

DESCRIPTION

METHOD FOR PRODUCING SECRETED β -GALACTOSIDASE

Technical Field

[0001]

The present invention relates to a method for producing a secreted β -galactosidase that is easily used in the production of a galactooligosaccharide.

Background Art

[0002]

A β -galactosidase is known to catalyze not only a reaction of hydrolyzing a β -D-galactoside bond in lactose or the like, but also a reaction of transferring a galactosyl group, and is used in the production of a galactooligosaccharide that selectively increases genus *Bifidobacterium* in the intestine.

[0003]

The present applicant has reported a technique for producing a galactooligosaccharide using a β -galactosidase derived from an over-producing mutant strain of *Sporobolomyces singularis* that is a basidiomycetous yeast (PTL 1).

[0004]

However, since the β -galactosidase used in the technique is a non-secreted (cell wall-binding) protein, it is necessary to use the β -galactosidase in the reaction as a cell concentrate containing cells of *Sporobolomyces singularis* that produces the β -galactosidase.

[0005]

Since the cell concentrate includes viable cells, it is easily deteriorated, and moreover, it is obtained by merely concentrating the cells, the specific activity is low, and there is also a problem that the cell debris leaks out into the galactooligosaccharide reaction solution to increase the purification cost, or the like

Citation List

Patent Literature

[0006]

PTL 1: JP-A-2006-223268

Summary of Invention

Technical Problem

[0007]

An object of the present invention is to provide a method for producing a β -galactosidase that solves the above-mentioned problems and is easily used in the production of a galactooligosaccharide.

Solution to Problem

[0008]

As a result of intensive studies to solve the above-mentioned problems, the present inventors found that a secreted β -galactosidase is produced by integrating a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae* and that the secreted β -galactosidase is easily used in the production of a galactooligosaccharide, and thus completed the present invention.

[0009]

That is, the present invention is directed to a method for producing a secreted β -galactosidase, characterized by integrating a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae* to produce a secreted β -galactosidase.

[0010]

In addition, the present invention is directed to a non-secreted β -galactosidase gene derived from a basidiomycetous yeast, which is a sequence represented by SEQ ID NO: 7, 13, or 19.

[0011]

Further, the present invention is directed to a transformant of *Aspergillus oryzae*, characterized in that a non-secreted β -galactosidase gene derived from a basidiomycetous yeast is integrated into *Aspergillus oryzae* to produce a secreted β -galactosidase.

[0012]

Still further, the present invention is directed to a method for producing a galactooligosaccharide, characterized by allowing a β -galactosidase produced by the above-mentioned method for producing

a β -galactosidase to react on a substrate containing at least lactose.
[0013]

In addition, the present invention is directed to a secreted β -galactosidase, obtained by integrating a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae*, and culturing the resulting *Aspergillus oryzae*.

Advantageous Effects of Invention

[0014]

According to the method for producing a secreted β -galactosidase of the present invention, a non-secreted β -galactosidase derived from a basidiomycetous yeast can be obtained as a secreted β -galactosidase.

[0015]

Therefore, a β -galactosidase obtained by the method for producing a secreted β -galactosidase of the present invention has a high β -galactosidase activity and also high thermostability, and further is easily separated and purified, and is easily used in the production of a galactooligosaccharide.

Brief Description of Drawings

[0016]

[Fig. 1] Fig. 1 is a view showing the results of SDS-PAGE and activity measurement using SsGal strain.

[Fig. 2] Fig. 2 is a view showing the results of SDS-PAGE and activity measurement using SmGal strain.

[Fig. 3] Fig. 3 is a view showing the results of SDS-PAGE and activity measurement using RmGal strain.

[Fig. 4] Fig. 4 is a view showing the results of SDS-PAGE and activity measurement using SeGal strain.

[Fig. 5] Fig. 5 is a view showing the results of SDS-PAGE and activity measurement (thermal inactivation) using SsGal strain.

[Fig. 6] Fig. 6 is a view showing the results of SDS-PAGE and activity measurement (thermal inactivation) using SmGal strain.

[Fig. 7] Fig. 7 is a view showing the results of SDS-PAGE and activity measurement (thermal inactivation) using RmGal strain.

[Fig. 8] Fig. 8 is a view showing the results of SDS-PAGE and

activity measurement (thermal inactivation) using SeGal strain.

[Fig. 9] Fig. 9 is a view showing the results of activity measurement after a heat treatment using SeGal strain and a parent strain.

[Fig. 10] Fig. 10 is a view showing a reaction time and a sugar composition in a solution in the production of a galactooligosaccharide using SsGal strain.

