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(54) **Trehalose releasing enzyme, DNA encoding therefor, their preparation and uses**

Trehalose-freisetzendes Enzym, dafür kodierende DNA, Herstellung und Verwendung

Enzyme libérant du tréhalose, ADN codant pour cela, leur préparation et utilisation

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- **BIOCHIMICA ET BIOPHYSICA ACTA, no. 1289, 1996, page 10-13 XP002016405 MARUTA K. ET AL.: "Cloning and Sequencing of Trehalose Biosynthesis Genes from Arthrobacter sp. Q36"**

EP 0 671 470 B9

Description

[0001] The present invention relates to a novel DNA encoding an enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, recombinant DNA containing the same, and a transformant, and further relates to a recombinant enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, as well as to preparations and uses thereof.

[0002] Trehalose is a disaccharide which consists of 2 glucose molecules which are linked together with their reducing groups, and, naturally, it is present in bacteria, fungi, algae, insects, etc., in an extremely small quantity. Having no reducing residue within the molecule, trehalose does not cause an unsatisfactory browning reaction even when heated in the presence of amino acids or the like, and because of this it can sweeten food products without fear of causing unsatisfactory coloration and deterioration. Trehalose, however, is far from being readily prepared in a desired amount by conventional methods, and, actually, it has not scarcely been used for sweetening food products.

[0003] Conventional methods are roughly classified into 2 groups, i.e. the one using cells of microorganisms and the other employing a multi-enzymatic system wherein enzymes are allowed to act on saccharides. The former, as disclosed in Japanese Patent Laid-Open No.154,485/75, is a method which comprises allowing to grow microorganisms such as bacteria and yeasts in a nutrient culture medium, and collecting trehalose from the proliferated cells in the resultant culture. The latter, as disclosed in Japanese Patent Laid-Open No.216,695/83, is a method which comprises providing maltose as a substrate, allowing a multi-enzymatic system using maltose- and trehalose-phosphorylases to act on maltose, and isolating the formed trehalose from the reaction system. Although the former facilitates the growth of microorganisms with a relative easiness, it requires a sequentially-complicated step for collecting trehalose from the microorganisms which contain at most 15 w/w % trehalose, on a dry solid basis (d.s.b.). While the latter enables the separation of trehalose itself with a relative easiness, but it is theoretically difficult to increase the trehalose yield by allowing enzymes to act on substrates at a considerably-high concentration because the enzymatic reaction per se is an equilibrium reaction of 2 different types of enzymes and the equilibrium point constantly inclines to the side of forming glucose phosphate.

[0004] In view of the foregoing, the present inventors energetically screened enzymes which form saccharides having a trehalose structure from amylaceous saccharides, and found that microorganisms such as those of the spices *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36 produce an absolutely novel enzyme which forms non-reducing saccharides having a trehalose structure as an end unit from reducing amylaceous saccharides having a degree of ***[deletion(s)]*** glucose polymerization of 3 or higher. Before or after this finding, it was revealed that such non-reducing saccharides are almost quantitatively hydrolyzed into trehalose and glucose and/or maltooligosaccharides by other enzymes produced from the same microorganisms of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36.

[0005] EP 0628630, which forms part of the state of the art only under Article 54(3) EPC, discloses a trehalose-releasing enzyme which specifically hydrolyses the linkage between a trehalose moiety and the remaining glycosyl moiety in a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerisation of 3 or higher.

[0006] Since the combination use of such enzymes enables to form a desired amount of trehalose with a relative easiness, the aforementioned objects relating to trehalose would be completely overcome. Insufficient producibility of such enzymes by the microorganisms results in a drawback that a relatively-large scale culture of the microorganisms is inevitable to industrially produce trehalose and/or non-reducing saccharides having a trehalose structure as an end unit.

[0007] Recombinant DNA technology has made a remarkable progress in recent years. At present, even an enzyme, whose total amino acid sequence has not yet been revealed, can be readily prepared in a desired amount, if a gene encoding the enzyme was once isolated and the base sequence was decoded, by preparing a recombinant DNA containing a DNA which encodes the enzyme, introducing the recombinant DNA into microorganisms or cells of plants or animals, and culturing the resultant transformants. Under these circumstances, urgently required are the finding of genes which encode these enzymes and the elucidation of their base sequences.

[0008] It is an aim of the present invention to provide a DNA which encodes an enzyme that releases trehalose from non-reducing saccharides having a trehalose structure as an end unit.

[0009] It is a further aim of the present invention to provide a replicable recombinant DNA containing the aforesaid DNA.

[0010] It is yet another aim of the present invention to provide a transformant which is prepared by introducing the recombinant DNA into an appropriate host.

[0011] It is a further aim of the present invention to prepare the aforesaid enzyme by the application of the recombinant DNA technology.

[0012] It is a further aim of the present invention to provide a preparation of the enzyme.

[0013] The first aim of the present invention is attained by a DNA which encodes an enzyme that releases trehalose

from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

[0014] The second aim of the present invention is attained by a replicable recombinant DNA which contains the aforesaid DNA and a self-replicable vector.

[0015] The third aim of the present invention is attained by a transformant prepared by introducing the aforesaid self-replicable vector into an appropriate host.

[0016] The fifth aim of the present invention is attained by a process to produce the recombinant enzyme comprising culturing a transformant capable of forming the enzyme in a nutrient culture medium, and recovering the formed enzyme from the resultant culture.

[0017] The present invention will now be described in further detail, by way of example only, with reference to the accompanying drawings, in which:

FIG. 1 shows the optimum temperature of an enzyme derived from *Rhizobium* sp. M-11.
FIG. 2 shows the optimum temperature of an enzyme derived from *Arthrobacter* sp. Q36.
FIG. 3 shows the optimum pH of an enzyme derived from *Rhizobium* sp. M-11.
FIG. 4 shows the optimum pH of an enzyme derived from *Arthrobacter* sp. Q36.
FIG. 5 shows the thermal stability of an enzyme derived from *Rhizobium* sp. M-11.
FIG. 6 shows the thermal stability of an enzyme derived from *Arthrobacter* sp. Q36.
FIG. 7 shows the pH stability of an enzyme derived from Rhizobium sp. M-11.
FIG. 8 shows the pH stability of an enzyme derived from *Arthrobacter* sp. Q36.
FIG. 9 shows the restriction map of the recombinant DNA pBMU27 according to the present invention. In the figure, the bold-lined part is a DNA encoding an enzyme derived from *Rhizobium* sp. M-11.
FIG. 10 shows the restriction map of the recombinant DNA pBRT32 according to the present invention. In the figure, the bold-lined part is a DNA encoding an enzyme derived from *Arthrobacter* sp. Q36.

[0018] The DNA according to the present invention exerts the production of the enzyme encoded by the DNA in a manner that the DNA is inserted into an appropriate self-replicable vector to form a replicable recombinant DNA, followed by introducing the recombinant DNA into a host, incapable of producing the enzyme per se but readily replicable, to form a transformant.

[0019] Although the recombinant DNA *per se* does not produce the enzyme, the production of the enzyme encoded by the DNA is attained by introducing the recombinant DNA into a host, incapable of producing the enzyme but replicable with a relative easiness, to form a transformant, and culturing the transformant to produce the enzyme.

[0020] The transformant according to the present invention produces the enzyme when cultured.

[0021] The recombinant enzyme according to the present invention releases trehalose when acts on non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

[0022] The recombinant enzyme is readily obtained in a desired amount by culturing the transformant according to the invention.

[0023] Non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher are converted into trehalose and glucose and/or maltooligosaccharides.

[0024] The present invention is based on the finding of a novel DNA encoding an enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. Such an enzyme can be obtained from cultures of microorganisms of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36, and the present inventors further characterised this enzyme by the combination use of conventional purification methods using column chromatography mainly, examined the properties and features, and revealed the reality, i.e. it is a polypeptide having the following physicochemical properties:

(1) Action
Releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher;
(2) Molecular weight
About 57,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE);
(3) Isoelectric point
About 3.3-4.6 on isoelectrophoresis;
(4) Optimum temperature
Exhibiting an optimum temperature of around 35-45°C when incubated at pH 7.0 for 30 min;
(5) Optimum pH
Exhibiting an optimum pH of around 6.0-7.5 when incubated at 40°C for 30 min;
(6) Thermal stability

Stable up to a temperature of around 30-45°C when incubated at pH 7.0 for 60 min; and

(7) pH Stability

Stable up to a pH of around 5.5-10.0 when incubated at 25°C for 16 hours.

[0025] Experiments, which were conducted to reveal the physicochemical properties of the enzymes produced by microorganisms of the species *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36 (the enzymes from *Rhizobium* sp. M-11 and *Arthrobacter* sp. Q36 are respectively designated as "enzyme M-11" and "enzyme Q36" hereinafter), are explained in the below:

Experiment 1

Purification of enzyme

Experiment 1-1

Purification of enzyme M-11

[0026] In 500-ml Erlenmeyer flasks were placed 100 ml aliquots of a liquid culture medium (pH 7.0) containing 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate, and 0.1 w/v % potassium dihydrogen phosphate, and the flasks were autoclaved at 120°C for 20 min to effect sterilization. After cooling the flasks a seed culture of *Rhizobium* sp. M-11 was inoculated into each liquid culture medium in each flask, followed by the incubation at 27°C for 24 hours under rotary-shaking conditions. Twenty L of a fresh preparation of the same liquid culture medium was put in a 30-L jar fermentor and sterilized, followed by inoculating one v/v % of the culture obtained in the above into the sterilized liquid culture medium in the jar fermentor, and incubating it at a pH of 6-8 and 30°C for 24 hours under aeration-agitation conditions.

[0027] Thereafter, about 18 L of the resultant culture was subjected to an ultra-high pressure cell disrupting apparatus to disrupt cells. The resultant suspension was centrifuged to obtain a supernatant, and to about 16 L of which was added ammonium sulfate to give a 20 w/v % saturation, followed by the standing at 4°C for one hour and the centrifugation to remove sediment. To the resultant supernatant was added ammonium sulfate to give a 60 w/v % saturation, and the solution was allowed to stand at 4°C for 24 hours and centrifuged to collect sediment which was then dissolved in a minimum amount of 10 mM phosphate buffer (pH 7.0). The solution thus obtained was dialyzed against 10 mM phosphate buffer (pH 7.0) for 24 hours, and centrifuged to remove insoluble substances. The resultant supernatant was fed to a column packed with "DEAE-TOYOPEARL®", a product for ion-exchange chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 10 mM phosphate buffer (pH 7.0), followed by feeding to the column a linear gradient buffer of sodium chloride ranging from 0 M to 0.5 M in 10 mM phosphate buffer (pH 7.0). Fractions containing the objective enzyme were collected from the eluate, pooled, dialyzed for 10 hours against 50 mM phosphate buffer (pH 7.0) containing 2 M ammonium sulfate, and centrifuged to remove insoluble substances. Thereafter, the resultant supernatant was fed to a column, which had been packed with "BUTYL TOYOPEARL®", a gel for hydrophobic column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, and equilibrated with 50 mM phosphate buffer (pH 7.0) containing 2 M ammonium sulfate, followed by feeding to the column a linear gradient buffer of ammonium sulfate ranging from 2 M to 0 M in 50 mM phosphate buffer (pH 7.0). Fractions containing the objective enzyme were collected from the eluate, pooled, fed to a column packed with "TOYOPEARL® HW-55", a product for gel filtration column chromatography commercialized by Tosoh Corporation, Tokyo, Japan, which had been previously equilibrated with 50 mM phosphate buffer (pH 7.0), followed by feeding to the column 50 mM phosphate buffer (pH 7.0) and collecting fractions containing the objective enzyme. The enzyme thus obtained had a specific activity of about 240 units/mg protein, and the yield was about 650 units per L of the culture.

[0028] Throughout the specification the enzyme activity is expressed by the value measured on the following assay: Place 4 ml of 50 mM phosphate buffer (pH 7.0) containing 1.25 w/v % maltotriosyltrehalose in a test tube, add one ml of an enzyme solution to the tube, and incubate the resultant solution at 40°C for 30 min to effect enzymatic reaction. Thereafter, one ml of the reaction mixture is mixed with 2 ml of copper reagent to suspend the enzymatic reaction, followed by assaying the reducing activity by the Somogyi-Nelson's method. As a control, an enzyme, which has been previously inactivated by heating at 100°C for 10 min, is similarly treated as above. One unit activity of the enzyme is defined as the amount of enzyme which increases the reducing power corresponding to one μmol glucose per min under the above conditions.

Experiment 1-2

Purification of enzyme Q36

[0029] Similarly as in Experiment 1-1, a seed culture of *Arthrobacter* sp.Q36 was cultured, and the resultant culture was treated to obtain a purified enzyme Q36 having a specific activity of about 450 units/mg protein in a yield of about 650 units per L of the culture.

Experiment 2

Physicochemical property of enzyme

Experiment 2-1

Action

[0030] According to the method disclosed in Japanese Patent Application No.349,216/93, a non-reducing saccharide containing 98 w/w % or higher, d.s.b., α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose or α-maltopentaosyltrehalose. Either of the non-reducing saccharides as a substrate was dissolved in 50 mM phosphate buffer (pH 7.0) into a 20 w/v % solution which was then mixed with 2 units/g substrate of the purified enzyme M-11 or Q36 in Experiment 1 and subjected to an enzymatic reaction at 40°C for 48 hours. The reaction mixture was desalted in usual manner, fed to "WB-T-330", a column for high-performance liquid chromatography (HPLC) commercialized by Wako Pure Chemical Industries, Ltd., Tokyo, Japan, followed by feeding to the column distilled water at a flow rate of 0.5 ml/min at ambient temperature to isolate saccharides contained in the reaction mixture while monitoring the saccharide concentration of the eluate with "MODEL RI-8012", a differential refractometer commercialized by Tosoh Corporation, Tokyo, Japan. As a control, an aqueous solution which contains either maltotriose, maltotetraose, maltopentaose, maltohexaose or maltoheptaose was similarly treated as above, and the resultant mixture was analyzed. The saccharide compositions of the reaction mixtures were tabulated in Tables 1 and 2.

Table 1

Substrate	Saccharide in reaction mixture	Saccharide composition (%)
α-Glucosyltrehalose	Trehalose	17.5
	Glucose	6.5
	α-Glucosyltrehalose	76.0
α-Maltosyltrehalose	Trehalose	44.3
	Maltose	44.4
	α-Maltosyltrehalose	11.3
α-Maltotriosyltrehalose	Trehalose	39.5
	Maltotriose	60.0
	α-Maltotriosyltrehalose	0.5
α-Maltotetraosyltrehalose	Trehalose	34.2
	Maltotetraose	65.5
	α-Maltotetraosyltrehalose	0.3
α-Maltopentaosyltrehalose	Trehalose	29.1
	Maltopentaose	70.6
	α-Maltopentaosyltrehalose	0.3
Maltotriose	Maltotriose	100.0
Maltotetraose	Maltotetraose	100.0
Maltopentaose	Maltopentaose	100.0
Maltohexaose	Maltohexaose	100.0
Maltoheptaose	Maltoheptaose	100.0

Table 2

Substrate	Saccharide in reaction mixture	Saccharide composition (%)
α-Glucosyltrehalose	Trehalose	19.3
	Glucose	10.2
	α-Glucosyltrehalose	70.5
α-Maltosyltrehalose	Trehalose	44.5
	Maltose	44.4
	α-Maltosyltrehalose	11.1
α-Maltotriosyltrehalose	Trehalose	38.8
	Maltotriose	60.7
	α-Maltotriosyltrehalose	0.5
α-Maltotetraosyltrehalose	Trehalose	34.1
	Maltotetraose	65.7
	α-Maltotetraosyltrehalose	0.2
α-Maltopentaosyltrehalose	Trehalose	29.3
	Maltopentaose	70.4
	α-Maltopentaosyltrehalose	0.3
Maltotriose	Maltotriose	100.0
Maltotetraose	Maltotetraose	100.0
Maltopentaose	Maltopentaose	100.0
Maltohexaose	Maltohexaose	100.0
Maltoheptaose	Maltoheptaose	100.0

[0031] As shown in Tables 1 and 2, enzymes M-11 and Q36 almost quantitatively released trehalose, glucose and maltooligosaccharides from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. These enzymes did not act on maltooligosaccharides, as a substrate, having a degree of glucose polymerization of 3 or higher. These facts indicate that these enzymes selectively act on non-reducing saccharides having a trehalose structure as an end unit and having a degree of polymerization degree of 3 or higher, and specifically hydrolyze the glycosidic bond between trehalose- and glycosyl-residues. Such an enzyme has never been reported and is estimated to have a novel enzymatic reaction mechanism.

Experiment 2-2

Molecular weight

[0032] In accordance with the method reported by U. K. Laemmli in Nature, Vol.227, pp.680-685 (1970), the purified enzymes M-11 and Q36 in Experiment 1 were respectively electrophoresed on sodium dodecyl sulfate polyacrylamide gel electrophoresis to show a single protein band at a position corresponding to about 57,000-68,000 daltons. The marker proteins used in this experiment were myosin (MW=200,000 daltons), β-galactosidase (MW=116,250 daltons), phosphorylase B (MW=97,400 daltons), serum albumin (MW=66,200 daltons) and ovalbumin (MW=45,000 daltons).

Experiment 2-3

Isoelectric point

[0033] The purified enzymes M-11 and Q36 obtained in Experiment 1 gave an isoelectric point of about 3.3-4.6 on isoelectrophoresis.

