; SEQ ID NO:6; SEQ ID NO:7; SEQ ID NO:8; SEQ ID NO:9; SEQ ID NO:10; SEQ ID NO:11; SEQ ID NO:12; SEQ ID NO:13; SEQ ID NO:14 and SEQ ID NO:15);

(b) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 (SEQ ID NO:16; SEQ ID NO:17; SEQ ID NO:18; SEQ ID NO:19; SEQ ID NO:20; SEQ ID NO:21; SEQ ID NO:22; SEQ ID NO:23; SEQ ID NO:24; SEQ ID NO:25; SEQ ID NO:26; SEQ ID NO:27; SEQ ID NO:28; SEQ ID NO:29 and SEQ ID NO:30);

(c) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 in which one or more amino acids thereof are deleted, substituted, inserted and/or added, and having an activity of imparting flocculation property to yeast;

(d) a polynucleotide comprising a polynucleotide encoding a protein having an amino acid sequence having 60% or higher identity with the amino acid sequence of any one of SEQ ID NO:16

through SEQ ID NO:30, and said protein having an activity of imparting flocculation property to yeast;

(e) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of any onr of SEQ ID NO:1 through SEQ ID NO:15 under stringent conditions, and which encodes a protein having an activity of imparting flocculation property to yeast; and

(f) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide encoding the protein having the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 under stringent conditions, and which encodes a protein having an activity of imparting flocculation property to yeast.

(2) The polynucleotide according to (1) above selected from the group consisting of:

(g) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30, or encoding the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 in which 1 to 10 amino acids thereof are deleted, substituted, inserted, and/or added, and wherein said protein has an activity of imparting flocculation property to yeast;

(h) a polynucleotide comprising a polynucleotide encoding a protein having 90% or higher identity with the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30, and having an activity of imparting flocculation property to yeast; and

(i) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15, or which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 under high stringent conditions, which encodes a protein having an activity of imparting flocculation property to yeast.

(3) The polynucleotide according to (1) above comprising a polynucleotide consisting of the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15.

(4) The polynucleotide according to (1) above comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30.

(5) The polynucleotide according to any one of (1) to (4) above, wherein the

polynucleotide is DNA.

(6) A polynucleotide selected from the group consisting of:

(j) a polynucleotide encoding RNA having a nucleotide sequence complementary to a transcript of the polynucleotide (DNA) according to (5) above;

(k) a polynucleotide encoding RNA that represses the expression of the polynucleotide (DNA) according to (5) above through an RNAi effect;

(l) a polynucleotide encoding RNA having an activity of specifically cleaving a transcript of the polynucleotide (DNA) according to (5) above; and

(m) a polynucleotide encoding RNA that represses the expression of the polynucleotide (DNA) according to (5) above through a co-suppression effect.

(7) A protein encoded by the polynucleotide according to any one of (1) to (5) above.

(8) A vector containing the polynucleotide according to any one of (1) to (5) above.

(8a) The vector of (8) above, which comprises the expression cassette comprising the following components:

(x) a promoter that can be transcribed in a yeast cell;

(y) any of the polynucleotides described in (1) to (5) above linked to the promoter in a sense or antisense direction; and

(z) a signal that can function in a yeast with respect to transcription termination and polyadenylation of a RNA molecule.

(8b) The vector of (8) above, which comprises the expression cassette comprising the following components:

(x) a promoter that can be transcribed in a yeast cell;

(y) any of the polynucleotides described in (1) to (5) above linked to the promoter in a sense direction; and

(z) a signal that can function in a yeast with respect to transcription termination and polyadenylation of a RNA molecule.

(8c) The vector of (8) above, which comprises the expression cassette comprising the following components:

(x) a promoter that can be transcribed in a yeast cell;

(y) any of the polynucleotides described in (1) to (5) above linked to the promoter in a antisense direction; and

(z) a signal that can function in a yeast with respect to transcription termination and polyadenylation of a RNA molecule.

(9) A vector containing the polynucleotide according to (6) above.

(10) A yeast into which the vector according to any one of (8) to (9) above has been introduced.

(11) The yeast according to (10) above, wherein the flocculation property is increased.

(12) The yeast according to (11) above, wherein the flocculation property is increased by increasing an expression level of the protein of (7) above.

(13) A yeast, wherein the expression of the polynucleotide (DNA) according to (5) above is repressed by:

(A) introducing the vector according to any one of (8) to (9) above;

(B) disrupting the gene according to the polynucleotide (DNA) of (5) above; or

(C) introducing a mutation into a promoter or genetically altering a promoter.

(14) The yeast according to (10) or (13) above, wherein the flocculation property is decreased.

(15) A method for producing an alcoholic beverage by using the yeast according to any one of (10) to (14) above.

(16) The method according to (15) above, wherein the brewed alcoholic beverage is a malt beverage.

(17) The method according to (15) above, wherein the brewed alcoholic beverage is wine.

(18) An alcoholic beverage produced by the method according to any one of (15) to (17) above.

(19) A method for assessing a test yeast for its flocculation property, comprising using a primer or probe designed based on the nucleotide sequence of a gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast.

(19a) A method for selecting a yeast having a high or low flocculation property by using the method in (19) above.

(19b) A method for producing an alcoholic beverage (for example, beer) by using the yeast selected with the method in (19a) above.

(20) A method for assessing a test yeast for its flocculation property, comprising: culturing the test yeast; and measuring the expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an

activity of imparting flocculation property to yeast.

(20a) A method for selecting a yeast, which comprises assessing a test yeast by the method described in (20) above and selecting a yeast having a high or low expression level of gene encoding a protein having an activity of imparting flocculation property to yeast.

(20b) A method for producing an alcoholic beverage (for example, beer) by using the yeast selected with the method in (20a) above.

(21) A method for selecting a yeast, comprising: culturing test yeasts; quantifying the protein of (7) above or measuring the expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast; and selecting a test yeast having an amount of the protein or the gene expression level according to the flocculation property of interest.

(22) The method for selecting a yeast according to (21) above, comprising: culturing a reference yeast and test yeasts; measuring for each yeast the expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast; and selecting a test yeast having the gene expression higher or lower than that in the reference yeast.

(23) The method for selecting a yeast according to (21) above, comprising: culturing a reference yeast and test yeasts; quantifying the protein according to (7) above in each yeast; and selecting a test yeast having a larger or smaller amount of the protein than that in the reference yeast. That is, the method for selecting a yeast of (21) above, comprising: culturing plural yeasts; quantifying the protein of (7) above in each yeast; and selecting a yeast having a larger or smaller amount of the protein among them.

(24) A method for producing an alcoholic beverage comprising: conducting fermentation for producing an alcoholic beverage using the yeast according to any one of (10) to (14) above or a yeast selected by the methods according to any one of (21) to (23) above, and adjusting flocculation property of yeast.

According to the method for producing alcoholic beverages using transformed yeast of the present invention, alcoholic beverages can be produced highly-efficiently by using yeasts having flocculation property suitable for production of desired alcoholic beverages.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the cell growth with time upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents optical density at 660 nm (OD660).

Figure 2 shows the extract (sugar) consumption with time upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents apparent extract concentration (w/w%).

Figure 3 shows the expression profile of non-ScECM25 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 4 shows the expression profile of non-ScYJL083W gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 5 shows the expression profile of non-ScG0N7 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 6 shows the expression profile of non-ScYUR1 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 7 shows the expression profile of non-ScMNN11 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 8 shows the expression profile of non-ScTIR1 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 9 shows the expression profile of non-ScTIR2 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 10 shows the expression profile of non-ScTIR3 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 11 shows the expression profile of non-ScSAC1 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 12 shows the expression profile of non-ScHYM1 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 13 shows the expression profile of non-ScYKL061W gene in yeasts upon beer

fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 14 shows the expression profile of non-ScDCW1 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 15 shows the expression profile of non-ScZRT3 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 16 shows the expression profile of non-ScLHS1 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

Figure 17 shows the expression profile of non-ScMNN4 gene in yeasts upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents the intensity of detected signal.

BEST MODES FOR CARRYING OUT THE INVENTION

The present inventors isolated and identified genes encoding a protein responsible for flocculation property of brewery yeast based on the lager brewing yeast genome information mapped according to the method disclosed in Japanese Patent Application Laid-Open No. 2004-283169. The nucleotide sequences of the genes are represented by SEQ ID NO:1 through SEQ ID NO:15. Further, the amino acid sequences of the proteins encoded by the genes are represented by SEQ ID NO:16 through SEQ ID NO:30.

1. Polynucleotide of the invention

First of all, the present invention provides (a) a polynucleotide comprising a polynucleotide of the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15; and (b) a polynucleotide comprising a polynucleotide encoding a protein of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30. The polynucleotide can be DNA or RNA.

The target polynucleotide of the present invention is not limited to the polynucleotide encoding a protein responsible for flocculation property derived from lager brewing yeast described above and may include other polynucleotides encoding proteins having equivalent functions to said protein. Proteins with equivalent functions include, for example, (c) a protein of an amino acid

sequence of any one of SEQ ID NO:16 through SEQ ID NO:30. with one or more amino acids thereof being deleted, substituted, inserted and/or added and imparting flocculation property to yeast.

Such proteins include a protein consisting of an amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 with, for example, 1 to 100, 1 to 90, 1 to 80, 1 to 70, 1 to 60, 1 to 50, 1 to 40, 1 to 39, 1 to 38, 1 to 37, 1 to 36, 1 to 35, 1 to 34, 1 to 33, 1 to 32, 1 to 31, 1 to 30, 1 to 29, 1 to 28, 1 to 27, 1 to 26, 1 to 25, 1 to 24, 1 to 23, 1 to 22, 1 to 21, 1 to 20, 1 to 19, 1 to 18, 1 to 17, 1 to 16, 1 to 15, 1 to 14, 1 to 13, 1 to 12, 1 to 11, 1 to 10, 1 to 9, 1 to 8, 1 to 7, 1 to 6 (1 to several amino acids), 1 to 5, 1 to 4, 1 to 3, 1 to 2, or 1 amino acid residues thereof being deleted, substituted, inserted and/or added and imparting flocculation property to yeast. In general, the number of deletions, substitutions, insertions, and/or additions is preferably smaller. In addition, such proteins include (d) a protein having an amino acid sequence with about 60% or higher, about 70% or higher, 71% or higher, 72% or higher, 73% or higher, 74% or higher, 75% or higher, 76% or higher, 77% or higher, 78% or higher, 79% or higher, 80% or higher, 81% or higher, 82% or higher, 83% or higher, 84% or higher, 85% or higher, 86% or higher, 87% or higher, 88% or higher, 89% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher, or 99.9% or higher identity with the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30., and imparting flocculation property to yeast. In general, the percentage identity is preferably higher.