[Fig. 11] Fig. 11 is a view showing a reaction time and a sugar composition in a solution in the production of a galactooligosaccharide using SmGal strain.

[Fig. 12] Fig. 12 is a view showing a reaction time and a sugar composition in a solution in the production of a galactooligosaccharide using SeGal strain.

[Fig. 13] Fig. 13 is a view showing a reaction time and a sugar composition in a solution in the production of a galactooligosaccharide using SeGal strain ((a): 70°C, (b): 80°C).

[Fig. 14] Fig. 14 is a view showing a reaction time and a sugar composition in a solution in the production of a galactooligosaccharide using SeGal strain ((c): 90°C)

Description of Embodiments

[0017]

The method for producing a secreted β -galactosidase of the present invention (hereinafter referred to as "the production method of the present invention") is a method including integrating a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae* to produce a secreted β -galactosidase.

[0018]

The non-secreted β -galactosidase gene derived from a basidiomycetous yeast used in the production method of the present invention is a gene encoding a non-secreted β -galactosidase produced by a basidiomycetous yeast. Here, the "non-secreted" means to have cell wall binding affinity, which can be confirmed by activity staining or the like.

[0019]

Further, the basidiomycetous yeast that produces a non-secreted

β -galactosidase is not particularly limited, but examples thereof include basidiomycetous yeasts belonging to the genus *Sporobolomyces* such as *Sporobolomyces singularis*, the genus *Sirobasidium* such as *Sirobasidium magnum*, the genus *Rhodotorula* such as *Rhodotorula minuta*, the genus *Sterigmatomyces* such as *Sterigmatomyces elviae*, the genus *Cryptococcus* such as *Cryptococcus laurentii*, and the like. Among these basidiomycetous yeasts, a yeast belonging to the genus *Sporobolomyces* or the genus *Sterigmatomyces* is preferred, and *Sporobolomyces singularis* or *Sterigmatomyces elviae* is more preferred.

[0020]

Further, as the gene encoding a non-secreted β -galactosidase produced by a basidiomycetous yeast, first, a gene cloned from the above-mentioned basidiomycetous yeast that produces a non-secreted β -galactosidase according to a conventional method such as PCR is exemplified. Note that as the gene, a gene obtained by total synthesis in accordance with a host from the information of a gene obtained as described above is preferred.

[0021]

Specifically, the following genes are exemplified. Note that in the genes, a signal sequence is also included.

[0022]

- a β -galactosidase gene derived from *Sporobolomyces singularis* composed of a base sequence represented by SEQ ID NO: 1 (a sequence at positions 1 to 57 in the sequence is a signal sequence)

- a β -galactosidase gene derived from *Sirobasidium magnum* composed of a base sequence represented by SEQ ID NO: 7 (a sequence at positions 1 to 48 in the sequence is a signal sequence)

- a β -galactosidase gene derived from *Rhodotorula minuta* composed of a base sequence represented by SEQ ID NO: 13 (a sequence at positions 1 to 57 in the sequence is a signal sequence)

- a β -galactosidase gene derived from *Sterigmatomyces elviae* composed of a base sequence represented by SEQ ID NO: 19 (a sequence at positions 1 to 57 in the sequence is a signal sequence)

[0023]

Further, a preferred gene of the above-mentioned gene is a gene in which the signal sequence of each of the basidiomycetous yeasts is

substituted with a signal sequence of *Aspergillus oryzae*. Examples of the signal sequence of *Aspergillus oryzae* include a secretory signal (TAA signal) sequence of α -amylase (Taka-amylase: TAA) of *Aspergillus oryzae* (Okazaki, F., Aoki, J., Tabuchi, S., Tanaka, T., Ogino, C., and Kondo, A., Efficient heterologous expression and secretion in *Aspergillus oryzae* of a llama variable heavy-chain antibody fragment V(HH) against EGFR. *Appl Microbiol Biotechnol* 96, 81-88 (2012)), and a secretory signal of lipase of *Rhizopus oryzae* (Hama, S., Tamalampudi, S., Shindo, N., Numata, T., Yamaji, H., Fukuda, H., and Kondo, A., Role of N-terminal 28-amino-acid region of *Rhizopus oryzae* lipase in directing proteins to secretory pathway of *Aspergillus oryzae*. *Appl Microbiol Biotechnol* 79, 1009-1018 (2008)). The substitution of such a signal sequence can be carried out according to a conventional method. [0024]

As a preferred gene among such β -galactosidase genes in which the signal sequence of each of the basidiomycetous yeasts is substituted with the signal sequence of *Aspergillus oryzae*, the following genes are exemplified. The sequences of these genes are each composed of a secretory signal (TAA signal) sequence of *Aspergillus oryzae* and a sequence encoding a native β -galactosidase.