Experiment 2-4

Optimum temperature

[0034] The optimum temperature of the purified enzymes M-11 and Q36 obtained in Experiment 1 was about 35-45°C as shown in FIGs. 1 and 2 when incubated in usual manner in 50 mM phosphate buffer (pH 7.0) for 30 min.

Experiment 2-5

Optimum pH

[0035] The optimum pH of the purified enzymes M-11 and Q36 obtained in Experiment 1 was about 6.0-7.5 as shown in FIGs. 3 and 4 when experimented in usual manner by incubating them at 40°C for 30 min in 50 mM acetate buffer, phosphate buffer or sodium carbonate-sodium hydrogen carbonate buffer having different pHs.

Experiment 2-6

Thermal stability

[0036] The purified enzymes M-11 and Q36 obtained in Experiment 1 were stable up to a temperature of about 30-45°C as shown in FIGs. 5 and 6 when experimented in usual manner by incubating them in 50 mM phosphate buffer (pH 7.0) for 60 min.

Experiment 2-7

pH Stability

[0037] The purified enzymes M-11 and Q36 obtained in Experiment 1 were stable up to a pH of about 5.5-10.0 as shown in FIGs. 7 and 8 when experimented in usual manner by incubating them at 25°C for 16 hours in 50 mM acetate buffer, phosphate buffer or sodium carbonate-sodium hydrogen carbonate buffer having different pHs.

Experiment 2-8

Amino acid sequence containing the N-terminal

[0038] The amino acid sequence containing the N-terminal of the purified enzyme M-11 obtained in Experiment 1 was analyzed on "MODEL 470A", a gas-phase protein sequencer commercialized by Applied Biosystems, Inc., Foster City, USA, to reveal that it has the amino acid sequence as shown in SEQ ID NO:5.

[0039] The amino acid sequence containing the N-terminal of the purified enzyme Q36 was analyzed similarly as above to reveal that it has the amino acid sequence as shown in SEQ ID NO:6.

Experiment 2-9

Partial amino acid sequence

[0040] An adequate amount of the purified enzyme M-11 obtained in Experiment 1-1 was weighed, dialyzed against 10 mM Tris-HCl buffer (pH 9.0) at 4°C for 18 hours, and admixed with 10 mM Tris-HCl buffer (pH 9.0) to give a concentration of about one mg/ml of the enzyme. About one ml of the resultant solution was placed in a container, admixed with 10 μg lysyl endopeptidase, and incubated at 30°C for 22 hours to partially hydrolyze the enzyme. The resultant hydrolysate was applied to "CAPCELL-PAK C18", a column for reverse-phase high-performance liquid chromatography commercialized by Shiseido Co., Ltd., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % trifluoroacetate containing 16 v/v % aqueous acetonitrile, followed by feeding to the column 0.1 v/v % trifluoroacetate at a flow rate of 0.9 ml/min while increasing the concentration of acetonitrile from 16 v/v % to 64 v/v % to separately collect fractions containing a peptide fragment eluted about 43 min or about 57 min after the initiation of feeding (the peptide fragments were respectively named "peptide fragment A" and "peptide fragment B"). Fractions containing the peptide fragment A or B were separately pooled, dried *in vacuo*, and dissolved in 0.1 v/v % trifluoroacetate containing 50 v/v % aqueous acetonitrile. Similarly as in Experiment 2-8, the peptide fragments A and B were analyzed to reveal that they have the amino acid sequences as shown in SEQ ID NOs:7 and 8, respectively.

[0041] Similarly as in enzyme M-11, enzyme Q36 obtained in Experiment 1-2 was partially hydrolyzed, and the resultant was fed to "μBONDAPAK C18", a column for reverse-phase high-performance liquid chromatography commercialized by Japan Millipore Ltd., Tokyo, Japan, which had been previously equilibrated with 0.1 v/v % trifluoroacetate containing 24 v/v % aqueous acetonitrile, followed by feeding to the column 0.1 v/v % trifluoroacetate containing 24 v/v % aqueous acetonitrile while increasing the concentration of aqueous acetonitrile from 24 v/v % to 44 v/v % at a flow rate of 0.9 ml/ml. Fractions containing a peptide fragment eluted about 4 min or about 24 min after the initiation of feeding (the fractions were respectively called "peptide fragment C" and "peptide fragment D" hereinafter) were respectively collected, pooled, dried *in vacuo*, and dissolved in 0.1 v/v % trifluoroacetate containing 50 v/v % aqueous acetonitrile. Analyses of the peptide fragments C and D conducted similarly as above have revealed that they have amino acid sequences as shown in SEQ ID NOs:9 10 respectively.

[0042] No enzyme having these physicochemical properties has been known, and this concluded that it is a novel substance. Referring to *Rhizobium* sp. M-11, it is a microorganism which was isolated from a soil of Okayama-city, Okayama, Japan, deposited on December 24, 1992, in National Institute of Bioscience and Human-Technology Agency of Industrial Science and Technology, Tsukuba, Ibaraki, Japan, and accepted under the accession number of FERM BP-4130, and it has been maintained by the institute. *Arthrobacter* sp. Q36 is a microorganism which was isolated from a soil of Soja-city, Okayama, Japan, deposited on June 3, 1993, in the same institute, and accepted under the accession number of FERM BP-4316, and it has been maintained by the institute. Japanese Patent Application No. 340,343/93, applied by the same applicant (EP 628 630), discloses the properties and features of the non-reducing saccharide-forming enzyme as well as the detailed bacteriological properties of these microorganisms.

[0043] The present inventors energetically screened the chromosomal DNA of *Rhizobium* sp. M-11 by using an oligonucleotide as a probe which had been chemically synthesized based on the partial amino acid sequence of enzyme M-11 as revealed in Experiment 2-8 or 2-9, and obtained a DNA fragment which consists of 1,767 base pairs having the base sequence as shown in the following SEQ ID NO:1 that initiates from the 5'-terminus. The decoding of the base sequence of the enzyme has revealed that it has an amino acid sequence consisting of 589 amino acids as shown in SEQ ID NO:2.

[0044] Similarly as in enzyme M-11, the chromosomal DNA of enzyme Q36 was screened by using an oligonucleotide as a probe which had been chemically synthesized based on a partial amino acid sequence of enzyme Q36, and this yielded a DNA fragment having a base sequence consisting of 1,791 base pairs as shown in SEQ ID NO:3. The base sequence was decoded to reveal that enzyme Q36 has an amino acid sequence consisting of 597 amino acids as shown in SEQ ID NO:4.

[0045] The sequential experimental steps used for revealing the base sequence and amino acid sequence as shown in SEQ ID NOs:1 to 4 are summarized as below:

(1) The enzyme was isolated from a culture of a donor microorganism and highly purified. The purified enzyme was partially hydrolyzed with protease, and the resultant 2 different types of peptide fragments were isolated and determined their amino acid sequences;

(2) Separately, a chromosomal DNA was isolated from a donor microorganism's cell, purified and partially digested by a restriction enzyme to obtain a DNA fragment consisting of about 2,000-6,000 base pairs. The DNA fragment was ligated by DNA ligase to a plasmid vector, which had been previously cut with a restriction enzyme, to obtain a recombinant DNA;

(3) The recombinant DNA was introduced into *Escherichia coli* to obtain transformants, and from which an objective transformant containing a DNA encoding the enzyme was selected by the colony hybridization method using an oligonucleotide, as a probe, which had been chemically synthesized based on the aforesaid partial amino acid sequence; and

(4) The recombinant DNA was obtained from the selected transformant and annealed with a primer, followed by allowing a DNA polymerase to act on the resultant to extend the primer, and determining the base sequence of the resultant complementary chain DNA by the dideoxy chain termination method. The comparison of an amino acid sequence, estimable from the determined base sequence with the aforesaid amino acid sequence, confirmed that the base sequence encodes the enzyme.

[0046] The recombinant enzyme as referred to in the specification mean the whole recombinant enzymes which are preparable by the recombinant DNA technology and capable of releasing trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. Generally, the recombinant enzyme according to the present invention has a revealed amino acid sequence, and, as an example, the amino acid sequence as shown in SEQ ID NO:2 or 4 which initiates from the N-terminal, as well as homologous ones to it, can be mentioned. Variants having amino acid sequences homologous to the one as shown in SEQ ID NO: 2 or 4 can be obtained by replacing one or more bases in SEQ ID NO:2 or 4 with other bases without substantially alternating the inherent activity of the enzyme. Although even when used the same DNA and it also depends on hosts

into which the DNA is introduced, as well as on ingredients and components of nutrient culture media used for culturing transformants, and their cultivation temperature and pH, there may be produced modified enzymes which have amino acid sequences similar to that of SEQ ID NO:2 or 4, as well as having the enzymatic activity inherent to the enzyme encoded by the DNA but defective one or more amino acids located near to the N-terminal of the amino acid sequence of SEQ ID NO:2 or 4 and/or having one or more amino acids newly added to the N-terminal by the modification of intracellular enzymes of hosts after the DNA expression. In view of the technical background in the art, the enzyme as referred to in the present invention includes those which have the amino acid sequence corresponding to that of SEQ ID NO:2 or 4, and those which substantially have the amino acid sequence as shown in SEQ ID NO:2 or 4 except that one or more amino acids in the amino acid sequence are defected, newly added to or replaced with other amino acids, as long as they release trehalose form non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher.

[0047] In this field, it is known that one or more bases in DNAs can be replaced with other bases by the degeneracy of genetic code without alternating the amino acid sequences encoded by the DNAs. Based on this the DNA according to the present invention includes DNAs which contain the amino acid sequence of SEQ ID NO:1 or 3 and other DNAs, wherein one or more bases are replaced with other bases by degeneracy of genetic code, as long as they encode enzymes having the amino acid sequence as shown in SEQ ID NO:2 or 4 and homologous variants thereof.

[0048] According to the today' s recombinant DNA technology, the determination of base sequences from the 5'-termini of DNAs define their complementary base sequences. Therefore, the DNA according to the present invention also includes complementary base sequences corresponding to any one of the aforesaid base sequences. Needless to say, one or more bases in the base sequence, which encodes the enzyme or their variants, can be readily replaced with other bases to allow the DNA to actually express the enzyme production in hosts.

[0049] The DNA according to the present invention is as described above, and any DNA derived from natural resources and those artificially synthesized can be used in the present invention as long as they have the aforementioned base sequences. The natural resources of the DNA according to the present invention are, for example, microorganisms of the genera *Rhizobium, Arthrobacter, Brevibacterium* and *Micrococcus*, i.e. *Rhizobium* sp. M-11 (FERM BP-4130), *Arthrobacter* sp. Q36 (FERM BP-4316), *Brevibacterium helovolum* (ATCC 11822) and *Micrococcus* roseus (ATCC 186) from which genes containing the present DNA can be obtained. These microorganisms can be inoculated in nutrient culture media and cultured for about 1-3 days under aerobic conditions, and the resultant cells were collected from the cultures and subjected to ultrasonication or treated with a cell-wall lysis enzyme such as lysozyme or β-glucanase to extract genes containing the present DNA. In this case, a proteolytic enzyme such as protease can be used along with the cell-wall lysis enzyme, and, in the case of treating the cells with ultrasonication, they may be treated in the presence of a surfactant such as sodium dodecyl sulfate (SDS) or treated with freezing- and thawing-methods. The objective DNA is obtainable by treating the resultant with phenol extraction, alcohol sedimentation, centrifugation, protease treatment and/or ribonuclease treatment used in general in the art.

[0050] To artificially synthesize the DNA according to the present invention, it can be chemically synthesized by using the base sequence as shown in SEQ ID NO:1 or 3, or can be obtained in plasmid form by inserting a DNA, which encodes the amino acid sequence as shown in SEQ ID NO:2 or 4, into an appropriate self-replicable vector to obtain a recombinant DNA, introducing the recombinant DNA into an appropriate host to obtain a transformant, culturing the transformant, separating the proliferated cells from the resultant culture, and collecting plasmids containing the DNA from the cells.

[0051] The present invention further relates to replicable recombinant DNAs which express the production of the enzyme according to the invention when introduced into microorganisms as well as plant- and animal-cells which do not produce the enzyme inherently but are readily proliferative. Such a recombinant DNA, which generally contains the aforesaid DNA and a self-replicable vector, can be prepared by conventional method with a relative easiness when the material DNA is in hand. Examples of such a vector are plasmid vectors such as pBR322, pUC18, Bluescript II SK (+), pUB110, pTZ4, pC194, pHV14, TRp7, TEp7, pBS7, etc.; and phage vectors such as λgt·λC, λgt·λB, ρ11, ϕ1, ϕ105, etc. Among these plasmid- and phage-vectors, pBR322, pUC18, Bluescript II SK(+), λgt·λC and λgt·λB are satisfactorily used in case that the present DNA should be expressed in *Escherichia coli*, while pUB110, pTZ4, pC194, ρ11, ϕ1 and ϕ105 are satisfactorily used to express the DNA in microorganisms of the genus *Bacillus*. The plasmid vectors pHV14, TRp7, TEp7 and pBS7 are suitably used when the recombinant DNA is allowed to grow in 2 or more hosts.

[0052] The methods used to insert the present DNA into such vectors in the present invention may be conventional ones generally used in this field. A gene containing the present DNA and a self-replicable vector are first digested by a restriction enzyme and/or ultrasonic disintegrator, then the resultant DNA fragments and vector fragments are ligated. To digest DNAs and vectors, restriction enzymes which specifically act on nucleotides, particularly, type II restriction enzymes, more particularly, *Sau* 3AI, *Eco* RI, *Hind* III, *Bam* HI, *Sal* I, *Xba* I, *Sac* I, *Pst* I, etc., facilitate the ligation of the DNA fragments and vector fragments. The ligation of the DNA fragments and vector fragments is effected by annealing them first if necessary, then subjected to the action of a DNA ligase *in vivo* or *in vitro*. The recombinant DNA thus obtained is replicable without substantial limitation by introducing it into appropriate hosts, and culturing the re-

sultant transformants.

[0053] The recombinant DNA according to the present invention can be introduced into appropriate host microorganisms including *Escherichia coli* and those of the genus *Bacillus* as well as actinomyces and yeasts. In the case of using *Escherichia coli* as a host, it can be cultured in the presence of the recombinant DNA and calcium ion, while in the case of using the microorganisms of the genus *Bacillus* the competent cell method and the colony hybridization method can be employed. Desired transformants can be cloned by the colony hybridization method or by culturing a variety of transformants in nutrient culture media containing non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, and selecting the objective transformants which release trehalose form the non-reducing saccharides.

[0054] The transformants thus obtained extracellularly produce the objective enzyme when cultured in nutrient culture media. Generally, liquid media in general supplemented with carbon sources, nitrogen sources and minerals, and, if necessary, further supplemented with a small amount of amino acids and vitamins can be used as the nutrient culture media. Examples of the carbon sources are saccharides such as starch, starch hydrolysate, glucose, fructose and sucrose. Examples of the nitrogen sources are organic- and inorganic-substances containing nitrogen such as ammonia, ammonium salts, urea, nitrate, peptone, yeast extract, defatted soy been, corn steep liquor and beef extract. Cultures containing the objective enzyme can be prepared by inoculating the transformants into nutrient culture media, and incubating them at a temperature of 25-65°C and a pH of 2-8 for about 1-6 days under aerobic aeration-agitation conditions. Such a culture can be used intact as an enzyme preparation, and, usually, it may be disrupted with ultrasonic disintegrator and/or cell-wall lysis enzymes prior to use, followed by separating the enzyme from the intact cells and cell debris by filtration and/or centrifugation, and purifying the enzyme. The methods used for purifying the enzyme in the invention include conventional ones in general. From cultures the intact cells and cell debris are eliminated and subjected to one or more methods such as concentration, salting out, dialysis, separately sedimentation, gel filtration chromatography, ion exchange chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectric point electrophoresis.

[0055] As is described above, the enzyme exerts a distinct activity of forming trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher, and such an activity has not yet been found in any conventional enzymes. Therefore, the use of the enzyme facilitates the preparation of trehalose in a relatively-high yield and efficiency from non-reducing saccharides such as α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose and α-maltopentaosyltrehalose in a considerably-high yield. These non-reducing saccharides can be obtained in a satisfactorily-high yield from starch hydrolysates, which are obtained by treating amylaceous substances such as starch, amylose and amylopectin prepared with acids and/or amylases, by using non-reducing saccharide-forming enzyme as disclosed in Japanese Patent Application No.349,216/93. Thus, trehalose, whose industrial preparation has been difficult, can be prepared from starch and amylaceous substances as a material with a relative easiness and in a desired amount when the present enzyme and the non-reducing saccharide-forming enzyme, as disclosed in Japanese Patent Application No. 349,216/93, are used in combination.

[0056] As described in "*Handbook of Amylases and Related Enzymes*", 1st edition, edited by The Amylase Research Society of Japan, published by Pergamon Press plc, Oxford, England (1988), α-amylase, maltotetraose-forming amylase, maltopentaose-forming amylase and maltohexaose-forming amylase are especially useful to prepare the reducing amylaceous saccharides used in the invention, and, the use of any one of these amylases readily yields amylaceous saccharide mixtures rich in reducing amylaceous saccharides having a degree of glucose polymerization of 3 or higher in a considerably-high yield. If necessary, the combination use of such an amylase and a starch debranching enzyme such as pullulanase or isoamylase can increase the yield of the reducing amylaceous saccharides usable as a substrate for the non-reducing saccharide-forming enzyme, i.e. the non-reducing saccharides can be obtained by coexisting the non-reducing saccharide-forming enzyme in an aqueous solution containing as a substrate one or more of the reducing amylaceous saccharides in an amount up to a concentration of 50 w/v %, and subjecting the solution to an enzymatic reaction at a temperature of about 40-55°C and a pH of about 6-8 until a desired amount of the objective non-reducing saccharides are formed.

