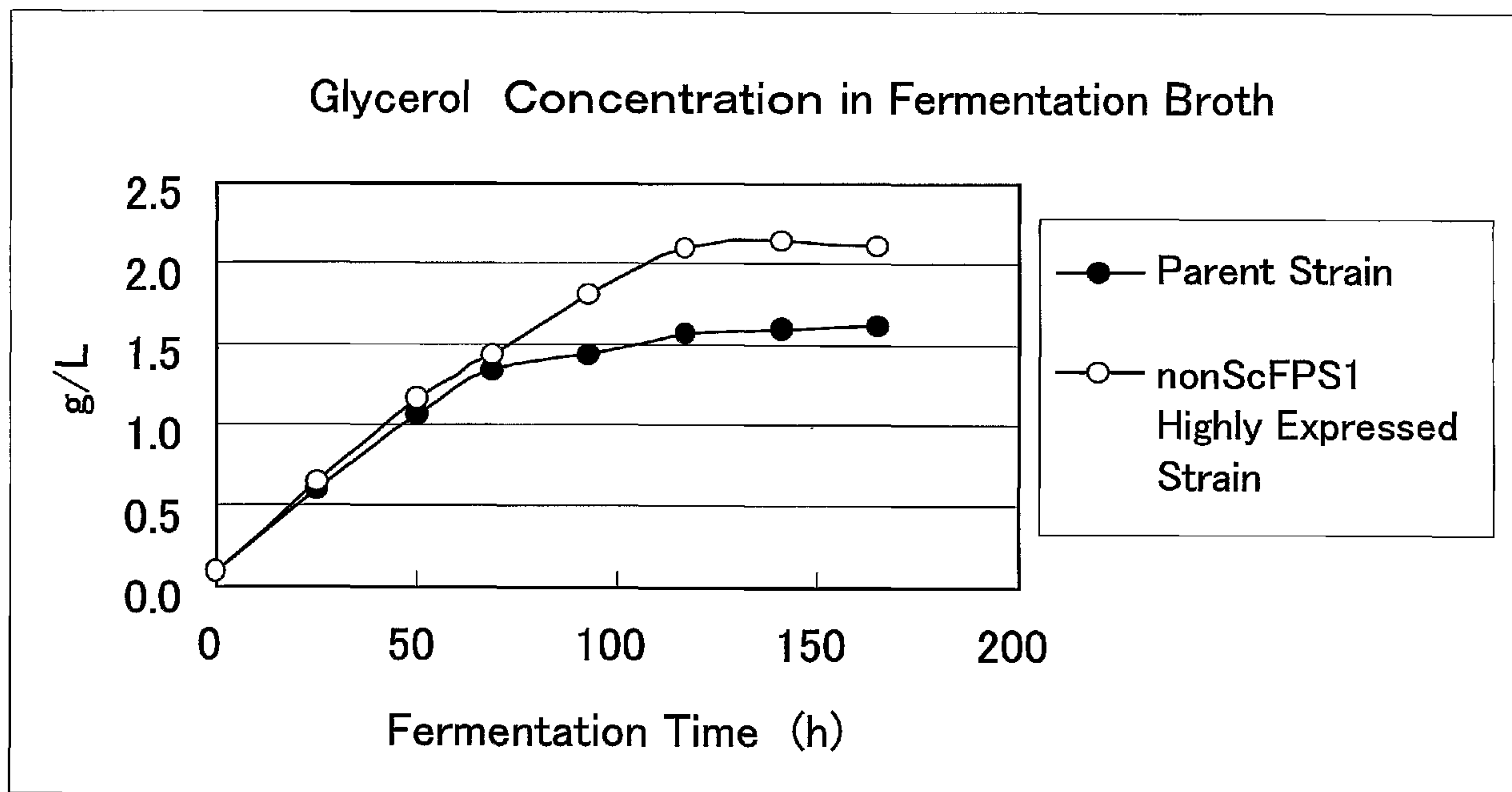




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(57) **Abrégé/Abstract:**

The present invention relates to a glycerol channel gene and its uses, specifically, a brewery yeast producing alcoholic beverages with excellent body and mellowness, alcoholic beverages produced using the yeast, a process for producing the alcoholic beverages. More particularly, the present invention relates to a yeast whose capability of producing glycerol, which contribute to body and mellowness of products, was controlled by controlling expression level of FPS1 gene encoding brewery yeast glycerol channel Fps1p, particularly non-ScFPS1 gene specific to lager brewing yeast, and to a method for producing alcoholic beverages with the yeast.

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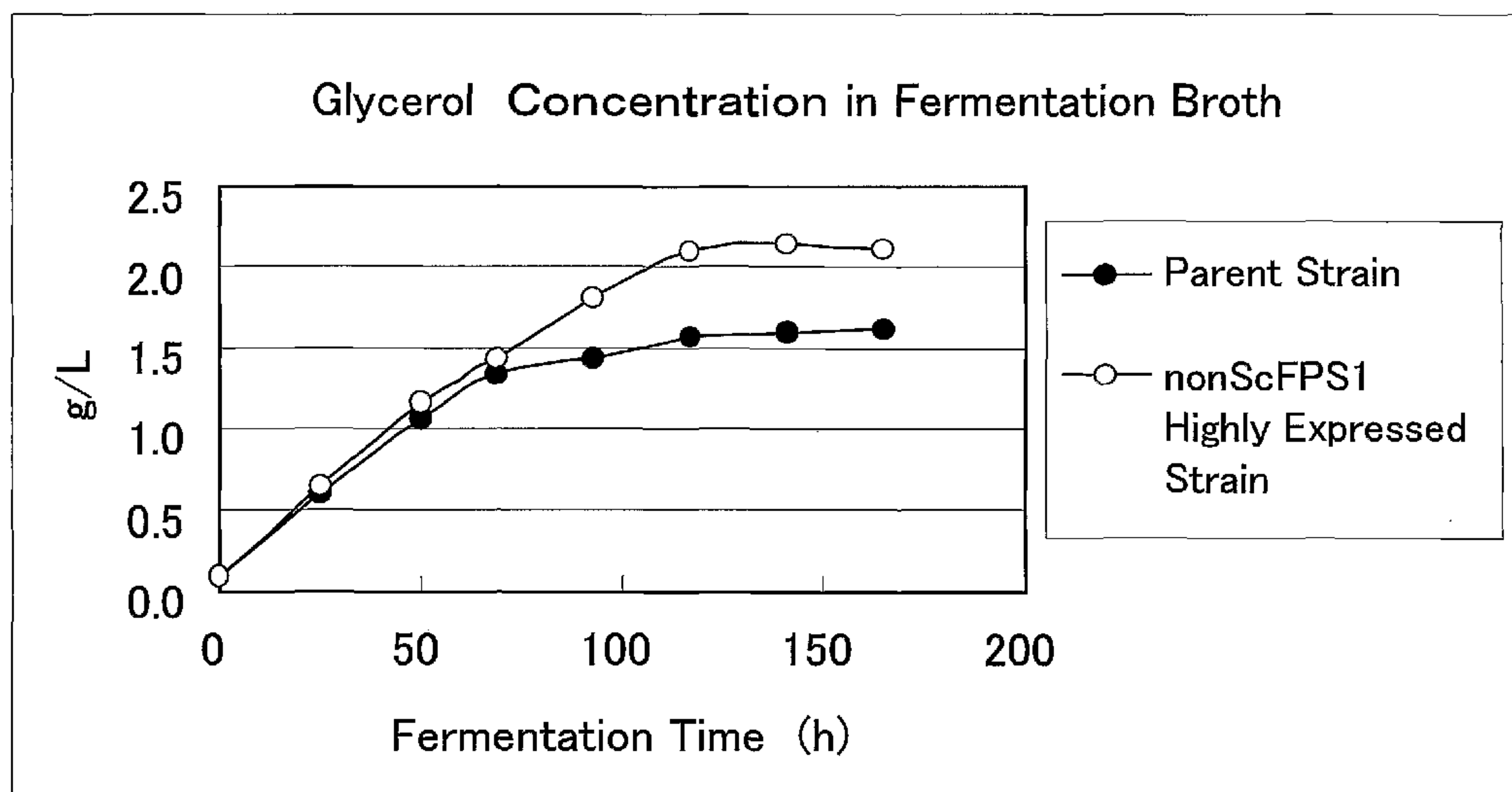
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(54) Title: GLYCEROL CHANNEL GENE AND USE THEREOF