[0025]

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 3 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 9 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 15 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 21 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

[0026]

Among the above-mentioned genes, a gene in which a codon of a sequence encoding a native β -galactosidase is changed within a range

where the amino acid sequence of the β -galactosidase is not changed is preferred. As such a β -galactosidase gene, the following genes are exemplified. The sequences of these genes are each composed of a secretory signal (TAA signal) sequence of *Aspergillus oryzae* and a sequence in which a codon of a sequence encoding a native β -galactosidase is changed within a range where the amino acid sequence of the β -galactosidase is not changed.

[0027]

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 5 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 11 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 17 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

- a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 23 (a sequence at positions 1 to 63 in the sequence is a secretory signal sequence)

[0028]

Among the above-mentioned genes, a β -galactosidase gene composed of a base sequence represented by SEQ ID NO: 5, 11, or 23 is preferred.

[0029]

The *Aspergillus oryzae*, which is used in the production method of the present invention, and into which the β -galactosidase gene is integrated, is not particularly limited, but, for example, *Aspergillus oryzae* NS4 strain deficient in ATP sulfurylase gene (sC) and nitrate reductase gene (niaD) (available from the National Research Institute of Brewing, 3-7-1, Kagamiyama, Higashihiroshima-shi, Hiroshima, 739-0046), *Aspergillus oryzae* nia D300, *Aspergillus oryzae* RIB40, and *Aspergillus oryzae* ATCC 11488 are exemplified. Among these, *Aspergillus oryzae* NS4 strain is preferred.

[0030]

In the production method of the present invention, a method for integrating the above-mentioned gene into *Aspergillus oryzae* is not

particularly limited, but for example, the above-mentioned gene need only be integrated into an expression vector by a conventional method. The type of the expression vector is not particularly limited, but an expression vector derived from *Aspergillus oryzae* is preferred, and in particular, a high expression vector containing an improved promoter using cis-element (Region III) involved in the regulation of expression of amylase genes (Improvement of the *Aspergillus oryzae* Enolase Promoter by the Introduction of cis-Element, Tsuboi, H. et al., Biosci. Biotechnol. Biochem., 69, 206-208 (2005)) and a 5'UTR sequence with high translation efficiency (Japanese Patent No. 4413557) is preferred. Further, in such a vector, an antibiotic resistance gene such as ampicillin may be integrated for selection of a transformant, or an ATP sulfurylase expression cassette or the like may be integrated as a marker.

[0031]

The above-mentioned expression vector may be prepared based on the method described in the above-mentioned document, or, for example, may be produced by Contract Protein Expression Service of Ozeki Co., Ltd. (4-9, Imazudezaike-cho, Nishinomiya-shi, Hyogo, 663-8227).

[0032]

After integrating the gene into the expression vector, the expression vector is integrated into *Aspergillus oryzae* to transform *Aspergillus oryzae*. A method for transforming *Aspergillus oryzae* is not particularly limited, and for example, the transformation may be carried out by a conventional method such as a protoplast-PEG method or an electroporation method. After the transformation, washing, selection, cell collection, and the like may be appropriately performed according to a conventional method.

[0033]

In this manner, a transformant of *Aspergillus oryzae* that produces a secreted β -galactosidase can be obtained by introducing a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae*. By appropriately culturing the transformant in DPY medium, CDD medium, or the like, a secreted β -galactosidase is produced from *Aspergillus oryzae*.

[0034]

Since the β -galactosidase obtained above is a secreted protein, and therefore, in the purification, for example, it is only necessary to separate the culture solution after culture by filtration, centrifugation, or the like, and collect the supernatant. Further, it is also possible to concentrate the supernatant using an ultrafilter or the like. The β -galactosidase has characteristics that the β -galactosidase activity is high, the thermostability is also high, and the content of impurities is low.

[0035]

Preferred examples of the amino acid sequence of such a secreted β -galactosidase include the following sequences.