Flocculation property of yeast may be measured, for example, by a method described in Japanese Patent Application Laid-Open No. H8-205890.

Furthermore, the present invention also contemplates (e) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 under stringent conditions and which encodes a protein imparting flocculation property to yeast; and (f) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide complementary to a nucleotide sequence of encoding a protein of any one of SEQ ID NO:16 through SEQ ID NO:30 under stringent conditions, and which encodes a protein imparting flocculation property to yeast.

Herein, "a polynucleotide that hybridizes under stringent conditions" refers to nucleotide sequence, such as a DNA, obtained by a colony hybridization technique, a plaque hybridization technique, a southern hybridization technique or the like using all or part of polynucleotide of a

nucleotide sequence complementary to the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 or polynucleotide encoding the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 as a probe. The hybridization method may be a method described, for example, in MOLECULAR CLONING 3rd Ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons 1987-1997, and so on.

The term "stringent conditions" as used herein may be any of low stringency conditions, moderate stringency conditions or high stringency conditions. "Low stringency conditions" are, for example, 5 x SSC, 5 x Denhardt's solution, 0.5% SDS, 50% formamide at 32°C. "Moderate stringency conditions" are, for example, 5 x SSC, 5 x Denhardt's solution, 0.5% SDS, 50% formamide at 42°C. "High stringency conditions" are, for example, 5 x SSC, 5 x Denhardt's solution, 0.5% SDS, 50% formamide at 50°C. Under these conditions, a polynucleotide, such as a DNA, with higher homology is expected to be obtained efficiently at higher temperature, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and one skilled in the art may appropriately select these factors to realize similar stringency.

When a commercially available kit is used for hybridization, for example, Alkphos Direct Labeling Reagents (Amersham Pharmacia) may be used. In this case, according to the attached protocol, after incubation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55°C, thereby detecting hybridized polynucleotide, such as DNA.

Other polynucleotides that can be hybridized include polynucleotides having about 60% or higher, about 70% or higher, 71% or higher, 72% or higher, 73% or higher, 74% or higher, 75% or higher, 76% or higher, 77% or higher, 78% or higher, 79% or higher, 80% or higher, 81% or higher, 82% or higher, 83% or higher, 84% or higher, 85% or higher, 86% or higher, 87% or higher, 88% or higher, 89% or higher, 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity to polynucleotide encoding the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 as calculated by homology search

software, such as FASTA and BLAST using default parameters.

Identity between amino acid sequences or nucleotide sequences may be determined using algorithm BLAST by Karlin and Altschul (*Proc. Natl. Acad. Sci. USA*, 87: 2264-2268, 1990; *Proc. Natl. Acad. Sci. USA*, 90: 5873, 1993). Programs called BLASTN and BLASTX based on BLAST algorithm have been developed (Altschul SF et al., *J. Mol. Biol.* 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score = 100 and word length = 12. When an amino acid sequence is sequenced using BLASTX, the parameters are, for example, score = 50 and word length = 3. When BLAST and Gapped BLAST programs are used, default parameters for each of the programs are employed.

The polynucleotide of the present invention includes (j) a polynucleotide encoding RNA having a nucleotide sequence complementary to a transcript of the polynucleotide (DNA) according to (5) above; (k) a polynucleotide encoding RNA that represses the expression of the polynucleotide (DNA) according to (5) above through RNAi effect; (l) a polynucleotide encoding RNA having an activity of specifically cleaving a transcript of the polynucleotide (DNA) according to (5) above; and (m) a polynucleotide encoding RNA that represses expression of the polynucleotide (DNA) according to (5) above through co-supression effect. These polynucleotides may be incorporated into a vector, which can be introduced into a cell for transformation to repress the expression of the polynucleotides (DNA) of (a) to (i) above. Thus, these polynucleotides may suitably be used when repression of the expression of the above DNA is preferable.

The phrase "polynucleotide encoding RNA having a nucleotide sequence complementary to the transcript of DNA" as used herein refers to so-called antisense DNA. Antisense technique is known as a method for repressing expression of a particular endogenous gene, and is described in various publications (see e.g., Hirajima and Inoue: New Biochemistry Experiment Course 2 Nucleic Acids IV Gene Replication and Expression (Japanese Biochemical Society Ed., Tokyo Kagaku Dozin Co., Ltd.) pp.319-347, 1993). The sequence of antisense DNA is preferably complementary to all or part of the endogenous gene, but may not be completely complementary as long as it can effectively repress the expression of the gene. The transcribed RNA has preferably 90% or higher, and more preferably 95% or higher complementarity to the transcript of the target gene. The length of the antisense DNA is at least 15 bases or more, preferably 100 bases or more, and more preferably

500 bases or more.

The phrase "polynucleotide encoding RNA that represses DNA expression through RNAi effect" as used herein refers to a polynucleotide for repressing expression of an endogenous gene through RNA interference (RNAi). The term "RNAi" refers to a phenomenon where when double-stranded RNA having a sequence identical or similar to the target gene sequence is introduced into a cell, the expressions of both the introduced foreign gene and the target endogenous gene are repressed. RNA as used herein includes, for example, double-stranded RNA that causes RNA interference of 21 to 25 base length, for example, dsRNA (double strand RNA), siRNA (small interfering RNA) or shRNA (short hairpin RNA). Such RNA may be locally delivered to a desired site with a delivery system such as liposome, or a vector that generates the double-stranded RNA described above may be used for local expression thereof. Methods for producing or using such double-stranded RNA (dsRNA, siRNA or shRNA) are known from many publications (see, e.g., Japanese National Phase PCT Laid-open Patent Publication No. 2002-516062; US 2002/086356A; Nature Genetics, 24(2), 180-183, 2000 Feb.; Genesis, 26(4), 240-244, 2000 April; Nature, 407:6802, 319-20, 2002 Sep. 21; Genes & Dev., Vol.16, (8), 948-958, 2002 Apr.15; Proc. Natl. Acad. Sci. USA., 99(8), 5515-5520, 2002 Apr. 16; Science, 296(5567), 550-553, 2002 Apr. 19; Proc Natl. Acad. Sci. USA, 99:9, 6047-6052, 2002 Apr. 30; Nature Biotechnology, Vol.20 (5), 497-500, 2002 May; Nature Biotechnology, Vol. 20(5), 500-505, 2002 May; Nucleic Acids Res., 30:10, e46,2002 May 15).

The phrase "polynucleotide encoding RNA having an activity of specifically cleaving transcript of DNA" as used herein generally refers to a ribozyme. Ribozyme is an RNA molecule with a catalytic activity that cleaves a transcript of a target DNA and inhibits the function of that gene. Design of ribozymes can be found in various known publications (see, e.g., FEBS Lett. 228: 228, 1988; FEBS Lett. 239: 285, 1988; Nucl. Acids. Res. 17: 7059, 1989; Nature 323: 349, 1986; Nucl. Acids. Res. 19: 6751, 1991; Protein Eng 3: 733, 1990; Nucl. Acids Res. 19: 3875, 1991; Nucl. Acids Res. 19: 5125, 1991; Biochem Biophys Res Commun 186: 1271, 1992). In addition, the phrase "polynucleotide encoding RNA that represses DNA expression through co-supression effect" refers to a nucleotide that inhibits functions of target DNA by "co-supression".

The term "co-supression" as used herein, refers to a phenomenon where when a gene

having a sequence identical or similar to a target endogenous gene is transformed into a cell, the expressions of both the introduced foreign gene and the target endogenous gene are repressed. Design of polynucleotides having a co-supression effect can also be found in various publications (see, e.g., Smyth DR: Curr. Biol. 7: R793, 1997, Martienssen R: Curr. Biol. 6: 810, 1996).

2. Protein of the present invention

The present invention also provides proteins encoded by any of the polynucleotides (a) to (i) above. A preferred protein of the present invention comprises an amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 with one or several amino acids thereof being deleted, substituted, inserted and/or added, and imparts flocculation property to yeast.

Such protein includes those having an amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 with amino acid residues thereof of the number mentioned above being deleted, substituted, inserted and/or added and imparting flocculation property to yeast. In addition, such protein includes those having homology as described above with the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 and imparting flocculation property to yeast.

Such proteins may be obtained by employing site-directed mutation described, for example, in MOLECULAR CLONING 3rd Ed., CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, *Nuc. Acids. Res.*, 10: 6487 (1982), *Proc. Natl. Acad. Sci. USA* 79: 6409 (1982), *Gene* 34: 315 (1985), *Nuc. Acids. Res.*, 13: 4431 (1985), *Proc. Natl. Acad. Sci. USA* 82: 488 (1985).

Deletion, substitution, insertion and/or addition of one or more amino acid residues in an amino acid sequence of the protein of the invention means that one or more amino acid residues are deleted, substituted, inserted and/or added at any one or more positions in the same amino acid sequence. Two or more types of deletion, substitution, insertion and/or addition may occur concurrently.

Hereinafter, examples of mutually substitutable amino acid residues are enumerated. Amino acid residues in the same group are mutually substitutable. The groups are provided below.

Group A: leucine, isoleucine, norleucine, valine, norvaline, alanine, 2-aminobutanoic acid, methionine, o-methylserine, t-butylglycine, t-butylalanine, cyclohexylalanine; Group B: asparatic acid, glutamic acid, isoasparatic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid; Group C: asparagine, glutamine; Group D: lysine, arginine, ornithine, 2,4-diaminobutanoic acid, 2,3-diaminopropionic acid; Group E: proline, 3-hydroxyproline, 4-hydroxyproline; Group F: serine,

threonine, homoserine; and Group G: phenylalanine, tyrosine.

The protein of the present invention may also be produced by chemical synthesis methods such as Fmoc method (fluorenylmethyloxycarbonyl method) and tBoc method (t-butyloxycarbonyl method). In addition, peptide synthesizers available from, for example, Advanced ChemTech, PerkinElmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimadzu Corp. can also be used for chemical synthesis.

3. Vector of the invention and yeast transformed with the vector

The present invention then provides a vector comprising the polynucleotide described above. The vector of the present invention is directed to a vector including any of the polynucleotides described in (a) to (i) above or any of the polynucleotides described in (j) to (m) above. Generally, the vector of the present invention comprises an expression cassette including as components (x) a promoter that can transcribe in a yeast cell; (y) a polynucleotide described in any of (a) to (i) above that is linked to the promoter in sense or antisense direction; and (z) a signal that functions in the yeast with respect to transcription termination and polyadenylation of RNA molecule.

According to the present invention, in order to highly express the protein of the invention described above upon brewing alcoholic beverages (e.g., beer) described below, these polynucleotides are introduced in the sense direction to the promoter to promote expression of the polynucleotide (DNA) described in any of (a) to (i) above. Further, in order to repress the above protein of the invention upon brewing alcoholic beverages (e.g., beer) described below, these polynucleotides are introduced in the antisense direction to the promoter to repress the expression of the polynucleotide (DNA) described in any of (a) to (i) above.