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DEMANDE OU BREVET VOLUMINEUX

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CECI EST LE TOME 1 DE 2
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DESCRIPTION

GLYCEROL CHANNEL GENE AND USE THEREOF

5 TECHNICAL FIELD

The present invention relates to a glycerol channel gene and to uses of the gene. The invention relates in particular to a brewer's yeast which produces alcoholic beverages with excellent body and mellowness, alcoholic beverages produced using such a yeast, and a method of producing such alcoholic beverages. More specifically, the invention relates to FPS1 gene which codes for
10 the glycerol channel Fps1p in brewer's yeast, particularly to a yeast which can control the body and mellowness of product by controlling the level of expression of the non-ScFPS1 gene characteristic to beer yeast and to a method of producing alcoholic beverages using such a yeast.

BACKGROUND ART

15 Glycerol, which is said to contribute to body and mellowness as well as sweetness, is one of the important taste components.

Glycerol high-producing yeasts have been developed to increase glycerol levels in alcoholic beverages. A method employing resistant property to allyl alcohol or pyrazole as an indicator (Japanese Examined Patent Publication (Kokoku) No. H7-89901), a method employing
20 resistant property to glycerol monochlorohydrin as an indicator (Japanese Patent Application Laid-open No. H10-210968), and a method employing resistant property to salts as an indicator (Japanese Patent Application Laid-open No. H7-115956), a method employing resistant property to amino acid analogues as a indicator (J. Ferment. Bioeng., 80, 218-222 (1995)) for mutating yeast and isolating glycerol high-producing yeasts effectively have been reported.

25 On the other hand, a method in which a Glycerol-3-phosphate dehydrogenase GPD1 was highly expressed in beer yeasts or wine yeasts was reported as a method employing development of yeast by gene manipulation technology (FEMS Yeast Res. 2: 225-232 (2002), Appl. Environ. Microbiol. 65: 143-149 (1999)).

30 DISCLOSURE OF INVENTION

As noted above, variant strains have been developed in order to increase the glycerol level in the final product. As a result, however, unexpected delays in fermentation and increases in undesirable flavor components have been observed in some cases, which makes the practical use of such yeasts questionable. There were thus demands for a method of developing yeast which can

produce sufficient amount of glycerol without compromising either the fermentation rate or the product quality.

The present inventors made exhaustive studies to solve the above problems and as a result, succeeded in identifying and isolating a gene encoding a glycerol channel which has advantageous effects than the existing proteins from lager brewing yeast. Moreover, a yeast in which the obtained gene was transformed and expressed was produced to confirm elevation of the amount of glycerol production, thereby completing the present invention.

Thus, the present invention relates to a novel glycerol channel gene existing specifically in a lager brewing 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 the amount of glycerol in a product by using a yeast in which the expression of said gene is controlled. More specifically, the present invention provides the following polynucleotides, a vector comprising said polynucleotide, a transformed yeast introduced with said vector, a method for producing alcoholic beverages by using said transformed yeast, and the like.

- 15 (1) A polynucleotide selected from the group consisting of:
- (a) a polynucleotide comprising a polynucleotide consisting of the nucleotide sequence of SEQ ID NO:1;
 - (b) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO:2;
 - 20 (c) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO:2 with one or more amino acids thereof being deleted, substituted, inserted and/or added, and having a glycerol channel activity;
 - (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 SEQ ID NO:2, and having a glycerol channel activity;
 - 25 (e) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO:1 under stringent conditions, and which encodes a protein having a glycerol channel activity; 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 of the amino acid sequence of SEQ ID NO:2 under stringent conditions, and which encodes a protein having a glycerol channel activity.
 - 30

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

- (g) a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID

NO: 2, or encoding an amino acid sequence of SEQ ID NO: 2 wherein 1 to 10 amino acids thereof is deleted, substituted, inserted, and/or added, and wherein said protein has a glycerol channel activity;

(h) a polynucleotide encoding a protein having 90% or higher identity with the amino acid sequence of SEQ ID NO: 2, and having a glycerol channel activity; and

5 (i) a polynucleotide which hybridizes to SEQ ID NO: 1 or which hybridizes to a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 under high stringent conditions, and which encodes a protein having a glycerol channel activity.

(3) The polynucleotide of (1) above comprising a polynucleotide consisting of SEQ ID NO: 1.

10 (4) The polynucleotide of (1) above comprising a polynucleotide encoding a protein consisting of SEQ ID NO: 2.

(5) The polynucleotide of any one of (1) to (4) above, wherein the polynucleotide is DNA.

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

15 (j) a polynucleotide encoding RNA of 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

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

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

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

25 (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 direction; and

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

(9) A vector comprising the polynucleotide of (6) above.

(10) A yeast, wherein the vector of (8) or (9) above is introduced.

(11) The yeast of (10) above, wherein glycerol-producing ability is increased.

35 (12) A yeast, wherein an expression of the polynucleotide (DNA) of (5) above is repressed by introducing the vector of (9) above, or by disrupting a gene related to the polynucleotide (DNA)

of (5) above.

(13) The yeast of (10) above, wherein a glycerol-producing ability is increased by increasing an expression level of the protein of (7) above.

5 (14) A method for producing an alcoholic beverage by using the yeast of any one of (10) to (13) above.

(15) The method for producing an alcoholic beverage of (14) above, wherein the brewed alcoholic beverage is a malt beverage.

(16) The method for producing an alcoholic beverage of (14) above, wherein the brewed alcoholic beverage is a wine.