- a β -galactosidase derived from *Sporobolomyces singularis* composed of an amino acid sequence represented by SEQ ID NO: 2 (a sequence at positions 1 to 575 in the sequence) (the same applies to amino acid sequences represented by SEQ ID NOS: 4 and 6 (a sequence at positions 1 to 575 in each of the sequences))

- a β -galactosidase derived from *Sirobasidium magnum* composed of an amino acid sequence represented by SEQ ID NO: 8 (a sequence at positions 1 to 685 in the sequence) (the same applies to amino acid sequences represented by SEQ ID NOS: 10 and 12 (a sequence at positions 1 to 685 in each of the sequences))

- a β -galactosidase derived from *Rodotorula minuta* composed of an amino acid sequence represented by SEQ ID NO: 14 (a sequence at positions 1 to 581 in the sequence) (the same applies to amino acid sequences represented by SEQ ID NOS: 16 and 18 (a sequence at positions 1 to 581 in each of the sequences))

- a β -galactosidase derived from *Sterigmatomyces elviae* composed of an amino acid sequence represented by SEQ ID NO: 20 (a sequence at positions 1 to 581 in the sequence) (the same applies to amino acid sequences represented by SEQ ID NOS: 22 and 24 (a sequence at positions 1 to 581 in each of the sequences))

[0036]

Among the above-mentioned β -galactosidases, the β -galactosidase derived from *Sporobolomyces singularis* composed of the amino acid sequence represented by SEQ ID NO: 2, the β -galactosidase derived from *Sirobasidium magnum* composed of the amino acid sequence represented

by SEQ ID NO: 8, or the β -galactosidase derived from *Sterigmatomyces elviae* composed of the amino acid sequence represented by SEQ ID NO: 20 is preferred.

[0037]

The β -galactosidase has a property of being secreted outside the cell, and also has a property of having favorable thermostability and storage stability without lowering the β -galactosidase activity even if it is stored for a long time. Note that the β -galactosidase activity can be confirmed by the method described in the below-mentioned Examples. In general, in order to efficiently produce a galactooligosaccharide, a plurality of β -galactosidases are sometimes used, however, the β -galactosidase obtained above can efficiently produce a galactooligosaccharide even by itself.

[0038]

The β -galactosidase obtained above can be used, for example, in the production of a galactooligosaccharide by allowing the β -galactosidase to act on a substrate containing at least lactose in the same manner as a conventionally known β -galactosidase. Note that the β -galactosidase is a secreted protein, and therefore, it is also not necessary to particularly remove cells or the like when producing a galactooligosaccharide.

[0039]

Specifically, in order to allow the β -galactosidase obtained above to act on a substrate containing at least lactose, it is only necessary to add the β -galactosidase to the substrate containing at least lactose and maintain a predetermined temperature. The addition amount of the β -galactosidase is not particularly limited, but is, for example, from 1 to 50 U, and preferably from 5 to 10 U with respect to 100 g of lactose. In addition, the temperature at which the β -galactosidase is allowed to act on the substrate is not particularly limited, but is from 30 to 90°C, and preferably from 60 to 90°C, and the maintenance time may only be set as appropriate. To the substrate containing at least lactose, a saccharide to be galactosylated may be added. Such a saccharide is not particularly limited, and examples thereof include galactose, mannose, ribose, xylose, arabinose, rhamnose, N-acetylglucosamine, α -methylmannoside,

α -methylgalactoside, α -methylglucoside, 2-deoxyglucose, and 2-deoxygalactose.

[0040]

The galactooligosaccharide produced as described above contains a galactooligosaccharide that is a pentasaccharide or a lower saccharide, particularly a galactooligosaccharide that is a trisaccharide in a large amount.

[0041]

Further, the galactooligosaccharide produced as described above may be as it is, but may be separated and purified using a general purification method. The purification method is not particularly limited, but specifically, purification can be carried out by subjecting the galactooligosaccharide to various types of chromatography such as ion exchange, gel filtration, activated carbon, and affinity chromatography.

[0042]

The thus obtained galactooligosaccharide can be used in useful food materials, pharmaceutical raw materials, and reagents.

Examples

[0043]

Hereinafter, the present invention will be described in detail with reference to Examples, however, the present invention is by no means limited to these Examples.

[0044]

The deposit numbers of the basidiomycetous yeasts used in these Examples are as follows.