In order to repress the above protein of the invention, the polynucleotide may be introduced into vectors such that the polynucleotide of any of the (j) to (m) is to be expressed. According to the present invention, the target gene (DNA) may be disrupted to repress the expression of the DNA described above or the expression of the protein described above. A gene may be disrupted by adding or deleting one or more bases to or from a region involved in expression of the gene product in the target gene, for example, a coding region or a promoter region, or by deleting these regions

entirely. Such disruption of gene may be found in known publications (see, e.g., Proc. Natl. Acad. Sci. USA, 76, 4951(1979) , Methods in Enzymology, 101, 202(1983), Japanese Patent Application Laid-Open No.6-253826).

Further, in the present invention, the expression level of a target gene can be controlled by introducing a mutation to a promoter or genetically altering a promoter by homologous recombination. Such mutation introducing method is described in Nucleic Acids Res. 29, 4238-4250 (2001), and such alteration of a promoter is described in, for example, Appl Environ Microbiol.,72, 5266-5273 (2006).

A vector introduced in the yeast may be any of a multicopy type (YEp type), a single copy type (YCp type), or a chromosome integration type (YIp type). For example, YEp24 (J. R. Broach et al., EXPERIMENTAL MANIPULATION OF GENE EXPRESSION, Academic Press, New York, 83, 1983) is known as a YEp type vector, YCp50 (M. D. Rose et al., *Gene* 60: 237, 1987) is known as a YCp type vector, and YIp5 (K. Struhl et al., *Proc. Natl. Acad. Sci. USA*, 76: 1035, 1979) is known as a YIp type vector, all of which are readily available.

Promoters/terminators for adjusting gene expression in yeast may be in any combination as long as they function in the brewery yeast and they are not influenced by constituents in fermentation broth. For example, a promoter of glyceraldehydes 3-phosphate dehydrogenase gene (TDH3), or a promoter of 3-phosphoglycerate kinase gene (PGK1) may be used. These genes have previously been cloned, described in detail, for example, in M. F. Tuite et al., *EMBO J.*, 1, 603 (1982), and are readily available by known methods.

Since an auxotrophy marker cannot be used as a selective marker upon transformation for a brewery yeast, for example, a geneticin-resistant gene (G418r), a copper-resistant gene (CUP1) (Marin et al., *Proc. Natl. Acad. Sci. USA*, 81, 337 1984) or a cerulenin-resistant gene (fas2m, PDR4) (Junji Inokoshi et al., *Biochemistry*, 64, 660, 1992; and Hussain et al., *Gene*, 101: 149, 1991, respectively) may be used.

A vector constructed as described above is introduced into a host yeast. Examples of the host yeast include any yeast that can be used for brewing, for example, brewery yeasts for beer, wine and sake. Specifically, yeasts such as genus *Saccharomyces* may be used. According to the present invention, a lager brewing yeast, for example, *Saccharomyces pastorianus* W34/70, etc.,

Saccharomyces carlsbergensis NCYC453 or NCYC456, etc., or *Saccharomyces cerevisiae* NBRC1951, NBRC1952, NBRC1953 or NBRC1954, etc., may be used. In addition, whisky yeasts such as *Saccharomyces cerevisiae* NCYC90, wine yeasts such as wine yeasts #1, 3 and 4 from the Brewing Society of Japan, and sake yeasts such as sake yeast #7 and 9 from the Brewing Society of Japan may also be used but not limited thereto. In the present invention, lager brewing yeasts such as *Saccharomyces pastorianus* may be used preferably.

A yeast transformation method may be a generally used known method. For example, methods that can be used include but not limited to an electroporation method (*Meth. Enzym.*, 194: 182 (1990)), a spheroplast method (*Proc. Natl. Acad. Sci. USA*, 75: 1929(1978)), a lithium acetate method (*J. Bacteriology*, 153: 163 (1983)), and methods described in *Proc. Natl. Acad. Sci. USA*, 75: 1929 (1978), METHODS IN YEAST GENETICS, 2000 Edition: A Cold Spring Harbor Laboratory Course Manual.

More specifically, a host yeast is cultured in a standard yeast nutrition medium (e.g., YEPD medium (Genetic Engineering. Vol. 1, Plenum Press, New York, 117(1979)), etc.) such that OD600 nm will be 1 to 6. This culture yeast is collected by centrifugation, washed and pre-treated with alkali metal ion, preferably lithium ion at a concentration of about 1 to 2 M. After the cell is left to stand at about 30°C for about 60 minutes, it is left to stand with DNA to be introduced (about 1 to 20 μg) at about 30°C for about another 60 minutes. Polyethyleneglycol, preferably about 4,000 Dalton of polyethyleneglycol, is added to a final concentration of about 20% to 50%. After leaving at about 30°C for about 30 minutes, the cell is heated at about 42°C for about 5 minutes. Preferably, this cell suspension is washed with a standard yeast nutrition medium, added to a predetermined amount of fresh standard yeast nutrition medium and left to stand at about 30°C for about 60 minutes. Thereafter, it is seeded to a standard agar medium containing an antibiotic or the like as a selective marker to obtain a transformant.

Other general cloning techniques may be found, for example, in MOLECULAR CLONING 3rd Ed., and METHODS IN YEAST GENETICS, A LABORATORY MANUAL (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

4. Method of producing alcoholic beverages according to the present invention and alcoholic beverages produced by the method

A yeast having flocculation property suitable for a target alcoholic beverages can be

obtained by introducing the vector of the present invention or DNA fragments described above to a yeast suitable for brewing target alcoholic beverages to control expression level of the gene. Thus, alcoholic beverages can be produced highly-efficiently. That is to say, desired kind of alcoholic beverages can be produced highly-efficiently by controlling (elevating or reducing) flocculation property using yeasts into which the vector or DNA fragment of the present invention was introduced described above, yeasts in which expression of the polynucleotide (DNA) of the present invention described above was regulated (promoted or suppressed) or yeasts selected by the yeast assessment method of the invention described below for fermentation to produce alcoholic beverages. The target alcoholic beverages include, for example, but not limited to beer, beer-taste beverages such as sparkling liquor (*happoushu*), wine, whisky, sake and the like. Further, alcohol for practical use such as alcohol for fuel is also included among them.

In order to produce these alcoholic beverages, a known technique can be used except that a brewery yeast obtained according to the present invention is used in the place of a parent strain. Since materials, manufacturing equipment, manufacturing control and the like may be exactly the same as the conventional ones, there is no need of increasing the cost for producing alcoholic beverages. Thus, according to the present invention, alcoholic beverages can be produced highly-efficiently using the existing facility without increasing the cost.

5. Yeast assessment method of the invention

The present invention relates to a method for assessing a test yeast for its flocculation property by using a primer or a probe designed based on a nucleotide sequence of a gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein imparting flocculation property to yeast. General techniques for such assessment method is known and is described in, for example, WO01/040514, Japanese Laid-Open Patent Application No. H8-205900 or the like. This assessment method is described in below.

First, genome of a test yeast is prepared. For this preparation, any known method such as Hereford method or potassium acetate method may be used (e.g., METHODS IN YEAST GENETICS, Cold Spring Harbor Laboratory Press, 130 (1990)). Using a primer or a probe designed based on a nucleotide sequence (preferably, ORF sequence) of the gene encoding a protein imparting

flocculation property to yeast, the existence of the gene or a sequence specific to the gene is determined in the test yeast genome obtained. The primer or the probe may be designed according to a known technique.

Detection of the gene or the specific sequence may be carried out by employing a known technique. For example, a polynucleotide including part or all of the specific sequence or a polynucleotide including a nucleotide sequence complementary to said nucleotide sequence is used as one primer, while a polynucleotide including part or all of the sequence upstream or downstream from this sequence or a polynucleotide including a nucleotide sequence complementary to said nucleotide sequence, is used as another primer to amplify a nucleic acid of the yeast by a PCR method, thereby determining the existence of amplified products and molecular weight of the amplified products. The number of bases of polynucleotide used for a primer is generally 10 base pairs (bp) or more, and preferably 15 to 25 bp. In general, the number of bases between the primers is suitably 300 to 2000 bp.

The reaction conditions for PCR are not particularly limited but may be, for example, a denaturation temperature of 90 to 95°C, an annealing temperature of 40 to 60°C, an elongation temperature of 60 to 75°C, and the number of cycle of 10 or more. The resulting reaction product may be separated, for example, by electrophoresis using agarose gel to determine the molecular weight of the amplified product. This method allows prediction and assessment of the flocculation property of yeast as determined by whether the molecular weight of the amplified product is a size that contains the DNA molecule of the specific part. In addition, by analyzing the nucleotide sequence of the amplified product, the property may be predicted and/or assessed more precisely.

Moreover, in the present invention, a test yeast is cultured to measure an expression level of the gene encoding a protein imparting flocculation property to yeast having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ IDNO:15 to assess the test yeast for its flocculation property. In this case, the test yeast is cultured and then mRNA or a protein resulting from the gene encoding a protein imparting flocculation property to yeast is quantified. The quantification of mRNA or protein may be carried out by employing a known technique. For example, mRNA may be quantified, by Northern hybridization or quantitative RT-PCR, while protein may be quantified, for example, by Western blotting (CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley &

Sons 1994-2003).

Furthermore, test yeasts are cultured and expression levels of the gene encoding a protein imparting flocculation property to yeast having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 are measured to select a test yeast with the gene expression level according to the target flocculation property, thereby selecting a yeast favorable for brewing desired alcoholic beverages. In addition, a reference yeast and a test yeast may be cultured so as to measure and compare the expression level of the gene in each of the yeasts, thereby selecting a favorable test yeast. More specifically, for example, a reference yeast and one or more test yeasts are cultured and an expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 is measured in each yeast. By selecting a test yeast with the gene expressed higher (i.e., expression level is enhanced) or lower (i.e., expression level is suppressed) than that in the reference yeast, a yeast suitable for brewing alcoholic beverages can be selected.

Alternatively, test yeasts are cultured and a yeast with a high or low flocculation property is selected, thereby selecting a yeast suitable for brewing desired alcoholic beverages.

In these cases, the test yeasts or the reference yeast may be, for example, a yeast introduced with the vector or DNA fragment of the invention, a yeast in which an expression of a polynucleotide (DNA) of the invention has been controlled, an artificially mutated yeast or a naturally mutated yeast. The flocculation property of yeast can be measured by, for example, a method described in Japanese Patent Application Laid-Open No. H8-205890. The mutation treatment may employ any methods including, for example, physical methods such as ultraviolet irradiation and radiation irradiation, and chemical methods associated with treatments with drugs such as EMS (ethylmethane sulphonate) and N-methyl-N-nitrosoguanidine (*see, e.g.,* Yasuji Oshima Ed., BIOCHEMISTRY EXPERIMENTS vol. 39, *Yeast Molecular Genetic Experiments*, pp. 67-75, JSSP).