10 (17) An alcoholic beverage, which is produced by the method of any one of (14) to (16) above.

(18) A method for assessing a test yeast for its glycerol-producing capability, comprising using a primer or a probe designed based on a nucleotide sequence of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1.

15 (18a) A method for selecting a yeast having a high or low glycerol-producing capability by using the method in (18) above.

(18b) A method for producing an alcoholic beverage (for example, beer) by using the yeast selected with the method in (18a) above.

20 (19) A method for assessing a test yeast for its glycerol-producing capability, comprising: culturing a test yeast; and measuring an expression level of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1.

25 (20) A method for selecting a yeast, comprising: culturing test yeasts; quantifying the protein of (7) above or measuring an expression level of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1; and selecting a test yeast having said protein amount or said gene expression level according to a target capability of producing glycerol.

(20a) A method for selecting a yeast, comprising: culturing test yeasts; measuring a glycerol-producing capability or a glycerol channel activity; and selecting a test yeast having a target capability of producing glycerol or a target glycerol channel activity.

30 (21) The method for selecting a yeast of (20) above, comprising: culturing a reference yeast and test yeasts; measuring an expression level of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1 in each yeast; and selecting a test yeast having the gene expressed higher or lower than that in the reference yeast.

35 (22) The method for selecting a yeast of (20) above comprising: culturing a reference yeast and test yeasts; quantifying the protein of (7) above in each yeast; and selecting a test yeast having said protein for a larger or smaller amount than that in the reference yeast. That is, the method for

selecting a yeast of (20) above comprising: culturing plural yeasts; quantifying the protein of (7) above in each yeast; and selecting a test yeast having a large or small amount of the protein from them.

(23) A method for producing an alcoholic beverage comprising: conducting fermentation
5 for producing an alcoholic beverage using the yeast according to any one of (10) to (13) or a yeast selected by the method according to any one of (20) to (22); and adjusting the production amount of glycerol.

BRIEF DESCRIPTION OF DRAWINGS

10 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%).

15 Figure 3 shows the expression profile of non-ScFPS1 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 cell growth with time upon fermentation test. The horizontal axis represents fermentation time while the vertical axis represents optical density at 660 nm (OD660).

20 Figure 5 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 6 shows the glycerol production with time upon beer fermentation test. The horizontal axis represents fermentation time while the vertical axis represents glycerol level (g/L).

25

BEST MODES FOR CARRYING OUT THE INVENTION

The present inventors conceived that it is possible to control glycerol in products by increasing or decreasing a glycerol channel activity of the yeast. The present inventors have studied based on this conception and as a result, isolated and identified a non-ScFPS1 gene encoding a
30 glycerol channel unique to lager brewing 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 sequence of the gene is represented by SEQ ID NO: 1. Further, an amino acid sequence of a protein encoded by the gene is represented by SEQ ID NO: 2.

35 1. Polynucleotide of the invention

First of all, the present invention provides (a) a polynucleotide comprising a polynucleotide of the nucleotide sequence of SEQ ID NO:1; and (b) a polynucleotide comprising a polynucleotide encoding a protein of the amino acid sequence of SEQ ID NO:2. The polynucleotide can be DNA or RNA.

5 The target polynucleotide of the present invention is not limited to the polynucleotide encoding a glycerol channel gene 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 SEQ ID NO: 2 with one or more amino acids thereof being deleted, substituted, inserted and/or
10 added and having a glycerol channel activity.

Such proteins include a protein consisting of an amino acid sequence of SEQ ID NO: 2 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,
15 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 having a glycerol channel activity. 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%
20 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
25 higher, 99.7% or higher, 99.8% or higher, or 99.9% or higher identity with the amino acid sequence of SEQ ID NO: 2, and having a glycerol channel activity. In general, the percentage identity is preferably higher.

Glycerol channel activity may be measured, for example, by a method described in *Eur. J. Biochem.* 271:771-779, 2004.

30 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 SEQ ID NO: 1 under stringent conditions and which encodes a protein having a glycerol channel activity; and (f) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide complementary to a nucleotide sequence of
35 encoding a protein of SEQ ID NO: 2 under stringent conditions, and which encodes a protein having

a glycerol channel activity.

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 SEQ ID NO: 1 or polynucleotide encoding the amino acid sequence of SEQ ID NO: 2 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 SEQ ID NO: 2 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-suppression 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; 5 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 10 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, 15 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-suppression effect" refers to a nucleotide that inhibits functions of target DNA by "co-suppression".

20 The term "co-suppression" 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-suppression 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).

25

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 SEQ ID NO:2 with one or several amino acids thereof being deleted, substituted, inserted and/or added, 30 and has a glycerol channel activity.

Such protein includes those having an amino acid sequence of SEQ ID NO: 2 with amino acid residues thereof of the number mentioned above being deleted, substituted, inserted and/or added and having a glycerol channel activity. In addition, such protein includes those having homology as described above with the amino acid sequence of SEQ ID NO: 2 and having a glycerol 35 channel activity.

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).

5 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.

10 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: aspartic acid, glutamic acid, isoaspartic acid, isoglutamic acid, 2-aminoadipic acid, 2-aminosuberic acid; 15 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 20 method). In addition, peptide synthesizers available from, for example, Advanced ChemTech, PerkinElmer, Pharmacia, Protein Technology Instrument, Synthecell-Vega, PerSeptive, Shimazu Corp. can also be used for chemical synthesis.

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

25 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 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 30 (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 35 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).

A vector introduced in the yeast may be any of a multicopy type (YE_p type), a single copy type (YC_p type), or a chromosome integration type (YI_p type). For example, YE_p24 (J. R. Broach et al., *EXPERIMENTAL MANIPULATION OF GENE EXPRESSION*, Academic Press, New York, 83, 1983) is known as a YE_p type vector, YC_p50 (M. D. Rose et al., *Gene* 60: 237, 1987) is known as a YC_p type vector, and YI_p5 (K. Struhl et al., *Proc. Natl. Acad. Sci. USA*, 76: 1035, 1979) is known as a YI_p 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 (G418^r), a copper-resistant gene (CUP1) (Marin et al., *Proc. Natl. Acad. Sci. USA*, 81, 337 1984) or a cerulenin-resistant gene (fas2^m, 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*. Vo1. 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

The vector of the present invention described above is introduced into a yeast suitable for brewing a target alcoholic product. This yeast can be used to produce a desired alcoholic beverage with enhanced body and mellowness with an elevated content of glycerol. In addition, yeasts to be selected by the yeast assessment method of the present invention described below can also be used. 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, according to the present invention, desired alcoholic beverages with reduced glycerol level can be produced using brewery yeast in which the expression of the target gene was suppressed, if needed. That is to say,

desired kind of alcoholic beverages with controlled (elevated or reduced) level of glycerol can be produced by controlling (elevating or reducing) production amount of glycerol using yeasts into which the vector of the present invention was introduced described above, yeasts in which expression of the polynucleotide (DNA) of the present invention described above was suppressed or yeasts selected by the yeast assessment method of the invention described below for fermentation to produce alcoholic beverages.