- *Sporobolomyces singularis* ATCC 24193
- *Rodotorula minuta* CBS 319
- *Sterigmatomyces elviae* IFO 1843
- *Sirobasidium magnum* CBS 6803

ATCC: 10801 University Boulevard Manassas, VA 20110 USA

CBS: Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands

IFO: 2-17-85 Jusohonmachi, Yodogawa-ku, Osaka-shi 532-8686

[0045]

Example 1

Acquisition of β -Galactosidase Gene Derived from *Sporobolomyces Singularis*:

A β -galactosidase gene derived from *Sporobolomyces singularis* was obtained based on the literature (Ishikawa, E., Sakai, T., Ikemura, H., Matsumoto, K., and Abe, H., Identification, cloning, and characterization of a *Sporobolomyces singularis* beta-galactosidase-like enzyme involved in galacto-oligosaccharide production. J Biosci Bioeng 99, 331-339 (2005)) (SEQ ID NO: 1). The gene is composed of a signal sequence and a sequence encoding a β -galactosidase. A sequence (SEQ ID NO: 3) in which the signal sequence of the gene was substituted with the TAA signal sequence of *Aspergillus oryzae* was obtained on a computer, and further, a sequence (SEQ ID NO: 5) in which a codon of a sequence encoding a native β -galactosidase was changed within a range where the amino acid sequence of the β -galactosidase is not changed was obtained (SsGal). The SsGal was contracted out to GenScript, Inc. and totally synthesized.

[0046]

Example 2

Acquisition of β -Galactosidase Gene Derived from *Sirobasidium Magnum*:

Degenerate primers (Table 1) (SEQ ID NOS: 25 to 29) were designed from a conserved region, and partial sequences were cloned through RT-PCR in six combinations in total using 2 types of forward primers and 3 types of reverse primers. From the partial sequences, 5' RACE and 3' RACE were performed, whereby a full-length cDNA was obtained.

[0047]

[Table 1]

SEQ ID NO	Name	Direction	Base sequence of degenerate primer
25	F1	forward	gccggcgcggtatgcargtngarggngcn
26	F2	forward	gtcaagacntggattyacnttyaaygarccn
27	R1	reverse	ctcgcccaccrcraaytcnswraartadat
28	R2	reverse	ccattcccarttrtcnacraanswcca
29	C-R70	reverse	gacgaggccnswrtccaytcraarttrtc

[0048]

Based on the above-prepared full-length cDNA, a β -galactosidase gene derived from *Sirobasidium magnum* (SEQ ID NO: 7) was obtained by analogy from the start codon (ATG) in the upstream region. The gene

is composed of a signal sequence and a sequence encoding a β -galactosidase. A sequence (SEQ ID NO: 9) in which the signal sequence of the gene was substituted with the TAA signal sequence of *Aspergillus oryzae* was obtained on a computer, and further, a sequence (SEQ ID NO: 11) in which a codon of a sequence encoding a native β -galactosidase was changed within a range where the amino acid sequence of the β -galactosidase is not changed was obtained (SmGal). The SmGal was contracted out to GenScript, Inc. and totally synthesized.

[0049]

Example 3

Acquisition of β -Galactosidase Gene Derived from *Rodotorula Minuta*:

A β -galactosidase gene derived from *Rodotorula minuta* (SEQ ID NO: 13) was obtained in the same manner as the β -galactosidase gene derived from *Sirobasidium magnum*. The gene is composed of a signal sequence and a sequence encoding a β -galactosidase. A sequence (SEQ ID NO: 15) in which the signal sequence of the gene was substituted with the TAA signal sequence of *Aspergillus oryzae* was obtained on a computer, and further, a sequence (SEQ ID NO: 17) in which a codon of a sequence encoding a native β -galactosidase was changed within a range where the amino acid sequence of the β -galactosidase is not changed was obtained (RmGal). The RmGal was contracted out to GenScript, Inc. and totally synthesized.

[0050]

Example 4

Acquisition of β -Galactosidase Gene Derived from *Sterigmatomyces Elviae*:

A β -galactosidase gene of *Sterigmatomyces elviae* (SEQ ID NO: 19) was obtained in the same manner as the β -galactosidase gene derived from *Sirobasidium magnum*. The gene is composed of a signal sequence and a sequence encoding a β -galactosidase. A sequence (SEQ ID NO: 21) in which the signal sequence of the gene was substituted with the TAA signal sequence of *Aspergillus oryzae* was obtained on a computer, and further, a sequence (SEQ ID NO: 23) in which a codon of a sequence encoding a native β -galactosidase was changed within a range where the amino acid sequence of the β -galactosidase is not changed was obtained

(SeGal). The SeGal was contracted out to GenScript, Inc. and totally synthesized.

[0051]

Example 5

Acquisition of SsGal Transformant

The SsGal obtained in Example 1 was sent to Contract Protein Expression Service of Ozeki Co., Ltd. (4-9, Imazudezaike-cho, Nishinomiya-shi, Hyogo, 663-8227), and integrated into an expression vector.