[0057] Usually, in the present conversion method, the recombinant enzyme according to the present invention is allowed to coexist in the aforesaid aqueous solution containing one or more of the non-reducing amylaceous saccharides, and to enzymatically react with the saccharides while keeping at a prescribed temperature and pH until a desired amount of trehalose is released.

[0058] Although the enzymatic reaction proceeds even below a concentration of 0.1 w/v % of a substrate, a higher concentration of 2 w/v %, preferably, 5-50 w/v % of a substrate can be satisfactorily used to apply the present conversion method to an industrial-scale production. The temperature and pH used in the enzymatic reaction are set within the ranges of which do not inactivate the recombinant enzyme and allow the recombinant enzyme to effectively act on substrates, i.e. a temperature up to about 55°C, preferably, a temperature in the range of about 40-55°C, and a pH of 5-10, preferably, a pH in the range of about 6-8. The amount and reaction time of the present recombinant enzyme

are chosen dependently on the enzymatic reaction conditions. The enzymatic reaction effectively converts non-reducing saccharides into saccharide compositions containing trehalose and glucose and/or maltooligosaccharides, and, in the case of using α-maltotriosyltrehalose as a substrate, the conversion rate reaches to approximately 100%. In the case of simultaneously subjecting starch hydrolysates to the action of either of the above amylases together with the non-reducing saccharide-forming enzyme and the present recombinant enzyme, non-reducing saccharides are formed from the hydrolysates while hydrolyzed into glucose and/or maltooligosaccharides, and because of this saccharide compositions with a relatively-high trehalose content can be effectively obtained in a relatively-high yield.

[0059] The reaction products obtained by the present conversion reaction can be used intact, and, usually, they are purified prior to use: Insoluble substances are eliminated from the reaction products by filtration and centrifugation, and the resultant solutions are decolored with activated charcoal, desalted and purified on ion exchangers, and concentrated into syrupy products. Dependently on their use, the syrupy products are dried *in vacuo* and spray-dried into solid products. In order to obtain products which substantially consist of non-reducing saccharides, the above mentioned syrupy products are subjected to one or more methods such as chromatography using an ion exchanger, activated charcoal and silica gel to separate saccharides, separately sedimentation using alcohol and/or acetone, membrane filtration, fermentation by yeasts, and removal and decomposition of reducing saccharides by alkalis. The methods to treat a large amount of reaction mixture are, for example, fixed bed- or pseudomoving bed-ion exchange column chromatography as disclosed in Japanese Patent Laid-Open Nos.23,799/83 and 72,598/83, and such a method enables an effective industrial-scale production of products with a relatively-high trehalose content.

[0060] These trehalose and compositions containing the same have a wide applicability to a variety of products which are apt to be readily damaged by the reducibility of saccharide sweeteners: For example, they can be satisfactorily used as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant in food products in general, cosmetics and pharmaceuticals.

[0061] The following examples explain the present invention in more detail, and the techniques themselves used in the examples are conventional ones in this field, for example, those described by J. Sumbruck et al. in "*Molecular Cloning A Laboratory Manual*", 2nd edition, published by Cold Spring Harbor Laboratory Press (1989).

Example 1

Preparation of recombinant DNA containing DNA encoding enzyme M-11 and transformant

Example 1-1

Preparation of chromosomal DNA

[0062] A seed culture of *Rhizobium* sp. M-11 was inoculated into bacto nutrient broth medium (pH 7.0), and cultured at 27°C for 24 hours with a rotary shaker. The cells were separated from the resultant culture by centrifugation, suspended in TES buffer (pH 8.0), admixed with 0.05 w/v % lysozyme, and incubated at 37°C for 30 min. The resultant was freezed at -80°C for one hour, admixed with TSS buffer (pH 9.0), heated to 60°C, and further admixed with a mixture solution of TES buffer and phenol, and the resultant solution was chilled with ice, followed by centrifugally collecting the precipitated crude chromosomal DNA. To the supernatant was added 2 fold volumes of cold ethanol, and the reprecipitated crude chromosomal DNA was collected, suspended in SSC buffer (pH 7.1), admixed with 7.5 μg ribonuclease and 125 μg protease, and incubated at 37°C for one hour. Thereafter, a mixture solution of chloroform and isoamyl alcohol was added to the reaction mixture to extract the objective chromosomal DNA, and admixed with cold ethanol, followed by collecting the formed sediment containing the chromosomal DNA. The purified chromosomal DNA thus obtained was dissolved in SSC buffer (pH 7.1) to give a concentration of about one mg/ml, and the resultant solution was freezed at -80°C.

Example 1-2

Preparation of recombinant DNA pBMU27 and transformant BMU27

[0063] About one ml of the purified chromosomal DNA obtained in Example 1-1 was placed in a container, admixed with about 35 units of *Sau* 3AI, a restriction enzyme, and enzymatically reacted at 37°C for about 20 min to partially digest the chromosomal DNA, followed by recovering a DNA fragment consisting of about 2,000-6,000 base pairs by means of sucrose density-gradient ultracentrifugation. One μg of Bluescript II SK(+), a plasmid vector, was provided, subjected to the action of *Bam* HI, a restriction enzyme, to completely digest the plasmid vector, admixed with 10 μg of the DNA fragment and 2 units of T4 DNA ligase, and allowed to stand at 4°C overnight to ligate the DNA fragment to the vector fragment. To the resultant recombinant DNA was added 30 μl of "Epicurian Coli® XLI-Blue", competent

cell commercialized by Toyobo Co., Ltd., Tokyo, Japan, allowed to stand under ice-chilling conditions for 30 min, heated to 42°C, admixed with SOC broth, and incubated at 37°C for one hour to introduce the recombinant DNA into *Escherichia coli*.

[0064] The resultant transformant was inoculated into agar plate (pH 7.0) containing 50 μg/ml of 5-bromo-4-chloro-3-indolyl-β-galactoside, and cultured at 37°C for 18 hours, followed by placing a nylon film on the agar plate to fix thereon about 6,000 colonies formed on the agar plate. Based on the amino acid sequence located at positions from 8 to 13 as shown in SEQ ID NO:7, i.e. Phe-Asp-Ile-Trp-Ala-Pro, the base sequence of probe 1 represented by 5'-TTY-GAYATHTGGGCNCC-3' was chemically synthesized, labelled with ^{32}P, and hybridized with the colonies of transformants fixed on the nylon film, followed by selecting 14 transformants which exhibited a strong hybridization.

[0065] The objective recombinant DNA was selected in usual manner from the 14 transformants, and, in accordance with the method described by E. M. Southern in *Journal of Molecular Biology*, Vol.98, pp.503-517 (1975), the recombinant DNA was hybridized with probe 2 having the base sequence as shown in SEQ ID NO:8, which had been chemically synthesized based on the amino acid sequence located at positions from 2 to 6, i.e. Asp-Trp-Ala-Glu-Ala, in SEQ ID NO:8, followed by selecting a recombinant DNA strongly hybridized with the probe 2. The recombinant DNA and transformant thus selected were respectively named "**pBMU27**" and "**BMU27**".

[0066] The transformant BMU27 was inoculated into L-broth (pH 7.0) containing 100 μg/ml ampicillin, and cultured at 37°C for 24 hours by a rotary shaker. After completion of the culture, the resultant cells were collected from the culture by centrifugation, and treated with the alkaline method in general to extracellularly extract a recombinant DNA. The extract was in usual manner purified and analyzed to reveal that the recombinant DNA pBMU27 consists of about 5,700 base pairs and has the structure expressed by the restriction map as shown in FIG. 9. It was found that, as shown in FIG. 9, the DNA which consists of 1,767 base pairs for encoding the enzyme M-11 is positioned in the downstream near to the digested site of *Eco* RV, a restriction enzyme.

Example 1-3

Production of enzyme by transformant BMU27

[0067] A liquid nutrient culture medium consisting of 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate was adjusted to pH 7.0, admixed with 50 μg/ml ampicillin, autoclaved at 120°C for 20 min, cooled and inoculated with a seed culture of transformant BMU27 obtained in Example 1-2, followed by culturing the transformant at 37°C for 24 hours by a rotary shaker. The resultant culture was treated with ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances. The supernatant thus obtained was assayed for the enzyme activity to find that one L of the culture yielded about 4,000 units of the enzyme.

[0068] As a control, a seed culture of *Escherichia coli* XLI-Blue or *Rhizobium* sp. M-11 was inoculated in the same fresh preparation of the same liquid nutrient culture medium but free of ampicillin, and, in the case of culturing *Rhizobium* sp. M-11, it was cultured and treated similarly as above except that the cultivation temperature was set to 30°C. Assaying the resultant activity, one L culture of *Rhizobium* sp. M-11 yielded about 2,000 units of the enzyme, and the yield was significantly lower than that of transformant BMU27. *Escherichia coli* XLI-Blue used as a host did not form the enzyme.

[0069] Thereafter, the enzyme produced by the transformant MBU27 was purified similarly as in Experiment 1-1, and examined on the properties and characters. As a result, it was revealed that it has substantially the same physicochemical properties as enzyme M-11, i.e. it has a molecular weight of about 57,000-68,000 daltons on SDS-PAGE and an isoelectric point of about 3.3-4.6 on isoelectrophoresis. The results indicate that the present enzyme can be prepared by the recombinant DNA technology, and the yield can be significantly increased thereby.

Example 2

Preparation of complementary chain DNA derived from *Rhizobium* sp. M-11, and determination for its base sequence and amino acid sequence

[0070] Two μg of the recombinant DNA pBMU27 obtained in Example 1-2 was provided, admixed with 2 M aqueous sodium hydroxide solution to effect degeneration, and admixed with an adequate amount of cold ethanol, followed by collecting the formed sediment containing a template DNA and drying the sediment *in vacuo*. To the template DNA were added 50 pmole/ml of a chemically synthesized primer 1 represented by 5'-GTAAAACGACGGCCAGT-3', 10 μl of 40 mM Tris-HCl buffer (pH 7.5) containing 20 mM magnesium chloride and 20 mM sodium chloride, and the mixture was incubated at 65°C for 2 min to effect annealing and admixed with 2 μl of an aqueous solution containing dATP, dGTP and dTTP in respective amounts of 7.5 μM, 0.5 μl of [α-^{32}P]dCTP (2 mCi/ml), one μl of 0.1 M dithiothreitol, and

2 μl of 1.5 units/ml T7 DNA polymerase, followed by incubating the resultant mixture at 25°C for 5 min to extend the primer 1 from the 5'-terminus to the 3'-terminus. Thus, a complementary chain DNA was formed.

[0071] The reaction product containing the complementary chain DNA was divided into quarters, to each of which 2.5 μl of 50 mM aqueous sodium chloride solution containing 80 μM dNTP and 8 μM ddATP, ddCTP, ddGTP or ddTTP was added, and the resultant mixture was incubated at 37°C for 5 min, followed by suspending the reaction by the addition of 4 μl of 98 v/v % aqueous formamide solution containing 20 mM EDTA, 0.05 w/v % bromophenol blue, and 0.05 w/v % xylene cyanol. The reaction mixture was heated with a boiling-water bath for 3 min, and a portion of which was placed on a gel containing 6 w/v % polyacrylamide, and electrophoresed by energizing the gel with a constant voltage of about 2, 000 volts to separate DNA fragments, followed by fixing the gel in usual manner, drying the gel and subjecting the resultant gel to autoradiography.

[0072] Analyses of the DNA fragments separated on the radiogram revealed that the complementary chain DNA contains the base sequence consisting of about 2,161 base pairs as shown in SEQ ID NO:11. An amino acid sequence estimable from the base sequence was as shown in SEQ ID NO:11 and was compared with the amino acid sequence containing the N-terminal or the partial amino acid sequence of enzyme M-11 as shown in SEQ ID NO:5, 7 or 8. As a result, it was found that the amino acid sequence containing the N-terminal of SEQ ID NO:5 corresponds to the amino acid sequence located at positions from 8 to 27 in SEQ ID NO:11, and the partial amino acid sequence of SEQ ID NO: 7 or 8 corresponds to the amino acid sequence located at positions from 10 to 30 or at positions from 493 to 509 in SEQ ID NO:11. These results indicate that enzyme M-11 has the amino acid sequence of SEQ ID NO:2, and it is encoded by the DNA having the base sequence as shown in SEQ ID NO:1.

Example 3

Preparation of recombinant DNA, containing DNA derived from *Arthrobacter* sp. Q36, and transformant

Example 3-1

Preparation of chromosomal DNA

[0073] Similarly as in Example 1-1, a chromosomal DNA was isolated from Arthrobacter sp. Q36, purified and dissolved in SSC buffer (pH 7.1) to give a concentration of about one mg/ml, and the resultant solution was freezed at -80°C for storage.

Example 3-2

Preparation of recombinant DNA pBRT32 and transformant BRT32

[0074] The purified chromosomal DNA obtained in Example 3-1 was partially digested similarly as in Example 1-2, followed by recovering a DNA fragment consisting of about 2,000-6,000 base pairs by sucrose density gradient ultracentrifugation. The DNA fragment was ligated to a lysate of Bluescript II SK(+) which had been treated with *Bam* HI, and the resultant recombinant DNA was introduced into *Escherichia coli* XLI-Blue. The transformants thus obtained were cultured similarly as in Example 1-2 on agar plates containing 5-bromo-4-chloro-3-indolyl-β-galactoside, and the formed about 5,000 colonies were fixed on a nylon film, while the probe 3 represented by 5'-ATGGGNTGGGAYCCNGC-3' was chemically synthesized based on the amino acid sequence of Met-Gly-Trp-Asp-Pro-Ala located at positions from 5 to 10 in SEQ ID NO:9, labelled with ^{32}P, and hybridized with transformant colonies which had been fixed on the nylon film, followed by selecting 10 transformants which strongly hybridized with the probe 3.

[0075] Similarly as in Example 1-2, the objective recombinant DNA was selected from 10 transformants, and hybridized with probe 4 represented by 5'-TAYGAYGTNTGGGC-3' which had been chemically synthesized based on the amino acid sequence of Tyr-Asp-Val-Trp-Ala located at positions from 8 to 12 in SEQ ID NO:10, followed by selecting a recombinant DNA which strongly hybridized with probe 4. The recombinant DNA and transformant thus selected were respectively named "**pBRT32**" and "**BRT32**".

[0076] The transformant BRT32 was inoculated into L-broth containing ampicillin, and cultured similarly as in Example 1-2, and the proliferated cells were collected from the resultant culture, and from which a recombinant DNA was extracted, purified and analyzed to reveal that the recombinant DNA pBRT32 consists of about 6, 200 base pairs and has the structure of the restriction map as shown in FIG. 10. As shown in FIG. 10, it was revealed that the DNA, which consists of 1,791 base pairs for encoding the DNA of enzyme Q36, is located in the downstream near to the cleavage site of Kpn I.

Example 3-3

Production of enzyme by transformant BRT32

[0077] A liquid nutrient culture medium consisting of 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate was adjusted to pH 7.0, admixed with 50 μg/ml ampicillin, autoclaved at 120°C for 20 min, cooled and inoculated with a seed culture of the transformant BRT32 obtained in Example 3-2, followed by culturing the transformant at 37°C for 24 hours by a rotary shaker. The resultant culture was treated with an ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances. The supernatant thus obtained was assayed for the present enzyme activity to find that one L of the culture yielded about 3,900 units of the enzyme.

[0078] As a control, a seed culture of *Escherichia coli* XLI-Blue or *Arthrobacter* sp. Q36 was inoculated into a fresh preparation of the same liquid nutrient culture medium but free of ampicillin, and, in the case of culturing *Arthrobacter* sp. Q36, it was cultured and treated similarly as above except that the cultivation temperature was set to 30°C. Assaying the enzyme activity, one L of the culture of *Arthrobacter* sp. Q36 yielded about 1,800 units of the enzyme, and the yield was significantly lower than that of the transformant BRT32. The *Escherichia coli* XLI-Blue used as a host did not form the enzyme.

[0079] Thereafter, the enzyme produced by the transformant BRT32 was purified similarly as in Experiment 1-1, and examined on the properties and characters to reveal that it has substantially the same physicochemical properties as that of enzyme Q36, i.e. it has a molecular weight of about 57,000-68,000 daltons on SDS-PAGE and an isoelectric point of about 3.3-4.6 on isoelectrophoresis. These results indicate that the enzyme can be prepared by the recombinant DNA technology, and the yield can be significantly increased thereby.

Example 4

Preparation of complementary chain DNA derived from *Arthrobacter* sp. Q36, and determination for its base sequence and amino acid sequence

[0080] The recombinant DNA pBRT32 obtained in Example 3-2 was similarly treated as in Example 2 to form a template DNA which was then annealed together with the primer 1, followed by allowing T7 DNA polymerase to act on the resultant to extend the primer 1 from the 5'-terminus to the 3'-terminus to obtain a complementary chain DNA. Similarly as in Example 2, the complementary chain DNA was subjected to the dideoxy chain terminator method to analyze DNA fragments which had been isolated on a radiogram. The result revealed that the complementary chain DNA contained a base sequence consisting of 2,056 base pairs as shown in SEQ ID NO:12. An amino acid sequence estimable from the base sequence was as shown in SEQ ID NO:12, and compared with the amino acid sequence containing the N-terminal or the partial amino acid sequence of SEQ ID NO:6, 9 or 10. As a result, it was found that the amino acid sequence of SEQ ID NO:6 corresponds to that located at positions from 2 to 21 in SEQ ID NO:12, and that the partial amino acid sequence in SEQ ID NO:9 or 10 corresponds to that located at positions from 470 to 489 or at positions from 11 to 30 in SEQ ID NO:12. These results indicate that enzyme Q36 has the amino acid sequence of SEQ ID NO:4, and it is encoded by the DNA having the base sequence as shown in SEQ ID NO:3.