In addition, examples of yeasts used as the reference yeast or the test yeasts include any yeasts that can be used for brewing, for example, brewery yeasts for beer, wine, sake and the like. More specifically, yeasts such as genus *Saccharomyces* may be used (*e.g., S. pastorianus, S. cerevisiae,* and *S. carlsbergensis*). According to the present invention, a lager brewing yeast, for example, *Saccharomyces pastorianus* W34/70; *Saccharomyces carlsbergensis* NCYC453 or NCYC456; or *Saccharomyces cerevisiae* NBRC1951, NBRC1952, NBRC1953 or NBRC1954, etc., may be used. Further, wine yeasts such as wine yeasts #1, 3 and 4 from the Brewing Society of

Japan; and sake yeasts such as sake yeast #7 and 9 from the Brewing Society of Japan may also be used but not limited thereto. In the present invention, lager brewing yeasts such as *Saccharomyces pastorianus* may preferably be used. The reference yeast and the test yeasts may be selected from the above yeasts in any combination.

EXAMPLES

Hereinafter, the present invention will be described in more detail with reference to working examples. The present invention, however, is not limited to the examples described below.

Example 1: Cloning of Gene Encoding Protein Responsible for Flocculation Property of Yeast (non-ScECM25, non-ScYJL083W, non-ScG0N7, non-ScYUR1, non-ScMNN11, non-ScTIR1, non-ScTIR2, non-ScTIR3, non-ScSAC1, non-ScHYM1, non-ScYKL061W, non-ScDCW1, non-ScZRT3, non-ScLHS1 and non-ScMNN4)

Genes encoding a protein responsible for flocculation property of brewery yeast: non-ScECM25 (SEQ ID NO:1), non-ScYJL083W (SEQ ID NO:2), non-ScG0N7 (SEQ ID NO: 3), non-ScYUR1(SEQ ID NO: 4), non-ScMNN11 (SEQ ID NO: 5), non-ScTIR1 (SEQ ID NO: 6), non-ScTIR2 (SEQ ID NO:7), non-ScTIR3 (SEQ ID NO:8), non-ScSAC1 (SEQ ID NO:9), non-ScHYM1 (SEQ ID NO:10), non-ScYKL061W (SEQ ID NO:11), non-ScDCW1 (SEQ ID NO:12), non-ScZRT3(SEQ ID NO:13), non-ScLHS1(SEQ ID NO:14) and non-ScMNN4 (SEQ ID NO:15) were found as a result of a search utilizing the comparison database described in Japanese Patent Application Laid-Open No. 2004-283169.

Based on the acquired nucleotide sequence information, primers non-ScECM25_F (SEQ ID NO:31), non-ScECM25_R (SEQ ID NO:32), non-ScYJL083W_F (SEQ ID NO:33), non-ScYJL083W_R (SEQ ID NO:34), non-ScG0N7_F (SEQ ID NO:35), non-ScG0N7_R (SEQ ID NO:36), non-ScYUR1_F (SEQ ID NO: 37), non-ScYUR1_R (SEQ ID NO: 38), non-ScMNN11_F (SEQ ID NO: 39), non-ScMNN11 _R(SEQ ID NO:40), non-ScTIR1_F (SEQ ID NO:41), non-ScTIR1_R (SEQ ID NO:42), non-ScTIR2_F (SEQ ID NO:43), non-ScTIR2_R (SEQ ID NO: 44), non-ScTIR3_F (SEQ ID NO: 45), non-ScTIR3_R (SEQ ID NO: 46), non-ScSAC1_F (SEQ ID NO:47), non-ScSAC1 _R(SEQ ID NO:48), non-ScHYM1_F (SEQ ID NO:49), non-ScHYM1_R

(SEQ ID NO:50), non-ScYKL061W_F (SEQ ID NO:51), non-ScYKL061W_R (SEQ ID NO:52), non-ScDCW1_F (SEQ ID NO:53), non-ScDCW1_R (SEQ ID NO:54), non-ScZRT3_F (SEQ ID NO:55), non-Sc ZRT3_R (SEQ ID NO:56), non-ScLHS1_F (SEQ ID NO:57), non-Sc LHS1_R (SEQ ID NO:58), non-ScMNN4_F (SEQ ID NO:59) and non-ScMNN4_R (SEQ ID NO:60) were designed to amplify the full-length of the gene. PCR was carried out using chromosomal DNA of a genome sequencing strain, Saccharomyces pastorianus Weihenstephan 34/70 (sometimes abbreviated as "W34/70 strain"), as a template to obtain DNA fragments including the full-length gene of non-ScECM25, non-ScYJL083W, non-ScG0N7, non-ScYUR1, non-ScMNN11, non-ScTIR1, non-ScTIR2, non-ScTIR3, non-ScSAC1, non-ScHYM1, non-ScYKL061W, non-ScDCW1, non-ScZRT3, non-ScLHS1 and non-ScMNN4.

The non-ScECM25, non-ScYJL083W, non-ScG0N7, non-ScYUR1, non-ScMNN11, non-ScTIR1, non-ScTIR2, non-ScTIR3, non-ScSAC1, non-ScHYM1, non-ScYKL061W, non-ScDCW1, non-ScZRT3, non-ScLHS1 and non-ScMNN4 genes fragments thus obtained were inserted into pCR2.1-TOPO vector (Invitrogen) by TA cloning. The nucleotide sequences of the genes were analyzed by Sanger's method (F. Sanger, Science, 214: 1215, 1981) to confirm the nucleotide sequence.

Example 2: Analysis of Expression of Gene during Beer Fermentation

A beer fermentation test was conducted using a lager brewing yeast, Saccharomyces pastorianus W34/70.

Wort extract concentration	12.69%
Wort content	70 L
Wort dissolved oxygen concentration	8.6 ppm
Fermentation temperature	15°C
Yeast pitching rate	12.8×10^6 cells/mL

The fermentation liquor was sampled over time, and the time-course changes in amount of yeast cell growth (Fig. 1) and apparent extract concentration (Fig. 2) were observed.

Simultaneously, yeast cells were sampled to prepare mRNA, and the prepared mRNA was labeled with biotin and was hybridized to a beer yeast DNA microarray. The signal was detected using the GeneChip Operating system (GCOS; GeneChip Operating Software 1.0, manufactured by Affymetrix Co). Expression patterns of the non-ScECM25, non-ScYJL083W, non-ScG0N7, non-ScYUR1, non-ScMNN11, non-ScTIR1, non-ScTIR2, non-ScTIR3, non-ScSAC1, non-ScHYM1, non-ScYKL061W, non-ScDCW1, non-ScZRT3, non-ScLHS1 and non-ScMNN4 genes are shown in Figures 3 to Figure 17. This result confirmed the expression of genes described above in the general beer fermentation.

Example 3: Construction of Strain in which Gene Encoding Protein Responsible For Flocculation Property of Yeast Is Highly Experssed

The pCR2.1-TOPO vector described in Example 1 was digested with the restriction enzymes SacI and NotI to prepare a DNA fragment containing the entire length of the protein-encoding region. This fragment was ligated to pYCGPYNot treated with the restriction enzymes SacI and NotI, thereby constructing the non-ScECM25/pYCGPYNot high expression vector non-ScYJL083W/pYCGPYNot, non-ScG0N7/pYCGPYNot, non-ScYUR1/pYCGPYNot, non-ScMNN11/pYCGPYNot, non-ScTIR1/pYCGPYNot, non-ScTIR2/pYCGPYNot, non-ScTIR3/pYCGPYNot, non-ScSAC1/pYCGPYNot, non-ScHYM1/pYCGPYNot, non-YKL061W/pYCGPYNot, non-ScDCW1/pYCGPYNot, non-ScZRT3/pYCGPYNot, non-ScLHS1/pYCGPYNot and non-ScMNN4/pYCGPYN.

pYCGPYNot is a YCp-type yeast expression vector. A gene inserted is highly expressed by the pyruvate kinase gene PYK1 promoter. The geneticin-resistant gene $G418^r$ is included as the selectable marker in the yeast, and the ampicillin-resistant gene Amp^r as the selectable marker in Escherichia coli.

Using the high expression vector prepared by the above method, the strain Saccharomyces pasteurianus Weihenstephaner 34/70 was transformed by the method described in Japanese Patent Application Laid-open No. H07-303475. The transformants were selected on a YPD plate medium (1% yeast extract, 2% polypeptone, 2% glucose and 2% agar) containing 300 mg/L of geneticin.

Example 4: Evaluation of Flocculation Property of Strain in which Gene Encoding Protein Responsible For Flocculation Property of Yeast Is Highly Experssed

Flocculation properties of the highly expressed strain obtained in Example 3 and the parent strain (W34/70 strain) are evaluated by a method described below.

The strains are inoculated into 50 mL of glucose CM medium (3% glucose, 1% bacto peptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.2% $MgSO_4.7H_2O$), then culture statically at 30°C for 2 days.

Whole amount of the culture is transferred to 450 mL of glucose CM medium and culture statically at 15°C for 4 days. The yeast cells are collected by centrifugation followed washing twice in cold water of 5°C. One point five grams (1.5 g) of the obtained wet yeast cells are accurately weighed, suspend in 20 mL of 0.1M acetate buffer (pH 4.5) containing 2mM Ca, and temperature equilibrated in an temperature controlled room at 15°C.

Subsequent measurement procedures are performed in a temperature controlled room at 15°C. The yeast suspension mixed for 30 seconds by vortex mixer is poured into a buret, then immediately 2 mL of the suspension is collected as a 0 min sample. After leaving 10 min at rest, 2 mL of the suspension is collected as a 10 min sample.