[0052]

As a host for transformation, NS4 strain that is a strain derived from *Aspergillus oryzae* and deficient in nitrate reductase gene (*niaD*) and ATP sulfurylase gene (*sC*) (obtained from the National Research Institute of Brewing, 3-7-1, Kagamiyama, Higashihiroshima-shi, Hiroshima, 739-0046) was used, and the expression vector was integrated into the host by a protoplast-PEG method, whereby a transformant was obtained (SsGal strain). Note that selection of the transformant was performed based on the complementation of trait of *sC* deficiency.

[0053]

Example 6

Acquisition of SmGal Transformant

A transfected expression vector and a transformant (SmGal strain) were obtained in the same manner as in Example 5 except that the SmGal obtained in Example 2 was used.

[0054]

Example 7

Acquisition of RmGal Transformant

A transfected expression vector and a transformant (RmGal strain) were obtained in the same manner as in Example 5 except that the RmGal obtained in Example 3 was used.

[0055]

Example 8

Acquisition of SeGal Transformant

A transfected expression vector and a transformant (SeGal strain) were obtained in the same manner as in Example 5 except that the SeGal obtained in Example 4 was used.

[0056]

Example 9

Evaluation of Production of β -Galactosidase by Transformant

(1) Activity Measurement

Among the respective transformants obtained in Examples 5 to 8, the SsGal strain was cultured in CDD medium (2% dextrin, 0.2% glucose, 0.2% NH_4Cl , 0.002% KCl , 0.001% K_2HPO_4 , 0.0005% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, $2 \times 10^{-5}\%$ $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, $1 \times 10^{-5}\%$ $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, $1 \times 10^{-6}\%$ $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $1 \times 10^{-6}\%$ $\text{MnSO}_4 \cdot 5\text{H}_2\text{O}$, $1 \times 10^{-6}\%$ AlCl_3 , 200 mM MOPS-NaOH buffer pH 7.0) at 30°C for 144 hours (15 mL/100 mL volume Erlenmeyer flask scale). The RmGal strain was cultured in 2×DPY medium (4% dextrin, 2% hipolypepton, 2% yeast extract, 1% KH_2PO_4 , 0.1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) at 30°C for 144 hours (150 mL/500 mL volume long-neck shake flask scale). The SmGal strain was cultured in 2×DPY medium at 30°C for 168 hours (150 mL/500 mL volume long-neck shake flask scale). The SeGal strain was cultured in DPY medium (2% dextrin, 1% hipolypepton, 1% yeast extract, 0.5% KH_2PO_4 , 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) at 30°C for 168 hours. The culture supernatant was recovered, and mixed with an equivalent amount of 2×sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 0.01% bromophenol blue, 4% SDS, 200 mM DTT), and the resulting mixture was treated at 100°C for 10 minutes and then subjected to SDS-PAGE (CBB staining).

[0057]

Further, the activity measurement was performed according to the following method using ONPG as the substrate. A solution was prepared by adding 2-nitrophenyl- β -galactoside (ONPG) to a 50 mM citrate phosphate buffer solution (pH 4.0) to give a concentration of 12.5 mM. To 0.8 mL of the solution, 0.2 mL of the culture supernatant containing the β -galactosidase diluted with a 50 mM citrate phosphate buffer solution (pH 4.0) so that the absorbance at 420 nm was 0.2 to 0.8 was added and allowed to react at 30°C for 10 minutes (test solution). After stopping the reaction by adding 4 mL of a 0.25 M sodium carbonate solution, centrifugation (3,000 g, 10 minutes) was performed, and the amount of released 2-nitrophenol contained in the supernatant was determined by measuring the absorbance at 420 nm using a spectrophotometer. On the other hand, a solution obtained by adding a 50 mM citrate phosphate buffer solution (pH 4.0) to a 2-nitrophenyl- β -galactoside solution was

used as a reagent blank, and a solution obtained by adding a sodium carbonate solution thereto in advance, and then, adding and mixing the culture supernatant containing the β -galactosidase therewith, and at the same time, stopping the reaction and performing color development was defined as a reaction initial solution (blind sample). One unit (U) of enzyme activity was defined as the amount of enzyme that releases 1 μ mol of 2-nitrophenol per minute under the conditions, and calculated according to the following formula.