Example 5

Preparation of recombinant enzyme

[0081] In 500-ml Erlenmeyer flasks were placed 100 ml aliquots of a liquid nutrient culture medium (pH 7.0) consisting of 2.0 w/v % "PINE-DEX #4", a starch hydrolysate commercialized by Matsutani Chemical Ind., Co., Ltd., Tokyo, Japan, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate, and to each flask was added 50 μg/ml ampicillin and autoclaved at 120°C for 20 min. Thereafter, the flasks were cooled and inoculated with a seed culture of the transformant BMU27 obtained in Example 1-2, followed by culturing the transformant at 27°C for 24 hours by a rotary shaker. Apart from this, 18 L of a fresh preparation of the same liquid culture medium was placed in a 30-L jar fermentor, admixed with 50 μg/ml ampicillin, sterilized at 120°C for 20 min, cooled and inoculated with one v/v % of the seed culture obtained in the above, followed by the culture at 37°C for 24 hours while keeping the pH at 6-8 under aeration-agitation conditions. The resultant culture was treated with an ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances. The supernatant thus obtained was assayed for the enzyme activity to reveal that one L of the culture yielded about 3,900 units of the enzyme. The supernatant was purified by the method in Experiment 1-1 to obtain an

about 67 ml aqueous solution containing an about 165 units/ml of a recombinant enzyme having a specific activity of about 290 units/mg protein.

Example 6

Preparation of recombinant enzyme

[0082] Recombinant BRT32 obtained by the method in Experiment 3-2 was cultured similarly as in Example 5, and the resultant culture was treated with an ultrasonic integrator to disrupt cells. The resultant suspension was centrifuged to remove insoluble substances, and the resultant supernatant was assayed for the enzyme activity to have an activity of about 4,000 units per L. The supernatant was purified by the method in Experiment 1-1 to obtain an about 55 ml aqueous solution containing about 200 units/ml of a recombinant enzyme with a specific activity of about 420 units/mg protein.

Example 7

Conversion of non-reducing saccharide by recombinant enzyme

Example 7-1 (a)

Preparation of non-reducing saccharide-forming enzyme

[0083] To 500-ml Erlenmeyer flasks were placed 100 ml aliquots of a liquid nutrient culture medium (pH 7.0) consisting of 2.0 w/v % maltose, 0.5 w/v % peptone, 0.1 w/v % yeast extract, 0.1 w/v % disodium hydrogen phosphate and 0.1 w/v % potassium dihydrogen phosphate, and the flasks were autoclaved at 120°C for 20 min. Thereafter, the flasks were cooled and inoculated with a seed culture of *Rhizobium* sp. M-11, followed by culturing it at 27°C for 24 hours by a rotary shaker. Apart from this, 20 L of a fresh preparation of the same liquid culture medium was placed in a 30-L jar fermentor, and sterilized, inoculated with one v/v % of the seed culture obtained in the above, followed by the culture at 30°C and at a pH of 7-8 for 24 hours under aeration-agitation conditions. Thereafter, the resultant culture was treated with an ultrasonic disintegrator to disrupt cells, and the resultant suspension was centrifuged to remove insoluble substances and purified according to the method in Experiment 1-1 to obtain a non-reducing saccharide-forming enzyme having a specific activity of about 195 units/mg protein in a yield of about 220 units per L of the culture.

[0084] Throughout the specification the activity of a non-reducing saccharide-forming enzyme is expressed by the value measured on the following assay: Place 4 ml of 50 mM phosphate buffer (pH 7.0) containing 1.25 w/v % maltopentaose in a test tube, add one ml of an enzyme solution to the test tube, and incubate the solution at 40°C for 60 min to effect enzymatic reaction. Thereafter, the reaction mixture is heated at 100°C for 10 min to suspend the enzymatic reaction, followed by diluting it with distilled water by 10 times and assaying the reducing activity by the Somogyi-Nelson's method. One unit activity of the non-reducing saccharide-forming enzyme is defined as the amount of enzyme which decreases the reducing power corresponding to one μmol maltopentaose per min under the above conditions.

Example 7-1(b)

Preparation of syrupy product containing trehalose

[0085] A potato starch was suspended in water to give a 15 w/w % suspension which was then mixed with 0.1 w/w % calcium carbonate. The mixture was adjusted its pH to 6.0, mixed with 0.2 w/w %, d.s.b., of "TERMAMYL 60L", an α-amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, and enzymatically reacted at 95°C for 15 min to effect gelatinization and liquefaction. The liquefied solution was autoclaved at 120°C for 30 min to inactivate the remaining enzyme, rapidly cooled to 45°C, 1,000 units/g starch, d.s.b., of pullulanase commercialized by Hayashibara Biochemical Laboratories., Inc., Okayama, Japan, 3.4 units/g starch, d.s.b., of the non-reducing saccharide-forming enzyme obtained in Example 7-1(a), and 4.2 units/g starch, d.s.b., of the recombinant enzyme obtained by the method in Example 5, followed the enzymatic reaction for 48 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion-exchange resin, and concentrated to obtain a syrupy product with a concentration of about 60 w/w % in a yield of about 92%, d.s.b.

[0086] Analysis of the syrup by the method of Experiment 2-1 revealed that it contained 70.2 w/w % trehalose, 2.4 w/w % α-glucosyltrehalose, 3.3 w/w % α-maltosyltrehalose, 0.7 w/w % glucose, 10.1 w/w % maltose, 12.9 w/w % maltotriose, and 0.4 w/w % maltooligosaccharides having a degree of glucose polymerization of 4 or higher. The prod-

uct, having a mild and moderate sweetness as well as an adequate viscosity and moisture-retaining ability, can be satisfactorily used in food products in general, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

Example 7-1(c)

Preparation of powdery product containing trehalose

[0087] To 4 jacketed-stainless steel columns, having a diameter of 5.4 cm and a length of 5 m each was packed homogeneity with "XT-1016 (Na^+-form)", a strong-acid cation exchange resin commercialized by Tokyo Organic Chemical Industries, Ltd., Tokyo, Japan, and the columns were cascaded in series to give a total column length of 20 m. The syrupy product obtained in Example 7-1(b) was fed to the columns at a rate of about 5 v/v % against the resin at an inner column temperature of 55°C, and the columns were fed with 55°C hot water at an SV (space velocity) 0.3 to fractionate saccharides in the syrupy product. Based on the analysis of the saccharide composition of the eluate, fractions rich in trehalose were collected, pooled, concentrated, dried *in vacuo* and pulverized to obtain a solid product containing about 97 w/w % trehalose in a yield of about 56% against the starting material, d.s.b.

[0088] The product, having a mild sweetness and substantially free of reducibility, can be satisfactorily used in food products in general, cosmetics and pharmaceuticals as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

Example 7-1(d)

Preparation of powdery crystalline trehalose

[0089] A portion of the trehalose rich fraction obtained in Example 7-1(c) was concentrated into an about 75 w/w % solution which was then transferred to a crystallizer, admixed with about 2 w/w %, d.s.b., hydrous crystalline trehalose as a seed crystal, and crystallized under gentle stirring conditions to obtain a massecuite with a crystallinity of about 45 w/w %. The massecuite was sprayed downward from a nozzle, equipped at the upper part of a spraying tower at a pressure of about 150 kg/cm^2 while about 85°C hot air was flowing downward from the upper part of the tower to accumulate a crystalline powder on a belt conveyer provided on the basement of the tower, followed by gradually transferring it out of the tower. Thereafter, the powder was transferred to an ageing tower and aged for 10 hours to complete the crystallization and drying while an about 40°C hot air was blowing to the contents. Thus, a powdery product containing hydrous crystalline trehalose was obtained in a yield of about 90 w/w % against the starting material, d.s.b.

[0090] The product, having a substantial non-hygroscopicity and a mild and high-quality sweetness, can be satisfactorily used in food products in general, cosmetics, pharmaceuticals and feeds as a sweetener, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

Example 8

Conversion of non-reducing saccharide by recombinant enzyme

[0091] Potato starch was suspended in water to give a concentration of 6 w/w %, d.s.b., and the suspension was admixed with 500 units/g starch of isoamylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, and enzymatically reacted for 20 hours. The reaction mixture was adjusted to a pH of 6.5, autoclaved at 120°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 95°C, admixed with 0.1 w/w % per g starch, d.s.b., of "TERMAMYL 60L", an α-amylase specimen commercialized by Novo Nordisk Bioindustri A/S, Copenhagen, Denmark, and enzymatically reacted for 15 min. The reaction mixture was heated at 130°C for 30 min to inactivate the remaining enzyme, rapidly cooled to 45°C, admixed with 4.1 units/g starch, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 7-1(a), and 4.9 units/g starch, d.s.b., of the present recombinant enzyme obtained by the method in Example 6, and enzymatically reacted for 64 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 55°C, adjusted to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", a glucoamylase specimen commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, and enzymatically reacted for 40 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion-exchange resin, and concentrated to obtain an about 60 w/w % syrupy product containing about 80.5 w/w % trehalose, d.s.b. The syrupy product was concentrated into an about 84 w/w % syrup which was then transferred to a crystallizer, admixed with an about 2 w/w % hydrous crystalline trehalose, d.s.b., and crystallized under gentle stirring

conditions to obtain a massecuite having a crystallinity of about 45 w/w %. The massecuite was distributed to plastic plain vessels which were then allowed to stand at ambient temperature for 3 days to effect solidification and aging, followed by detaching the resultant blocks from the vessels and pulverizing the blocks with a cutter to obtain a solid product containing hydrous crystalline trehalose in a yield of about 90 w/w % against the material starch, d.s.b.

[0092] The product, which is substantially free of hygroscopicity and readily handleable, can be arbitrarily used in food products in general, cosmetics, pharmaceuticals as a sweetening agent, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

Example 9

Conversion of non-reducing saccharide by recombinant enzyme

[0093] Potato starch was suspended in water to give a concentration of 6 w/w %, d.s.b., and the suspension was admixed with 0.01 w/w % "NEO-SPITASE", α-amylase commercialized by Nagase Biochemicals, Ltd., Kyoto, Japan, adjusted to pH 6.2, and enzymatically reacted at 85-90°C for 20 min to gelatinize and liquefy the starch. The liquefied starch was heated at 120°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 45°C, admixed with 500 units/g starch, d.s.b., of isoamylase commercialized by Hayashibara Biochemical Laboratories, Inc., Okayama, Japan, 3.2 units/g starch, d.s.b., of a non-reducing saccharide-forming enzyme obtained by the method in Example 7-1(a), and 5.0 units/g starch, d.s.b., of the present recombinant enzyme obtained by the method in Example 5, and enzymatically reacted for 48 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, rapidly cooled to 55°C, adjusted to pH 5.0, admixed with 10 units/g starch, d.s.b., of "GLUCOZYME", glucoamylase commercialized by Nagase Biochemicals Ltd., Kyoto, Japan, and enzymatically reacted for 40 hours. The reaction mixture was heated at 95°C for 10 min to inactivate the remaining enzyme, rapidly cooled, filtered, and, in usual manner, decolored with an activated charcoal, desalted and purified with an ion-exchange resin, and concentrated to give a concentration of about 60 w/w %, d.s.b., to obtain a syrupy product containing 78.3 w/w % trehalose, d.s.b. The syrupy product was fractionated similarly as in Example 7-1(c) except for using "CG6000(Na^+)", a strong-acid cation exchange resin commercialized by Japan Organo, Co., Ltd., Tokyo, Japan, to obtain a fraction containing abut 95 w/w % trehalose, d.s.b. The fraction was concentrated to give a concentration of about 75 w/w %, d.s.b., and, similarly as in Example 8, crystallized, and the resultant massecuite in the form of block was pulverized to obtain a powdery product containing hydrous crystalline trehalose in a yield of about 70 w/w % against the material starch, d.s.b.

[0094] The product, which is substantially free of hygroscopicity and readily handleable, can be arbitrarily used in food products in general, cosmetics, pharmaceuticals as a sweetening agent, taste-improving agent, quality-improving agent, stabilizer, filler, excipient and adjuvant.

[0095] As is described above, the present invention is based on the finding that a novel enzyme which releases trehalose from non-reducing saccharides having a trehalose structure as an end unit and having a degree of glucose polymerization of 3 or higher. The present invention is to explore a way to produce the enzyme in a relatively-large scale and in a considerably-high yield. The enzyme produced by the transformant according to the present invention is the one characterized by its revealed total amino acid sequence, and because of this it can be used for the preparations of trehalose which is premised on being used in food products without fear of causing side effects.

[0096] Therefore, the present invention is an useful invention which exerts the aforesaid significant action and effect as well as giving a great contribution to this field.

SEQUENCE LISTING

[0097]

(1) GENERAL INFORMATION:

(i) APPLICANT:
NAME:KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO

(ii) TITLE OF INVENTION:DNA ENCODING ENZYME, RECOMBINANT DNA AND ENZYME, TRANSFORMANT, AND THEIR PREPARATIONS AND USES

(iii) NUMBER OF SEQUENCES:20

(iv) ADDRESS:

(A) ADDRESSEE:KABUSHIKI KAISHA HAYASHIBARA SEIBUTSU KAGAKU KENKYUJO
(B) STREET:2-3, 1-CHOME, SHIMOISHII
(C) CITY:OKAYAMA
(E) COUNTRY:JAPAN
(F) POSTAL CODE (ZIP):700

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE:Floppy disk
(B) COMPUTER:IBM PC compatible
(C) OPERATING SYSTEM:PC-DOS/MS-DOS

(vii) PRIOR APPLICATION DATA:

(A1) APPLICATION NUMBER:JP 59840/94
(B1) FILING DATE:March 7, 1994
(A2) APPLICATION NUMBER:JP 59834/94
(B2) FILING DATE:March 7, 1994

(2)INFORMATION FOR SEQ ID NO:1:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:1767 base pairs
(B)TYPE:nucleic acid
(D)TOPOLOGY:linear

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:1:

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ACGCTGCTGG CCGGCGGGGA GCGCTACGAG ATGGGCCGCC GCCCCGGCAA CGGGCCGGCG  120
GACGAAGGCT GGTGGACGGC CGCGGATGCA CCGACAGGCG CGGACGTGGA CTACGGATAC  180
CTGCTCGACG GCGACGAAAT CCCGCTGCCG GACCCCCGGA CCCGCCGCCA GCCCGAAGGC  240
GTCCATGCCC TGTCCCGGAC CTTCGACCCC GGCGCCCACC GCTGGCAGGA CGCCGGGTGG  300
CAGGGCAGGG AACTCCAGGG CTCCGTGATT TACGAACTCC ACATCGGAAC GTTCACGCCG  360
GAAGGGACGC TGGACGCCGC CGCGGGCAAG CTGGACTACC TCGCCGGCCT GGGCATCGAC  420
TTCATTGAGC TGCTGCCCGT GAATGCCTTC AACGGCACGC ACAACTGGGG CTACGACGGC  480
GTCCAGTGGT TTGCCGTGCA TGAAGGCTAC GGCGGGCCTG CGGCGTACCA GCGGTTCGTG  540
GATGCGGCCC ACGCGGCCGG CCTCGGCGTC ATCCAGGACG TGGTCTACAA CCACCTCGGG  600
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TGGGGCGATT CGGTCAACCT GGACGGGCCG GGATCCGACC ACGTCCGCCA GTACATCCTG  720
GACAACGTGG CCATGTGGCT GCGCGACTAC CGGGTGGACG GCCTCCGCCT GGACGCCGTC  780
CACGCCCTGA AGGATGAGCG GGCCGTCCAC ATCCTGGAGG AGTTCGGCGC GCTGGCGGAC  840
GCCCTGTCGT CCGAAGGCGG CCGCCCGCTG ACCCTCATCG CCGAGTCCGA CCTCAACAAT  900
CCGCGGCTGC TGTACCCCCG GGATGTCAAC GGCTACGGAC TGGCCGGCCA GTGGAGCGAC  960
GACTTCCACC ACGCCGTGCA CGTCAACGTC AGCGGGGAAA CCACCGGCTA CTACAGCGAC 1020
TTCGACTCGC TCGGAGCCCT CGCCAAGGTC CTGCGTGACG GGTTCTTCCA CGACGGCAGC 1080
TACTCCAGCT TCCGCGGCCG CTGCCACGGC CGGCCGATCA ACTTCAGCGC CGTGCATCCG 1140
GCCGCGCTGG TGGTCTGCTC ACAGAACCAT GACCAGATCG GCAACCGGGC CACCGGGGAC 1200
CGGCTGTCCC AGTCACTTCC GTACGGCAGC CTGGCCCTGG CCGCCGTGCT GACCCTCACC 1260
GGTCCGTTCA CGCCCATGCT GTTCATGGGA GAGGAATACG GGGCCACCAC CCCGTGGCAG 1320
TTCTTCACCT CGCACCCTGA ACCCGAGCTG GGCAAGGCCA CGGCCGAGGG CAGGATCAGG 1380
GAGTTCGAGC GCATGGGGTG GGATCCCGCC GTCGTGCCCG ATCCGCAGGA TCCGGAGACC 1440
TTCACCCGCT CCAAACTGGA CTGGGCGGAA GCGTCCGCCG GCGATCATGC CCGCCTCCTG 1500
GAGCTGTACC GCTCGCTTAT CACGCTGCGG CGGTCAACTC CGGAGCTCGC GCGCCTGGGC 1560
TTTGCGGACA CCGCCGTCGA GTTCGACGAC GACGCCCGCT GGCTCCGTTA TTGGCGCGGA 1620
GGCGTGCAGG TGGTGCTGAA CTTCGCGGAC CGTCCCATCA GCCTGGACCG GCCGGGAACC 1680
GCGCTGCTGC TCGCCACCGA CGACGCCGTC CGGATGGACG GAGTCCAGGT GGAGCTGCCG 1740
CCGCTGAGCG CCGCGGTTCT GCGCGAC                                      1767
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(3)INFORMATION FOR SEQ ID NO:2:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:589
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:2:

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            20                  25                  30
Pro Gly Asn Gly Pro Ala Asp Glu Gly Trp Trp Thr Ala Ala Asp Ala Pro
        35                  40                  45                  50
Thr Gly Ala Asp Val Asp Tyr Gly Tyr Leu Leu Asp Gly Asp Glu Ile Pro
                55                  60                  65
Leu Pro Asp Pro Arg Thr Arg Arg Gln Pro Glu Gly Val His Ala Leu Ser
    70                  75                  80                  85
Arg Thr Phe Asp Pro Gly Ala His Arg Trp Gln Asp Ala Gly Trp Gln Gly
                90                  95                  100
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Arg Glu Leu Gln Gly Ser Val Ile Tyr Glu Leu His Ile Gly Thr Phe Thr
            105                 110                 115
Pro Glu Gly Thr Leu Asp Ala Ala Ala Gly Lys Leu Asp Tyr Leu Ala Gly
120                 125                 130                 135
Leu Gly Ile Asp Phe Ile Glu Leu Leu Pro Val Asn Ala Phe Asn Gly Thr
                140                 145                 150
His Asn Trp Gly Tyr Asp Gly Val Gln Trp Phe Ala Val His Glu Gly Tyr
    155                 160                 165                 170
Gly Gly Pro Ala Ala Tyr Gln Arg Phe Val Asp Ala Ala His Ala Ala Gly
                175                 180                 185
Leu Gly Val Ile Gln Asp Val Val Tyr Asn His Leu Gly Pro Ser Gly Asn
            190                 195                 200
Tyr Leu Pro Arg Tyr Gly Pro Tyr Leu Lys His Gly Glu Gly Asn Thr Trp
205                 210                 215                 220
Gly Asp Ser Val Asn Leu Asp Gly Pro Gly Ser Asp His Val Arg Gln Tyr
                225                 230                 235
Ile Leu Asp Asn Val Ala Met Trp Leu Arg Asp Tyr Arg Val Asp Gly Leu
    240                 245                 250                 255
Arg Leu Asp Ala Val His Ala Leu Lys Asp Glu Arg Ala Val His Ile Leu
                260                 265                 270
Glu Glu Phe Gly Ala Leu Ala Asp Ala Leu Ser Ser Glu Gly Gly Arg Pro
            275                 280                 285
Leu Thr Leu Ile Ala Glu Ser Asp Leu Asn Asn Pro Arg Leu Leu Tyr Pro
290                 295                 300                 305
Arg Asp Val Asn Gly Tyr Gly Leu Ala Gly Gln Trp Ser Asp Asp Phe His
                310                 315                 320
His Ala Val His Val Asn Val Ser Gly Glu Thr Thr Gly Tyr Tyr Ser Asp
    325                 330                 335                 340
Phe Asp Ser Leu Gly Ala Leu Ala Lys Val Leu Arg Asp Gly Phe Phe His
                345                 350                 355
Asp Gly Ser Tyr Ser Ser Phe Arg Gly Arg Cys His Gly Arg Pro Ile Asn
            360                 365                 370
Phe Ser Ala Val His Pro Ala Ala Leu Val Val Cys Ser Gln Asn His Asp
375                 380                 385                 390
Gln Ile Gly Asn Arg Ala Thr Gly Asp Arg Leu Ser Gln Ser Leu Pro Tyr
                395                 400                 405
Gly Ser Leu Ala Leu Ala Ala Val Leu Thr Leu Thr Gly Pro Phe Thr Pro
    410                 415                 420                 425
Met Leu Phe Met Gly Glu Glu Tyr Gly Ala Thr Thr Pro Trp Gln Phe Phe
                430                 435                 440
Thr Ser His Pro Glu Pro Glu Leu Gly Lys Ala Thr Ala Glu Gly Arg Ile
            445                 450                 455
Arg Glu Phe Glu Arg Met Gly Trp Asp Pro Ala Val Val Pro Asp Pro Gln
460                 465                 470                 475
Asp Pro Glu Thr Phe Thr Arg Ser Lys Leu Asp Trp Ala Glu Ala Ser Ala
                480                 485                 490
Gly Asp His Ala Arg Leu Leu Glu Leu Tyr Arg Ser Leu Ile Thr Leu Arg
    495                 500                 505                 510
Arg Ser Thr Pro Glu Leu Ala Arg Leu Gly Phe Ala Asp Thr Ala Val Glu
                515                 520                 525
Phe Asp Asp Asp Ala Arg Trp Leu Arg Tyr Trp Arg Gly Gly Val Gln Val
            530                 535                 540
Val Leu Asn Phe Ala Asp Arg Pro Ile Ser Leu Asp Arg Pro Gly Thr Ala
545                 550                 555                 560
Leu Leu Leu Ala Thr Asp Asp Ala Val Arg Met Asp Gly Val Gln Val Glu
                565                 570                 575
Leu Pro Pro Leu Ser Ala Ala Val Leu Arg Asp
    580                 585
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(4)INFORMATION FOR SEQ ID NO:3:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:1791 base pairs
(B)TYPE:nucleic acid
(D)TOPOLOGY:linear

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:3:

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TGGGCGCCCA ACGCTGAATC CGTGACGCTG CTGGCCGGCG GGGAGCGCTA CGCCATGCAG 120

CGCCGGGCCG AGACCGGGCC GGAGGACGCC GGCTGGTGGA CCGCCGCCGG CGCGCCTACG 180

GATGGCAACG TGGACTACGG GTACCTTCTG GACGGCGACG AAACACCGCT TCCGGATCCA 240

CGGACCCGCC GCCAGCCCGA CGGCGTCCAC GCCCTGTCCC GCACGTTCGA CCCGTCCGCG 300

TACAGCTGGC AGGACGACGC CTGGCAGGGC AGGGAACTGC AGGGCGCCGT CATCTACGAG 360

CTCCACCTCG GAACATTCAC GCCCGAAGGG ACGCTGGAGG CGGCCGCCGG AAAGCTGGAC 420

TACCTCGCCG GCTTGGGCGT CGACTTCATC GAGCTGCTGC CGGTGAACGC TTTCAACGGC 480

ACGCACAACT GGGGTTACGA CGGTGTCCAG TGGTTCGCTG TGCACGAGGC ATACGGCGGG 540

CCGGAAGCGT ACCAGCGGTT CGTCGACGCC GCCCACGCCG CAGGCCTTGG CGTGATCCAG 600

GACGTGGTCT ACAACCACCT CGGCCCCAGC GGGAACTACC TGCCGCGGTT CGGGCCGTAC 660

CTCAAGCAGG GCGAGGGTAA CACGTGGGGC GACTCGGTGA ACCTGGACGG GCCCGGCTCC 720

GACCATGTGC GCCGGTACAT CCTGGACAAC CTGGCCATGT GGCTGCGTGA CTACCGGGTG 780

GACGGCCTGC GGCTGGACGC CGTCCACGCC CTGAAGGATG AGCGGGCGGT GCACATCCTG 840

GAGGACTTCG GGGCGCTGGC CGATCAGATC TCCGCCGAGG TGGGACGGCC GCTGACGCTC 900

ATCGCCGAGT CCGACCTCAA CAACCCGCGG CTGCTGTACC CGCGGGACGT CAACGGGTAC 960

GGGCTGGAAG GGCAGTGGAG CGACGACTTC CACCACGCCG TCCACGTCAA CGTCACCGGC 1020

GAAACCACCG GCTACTACAG TGACTTCGAC TCGCTGGCCG CCCTCGCCAA GGTGCTCCGG 1080

GACGGCTTCT TCCACGACGG CAGCTACTCC AGCTTCCGGG AACGCCACCA CGGACGGCCG 1140

ATTAATTTCA GCGCCGTACA CCCAGCCGCC CTGGTGGTCT GTTCGCAGAA CCACGACCAG 1200

ATCGGCAACC GTGCCACGGG GGACCGGCTC TCCCAGACCC TGCCGTACGG AAGCCTGGCC 1260

CTCGCTGCGG TGCTGACCCT GACGGGACCC TTCACGCCCA TGCTGCTCAT GGGCGAGGAG 1320

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CCCGATCCCC AGGATCCTGA GACGTTCCGC CGGTCCAAGC TGGACTGGGC GGAAGCCGCC 1500

GAAGGCGACC ATGCCCGGCT GCTGGAGCTG TACCGTTCGC TCACCGCCCT GCGCCGCTCC 1560

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CGCTGGCTGC GGTTCCGCCG GGGTGGCGTG CAGGTGCTGC TCAACTTCTC GGAACAGCCC 1680

GTGAGCCTGG ACGGGGCGGG CACGGCCCTG CTGCTGGCCA CCGACGACGC CGTCCGGCTA 1740

GAAGGTGAGC GTGCGGAACT CGGTCCGCTG AGCGCCGCCG TCGTCAGCGA C 1791

(5)INFORMATION FOR SEQ ID NO:4:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:597
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:4:

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            20                  25                  30
Glu Arg Tyr Ala Met Gln Arg Arg Ala Glu Thr Gly Pro Glu Asp Ala Gly
        35                  40                  45                  50
Trp Trp Thr Ala Ala Gly Ala Pro Thr Asp Gly Asn Val Asp Tyr Gly Tyr
                    55                  60                  65
Leu Leu Asp Gly Asp Glu Thr Pro Leu Pro Asp Pro Arg Thr Arg Arg Gln
    70                  75                  80                  85
Pro Asp Gly Val His Ala Leu Ser Arg Thr Phe Asp Pro Ser Ala Tyr Ser
                90                  95                  100
Trp Gln Asp Asp Ala Trp Gln Gly Arg Glu Leu Gln Gly Ala Val Ile Tyr
            105                 110                 115
Glu Leu His Leu Gly Thr Phe Thr Pro Glu Gly Thr Leu Glu Ala Ala Ala
        120                 125                 130                 135
Gly Lys Leu Asp Tyr Leu Ala Gly Leu Gly Val Asp Phe Ile Glu Leu Leu
                    140                 145                 150
Pro Val Asn Ala Phe Asn Gly Thr His Asn Trp Gly Tyr Asp Gly Val Gln
    155                 160                 165                 170
Trp Phe Ala Val His Glu Asp Tyr Gly Gly Pro Glu Ala Tyr Gln Arg Phe
                175                 180                 185
Val Asp Ala Ala His Ala Ala Gly Leu Gly Val Ile Gln Asp Val Val Tyr
            190                 195                 200
Asn His Leu Gly Pro Ser Gly Asn Tyr Leu Pro Arg Phe Gly Pro Tyr Leu
        205                 210                 215                 220
Lys Gln Gly Glu Gly Asn Thr Trp Gly Asp Ser Val Asn Leu Asp Gly Pro
                    225                 230                 235
Gly Ser Asp His Val Arg Arg Tyr Ile Leu Asp Asn Leu Ala Met Trp Leu
    240                 245                 250                 255
Arg Asp Tyr Arg Val Asp Gly Leu Arg Leu Asp Ala Val His Ala Leu Lys
                260                 265                 270
Asp Glu Arg Ala Val His Ile Leu Glu Asp Phe Gly Ala Leu Ala Asp Gln
            275                 280                 285
Ile Ser Ala Glu Val Gly Arg Pro Leu Thr Leu Ile Ala Glu Ser Asp Leu
290                 295                 300                 305
Asn Asn Pro Arg Leu Leu Tyr Pro Arg Asp Val Asn Gly Tyr Gly Leu Glu
                310                 315                 320
Gly Gln Trp Ser Asp Asp Phe His His Ala Val His Val Asn Val Thr Gly
    325                 330                 335                 340
Glu Thr Thr Gly Tyr Tyr Ser Asp Phe Asp Ser Leu Ala Ala Leu Ala Lys
                345                 350                 355
Val Leu Arg Asp Gly Phe Phe His Asp Gly Ser Tyr Ser Ser Phe Arg Glu
            360                 365                 370
Arg His His Gly Arg Pro Ile Asn Phe Ser Ala Val His Pro Ala Ala Leu
375                 380                 385                 390
Val Val Cys Ser Gln Asn His Asp Gln Ile Gly Asn Arg Ala Thr Gly Asp
                395                 400                 405
Arg Leu Ser Gln Thr Leu Pro Tyr Gly Ser Leu Ala Leu Ala Ala Val Leu
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        445                 450                 455
Lys Ala Thr Ala Glu Gly Arg Ile Lys Glu Phe Glu Arg Met Gly Trp Asp
460                 465                 470                 475
Pro Ala Val Val Pro Asp Pro Gln Asp Pro Glu Thr Phe Arg Arg Ser Lys
                480                 485                 490
Leu Asp Trp Ala Glu Ala Ala Glu Gly Asp His Ala Arg Leu Leu Glu Leu
    495                 500                 505                 510
Tyr Arg Ser Leu Thr Ala Leu Arg Arg Ser Thr Pro Asp Leu Thr Lys Leu
                515                 520                 525
Gly Phe Glu Asp Thr Gln Val Ala Phe Asp Glu Asp Ala Arg Trp Leu Arg
        530                 535                 540
Phe Arg Arg Gly Gly Val Gln Val Leu Leu Asn Phe Ser Glu Gln Pro Val
545                 550                 555                 560
Ser Leu Asp Gly Ala Gly Thr Ala Leu Leu Leu Ala Thr Asp Asp Ala Val
                565                 570                 575
Arg Leu Glu Gly Glu Arg Ala Glu Leu Gly Pro Leu Ser Ala Ala Val Val
    580                 585                 590                 595
Ser Asp
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(6)INFORMATION FOR SEQ ID NO:5:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:20
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:N-terminal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:5:

```
Ala Lys Pro Val Gln Gly Ala Gly Arg Phe Asp Ile Trp Ala Pro Glu Ala
1               5                   10                  15
Gly Thr Val
        20
```

(7)INFORMATION FOR SEQ ID NO:6:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:20
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:N-terminal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:6:

```
Thr His Thr Tyr Pro Arg Glu Ala Ala Lys Pro Val Leu Gly Pro Ala Arg
1               5                   10                  15
Tyr Asp Val
        20
```

(8)INFORMATION FOR SEQ ID NO:7:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:21
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:9:

```
Pro Val Gln Gly Ala Gly Arg Phe Asp Ile Trp Ala Pro Glu Ala Gly Thr
1               5                   10                  15
Val Thr Leu Leu
        20
```

(9)INFORMATION FOR SEQ ID NO:8:

(i)SEQUENCE CHARACTERISTICS:

(A) LENGTH:17
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:8:

```
Leu Asp Trp Ala Glu Ala Ser Ala Gly Asp His Ala Arg Leu Leu Glu Leu
1               5                   10                  15
```

(10)INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A)LENGTH:20
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(v) FRAGMENT TYPE:internal fragment
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:9:

```
Glu Phe Glu Arg Met Gly Trp Asp Pro Ala Val Val Pro Asp Pro Gln Asp
1               5                   10                  15
Pro Glu Thr
        20
```

(11)INFORMATION FOR SEQ ID NO:10:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:20
(B)TYPE:amino acid

(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(v)FRAGMENT TYPE:internal fragment
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:10:

```
Pro Val Leu Gly Pro Ala Arg Tyr Asp Val Trp Ala Pro Asn Ala Glu Ser
1               5                   10                  15
Val Thr Leu
        20
```

(12)INFORMATION FOR SEQ ID NO:11:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:2161 base pairs
(B)TYPE:nucleic acid
(C)strandedness:double
(D)TOPOLOGY:linear

(ii) MOLECULE TYPE:genomic DNA
(vi)ORIGINAL SOURCE:

(A)ORGANISM:Rhizobium sp.
(B)INDIVIDUAL ISOLATE:M-11 (FERM BP-4130)

(ix)FEATURE:

(A) NAME/KEY:5'UTR
(B)LOCATION:1..206
(C)IDENTIFICATION METHOD:E
(A)NAME/KEY:mat peptide
(B)LOCATION:207..1994
(C)IDENTIFICATION METHOD:S
(A)NAME/KEY:3'UTR
(B) LOCATION:1995..2161
(C)IDENTIFICATION METHOD:E