OD600 of the collected yeast suspensions are measured, respectively, and flocculation properties (Segmentation Index; SI) are calculated by formula below.

$$SI=(OD_{10min}-OD_{0min})/OD_{0min}*100$$

Example 5: Disruption of Gene Encoding Protein Responsible For Flocculation Property of Yeast

Fragments for gene disruption were prepared by PCR using a plasmid containing a drug resistance marker (pFA6a(G418r), pAG25(nat1), pAG32(hph)) as a template in accordance with a method described in literature (Goldstein et al., Yeast, 15, 1541 (1999)). Primers consisting of non-ScEC25_delta_F (SEQ ID NO. 61), non-ScEC25_delta_R (SEQ ID NO: 62), non-ScYJL083W_delta_F (SEQ ID NO: 63), non-ScEYJL083W_delta_R (SEQ ID NO: 64), non-ScG0N7_delta_F (SEQ ID NO: 65), non-ScG0N7_delta_R (SEQ ID NO: 66), non-ScYUR1_delta_F (SEQ ID NO: 67), non-YUR1_delta_R (SEQ ID NO: 68), non-ScMNN11_delta_F (SEQ ID NO: 69), non-ScMNN11_delta_R (SEQ ID NO: 70),

non-ScTIR1_delta_F (SEQ ID NO: 71), non-ScTIR1_delta_R (SEQ ID NO: 72), non-ScTIR2_delta_F (SEQ ID NO: 73), non-ScTIR2_delta_R (SEQ ID NO: 74), non-ScTIR3_delta_F (SEQ ID NO: 75), non-ScTIR3_delta_R (SEQ ID NO: 76), non-ScSAC1_delta_F (SEQ ID NO: 77), non-ScSAC1_delta_R (SEQ ID NO: 78), non-ScHYM1_delta_F (SEQ ID NO: 79), non-ScHYM1_delta_R (SEQ ID NO: 80), non-ScYKL061W_delta_F (SEQ ID NO: 81), non-ScYKL061W_delta_R (SEQ ID NO: 82), non-ScDCW1_delta_F (SEQ ID NO: 83), non-ScDCW1_delta_R (SEQ ID NO: 84), non-ScZRT3_delta_F (SEQ ID NO: 85), non-ScZRT3_delta_R (SEQ ID NO: 86), non-ScLHS1_delta_F (SEQ ID NO: 87), non-ScLHS1_delta_R (SEQ ID NO: 88), non-ScMNN4_delta_F (SEQ ID NO: 89) and non-ScMNN4_delta_R (SEQ ID NO: 90) were used for the PCR primers.

A spore clone (W34/70-2) isolated from lager brewing yeast Saccharomyces pastorianus strain W34/70 was transformed with the fragments for gene disruption prepared as described above. Transformation was carried out according to the method described in Japanese Patent Application Laid-open No. H07-303475, and transformants were selected on YPD plate medium (1% yeast extract, 2% polypeptone, 2% glucose, 2% agar) containing 300 mg/L of geneticin, 50 mg/L of nourseothricin or 200 mg/L of hygromycin B.

Example 6: Evaluation of Flocculation Property of Strain in which Gene Encoding Protein Responsible For Flocculation Property of Yeast Is Disrupted

Flocculation properties of the disrupted strain obtained in Example 5 and the parent strain (W34/70-2 strain) are evaluated by the same method as Example 4.

INDUSTRIAL APPLICABILITY

The method of producing alcoholic beverages of the present invention may allow for highly-efficient production of alcoholic beverages by using a yeast having suitable for production of desired alcoholic beverages, because the flocculation property of yeast during fermentation can be controlled.

CLAIMS

1. A polynucleotide selected from the group consisting of:

(a) a polynucleotide comprising a polynucleotide consisting of the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15;

(b) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30;

(c) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 in which one or more amino acids thereof are deleted, substituted, inserted and/or added, and having an activity of imparting flocculation property to yeast;

(d) a polynucleotide comprising a polynucleotide encoding a protein having an amino acid sequence having 60% or higher identity with the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30, and said protein having an activity of imparting flocculation property to yeast;

(e) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15, under stringent conditions, and which encodes a protein having an activity of imparting flocculation property to yeast; and

(f) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of the polynucleotide encoding the protein having the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30, under stringent conditions, and which encodes a protein having an activity of imparting flocculation property to yeast.

2. The polynucleotide according to Claim 1 selected from the group consisting of:

(g) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30, or encoding the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30 in which 1 to 10 amino acids

thereof are deleted, substituted, inserted, and/or added, and wherein said protein has an activity of imparting flocculation property to yeast;

(h) a polynucleotide comprising a polynucleotide encoding a protein having 90% or higher identity with the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30, and having an activity of imparting flocculation property to yeast; and

(i) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 or which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15, under high stringent conditions, which encodes a protein having an activity of imparting flocculation property to yeast.

3. The polynucleotide according to Claim 1 comprising a polynucleotide consisting of the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15.

4. The polynucleotide according to Claim 1 comprising a polynucleotide encoding a protein consisting of the amino acid sequence of any one of SEQ ID NO:16 through SEQ ID NO:30.

5. The polynucleotide according to any one of Claims 1 to 4, wherein the polynucleotide is DNA.

6. A polynucleotide selected from the group consisting of:

(j) a polynucleotide encoding RNA having a nucleotide sequence complementary to a transcript of the polynucleotide (DNA) according to Claim 5;

(k) a polynucleotide encoding RNA that represses the expression of the polynucleotide (DNA) according to Claim 5 through an RNAi effect;

(l) a polynucleotide encoding RNA having an activity of specifically cleaving a transcript of the polynucleotide (DNA) according to Claim 5; and

(m) a polynucleotide encoding RNA that represses the expression of the polynucleotide (DNA) according to Claim 5 through a co-suppression effect.

7. A protein encoded by the polynucleotide according to any one of Claims 1 to 5.

8. A vector containing the polynucleotide according to any one of Claims 1 to 5.

9. A vector containing the polynucleotide according to Claim 6.

10. A yeast into which the vector according to Claim 8 or 9 has been introduced.

11. The yeast according to Claim 10, wherein the flocculation property is increased.

12. The yeast according to Claim 11, wherein the flocculation property is increased by increasing an expression level of the protein of Claim 7.

13. A yeast, wherein the expression of the polynucleotide (DNA) according to Claim 5 is repressed by:

(A) introducing the vector according to Claim 8 or 9;

(B) disrupting the gene according to the polynucleotide (DNA) of Claim 5; or

(C) introducing a mutation into a promoter or genetically altering a promoter.

14. The yeast according to Claim 10 or 13, wherein the flocculation property is decreased.

15. A method for producing an alcoholic beverage by using the yeast according to any one of Claims 10 to 14.

16. The method according to Claim 15, wherein the brewed alcoholic beverage is a malt beverage.

17. The method according to Claim 15, wherein the brewed alcoholic beverage is wine.

18. An alcoholic beverage produced by the method according to any one of Claims 15 to 17.

19. A method for assessing a test yeast for its flocculation property, comprising using a primer or probe designed based on the nucleotide sequence of a gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast.

20. A method for assessing a test yeast for its flocculation property, comprising: culturing the test yeast; and measuring the expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast.

21. A method for selecting a yeast, comprising: culturing test yeasts; quantifying the protein of Claim 7 or measuring the expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast; and selecting a test yeast having an amount of the protein or the gene expression level according to the flocculation property of interest.

22. The method for selecting a yeast according to Claim 21, comprising: culturing a reference yeast and test yeasts; measuring for each yeast the expression level of the gene having the nucleotide sequence of any one of SEQ ID NO:1 through SEQ ID NO:15 and encoding a protein having an activity of imparting flocculation property to yeast; and selecting a test yeast having the gene expression higher or lower than that in the reference yeast.

23. The method for selecting a yeast according to Claim 21, comprising: culturing a reference yeast and test yeasts; quantifying the protein according to Claim 7 in each yeast; and selecting a test yeast having a larger or smaller amount of the protein than that in the reference yeast.

24. A method for producing an alcoholic beverage comprising: conducting fermentation for producing an alcoholic beverage using the yeast according to any one of Claims 10 to 14 or a yeast selected by the methods according to any one of Claims 21 to 23, and adjusting flocculation property of yeast.

Figure 1

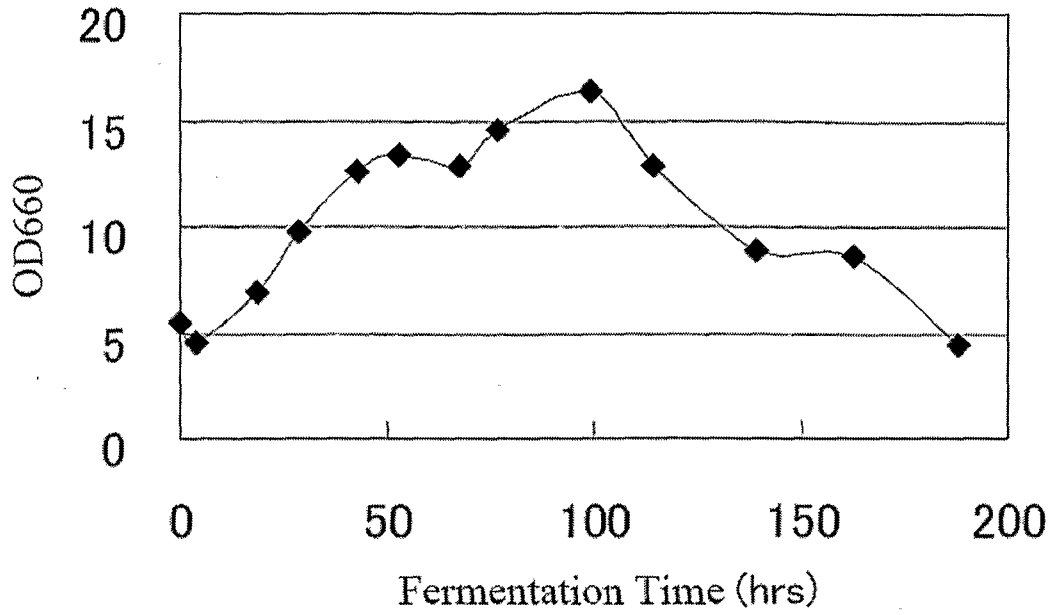
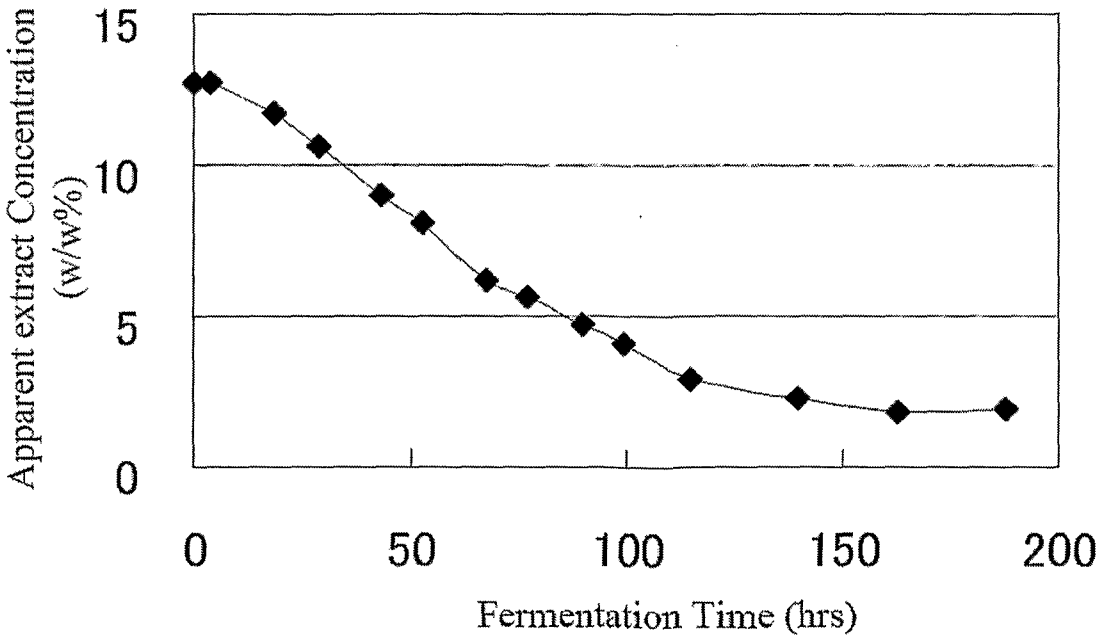