[0058]

[Math. 1]

Activity value (U/mL) = $\left[\frac{\{(\text{absorbance of test solution}) - (\text{absorbance of reagent blank}) - (\text{absorbance of blind sample})\}}{0.91(\text{extinction coefficient of 2-nitrophenol}) \times 0.2 \times 10} \right] \times \text{dilution factor}$

[0059]

The results of SDS-PAGE and activity measurement are shown in Figs. 1 to 4. By the CBB staining, in each of the RmGal strain, the SmGal strain, and the SeGal strain, a specific band that is not present in the parent strain was detected, and the band was presumed to be the β -galactosidase of each strain. In the SsGal strain, a specific band was not observed in the DPY medium, but when the strain was cultured in the CDD (pH 7.0) medium, a specific band that is not present in the parent strain was detected, and the band was presumed to be the β -galactosidase. The culture conditions in which the secretory productivity of each β -galactosidase becomes high were studied. As a result, the maximum activity was obtained under the conditions that the SsGal strain was cultured in CDD (pH 7.0) medium at 30°C for 144 hours, the RmGal strain was cultured in 2×DPY medium at 30°C for 144 hours, the SmGal strain was cultured in 2×DPY medium at 30°C for 168 hours, and the SeGal strain was cultured in DPY medium at 30°C for 168 hours. Further, the productivities of the SsGal strain, the RmGal strain, the SmGal strain, and the SeGal strain were estimated to be about 200 mg/L, about 200 mg/L, about 200 mg/L, and about 1 g/L, respectively, based on the concentrations of the bands of SDS-PAGE.

[0060]

(2) Estimation of Copy Number

Further, estimation of the number of expression cassettes

integrated into the transformant was performed by a real-time PCR method.

[0061]

From the results of the PCR, it was estimated that the SsGal strain, the RmGal strain, and the SmGal strain are each a strain in which one copy of expression cassette was inserted, and the SeGal strain is a strain in which two copies of expression cassettes were inserted.

[0062]

(3) Thermal Inactivation Test

1 mL of each of the culture solutions of the respective transformants and the parent strain (NS4 strain) cultured under the culture conditions described in (1) was incubated at 40°C, 50°C, 60°C, 70°C, or 80°C for 1 hour, and the measurement of enzyme activity and SDS-PAGE were performed.

[0063]

The results of SDS-PAGE and activity measurement are shown in Figs. 5 to 8. The SsGal strain maintained its activity up to 40°C, but when the SsGal strain was incubated at 50°C for 1 hour, the activity was decreased by about 70%, and the activity was lost at 70°C. The activity of the parent strain cultured under the same conditions was slightly detected up to 60°C, and lost at 70°C. The RmGal strain maintained its activity up to 50°C, but when the RmGal strain was incubated at 60°C for 1 hour, the activity was lost. The activity of the parent strain cultured under the same conditions was detected up to 70°C, and lost at 80°C. The SmGal strain maintained its activity up to 50°C, but when the SmGal strain was incubated at 60°C for 1 hour, the activity was decreased by about 20%, and the activity was lost at 80°C. The activity of the parent strain cultured under the same conditions was detected up to 70°C, and lost at 80°C. The SeGal strain maintained its activity up to 70°C. When the SeGal strain was incubated at 80°C for 1 hour, the activity was decreased by about 97%. The activity of the parent strain cultured under the same conditions was detected up to 40°C, and lost at 50°C. Further, with respect to the SeGal, a treatment at 80°C was performed in a shorter time (5 minutes, 10 minutes, and 20 minutes) than 1 hour. As a result, the activity was decreased by about 37% by performing the treatment at 80°C for 5 minutes, and

decreased by about 98% by performing the treatment for 20 minutes. In addition, as a result of the activity measurement, all the strains exhibited a higher activity than the parent strain when being treated at 40°C and 50°C.

[0064]

From the above results, it was found that the SeGal strain can maintain its activity even at high temperature.

[0065]

Example 10

Removal of Contaminant Enzymes:

As shown in Example 9(3), it was found that the SeGal strain can maintain its activity even at high temperature. On the other hand, when a thermal inactivation test was performed in the same manner as in Example 9(3) for contaminant enzymes come from *Aspergillus oryzae* that is the parent strain of the SeGal strain, the contaminant enzymes could be inactivated by a heat treatment at 70°C. Therefore, it was found that the β -galactosidase produced by the SeGal strain can be purified by a heat treatment (Fig. 9).

[0066]

Example 11

Production of Galactooligosaccharide (1):

To 150 mL of a solution containing 66% (w/v) lactose, each of the culture supernatants of the SsGal strain, the SmGal strain, and the SeGal strain obtained in Example 9 in an amount corresponding to 10 U was added, and allowed to react at a predetermined temperature for a predetermined time, whereby a galactooligosaccharide was produced. The sugar composition and amount were measured by high-performance liquid chromatography. The reaction time and the sugar composition in the solution are shown in Figs. 10 to 12 (Fig. 10: SsGal strain, Fig. 11: SmGal strain, Fig. 12: SeGal strain).