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:11:

```
GGCGCCGGGG GAGTGCTGGC GCTTGCCACC CGGCTCCCCT ACGGGCTGGA ACAGTCGGGC   60

GGCTGGCGGG ACACCGCCGT CGAGCTTGAA GCCGCCATGA CGGACGAACT GACCGGCTCC  120

ACTTTCGGGC CGGGACCGGC GGCGCTGTCA GAAGTCTTCC GGGCCTACCC GGTGGCCTTG  180

TTGGTCCCCG CGACAGGAGG CAAGTC                                       206

ATG ACG CAG CCC AAC GAT GCG GCC AAG CCG GTG CAG GGA GCG GGG CGC     254
Met Thr Gln Pro Asn Asp Ala Ala Lys Pro Val Gln Gly Ala Gly Arg
1               5                  10                  15
TTC GAT ATC TGG GCG CCC GAG GCA GGC ACC GTA ACG CTG CTG GCC GGC     302
Phe Asp Ile Trp Ala Pro Glu Ala Gly Thr Val Thr Leu Leu Ala Gly
            20                  25                  30
GGG GAG CGC TAC GAG ATG GGC CGC CGC CCC GGC AAC GGG CCG GCG GAC     350
Gly Glu Arg Tyr Glu Met Gly Arg Arg Pro Gly Asn Gly Pro Ala Asp
        35                  40                  45
GAA GGC TGG TGG ACG GCC GCG GAT GCA CCG ACA GGC GCG GAC GTG GAC     398
Glu Gly Trp Trp Thr Ala Ala Asp Ala Pro Thr Gly Ala Asp Val Asp
    50                  55                  60
TAC GGA TAC CTG CTC GAC GGC GAC GAA ATC CCG CTG CCG GAC CCC CGG     446
Tyr Gly Tyr Leu Leu Asp Gly Asp Glu Ile Pro Leu Pro Asp Pro Arg
65                  70                  75                  80
ACC CGC CGC CAG CCC GAA GGC GTC CAT GCC CTG TCC CGG ACC TTC GAC     494
Thr Arg Arg Gln Pro Glu Gly Val His Ala Leu Ser Arg Thr Phe Asp
                85                  90                  95
CCC GGC GCC CAC CGC TGG CAG GAC GCC GGG TGG CAG GGC AGG GAA CTC     542
Pro Gly Ala His Arg Trp Gln Asp Ala Gly Trp Gln Gly Arg Glu Leu
            100                 105                 110
CAG GGC TCC GTG ATT TAC GAA CTC CAC ATC GGA ACG TTC ACG CCG GAA     590
Gln Gly Ser Val Ile Tyr Glu Leu His Ile Gly Thr Phe Thr Pro Glu
        115                 120                 125
GGG ACG CTG GAC GCC GCC GCG GGC AAG CTG GAC TAC CTC GCC GGC CTG     638
Gly Thr Leu Asp Ala Ala Ala Gly Lys Leu Asp Tyr Leu Ala Gly Leu
    130                 135                 140
GGC ATC GAC TTC ATT GAG CTG CTG CCC GTG AAT GCC TTC AAC GGC ACG     686
Gly Ile Asp Phe Ile Glu Leu Leu Pro Val Asn Ala Phe Asn Gly Thr
145                 150                 155                 160
CAC AAC TGG GGC TAC GAC GGC GTC CAG TGG TTT GCC GTG CAT GAA GGC     734
His Asn Trp Gly Tyr Asp Gly Val Gln Trp Phe Ala Val His Glu Gly
                165                 170                 175
TAC GGC GGG CCT GCG GCG TAC CAG CGG TTC GTG GAT GCG GCC CAC GCG     782
```

```
Tyr Gly Gly Pro Ala Ala Tyr Gln Arg Phe Val Asp Ala Ala His Ala
                180                 185                 190
GCC GGC CTC GGC GTC ATC CAG GAC GTG GTC TAC AAC CAC CTC GGG CCG    830
Ala Gly Leu Gly Val Ile Gln Asp Val Val Tyr Asn His Leu Gly Pro
        195                 200                 205
AGC GGG AAC TAC CTC CCC AGG TAC GGC CCG TAC CTC AAG CAC GGC GAA    878
Ser Gly Asn Tyr Leu Pro Arg Tyr Gly Pro Tyr Leu Lys His Gly Glu
    210                 215                 220
GGC AAC ACC TGG GGC GAT TCG GTC AAC CTG GAC GGG CCG GGA TCC GAC    926
Gly Asn Thr Trp Gly Asp Ser Val Asn Leu Asp Gly Pro Gly Ser Asp
225                 230                 235                 240
CAC GTC CGC CAG TAC ATC CTG GAC AAC GTG GCC ATG TGG CTG CGC GAC    974
His Val Arg Gln Tyr Ile Leu Asp Asn Val Ala Met Trp Leu Arg Asp
                245                 250                 255
TAC CGG GTG GAC GGC CTC CGC CTG GAC GCC GTC CAC GCC CTG AAG GAT   1022
Tyr Arg Val Asp Gly Leu Arg Leu Asp Ala Val His Ala Leu Lys Asp
            260                 265                 270
GAG CGG GCC GTC CAC ATC CTG GAG GAG TTC GGC GCG CTG GCG GAC GCC   1070
Glu Arg Ala Val His Ile Leu Glu Glu Phe Gly Ala Leu Ala Asp Ala
        275                 280                 285
CTG TCG TCC GAA GGC GGC CGC CCG CTG ACC CTC ATC GCC GAG TCC GAC   1118
Leu Ser Ser Glu Gly Gly Arg Pro Leu Thr Leu Ile Ala Glu Ser Asp
    290                 295                 300
CTC AAC AAT CCG CGG CTG CTG TAC CCC CGG GAT GTC AAC GGC TAC GGA   1166
Leu Asn Asn Pro Arg Leu Leu Tyr Pro Arg Asp Val Asn Gly Tyr Gly
305                 310                 315                 320
CTG GCC GGC CAG TGG AGC GAC GAC TTC CAC CAC GCC GTG CAC GTC AAC   1214
Leu Ala Gly Gln Trp Ser Asp Asp Phe His His Ala Val His Val Asn
                325                 330                 335
GTC AGC GGG GAA ACC ACC GGC TAC TAC AGC GAC TTC GAC TCG CTC GGA   1262
Val Ser Gly Glu Thr Thr Gly Tyr Tyr Ser Asp Phe Asp Ser Leu Gly
            340                 345                 350
GCC CTC GCC AAG GTC CTG CGT GAC GGG TTC TTC CAC GAC GGC AGC TAC   1310
Ala Leu Ala Lys Val Leu Arg Asp Gly Phe Phe His Asp Gly Ser Tyr
        355                 360                 365
TCC AGC TTC CGC GGC CGC TGC CAC GGC CGG CCG ATC AAC TTC AGC GCC   1358
Ser Ser Phe Arg Gly Arg Cys His Gly Arg Pro Ile Asn Phe Ser Ala
    370                 375                 380
GTG CAT CCG GCC GCG CTG GTG GTC TGC TCA CAG AAC CAT GAC CAG ATC   1406
Val His Pro Ala Ala Leu Val Val Cys Ser Gln Asn His Asp Gln Ile
385                 390                 395                 400
GGC AAC CGG GCC ACC GGG GAC CGG CTG TCC CAG TCA CTT CCG TAC GGC   1454
Gly Asn Arg Ala Thr Gly Asp Arg Leu Ser Gln Ser Leu Pro Tyr Gly
                405                 410                 415
AGC CTG GCC CTG GCC GCC GTG CTG ACC CTC ACC GGT CCG TTC ACG CCC   1502
Ser Leu Ala Leu Ala Ala Val Leu Thr Leu Thr Gly Pro Phe Thr Pro
            420                 425                 430
ATG CTG TTC ATG GGA GAG GAA TAC GGG GCC ACC ACC CCG TGG CAG TTC   1550
Met Leu Phe Met Gly Glu Glu Tyr Gly Ala Thr Thr Pro Trp Gln Phe
        435                 440                 445
TTC ACC TCG CAC CCT GAA CCC GAG CTG GGC AAG GCC ACG GCC GAG GGC   1598
Phe Thr Ser His Pro Glu Pro Glu Leu Gly Lys Ala Thr Ala Glu Glu
    450                 455                 460
AGG ATC AGG GAG TTC GAG CGC ATG GGG TGG GAT CCC GCC GTC GTG CCC   1646
Arg Ile Arg Glu Phe Glu Arg Met Gly Trp Asp Pro Ala Val Val Pro
465                 470                 475                 480
GAT CCG CAG GAT CCG GAG ACC TTC ACC CGC TCC AAA CTG GAC TGG GCG   1694
Asp Pro Gln Asp Pro Glu Thr Phe Thr Arg Ser Lys Leu Asp Trp Ala
                485                 490                 495
GAA GCG TCC GCC GGC GAT CAT GCC CGC CTC CTG GAG CTG TAC CGC TCG   1742
Glu Ala Ser Ala Gly Asp His Ala Arg Leu Leu Glu Leu Tyr Arg Ser
            500                 505                 510
CTT ATC ACG CTG CGG CGG TCA ACT CCG GAG CTC GCG CGC CTG GGC TTT   1790
```

```
Leu Ile Thr Leu Arg Arg Ser Thr Pro Glu Leu Ala Arg Leu Gly Phe
        515                 520                 525
GCG GAC ACC GCC GTC GAG TTC GAC GAC GAC GCC CGC TGG CTC CGT TAT   1838
Ala Asp Thr Ala Val Glu Phe Asp Asp Asp Ala Arg Trp Leu Arg Tyr
    530                 535                 540
TGG CGC GGA GGC GTG CAG GTG GTG CTG AAC TTC GCG GAC CGT CCC ATC   1886
Trp Arg Gly Gly Val Gln Val Val Leu Asn Phe Ala Asp Arg Pro Ile
545                 550                 555                 560
AGC CTG GAC CGG CCG GGA ACC GCG CTG CTG CTC GCC ACC GAC GAC GCC   1934
Ser Leu Asp Arg Pro Gly Thr Ala Leu Leu Leu Ala Thr Asp Asp Ala
                565                 570                 575
GTC CGG ATG GAC GGA GTC CAG GTG GAG CTG CCG CCG CTG AGC GCC GCG   1982
Val Arg Met Asp Gly Val Gln Val Glu Leu Pro Pro Leu Ser Ala Ala
            580                 585                 590
GTT CTG CGC GAC                                                  1994
Val Leu Arg Asp
        595
TGAGCGTGCG CGCCTTCGGG GCGGGCGTCC TTCCGGTGAC CGGATGCTGG ACGCCCGCCC 2054

CGCAGCTCCA CAGGCGCTGG CAGGATGGAA CGTATGACTT TTCTGGCAGC GGACAACCGC 2114

TACGAAACCA TGCCATACCG CCGCGTCGGA CGCAGCGGGC TGAAGCT               2161
```

(13)INFORMATION FOR SEQ ID NO:12:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:2056 base pairs
(B)TYPE:nucleic acid
(C)strandedness:double
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:genomic DNA
(vi)ORIGINAL SOURCE:

(A)ORGANISM:Arthrobacter sp.
(B)INDIVIDUAL ISOLATE:Q36 (FERM BP-4316)

(ix)FEATURE:

(A) NAME/KEY:5'UTR
(B)LOCATION:1..89
(C)IDENTIFICATION METHOD:E
(A)NAME/KEY:mat peptide
(B)LOCATION:90..1883
(C)IDENTIFICATION METHOD:S
(A)NAME/KEY:3'UTR
(B) LOCATION:1884..2056
(C)IDENTIFICATION METHOD:E

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:12:

```
GCCGGCTTCG GACCGGGGGC AGTGAAGATC GCCGACATCT TCCGGTCGTT CCCCGTTGCG   60

CTGCTGGTGC CGCAGACAGG AGGAGAGTC                                     89

ATG ACG CAC ACC TAC CCG CGG GAA GCC GCG AAA CCC GTC CTG GGC CCC    137
Met Thr His Thr Tyr Pro Arg Glu Ala Ala Lys Pro Val Leu Gly Pro
1               5                  10                  15
GCA CGC TAC GAC GTC TGG GCG CCC AAC GCT GAA TCC GTG ACG CTG CTG    185
Ala Arg Tyr Asp Val Trp Ala Pro Asn Ala Glu Ser Val Thr Leu Leu
            20                  25                  30
GCC GGC GGG GAG CGC TAC GCC ATG CAG CGC CGG GCC GAG ACC GGG CCG    233
Ala Gly Gly Glu Arg Tyr Ala Met Gln Arg Arg Ala Glu Thr Gly Pro
        35                  40                  45
GAG GAC GCC GGC TGG TGG ACC GCC GCC GGC GCG CCT ACG GAT GGC AAC    281
Glu Asp Ala Gly Trp Trp Thr Ala Ala Gly Ala Pro Thr Asp Gly Asn
```

```
     50                  55                  60
GTG GAC TAC GGG TAC CTT CTG GAC GGC GAC GAA ACA CCG CTT CCG GAT    329
Val Asp Tyr Gly Tyr Leu Leu Asp Gly Asp Glu Thr Pro Leu Pro Asp
65                  70                  75                  80
CCA CGG ACC CGC CGC CAG CCC GAC GGC GTC CAC GCC CTG TCC CGC ACG    377
Pro Arg Thr Arg Arg Gln Pro Asp Gly Val His Ala Leu Ser Arg Thr
                85                  90                  95
TTC GAC CCG TCC GCG TAC AGC TGG CAG GAC GAC GCC TGG CAG GGC AGG    425
Phe Asp Pro Ser Ala Tyr Ser Trp Gln Asp Asp Ala Trp Gln Gly Arg
            100                 105                 110
GAA CTG CAG GGC GCC GTC ATC TAC GAG CTC CAC CTC GGA ACA TTC ACG    473
Glu Leu Gln Gly Ala Val Ile Tyr Glu Leu His Leu Gly Thr Phe Thr
        115                 120                 125
CCC GAA GGG ACG CTG GAG GCG GCC GCC GGA AAG CTG GAC TAC CTC GCC    521
Pro Glu Gly Thr Leu Glu Ala Ala Ala Gly Lys Leu Asp Tyr Leu Ala
    130                 135                 140
GGC TTG GGC GTC GAC TTC ATC GAG CTG CTG CCG GTG AAC GCT TTC AAC    569
Gly Leu Gly Val Asp Phe Ile Glu Leu Leu Pro Val Asn Ala Phe Asn
145                 150                 155                 160
GGC ACG CAC AAC TGG GGT TAC GAC GGT GTC CAG TGG TTC GCT GTG CAC    617
Gly Thr His Asn Trp Gly Tyr Asp Gly Val Gln Trp Phe Ala Val His
                165                 170                 175
GAG GCA TAC GGC GGG CCG GAA GCG TAC CAG CGG TTC GTC GAC GCC GCC    665
Glu Asp Tyr Gly Gly Pro Glu Ala Tyr Gln Arg Phe Val Asp Ala Ala
            180                 185                 190
CAC GCC GCA GGC CTT GGC GTG ATC CAG GAC GTG GTC TAC AAC CAC CTC    713
His Ala Ala Gly Leu Gly Val Ile Gln Asp Val Val Tyr Asn His Leu
        195                 200                 205
GGC CCC AGC GGG AAC TAC CTG CCG CGG TTC GGG CCG TAC CTC AAG CAG    761
Gly Pro Ser Gly Asn Tyr Leu Pro Arg Phe Gly Pro Tyr Leu Lys Gln
    210                 215                 220
GGC GAG GGT AAC ACG TGG GGC GAC TCG GTG AAC CTG GAC GGG CCC GGC    809
Gly Glu Gly Asn Thr Trp Gly Asp Ser Val Asn Leu Asp Gly Pro Gly
225                 230                 235                 240
TCC GAC CAT GTG CGC CGG TAC ATC CTG GAC AAC CTG GCC ATG TGG CTG    857
Ser Asp His Val Arg Arg Tyr Ile Leu Asp Asn Leu Ala Met Trp Leu
                245                 250                 255
CGT GAC TAC CGG GTG GAC GGC CTG CGG CTG GAC GCC GTC CAC GCC CTG    905
Arg Asp Tyr Arg Val Asp Gly Leu Arg Leu Asp Ala Val His Ala Leu
            260                 265                 270
AAG GAT GAG CGG GCG GTG CAC ATC CTG GAG GAC TTC GGG GCG CTG GCC    953
Lys Asp Glu Arg Ala Val His Ile Leu Glu Asp Phe Gly Ala Leu Ala
        275                 280                 285
GAT CAG ATC TCC GCC GAG GTG GGA CGG CCG CTG ACG CTC ATC GCC GAG   1001
Asp Gln Ile Ser Ala Glu Val Gly Arg Pro Leu Thr Leu Ile Ala Glu
    290                 295                 300
TCC GAC CTC AAC AAC CCG CGG CTG CTG TAC CCG CGG GAC GTC AAC GGG   1049
Ser Asp Leu Asn Asn Pro Arg Leu Leu Tyr Pro Arg Asp Val Asn Gly
305                 310                 315                 320
TAC GGG CTG GAA GGG CAG TGG AGC GAC GAC TTC CAC CAC GCC GTC CAC   1097
Tyr Gly Leu Glu Gly Gln Trp Ser Asp Asp Phe His His Ala Val His
                325                 330                 335
GTC AAC GTC ACC GGC GAA ACC ACC GGC TAC TAC AGT GAC TTC GAC TCG   1145
Val Asn Val Thr Gly Glu Thr Thr Gly Tyr Tyr Ser Asp Phe Asp Ser
            340                 345                 350
CTG GCC GCC CTC GCC AAG GTG CTC CGG GAC GGC TTC TTC CAC GAC GGC   1193
Leu Ala Ala Leu Ala Lys Val Leu Arg Asp Gly Phe Phe His Asp Gly
        355                 360                 365
AGC TAC TCC AGC TTC CGG GAA CGC CAC CAC GGA CGG CCG ATT AAT TTC   1241
Ser Tyr Ser Ser Phe Arg Glu Arg His His Gly Arg Pro Ile Asn Phe
    370                 375                 380
AGC GCC GTA CAC CCA GCC GCC CTG GTG GTC TGT TCG CAG AAC CAC GAC   1289
Ser Ala Val His Pro Ala Ala Leu Val Val Cys Ser Gln Asn His Asp
```

```
385                 390                 395                 400
CAG ATC GGC AAC CGT GCC ACG GGG GAC CGG CTC TCC CAG ACC CTG CCG   1337
Gln Ile Gly Asn Arg Ala Thr Gly Asp Arg Leu Ser Gln Thr Leu Pro
                405                 410                 415
TAC GGA AGC CTG GCC CTC GCT GCG GTG CTG ACC CTG ACG GGA CCC TTC   1385
Tyr Gly Ser Leu Ala Leu Ala Ala Val Leu Thr Leu Thr Gly Pro Phe
            420                 425                 430
ACG CCC ATG CTG CTC ATG GGC GAG GAG TAC GGC GCC AGC ACG CCG TGG   1433
Thr Pro Met Leu Leu Met Gly Glu Glu Tyr Gly Ala Ser Thr Pro Trp
        435                 440                 445
CAG TTT TTC ACC TCG CAC CCG GAG CCG GAG CTC GGC AAG GCC ACC GCG   1481
Gln Phe Phe Thr Ser His Pro Glu Pro Glu Leu Gly Lys Ala Thr Ala
    450                 455                 460
GAG GGC CGG ATC AAG GAG TTC GAG CGC ATG GGG TGG GAT CCC GCC GTC   1529
Glu Gly Arg Ile Lys Glu Phe Glu Arg Met Gly Trp Asp Pro Ala Val
465                 470                 475                 480
GTG CCC GAT CCC CAG GAT CCT GAG ACG TTC CGC CGG TCC AAG CTG GAC   1577
Val Pro Asp Pro Gln Asp Pro Glu Thr Phe Arg Arg Ser Lys Leu Asp
                485                 490                 495
TGG GCG GAA GCC GCC GAA GGC GAC CAT GCC CGG CTG CTG GAG CTG TAC   1625
Trp Ala Glu Ala Ala Glu Gly Asp His Ala Arg Leu Leu Glu Leu Tyr
            500                 505                 510
CGT TCG CTC ACC GCC CTG CGC CGC TCC ACG CCG GAC CTC ACC AAG CTG   1673
Arg Ser Leu Thr Ala Leu Arg Arg Ser Thr Pro Asp Leu Thr Lys Leu
        515                 520                 525
GGC TTC GAG GAC ACG CAG GTG GCG TTC GAC GAG GAC GCC CGC TGG CTG   1721
Gly Phe Glu Asp Thr Gln Val Ala Phe Asp Glu Asp Ala Arg Trp Leu
    530                 535                 540
CGG TTC CGC CGG GGT GGC GTG CAG GTG CTG CTC AAC TTC TCG GAA CAG   1769
Arg Phe Arg Arg Gly Gly Val Gln Val Leu Leu Asn Phe Ser Glu Gln
545                 550                 555                 560
CCC GTG AGC CTG GAC GGG GCG GGC ACG GCC CTG CTG CTG GCC ACC GAC   1817
Pro Val Ser Leu Asp Gly Ala Gly Thr Ala Leu Leu Leu Ala Thr Asp
                565                 570                 575
GAC GCC GTC CGG CTA GAA GGT GAG CGT GCG GAA CTC GGT CCG CTG AGC   1865
Asp Ala Val Arg Leu Glu Gly Glu Arg Ala Glu Leu Gly Pro Leu Ser
            580                 585                 590
GCC GCC GTC GTC AGC GAC                                         1883
Ala Ala Val Val Ser Asp
        595
TGACGTTTTC TTGGGGGCGG CGTCCACCGC CGGTGACCGG ATGGTGGACG TCCGCCCCGA 1943

AGCCTCGGCG CGGCTGGCAG GATGGAACGC ATGACTTATG TGGCCTCGGA CACCCGCTAC 2003

GACACCATGC CCTACCGCCG CGTCGGACGC AGCGGCCTCA AACTGCCGGC CAT        2056
```