Figure 2



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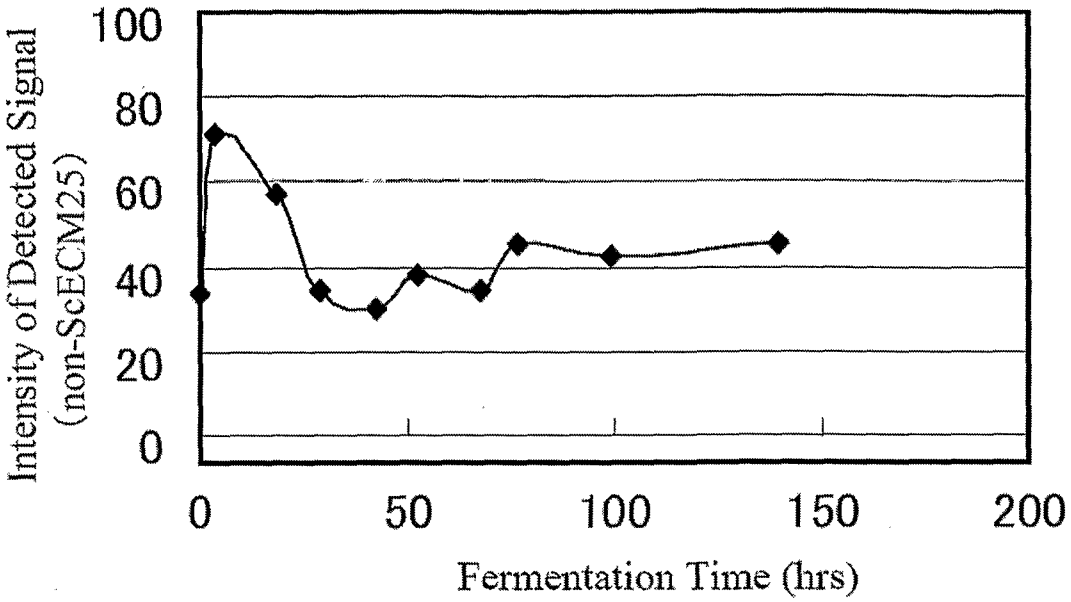

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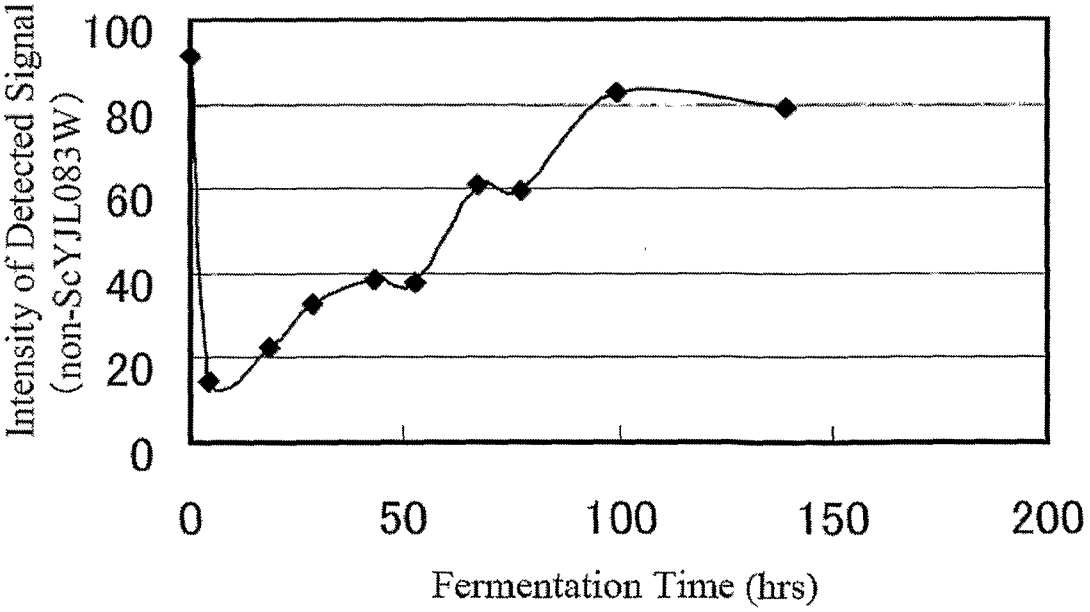

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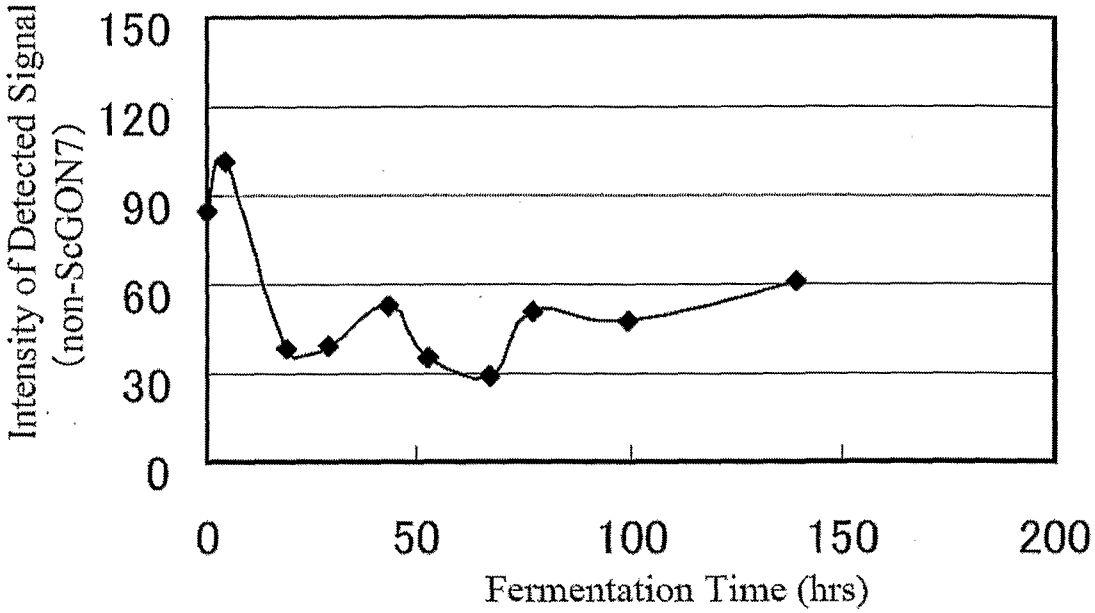

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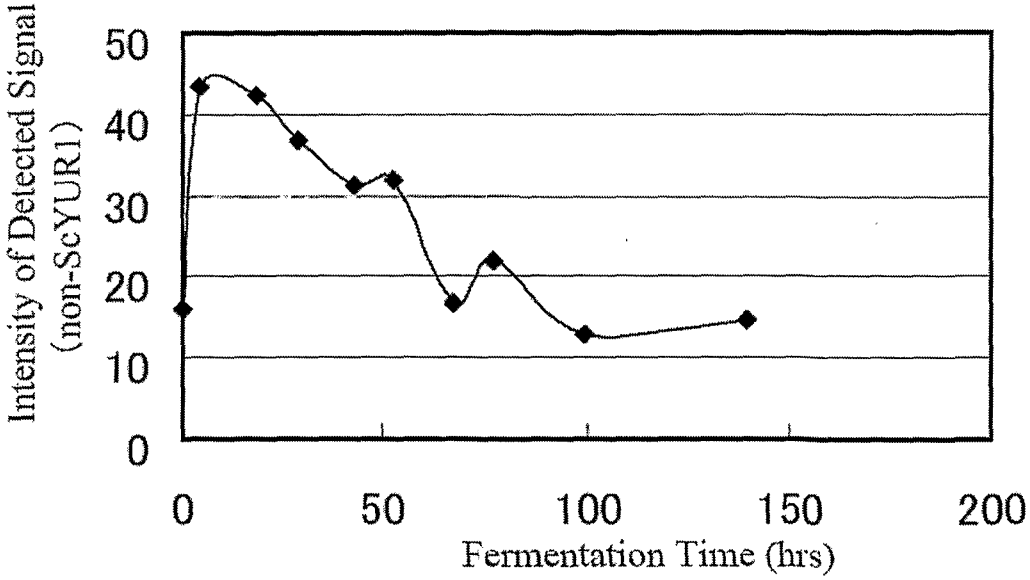

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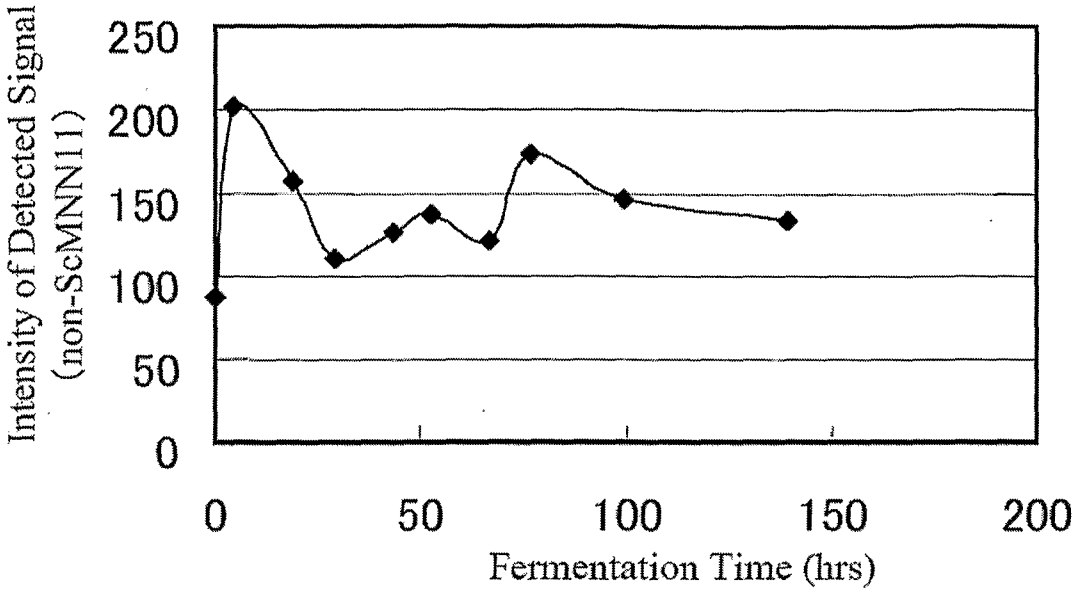