[0067]

From the drawings, it was found that by the β -galactosidases produced by the SsGal strain, the SmGal strain, and the SeGal strain, a galactooligosaccharide that is a trisaccharide can be mainly produced from lactose.

[0068]

Further, when a galactooligosaccharide was produced using the β -galactosidase produced by the SsGal strain, the galactooligosaccharide content was 56.0%, when a galactooligosaccharide was produced using the β -galactosidase produced by the SmGal strain, the galactooligosaccharide content was 66.7%, and when a galactooligosaccharide was produced using the β -galactosidase produced by the SeGal strain, the galactooligosaccharide content was 68.5%.

[0069]

Note that the above-mentioned β -galactosidases were secreted proteins, and therefore, it is not necessary to treat the cells after producing the galactooligosaccharide, and the galactooligosaccharide can be efficiently produced.

[0070]

Example 12

Production of Galactooligosaccharide (2):

To 150 mL of a solution containing 66% (w/v) lactose, the culture supernatant of the SeGal strain obtained in Example 9 in an amount corresponding to 1.0 U was added, and allowed to react at 70°C, 80°C, or 90°C for a predetermined time, whereby a galactooligosaccharide was produced. The sugar composition and amount were measured by high-performance liquid chromatography. The reaction time and the sugar composition in the solution are shown in Fig. 13 ((a): 70°C, (b): 80°C) and Fig. 14 ((c): 90°C).

[0071]

The β -galactosidase derived from the SeGal strain had high heat resistance, and could achieve GOS production at 70 to 90°C.

Industrial Applicability

[0072]

A β -galactosidase obtained by the method for producing a secreted β -galactosidase is easily separated and purified, and can be used in the production of a galactooligosaccharide.

CLAIMS

[Claim 1]

A method for producing a secreted β -galactosidase, characterized by integrating a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae* to produce a secreted β -galactosidase.

[Claim 2]

The method for producing a secreted β -galactosidase according to claim 1, wherein the basidiomycetous yeast belongs to the genus *Sporobolomyces*, the genus *Sirobasidium*, the genus *Rhodotorula*, or the genus *Sterigmatomyces*.

[Claim 3]

The method for producing a β -galactosidase according to claim 1 or 2, wherein the non-secreted β -galactosidase gene derived from a basidiomycetous yeast is composed of a signal sequence and a sequence encoding the β -galactosidase.

[Claim 4]

The method for producing a β -galactosidase according to claim 3, wherein the signal sequence of the non-secreted β -galactosidase gene derived from a basidiomycetous yeast is a signal sequence of *Aspergillus oryzae*.

[Claim 5]

The method for producing a β -galactosidase according to claim 3 or 4, wherein the sequence encoding the β -galactosidase is a sequence in which a codon of a sequence encoding a native β -galactosidase is changed within a range where the amino acid sequence of the β -galactosidase is not changed.

[Claim 6]

The method for producing a β -galactosidase according to claim 3, wherein the signal sequence and the sequence encoding the β -galactosidase are a sequence represented by SEQ ID NO: 1, 7, 13, or 19.

[Claim 7]

The method for producing a β -galactosidase according to claim 3 or 4, wherein the signal sequence and the sequence encoding the β -galactosidase are a sequence represented by SEQ ID NO: 3, 9, 15, or

21.

[Claim 8]

The method for producing a β -galactosidase according to claim 5, wherein the signal sequence and the sequence encoding the β -galactosidase are a sequence represented by SEQ ID NO: 5, 11, 17, or 23.

[Claim 9]

A non-secreted β -galactosidase gene derived from a basidiomycetous yeast, which is a sequence represented by SEQ ID NO: 7, 13, or 19.

[Claim 10]

A transformant of *Aspergillus oryzae*, characterized in that a non-secreted β -galactosidase gene derived from a basidiomycetous yeast is integrated into *Aspergillus oryzae* to produce a secreted β -galactosidase.

[Claim 11]

A method for producing a galactooligosaccharide, characterized by allowing a β -galactosidase produced by the method for producing a β -galactosidase according to any one of claims 1 to 8 to act on a substrate containing at least lactose.

[Claim 12]

A secreted β -galactosidase, obtained by integrating a non-secreted β -galactosidase gene derived from a basidiomycetous yeast into *Aspergillus oryzae*, and culturing the resulting *Aspergillus oryzae*.