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 with an elevated content of glycerol. Thus, according to the present invention, alcoholic beverages with excellent body and mellowness can be produced using the existing facility without increasing the cost.

15 **5. Yeast assessment method of the invention**

The present invention relates to a method for assessing a test yeast for its glycerol-producing capability by using a primer or a probe designed based on a nucleotide sequence of a glycerol channel gene having the nucleotide sequence of SEQ ID NO:1. General techniques for such assessment method is known and is described in, for example, WO01/040514, Japanese Laid-Open Patent Application No. 8-205900 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 glycerol channel gene, 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
5 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 capability of the yeast to produce glycerol as determined by whether the molecular weight of the amplified
10 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 capability 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 glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1 to assess the test yeast for its glycerol-producing capability. In measuring an expression level of the glycerol channel gene,
15 the test yeast is cultured and then mRNA or a protein resulting from the glycerol channel gene 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).

20 Furthermore, test yeasts are cultured and expression levels of the glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1 are measured to select a test yeast with the gene expression level according to the target capability of producing glycerol, 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,
25 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 glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1 is measured in each yeast. By selecting a test yeast with the gene expressed higher or lower than that in the reference yeast, a yeast suitable for brewing alcoholic beverages can be selected.

30 Alternatively, test yeasts are cultured and a yeast with a higher or lower glycerol-producing capability or with a higher or lower glycerol channel activity 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 of the invention, a yeast in which an expression of a polynucleotide (DNA) of the
35 invention has been controlled, an artificially mutated yeast or a naturally mutated yeast. The

glycerol-producing capability can be measured by, for example, a method described in Method of Enzymatic Analysis, vol.4 1825-1831,1974. Glycerol channel activity can be measured by, for example, a method described in Eur. J. Biochem. 271:771-779, 2004. 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.

20 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.

25 Example 1: Cloning of Glycerol Channel (non-ScFPS1) Gene

A specific novel glycerol channel gene (non-ScFPS1) (SEQ ID NO: 1) from a lager brewing yeast 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-ScFPS1_for (SEQ ID NO: 3) and non-ScFPS1_rev (SEQ ID NO: 4) were designed to amplify the full-length genes, respectively. PCR was carried out using chromosomal DNA of a genome sequencing strain, *Saccharomyces pastorianus* Weihenstephan 34/70 strain, as a template to obtain DNA fragments (about 2 kb) including the full-length gene of non-ScFPS1.

The thus-obtained non-ScFPS1 gene fragment was inserted into pCR2.1-TOPO vector (Invitrogen) by TA cloning. The nucleotide sequences of non-ScFPS1 gene were analyzed

according to Sanger's method (F. Sanger, *Science*, 214: 1215, 1981) to confirm the nucleotide sequence.

Example 2: Analysis of Expression of non-ScFPS1 Gene during Beer Fermentation

5 A beer fermentation test was conducted using a lager brewing yeast, *Saccharomyces pastorianus* W34/70 strain and then mRNA extracted from yeast cells during fermentation was analyzed by a DNA microarray.

10	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

15 Sampling of fermentation liquor was performed with time, and variation with time of yeast growth amount (Fig. 1) and apparent extract concentration (Fig. 2) was observed. Simultaneously, sampling of yeast cells was performed, and the prepared mRNA was subjected to be biotin-labeled and was hybridized to a beer yeast DNA microarray. The signal was detected using GCOS; GeneChip Operating Software 1.0 (manufactured by Affymetrix Co.). Expression pattern of
20 non-ScFPS1 gene is shown in Figure 3. As a result, it was confirmed that non-ScFPS1 gene was expressed in the general beer fermentation.

Example 3: Construction of non-ScFPS1 Gene Highly Expressed Strain

25 The non-ScFPS1/pCR2.1-TOPO described in Example 1 was digested using the restriction enzymes *SacI* and *NotI* so as 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-ScFPS1 high expression vector non-ScFPS1/pYCGPYNot. pYCGPYNot is the YCp-type yeast expression vector. The inserted gene is highly expressed by the pyruvate kinase gene *PYK1* promoter. The geneticin-resistant gene
30 *G418^r* is included as the selection marker in the yeast, and the ampicillin-resistant gene *Amp^r* is included as the selection 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. H7-303475. The transformant was selected in a YPD plate culture (1% yeast extract, 2% polypeptone, 2% glucose, 2% agar) containing 300 mg/L of geneticin.

5 **Example 4: Analysis of Amount of Glycerol Production in Beer Fermentation Test**

A fermentation test was carried out under the following conditions using the parent strain (34/70 strain) and the non-ScFPS1 highly expressed strain obtained in Example 3.

10	Wort extract concentration	12.8 %
	Wort content	1 L
	Wort dissolved oxygen concentration	approx. 10 ppm
	Fermentation temperature	15°C (fixed)
	Yeast pitching rate	6 g wet yeast cells/L of wort

15 The fermentation broth was sampled over time, and the change over time in the yeast growth rate (OD660) (FIG. 4), the amount of extract consumption (FIG. 5) and the amount of glycerol production (FIG. 6) were determined. Glycerol in the fermentation broth was quantified using F-kit glycerol (product number 148270, manufactured by Roche) (see Method of Enzymatic Analysis, vol.4 1825-1831, 1974, etc.). The amount of glycerol on the completion of fermentation
20 was 1.6 g/L for the parent strain, and was 2.1 g/L for non-ScFPS1 highly expressed strain, which was about 1.3-fold of the parent strain.

Example 5: Disruption of nonScFPS1 Gene

25 According to the publication (Goldstein et al., yeast. 15 1541 (1999)), PCR using a plasmid including a drug-resistant marker (pFA6a (*G418^r*) or pAG25 (*nat1*)) as a template is conducted to prepare a fragment for gene disruption.

30 With the prepared fragment for gene disruption, beer yeast *Saccharomyces pastorianus* W34/70 strain or spore cloning strain (W34/70-2) is transformed. The transformation is performed in accordance with the method described in Japanese Patent Application Laid-Open No. H07-303475. The concentrations of the drugs for selection are 300 mg/L for geneticin and 50 mg/L of nourseothricin, respectively.

Example 6: Analysis of Amounts of Glycerol Production in Beer Fermentation Test

Using parent strain and the non-ScFPS1-disrupted strain obtained in Example 5, under the following conditions, beer brewing testing is carried out.

5	Wort extract concentration	13%
	Wort content	1L
	Wort dissolved oxygen concentration	about 10 ppm
	Fermentation temperature	15°C constantly
	Yeast input	6 g of wet yeast cells/ L of wort

10 The fermentation broth is sampled with time to observe the cell growth (OD660), the sugar consumption and glycerol production with time. Glycerol in the fermentation broth is quantified using F-kit glycerol (product number 148270, manufactured by Roche) (see Method of Enzymatic Analysis, vol.4 1825-1831, 1974, etc.).