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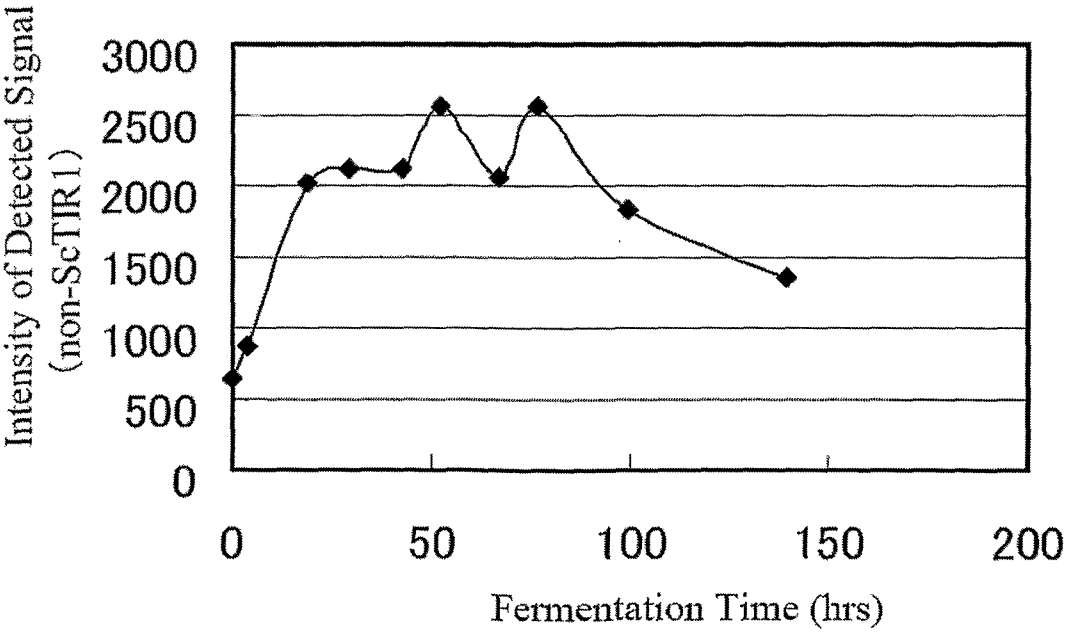

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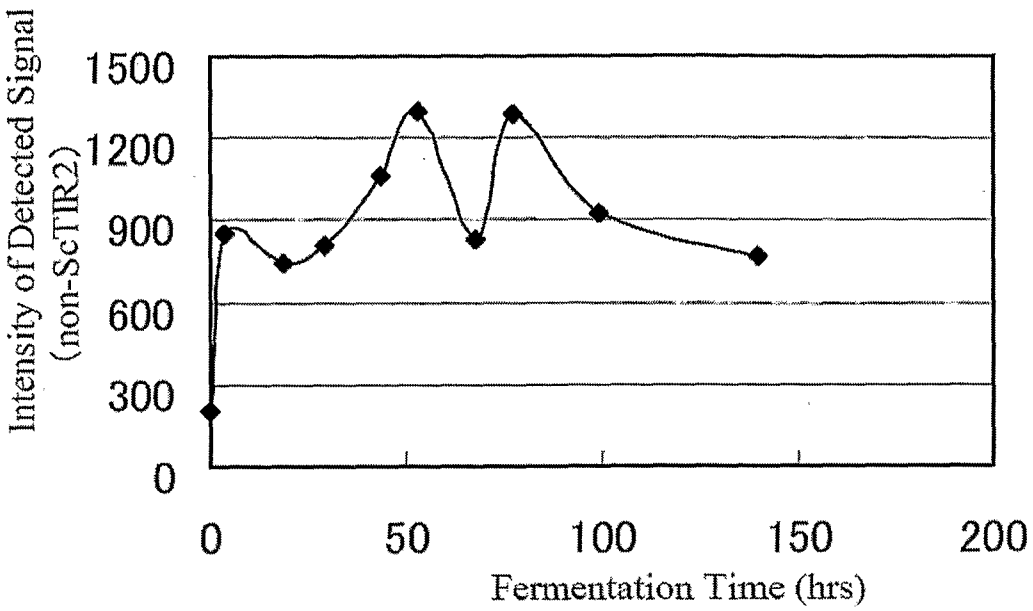