(14)INFORMATION FOR SEQ ID NO:13:

(i)SEQUENCE CHARACTERISTICS:

(A) LENGTH:6
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:13:

```
Phe Asp Ile Trp Ala Pro
  5
```

(15)INFORMATION FOR SEQ ID NO:14:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:17 base pairs
(B)TYPE:nucleic acid
(D)TOPOLOGY:linear

(xi)SEQUENCE DESCRIPTION:SEQ ID NO:14:

TTYGAYATHT GGGCNCC 17

(16)INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:5
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:15:

Asp Trp Ala Glu Ala
5

(17)INFORMATION FOR SEQ ID NO:16:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:17 base pairs
(B)TYPE:nucleic acid
(D)TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:16:

GTAAAACGAC GGCCAGT 17

(18)INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A)LENGTH:17 base pairs
(B)TYPE:nucleic acid
(D)TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:17:

ATGGGNTGGG AYCCNGC 17

(19)INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:6
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(xi) SEQUENCE DESCRIPTION:SEQ ID NO:18:

Met Gly Trp Asp Pro Ala
5

(20)INFORMATION FOR SEQ ID NO:19:

(i)SEQUENCE CHARACTERISTICS:

(A)LENGTH:14 base pairs
(B)TYPE:nucleic acid
(D)TOPOLOGY:linear

(xi) SEQUENCE DESCRIPTION:SEQ ID NO:19:

TAYGAYGTNT GGGC 14

(21)INFORMATION FOR SEQ ID NO:20:

(i)SEQUENCE CHARACTERISTICS:

(A) LENGTH:5
(B)TYPE:amino acid
(D)TOPOLOGY:linear

(ii)MOLECULE TYPE:peptide
(xi)SEQUENCE DESCRIPTION:SEQ ID NO:20:

Try Asp Val Trp Ala
5

Claims

1. An isolated DNA, which DNA encodes an enzyme obtainable from a microorganism of the genera *Rhizobium, Arthrobacter, Brevibacterium or Micrococcus*, said enzyme having:

i) the amino acid sequence of SEQ ID NO:2 or SEQ ID NO:4; or
ii) the amino acid sequence of a variant of SEQ ID NO:2 or SEQ ID NO:4 in which one or more amino acids in SEQ ID NO:2 or SEQ ID NO:4 are deleted or replaced with different amino acids, or one or more amino acids are added to SEQ ID NO:2 or SEQ ID NO:4;

wherein the enzyme has the following physicochemical properties:

(a) Activity
Releases trehalose from a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerisation of 3 or higher;
b) Molecular weight
57,000-68,000 daltons on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); and
c) Isoelectric point (pI)

3.3-4.6 on isoelectrophoresis.

2. The DNA according to claim 1, which has the base sequence of SEQ ID NO:1 or SEQ ID NO:3.

3. The DNA according to claim 1, which has the base sequence SEQ ID NO:11 or SEQ ID NO:12.

4. A replicable recombinant DNA containing the DNA defined in any preceding claim., and a self-replicable vector selected from the group consisting of pBR322, pUC18, Bluescript II SK(+), pUB110, pTZ4, pC194, pHV14, TRp7, TEp7, pBS7, λgt λC, λgt λB, p11, Φ1, and Φ105.

5. A transformant obtainable by introducing into a host said replicable recombinant DNA as defined in claim 4.

6. The transformant according to claim 5, wherein said host is a microorganism of the species *Escherichia coli*.

7. A process for producing a recombinant enzyme, which comprises culturing in a liquid culture medium the transformant defined in claim 5 or claim 6, so as to form said recombinant enzyme and optionally further isolating said recombinant enzyme.

8. The process according to claim 7, wherein the transformant is inoculated into said liquid culture medium having a pH of 2-8, and cultured at a temperature of 25-65°C for about 1-6 days.

9. The process according to claim 7 or claim 8, which process further comprises treating the liquid culture medium with an ultrasonic disintegrator and optionally centrifuging the liquid culture medium to remove insoluble substances.

10. The process according to any of claims 7-9, wherein the step of isolating comprising one or more methods selected from the group consisting of centrifugation, filtration, concentration, salting out, dialysis, ion-exchange chromatography, gel filtration chromatography, hydrophobic chromatography, affinity chromatography, gel electrophoresis and isoelectrophoresis.

11. A method for converting a non-reducing saccharide, which method comprises:

a) producing the recombinant enzyme by the process defined in any of claims 8-11; and
b) allowing the recombinant enzyme to act on a non-reducing saccharide, having a trehalose structure as an end unit and having a degree of glucose polymerisation of 3 or higher to release trehalose.

12. The method according to claim 11, wherein said non-recuding saccharide is prepared by successively treating a member selected from the group consisting of starch, amylopectin, amylose and mixtures thereof with acid together with or without amylase, and subjecting the resultant mixture to the action of a non-reducing saccharide-forming enzyme.

13. The method according to claim 11 or claim 12, wherein said non-reducing saccharide is a member selected from the group consisting of α-glucosyltrehalose, α-maltosyltrehalose, α-maltotriosyltrehalose, α-maltotetraosyltrehalose, α-maltopentaosyltrehalose, and mixtures thereof.

14. The method according to any of claims 11 to 13, wherein said non-reducing saccharide is in a solution form with a concentration of 50 w/v% or lower, and the step is carried out at a temperature of about 40-55°C and a pH of about 6-8.

15. A method of producing trehalose, which method comprises the following steps:

a) treating starch, amylopectin, amylose, or a mixture thereof with an acid and/or an amylase;
b) subjecting the resulting mixture to the action of a non-reducing saccharide forming enzyme to form a non-reducing saccharide having a trehalose structure as an end unit and having a degree of glucose polymerisation of 3 or higher;
c) allowing the recombinant enzyme, produced by a process as defined in any of claims 7 to 10, to act on the non-reducing saccharide produced in step (b) to release trehalose; and
d) collecting the trehalose produced in step (c).

Patentansprüche

1. Isolierte DNA, welche ein aus einem Mikroorganismus der Gattung *Rhizobium, Arthrobacter, Brevibacterium* oder *Micrococcus* erhältliches Enzym kodiert, wobei das Enzym

   i) die Aminosäuresequenz SEQ ID NO:2 oder SEQ ID NO:4; oder
   ii) die Aminosäuresequenz einer Variante von SEQ ID NO:2 oder SEQ ID NO:4, in welcher eine oder mehrere Aminosäuren in SEQ ID NO:2 oder SEQ ID NO:4 deletiert oder durch andere Aminosäuren ersetzt oder eine oder mehrere Aminosäuren zu SEQ ID NO:2 oder SEQ ID NO:4 hinzugefügt wurden;

   aufweist, und wobei das Enzym folgende physikalisch-chemischen Eigenschaften aufweist:

   (a) Aktivität
   Freisetzen von Trehalose aus einem nicht-reduzierenden Saccharid mit Trehalose-Struktur als Endeinheit und mit einem Grad an Glucosepolymerisation von 3 oder höher;
   (b) Molekulargewicht
   57.000 - 68.000 Dalton nach Natriumdodecylsulfat-Polyacrylamid-Gelelektrophorese (SDS-PAGE); und
   (c) Isoelektrischer Punkt (pI)
   3,3 - 4,6 nach Isoelektrophorese.

2. DNA nach Anspruch 1, welche die Basensequenz SEQ ID NO:1 oder SEQ ID NO:3 aufweist.

3. DNA nach Anspruch 1, welche die Basensequenz SEQ ID NO:11 oder SEQ ID NO:12 aufweist.

4. Replizierbare rekombinante DNA enthaltend die DNA nach einem der vorhergehenden Ansprüche sowie einen selbstreplizierbaren Vektor ausgewählt aus der Gruppe, welche aus pBR322, pUC18, Bluescript II SK(+), pUB110, pTZ4, pC194, pHV14, TRp7, TEp7, pBS7, λgt λC, λgt λB, p11, Φ 1 und Φ105 besteht.

5. Transformant erhältlich durch Einführung einer replizierbaren rekombinanten DNA gemäß Anspruch 4 in einen Wirt.

6. Transformant nach Anspruch 5, wobei der Wirt ein Mikroorganismus der Art *Escherichia coli* ist.

7. Verfahren zur Herstellung eines rekombinanten Enzyms, welches Kultivieren eines Transformanten gemäß Anspruch 5 oder 6 in einem flüssigen Kulturmedium zwecks Bildung des rekombinanten Enzyms und, optional, Isolierung des rekombinanten Enzyms umfasst.

8. Verfahren nach Anspruch 7, wobei der Transformant in das flüssige Kulturmedium mit einem pH von 2 - 8 eingeimpft und bei einer Temperatur von 25 - 65°C für etwa 1 - 6 Tage kultiviert wird.

9. Verfahren nach Anspruch 7 oder 8, das außerdem Behandeln des flüssigen Kulturmediums mit einem Ultraschall-Zerkleinerer und, optional, Zentrifugieren des Kulturmediums zur Entfernung unlöslicher Substanzen umfasst.

10. Verfahren nach einem der Ansprüche 7 bis 9, wobei der Verfahrensschritt der Isolierung einen oder mehrere Prozesse ausgewählt aus der Gruppe, welche aus Zentrifugation, Filtration, Konzentration, Aussalzen, Dialyse, Ionenaustauschchromatographie, Gelfiltrationschromatographie, hydrophobe Chromatographie, Affinitätschromatographie, Gelelektrophorese und Isoelektrophorese besteht, umfasst.

11. Verfahren zur Umsetzung eines nicht-reduzierenden Saccharids, umfassend:

    a) Herstellen des rekombinanten Enzyms durch ein Verfahren nach einem der Ansprüche 8 bis 11; und
    b) Ermöglichen des Einwirkens des rekombinanten Enzyms auf ein nicht-reduzierendes Saccharid mit einer Trehalose-Struktur als Endeinheit und mit einem Grad an Glucosepolymerisation von 3 oder höher zwecks Freisetzung von Trehalose.

12. Verfahren nach Anspruch 11, wobei das nicht-reduzierende Saccharid durch aufeinanderfolgendes Behandeln einer Verbindung ausgewählt aus der Gruppe, welche aus Stärke, Amylopektin, Amylose und Mischungen hiervon besteht, mit einer Säure zusammen mit oder ohne Amylase und Unterwerfen der sich daraus ergebenden Mi-

schung der Einwirkung eines nicht-reduzierendes Saccharid bildenden Enzyms hergestellt wird.

13. Verfahren nach Anspruch 11 oder 12, wobei das nicht-reduzierende Saccharid eine Verbindung ausgewählt aus der Gruppe, welche aus α-Glukosyltrehalose, α-Maltosyltrehalose, α-Maltotriosyltrehalose, α-Maltotetraosyltrehalose, α-Maltopentaosyltrehalose und Mischungen hiervon besteht, ist.

14. Verfahren nach einem der Ansprüche 11 bis 13, wobei das nicht-reduzierende Saccharid in Lösung mit einer Konzentration von 50 % (w/v) oder weniger vorliegt und der Verfahrensschritt bei einer Temperatur von etwa 40 - 55°C und einem pH von etwa 6 - 8 durchgeführt wird.

15. Verfahren zur Herstellung von Trehalose, umfassend folgende Schritte:

a) Behandeln von Stärke, Amylopektin, Amylose oder einer Mischung hiervon mit einer Säure und/oder einer Amylase;
b) Unterwerfen der sich daraus ergebenden Mischung der Einwirkung eines nicht-reduzierendes Saccharid bildenden Enzyms zwecks Bildung eines nicht-reduzierenden Saccharids mit einer Trehalose-Struktur als Endeinheit und mit einem Grad an Glucosepolymerisation von 3 oder höher;
c) Ermöglichen des Einwirkens des durch ein Verfahren nach einem der Ansprüche 7 bis 10 hergestellten rekombinanten Enzyms auf das in Schritt (b) hergestellte nicht-reduzierende Saccharid zwecks Freisetzung von Trehalose; und
d) Sammeln der in Schritt (c) hergestellten Trehalose.

Revendications

1. ADN isolé, lequel ADN code un enzyme pouvant être obtenu à partir d'un micro-organisme des genres *Rhizobium, Arthrobacter, Brevibacterium* ou *Micrococcus*, ledit enzyme ayant :

i) la séquence d'acides aminés de la SEQ ID NO : 2 ou de la SEQ ID NO : 4 ; ou
ii) la séquence d'acides aminés d'un variant de la SEQ ID NO : 2 ou de la SEQ ID NO : 4, dans laquelle un ou plusieurs acides aminés de la SEQ ID NO : 2 ou de la SEQ ID NO : 4 sont supprimés ou remplacés par différents acides aminés, ou un ou plusieurs acides aminés sont ajoutés à la SEQ ID NO : 2 ou à la SEQ ID NO : 4 ;

dans laquelle l'enzyme a les propriétés physico-chimiques suivantes :

(a) Activité
Libère du tréhalose à partir d'un saccharide non réducteur ayant une structure de tréhalose comme extrémité et ayant un degré de polymérisation du glucose supérieur ou égal à 3 ;
(b) Poids moléculaire
57 000 - 68 000 daltons, évalué par électrophorèse sur gel polyacrylamide - sulfate de dodécyle sodique (technique SDS-PAGE) ; et
(c) Point isoélectrique (pI)
3,3 - 4,6, évalué par isoélectrophorèse.