15 **INDUSTRIAL APPLICABILITY**

The inventive method of producing alcoholic beverages can be used to produce alcoholic beverages with excellent taste, because the method can control the amount of glycerol, which provides body or mellowness to the product.

DEMANDE OU BREVET VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2
CONTENANT LES PAGES 1 À 18

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME 1 OF 2
CONTAINING PAGES 1 TO 18

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NOTE POUR LE TOME / VOLUME NOTE:

CLAIMS

1. A polynucleotide selected from the group consisting of:

5 (a) a polynucleotide comprising a polynucleotide consisting of the nucleotide sequence of SEQ ID NO:1;

(b) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO:2;

10 (c) a polynucleotide comprising a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO:2 with one or more amino acids thereof being deleted, substituted, inserted and/or added, and having a glycerol channel activity;

(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 SEQ ID NO:2, and having a glycerol channel activity;

15 (e) a polynucleotide comprising a polynucleotide which hybridizes to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO:1 under stringent conditions, and which encodes a protein having a glycerol channel activity; and

20 (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 of the amino acid sequence of SEQ ID NO:2 under stringent conditions, and which encodes a protein having a glycerol channel activity.

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

25 (g) a polynucleotide encoding a protein consisting of the amino acid sequence of SEQ ID NO: 2, or encoding an amino acid sequence of SEQ ID NO: 2 wherein 1 to 10 amino acids thereof is deleted, substituted, inserted, and/or added, and wherein said protein has a glycerol channel activity;

(h) a polynucleotide encoding a protein having 90% or higher identity with the amino acid sequence of SEQ ID NO: 2, and having a glycerol channel activity; and

30 (i) a polynucleotide which hybridizes to SEQ ID NO: 1 or which hybridizes to a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1 under high stringent conditions, and which encodes a protein having a glycerol channel activity.

3. The polynucleotide of Claim 1 comprising a polynucleotide consisting of SEQ ID NO:

1.

35 4. The polynucleotide of Claim 1 comprising a polynucleotide encoding a protein

consisting of SEQ ID NO: 2.

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

5 6. A polynucleotide selected from the group consisting of:

(j) a polynucleotide encoding RNA of 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 RNAi effect;

10 (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 expression of the polynucleotide (DNA) according to Claim 5 through co-suppression effect.

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

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

9. A vector comprising the polynucleotide of Claim 6.

20

10. A yeast comprising the vector of Claim 8 or 9.

11. The yeast of Claim 10, wherein a glycerol-producing ability is increased.

25 12. A yeast, wherein an expression of the polynucleotide (DNA) of Claim 5 is repressed by introducing the vector of Claim 9, or by disrupting a gene related to the polynucleotide (DNA) of Claim 5.

30 13. The yeast of Claim 10, wherein a glycerol-producing ability is increased by increasing an expression level of the protein of Claim 7.

14. A method for producing an alcoholic beverage by using the yeast of any one of Claims 10 to 13.

35 15. The method for producing an alcoholic beverage of Claim 14, wherein the brewed

alcoholic beverage is a malt beverage.

16. The method for producing an alcoholic beverage of Claim 14, wherein the brewed alcoholic beverage is wine.

5

17. An alcoholic beverage produced by the method of any one of Claims 14 to 16.

18. A method for assessing a test yeast for its glycerol-producing capability, comprising using a primer or a probe designed based on a nucleotide sequence of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1.

10

19. A method for assessing a test yeast for its glycerol-producing capability, comprising: culturing a test yeast; and measuring an expression level of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1.

15

20. A method for selecting a yeast, comprising: culturing test yeasts; quantifying the protein according to Claim 7 or measuring an expression level of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1; and selecting a test yeast having said protein amount or said gene expression level according to a target capability of producing glycerol.

20

21. The method for selecting a yeast according to Claim 20, comprising: culturing a reference yeast and test yeasts; measuring an expression level of a glycerol channel gene having the nucleotide sequence of SEQ ID NO: 1 in each yeast; and selecting a test yeast having the gene expressed higher or lower than that in the reference yeast.

25

22. The method for selecting a yeast according to Claim 20, comprising: culturing a reference yeast and test yeasts; quantifying the protein according to Claim 7 in each yeast; and selecting a test yeast having said protein for a larger or smaller amount than that in the reference yeast.

30

23. 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 13 or a yeast selected by the method according to any one of Claims 20 to 22; and adjusting the production amount of glycerol.