Figure 10

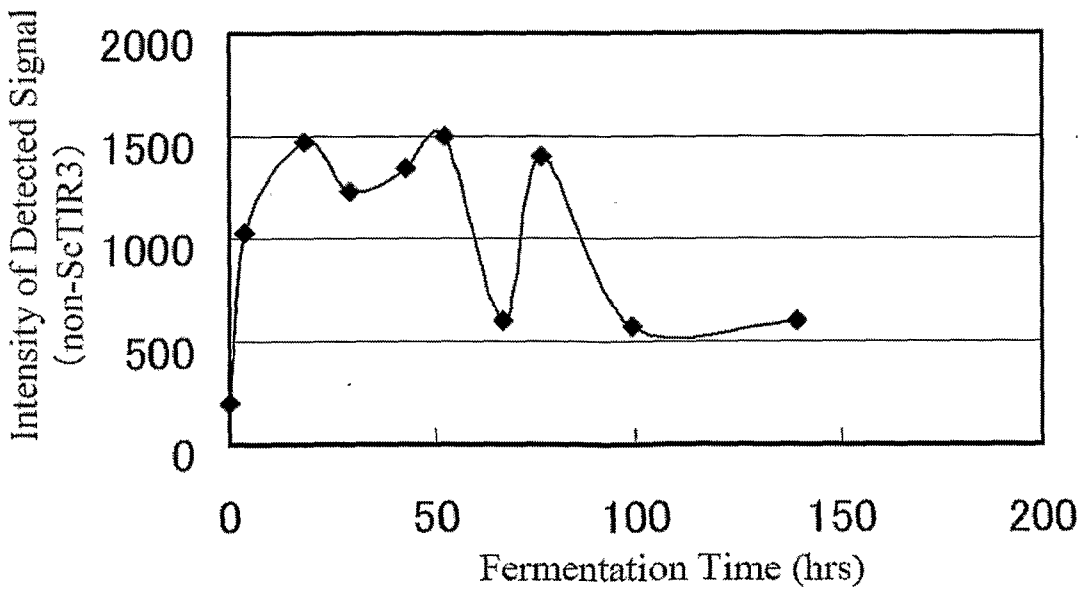


Figure 11

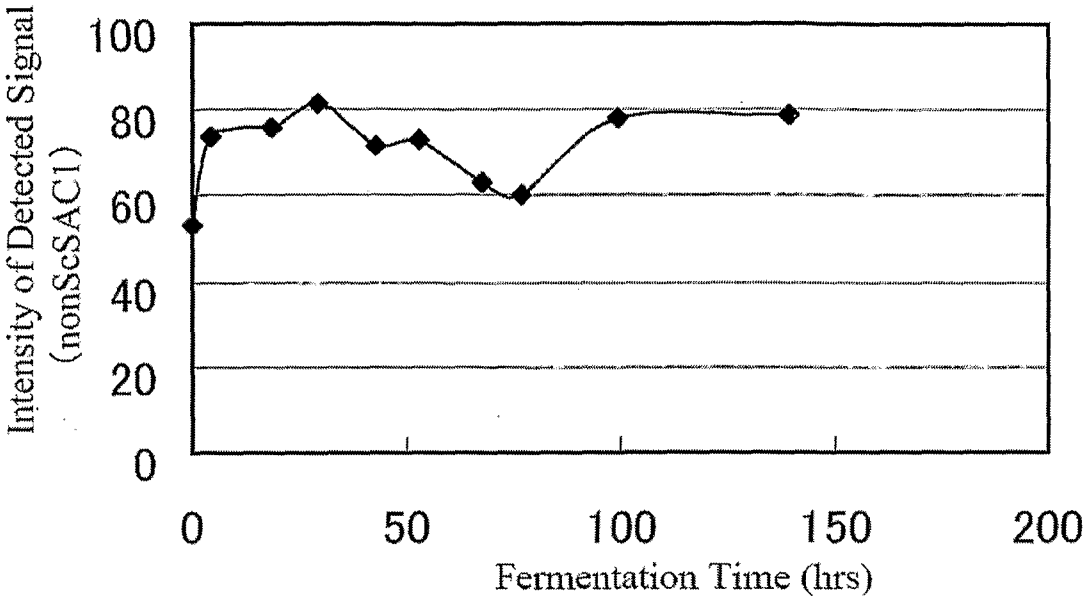


Figure 12

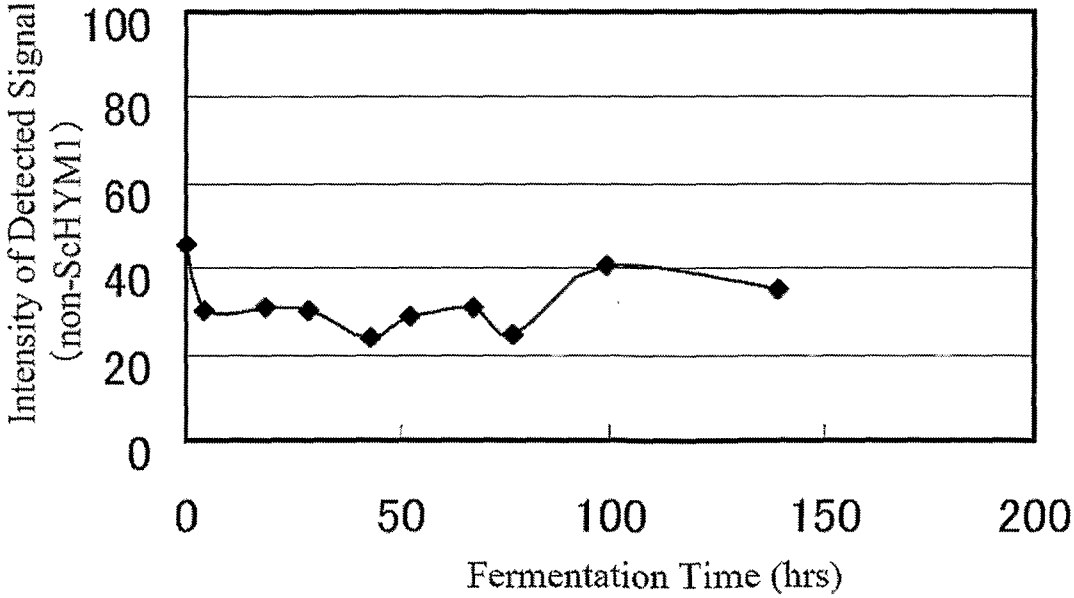


Figure 13

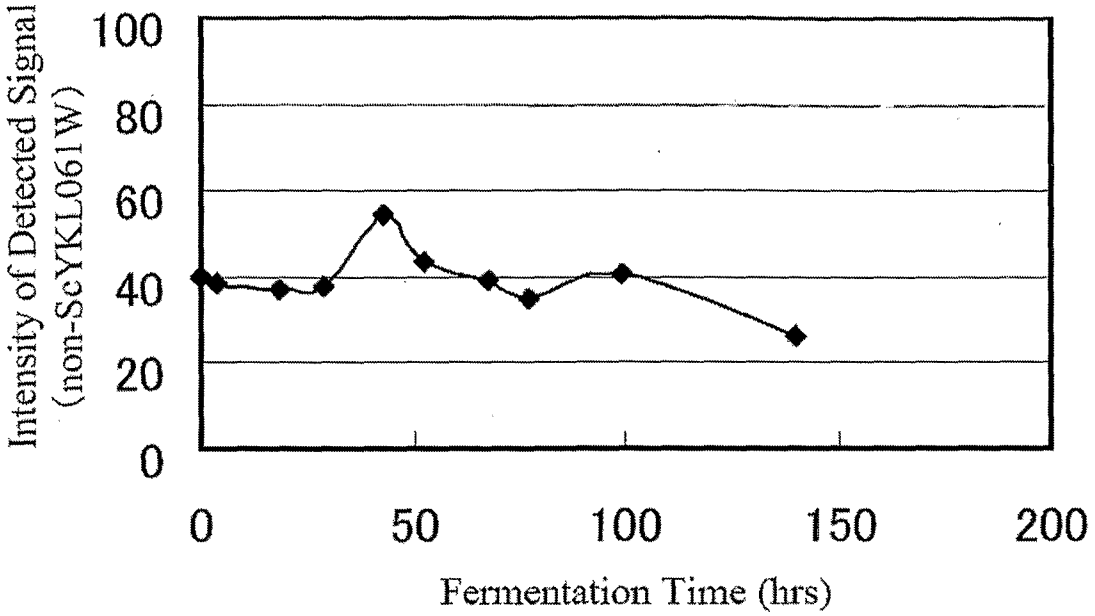
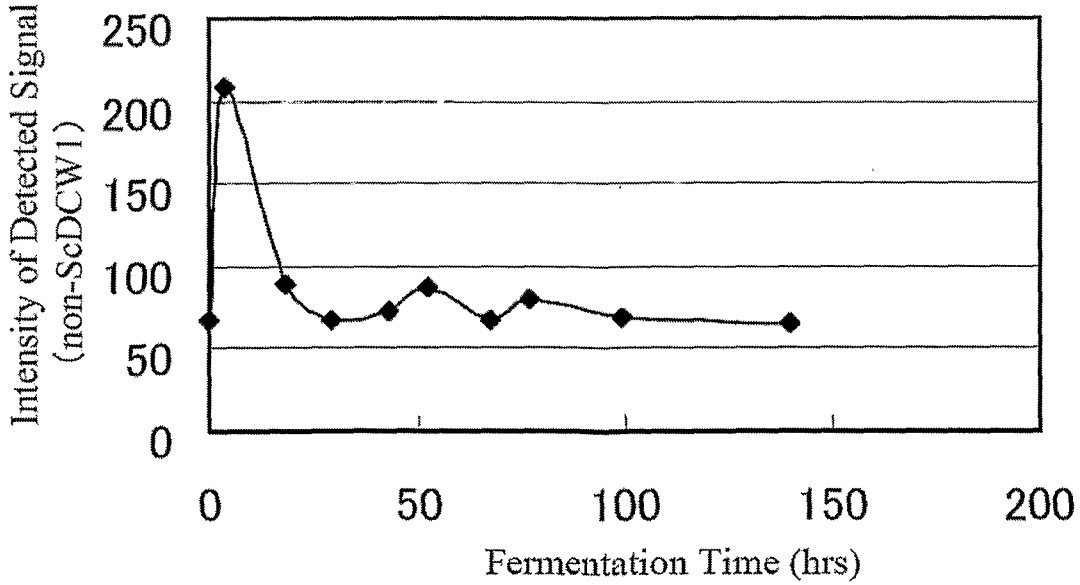


Figure 14



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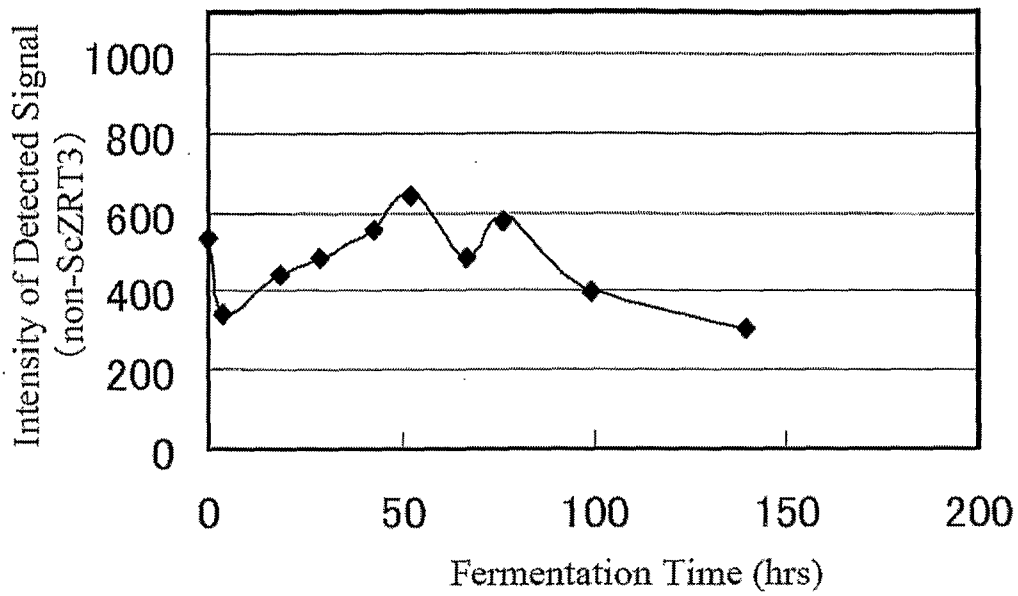


Figure 16

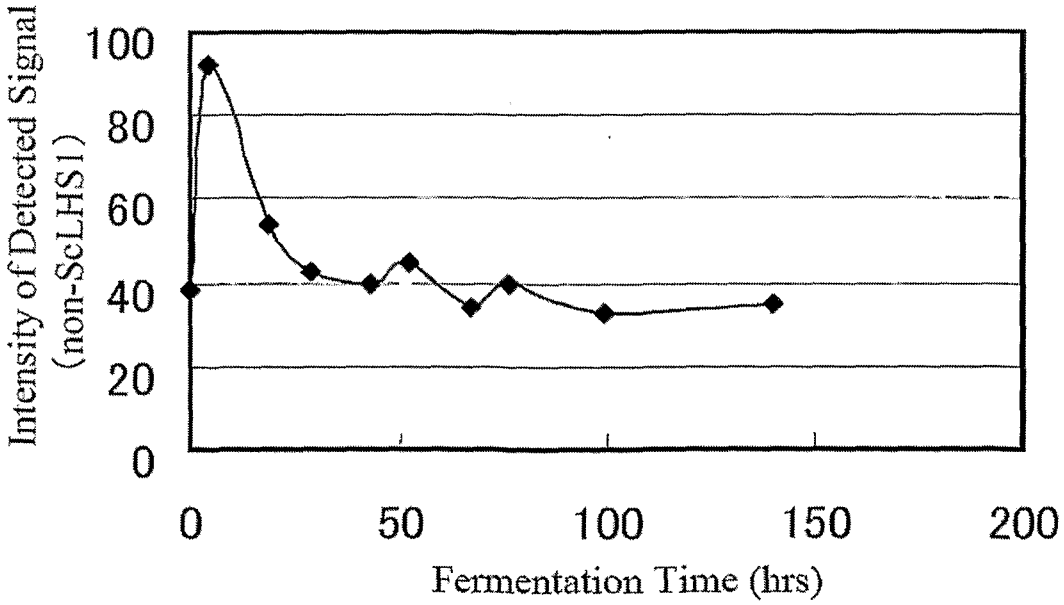
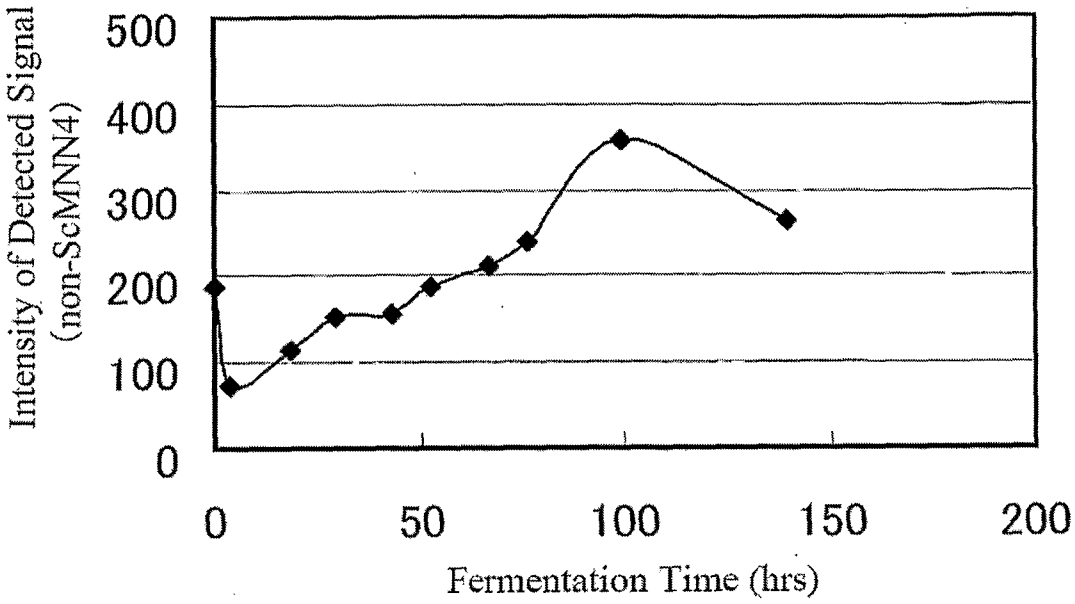


Figure 17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Fermentation Time (hrs)