FIG. 1

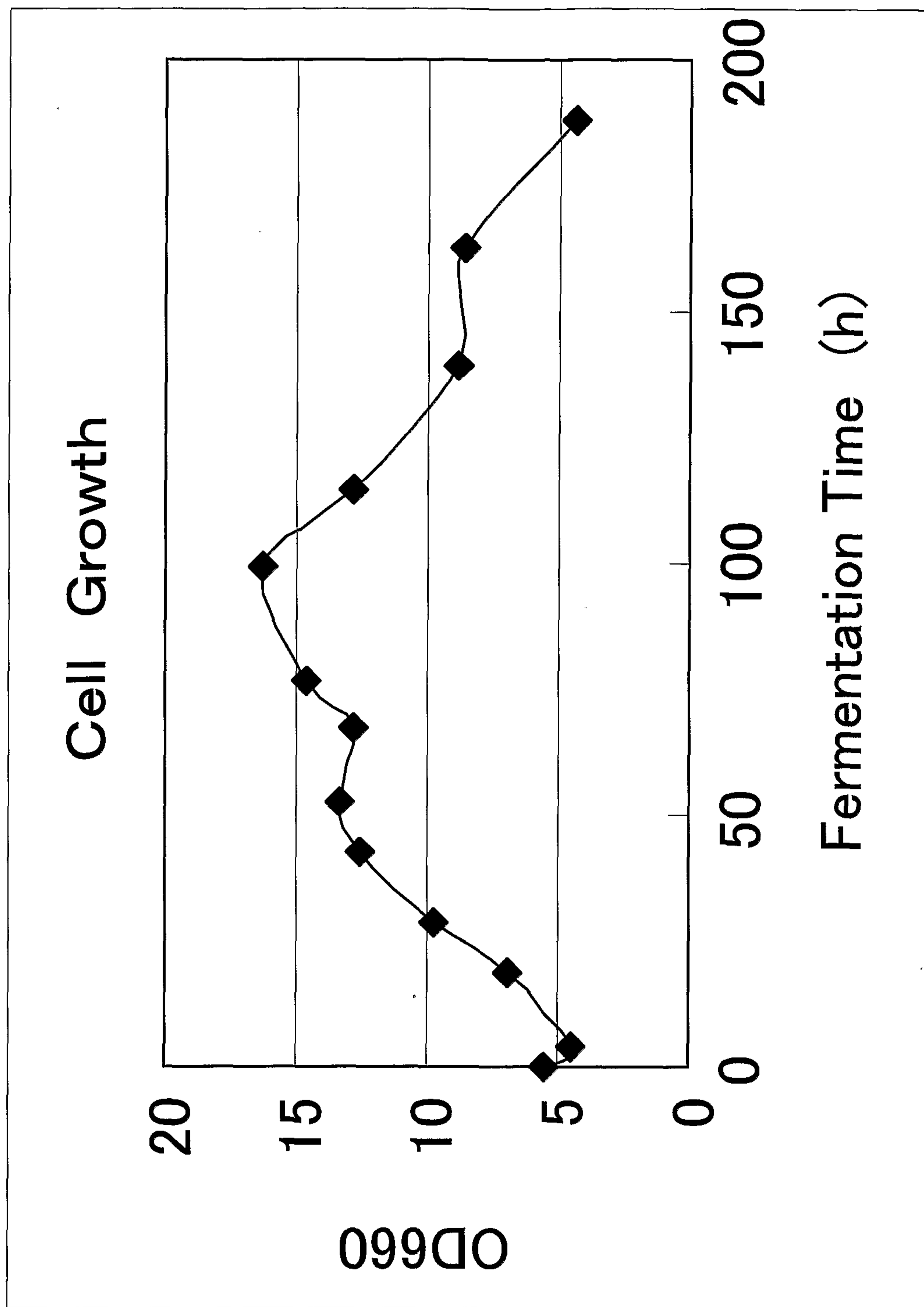
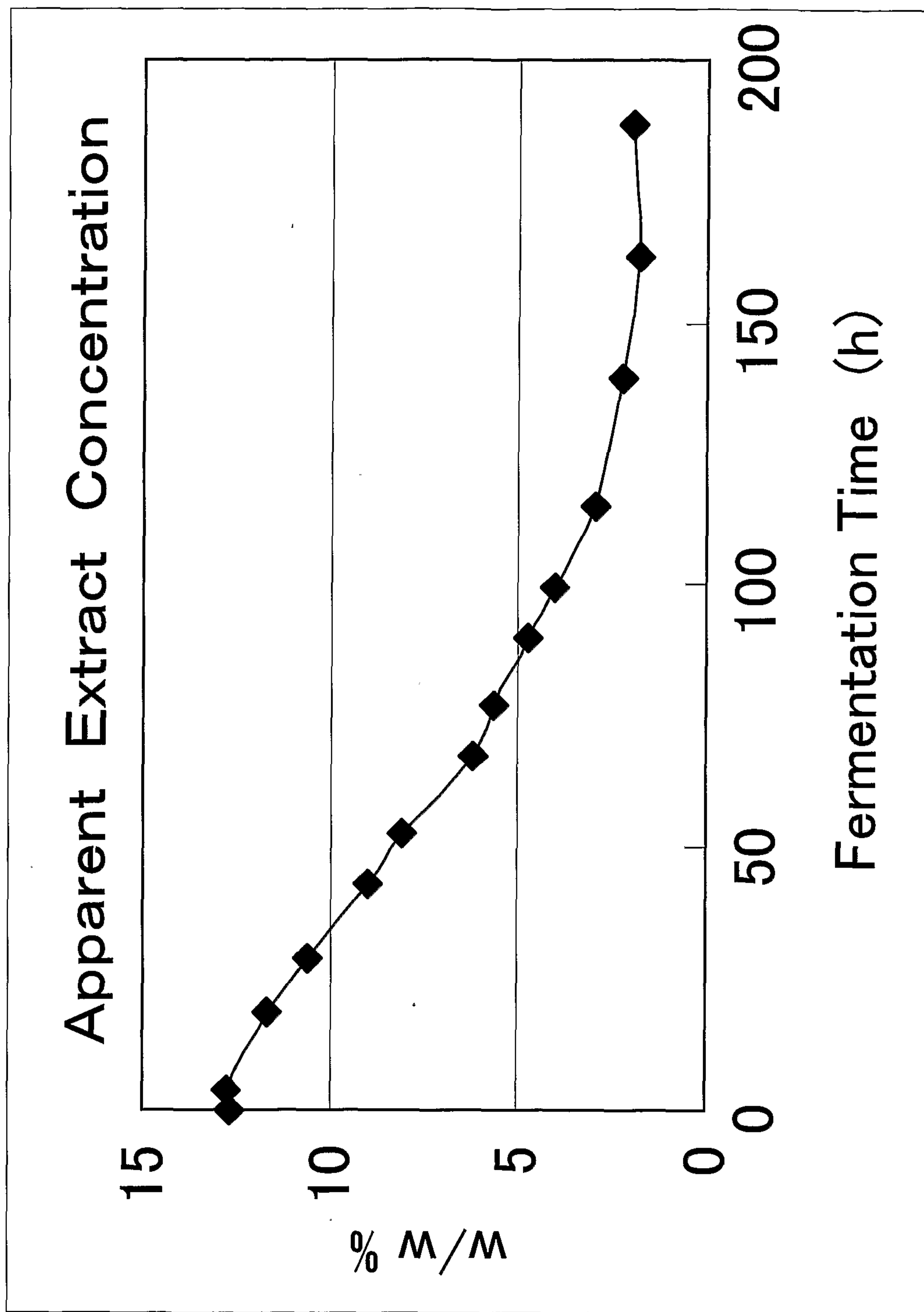


FIG. 2



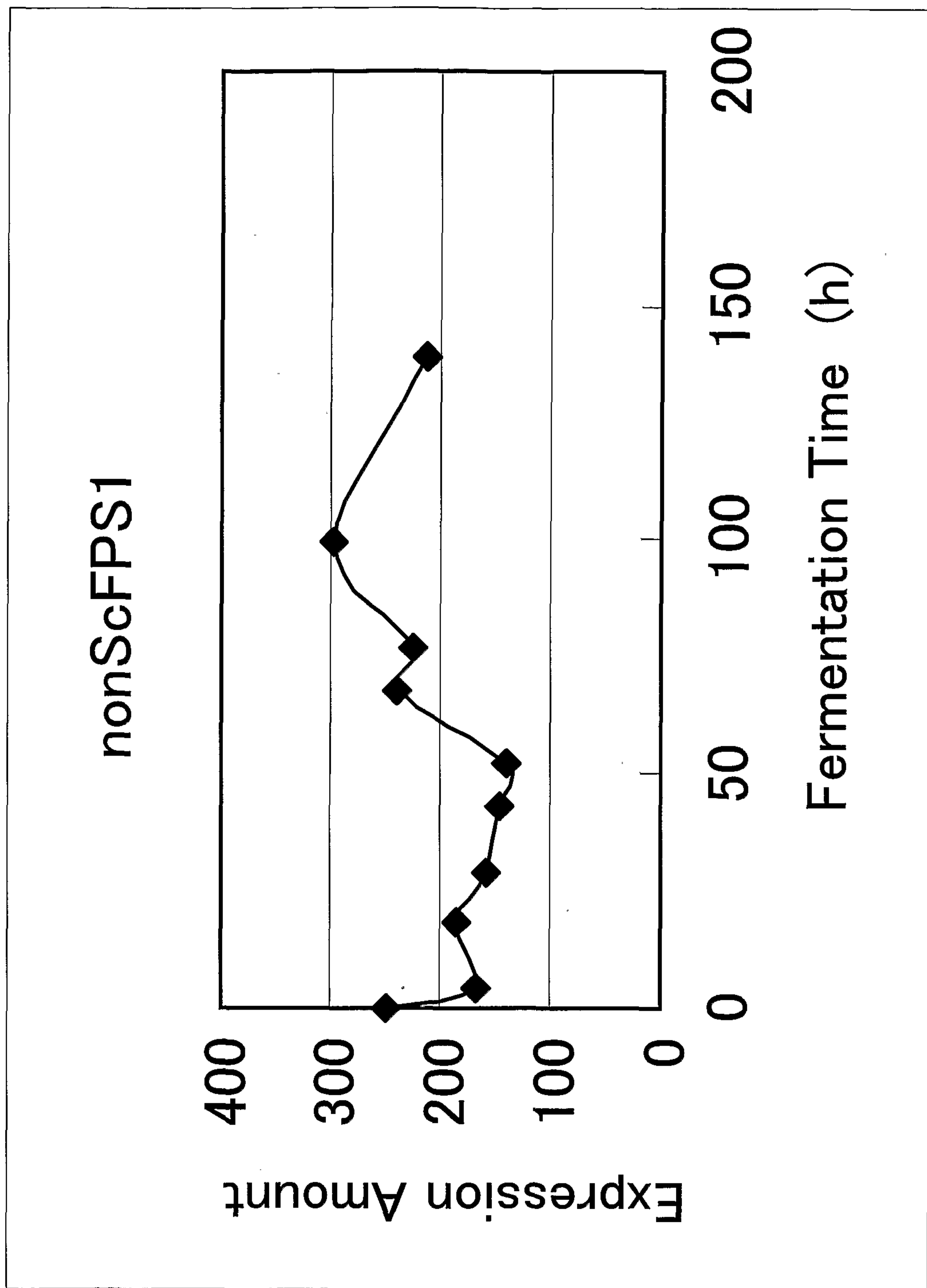


FIG. 3

FIG. 4

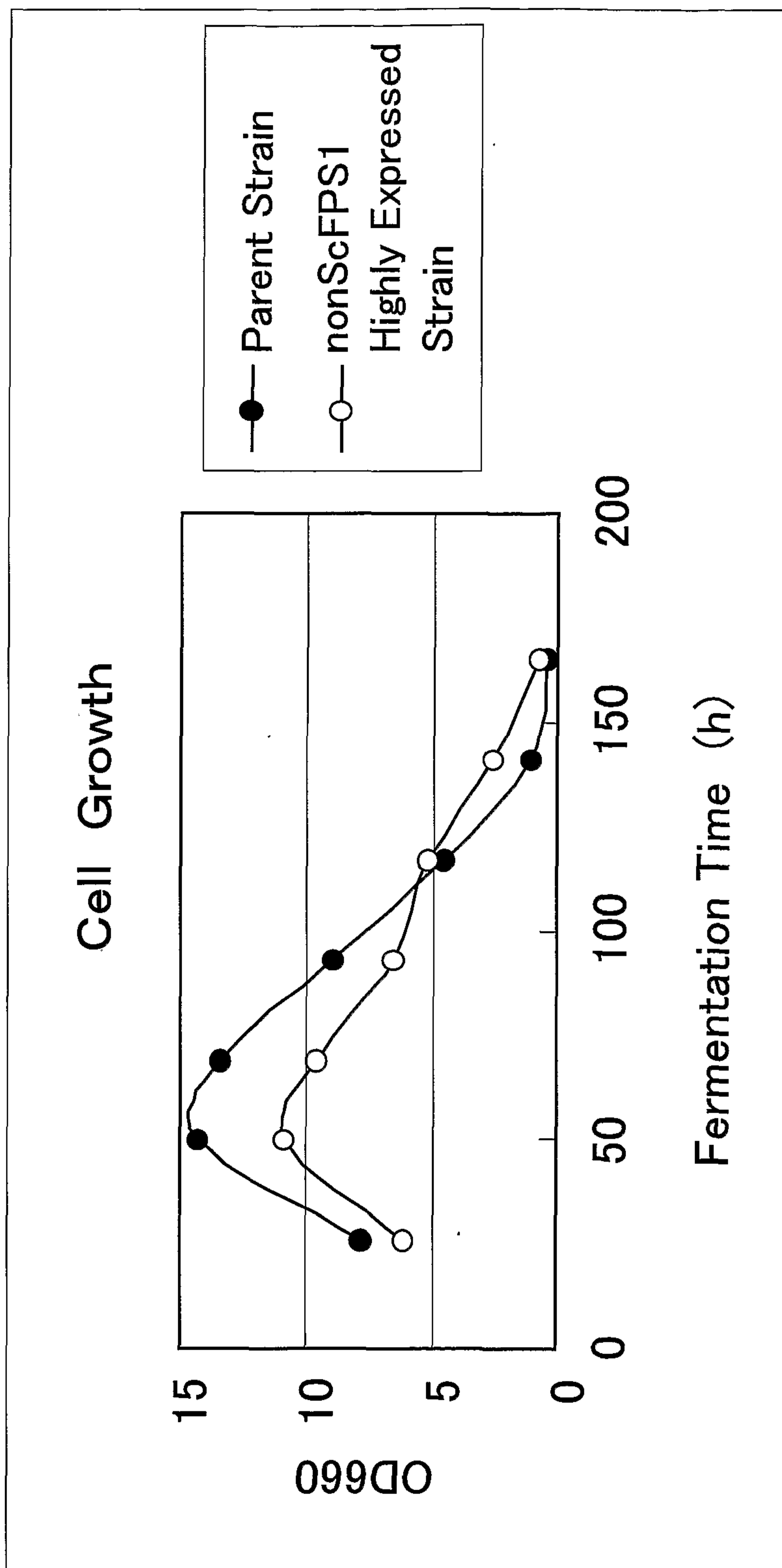


FIG. 5

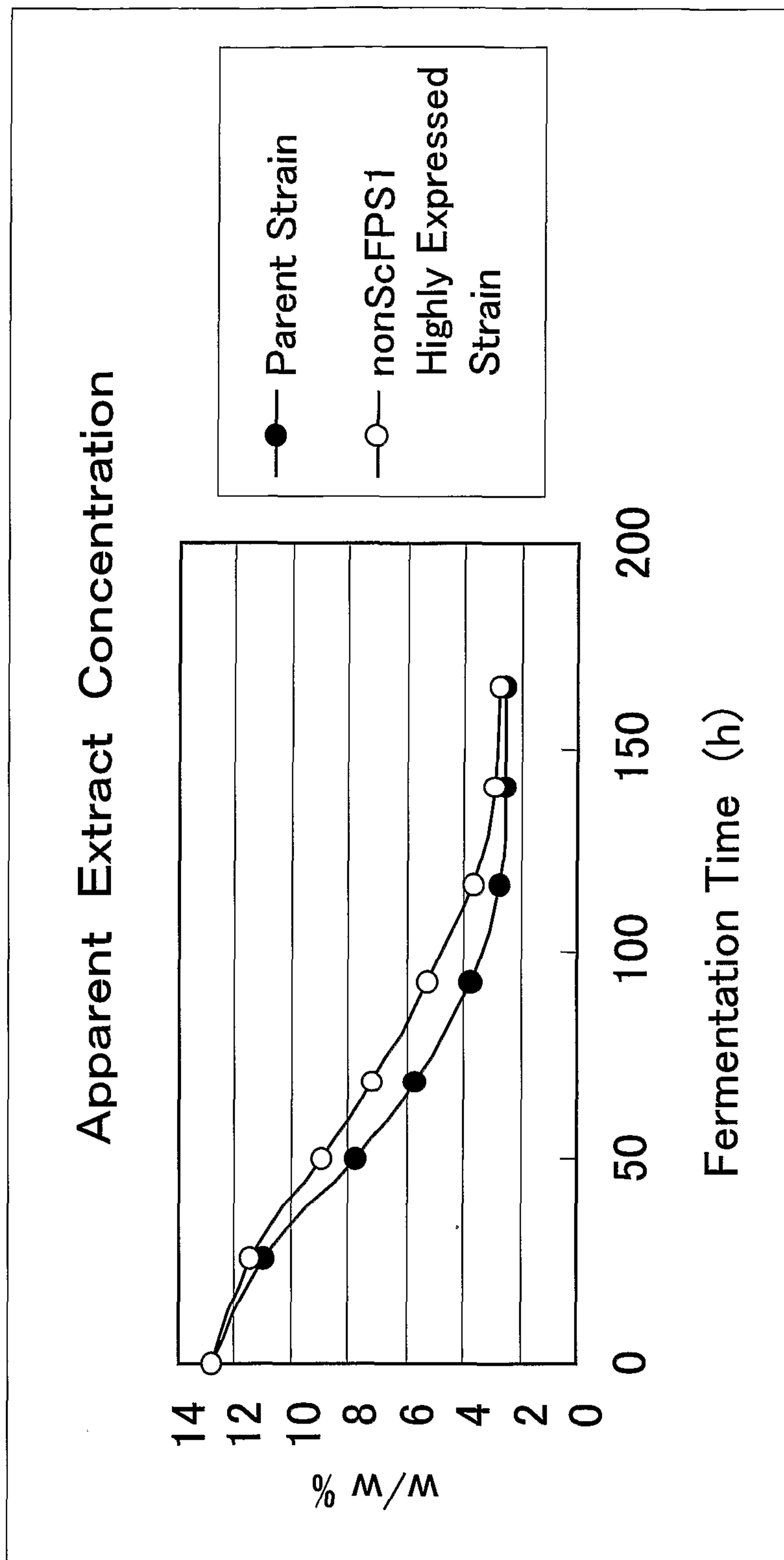
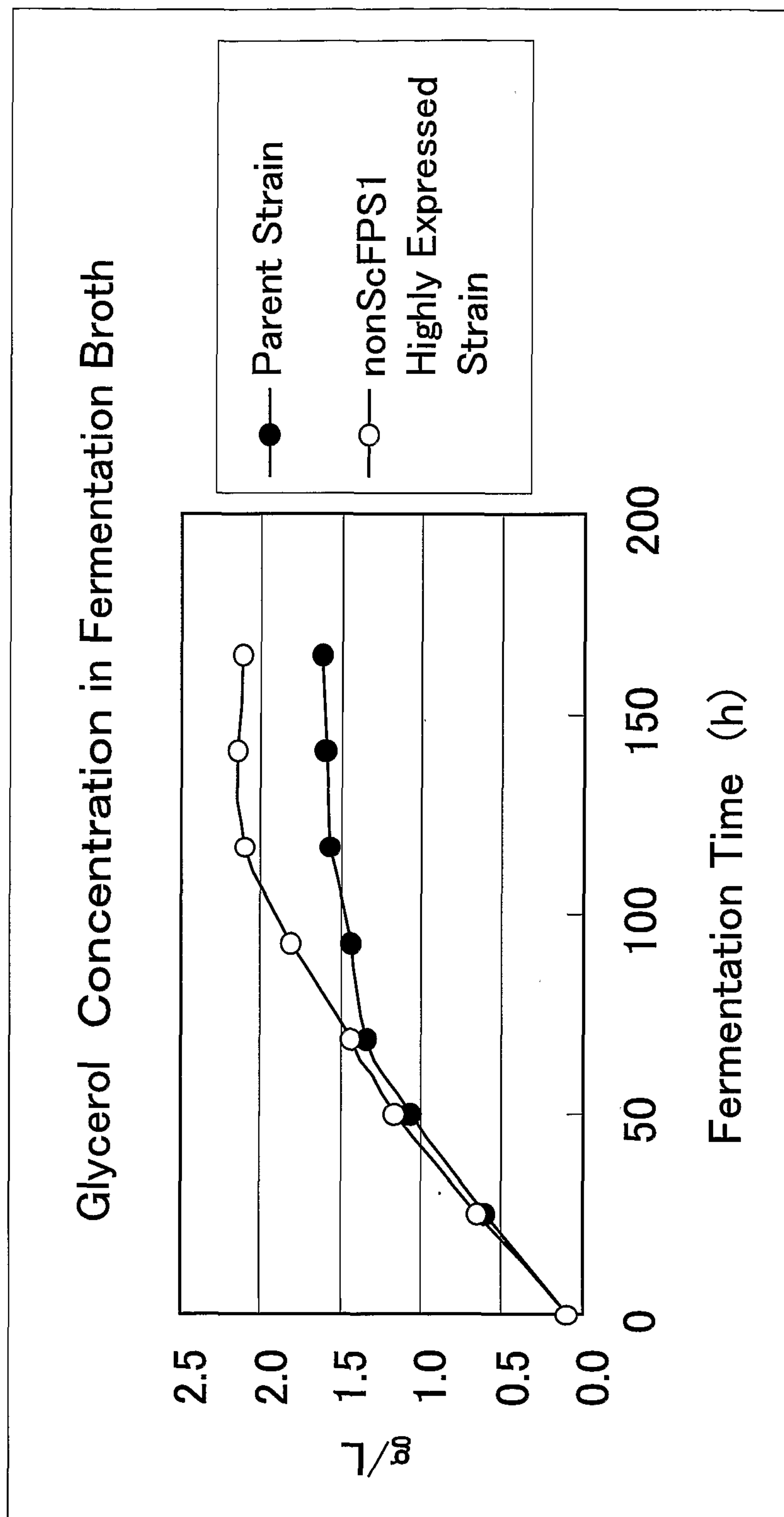


FIG. 6



Glycerol Concentration in Fermentation Broth