2. ADN selon la revendication 1, dont la séquence de base est la SEQ ID NO : 1 ou la SEQ ID NO : 3.

3. ADN selon la revendication 1, dont la séquence de base est la SEQ ID NO : 11 ou la SEQ ID NO : 12.

4. ADN recombinant réplicable, contenant l'ADN défini dans l'une quelconque des revendications précédentes, et un vecteur auto-réplicable choisi dans le groupe comprenant les pBR322, pUC18, Bluescript II SK (+), pUB110, pTZ4, pC194, pHV14, TRp7, TEp7, pBS7, λgt λC, λgt λB, p11, Φ1 et Φ105.

5. Transformant pouvant être obtenu en introduisant dans un hôte ledit ADN recombinant réplicable tel que défini dans la revendication 4.

6. Transformant selon la revendication 5, dans lequel ledit hôte est un micro-organisme de l'espèce *Escherichia coli*.

7. Procédé de production d'un enzyme recombinant, comprenant la mise en culture dans un milieu de culture liquide du transformant défini dans la revendication 5 ou dans la revendication 6, de manière à former ledit enzyme recombinant et, en outre, d'isoler éventuellement ledit enzyme recombinant.

8. Procédé selon la revendication 7, dans lequel le transformant est inoculé dans ledit milieu de culture liquide ayant un pH de 2 à 8, puis cultivé à une température de 25 à 65° C pendant environ 1 à 6 jours.

9. Procédé selon la revendication 7 ou la revendication 8, lequel procédé comprend, en outre, le traitement du milieu de culture liquide avec un désintégrateur à ultrasons et éventuellement la centrifugation du milieu de culture liquide afin de retirer les substances insolubles.

10. Procédé selon l'une quelconque des revendications 7 à 9, dans lequel l'étape d'isolement comprend un ou plusieurs méthodes choisies dans le groupe comprenant la centrifugation, la filtration, la concentration, le relargage, la dialyse, la chromatographie par échanges d'ions, la chromatographie d'exclusion diffusion, la chromatographie hydrophobe, la chromatographie d'affinité, l'électrophorèse sur gel et l'isoélectrophorèse.

11. Méthode de conversion d'un saccharide non réducteur, laquelle méthode comprend les étapes consistant à :

a) produire l'enzyme recombinant par le processus défini dans l'une quelconque des revendications 8 à 11 ; et
b) autoriser l'enzyme recombinant à agir sur un saccharide non réducteur, ayant une structure de tréhalose comme extrémité et ayant un degré de polymérisation du glucose supérieur ou égal à 3 pour libérer du tréhalose.

12. Méthode selon la revendication 11, dans laquelle ledit saccharide non réducteur est préparé en traitant successivement un élément choisi parmi le groupe comprenant l'amidon, l'amylopectine, l'amylose, et leurs mélanges, avec un acide et conjointement avec ou sans amylase, puis en soumettant le mélange obtenu à l'action de l'enzyme de formation du saccharide non réducteur.

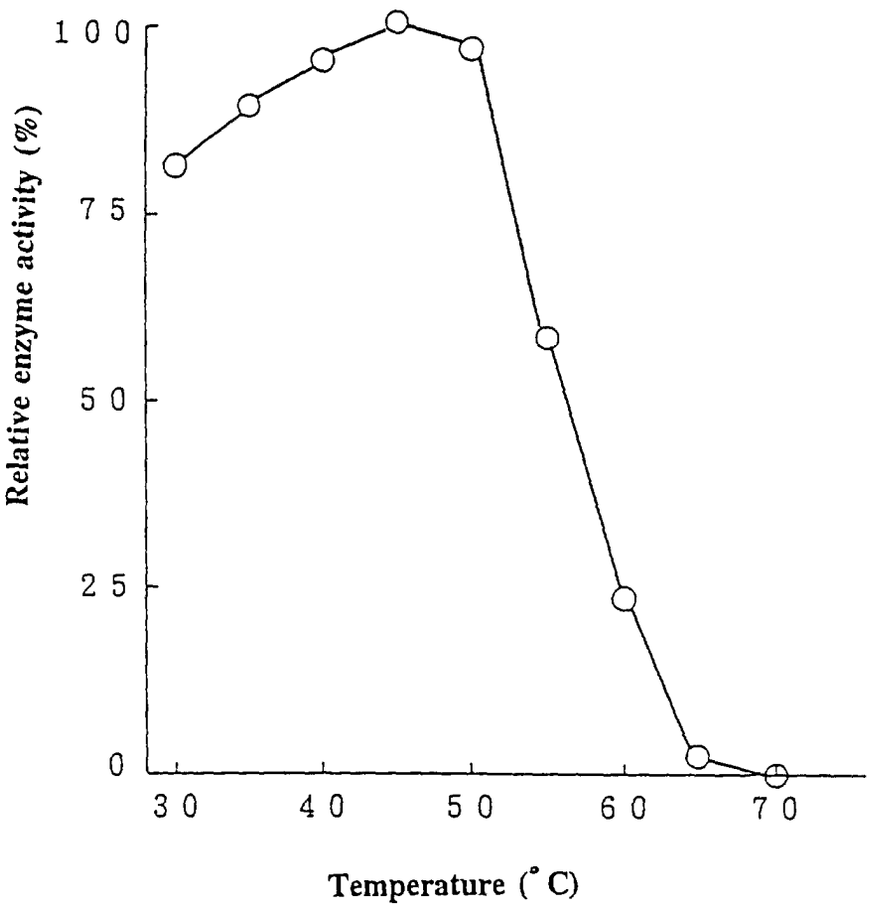
13. Méthode selon la revendication 11 ou la revendication 12, dans laquelle ledit saccharide non réducteur est un élément sélectionné dans le groupe comprenant les α-glucosyltréhalose, α-maltosyltréhalose, α-maltotriosyltréhalose, α-maltotétraosyltréhalose, α-maltopentaosyltréhalose et leurs mélanges.

14. Méthode selon l'une quelconque des revendications 11 à 13, dans laquelle ledit saccharide non réducteur est sous forme de solution ayant une concentration inférieure ou égale à 50 % en poids/volume, et cette étape est réalisée à une température d'environ 40 à 55° C et avec un pH d'environ 6 à 8.

15. Méthode de production du tréhalose, laquelle méthode comprend les étapes suivantes consistant à :

a) traiter l'amidon, l'amylopectine, l'amylose, ou leurs mélanges, avec un acide et/ou une amylase ;
b) soumettre le mélange obtenu à l'action d'un enzyme de formation de saccharide non réducteur afin de former un saccharide non réducteur ayant une structure de tréhalose comme extrémité et ayant un degré de polymérisation du glucose supérieur ou égal à 3 ;
c) autoriser l'enzyme recombinant produit par un procédé tel que défini dans l'une quelconque des revendications 7 à 10, à agir sur le saccharide non réducteur produit à l'étape b) afin de libérer du tréhalose ; et
d) recueillir le tréhalose produit à l'étape (e).

FIG. 1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75
50
25
0
Relative enzyme activity (%)
30
40
50
60
70
Temperature (°C)

FIG. 2

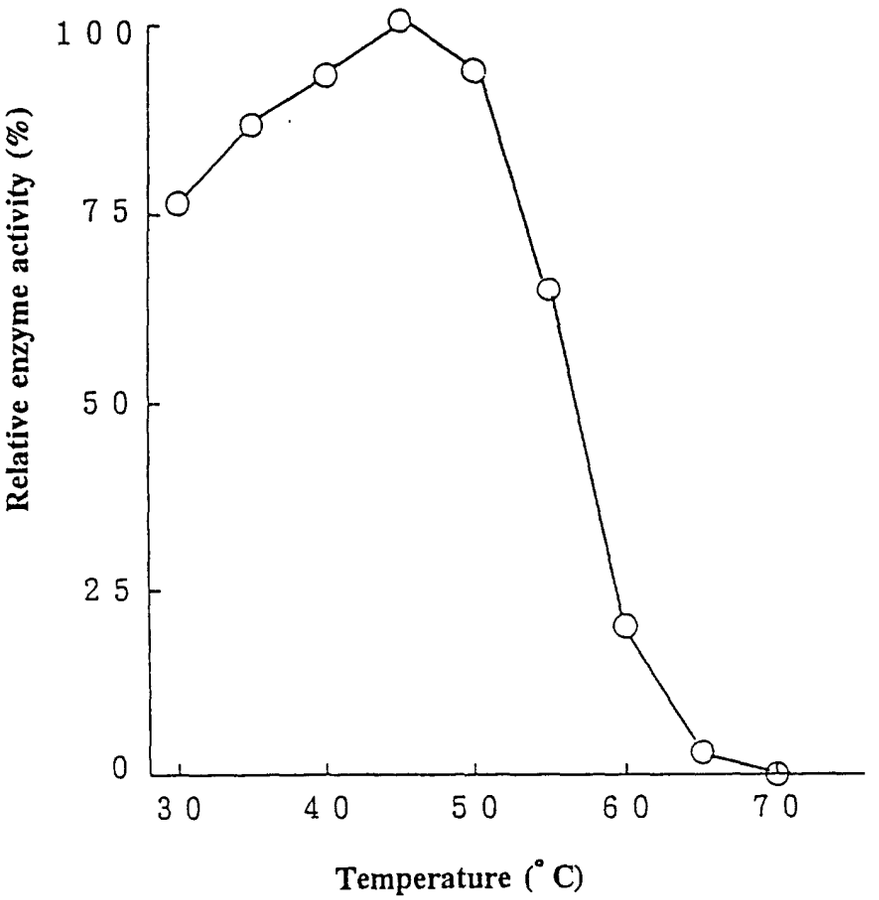

100
75
50
25
0
Relative enzyme activity (%)
30
40
50
60
70
Temperature (°C)

FIG. 3

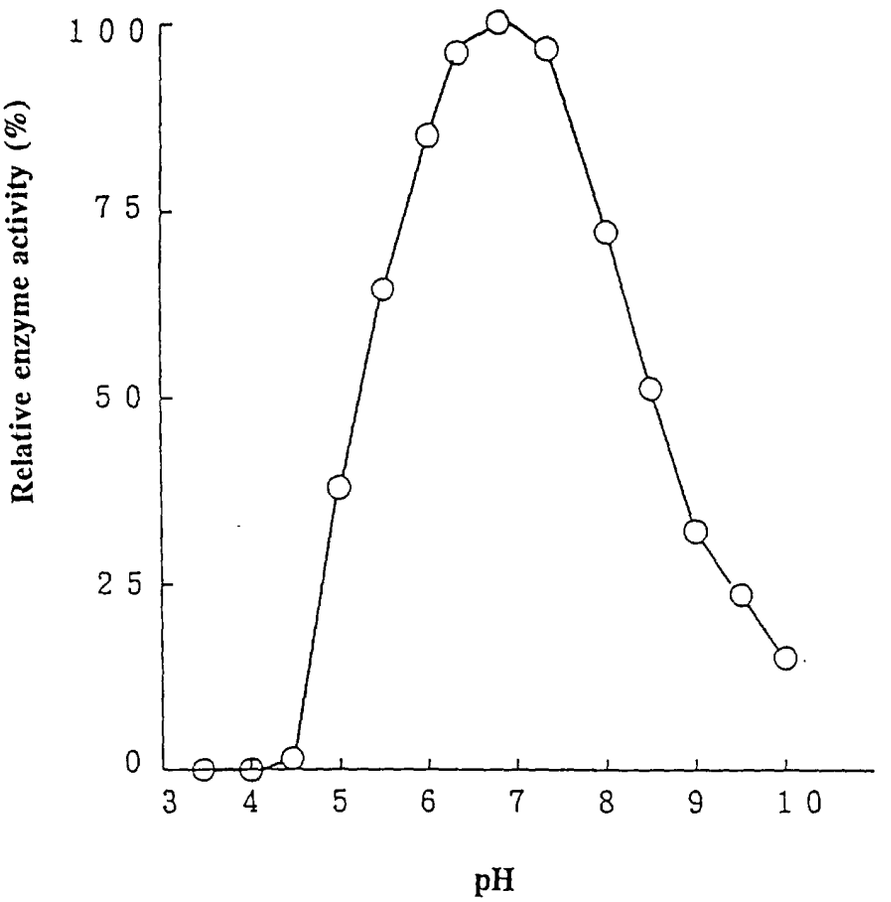


FIG. 4

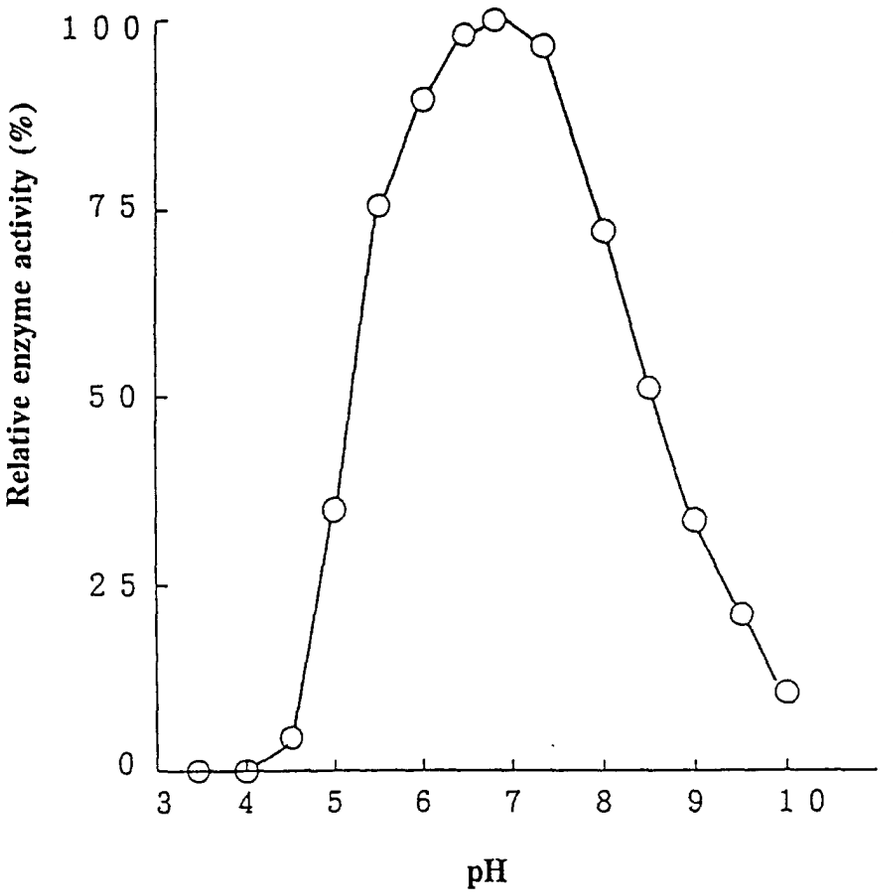
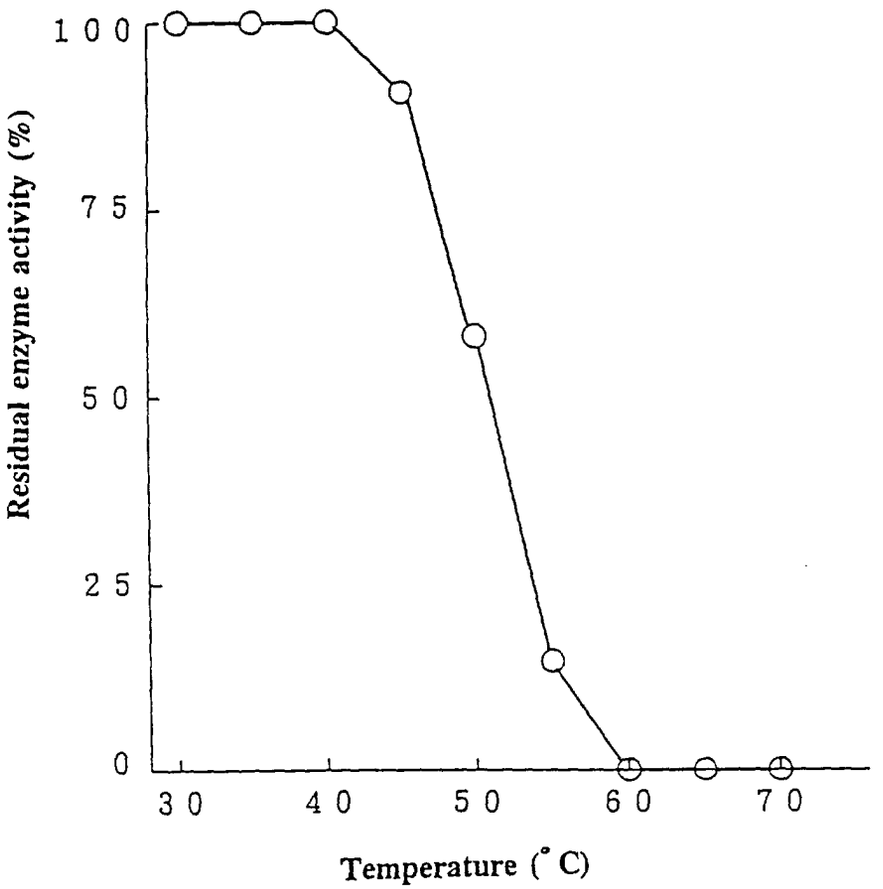


FIG. 5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75
50
25
0
Residual enzyme activity (%)
30
40
50
60
70
Temperature (° C)

FIG. 6

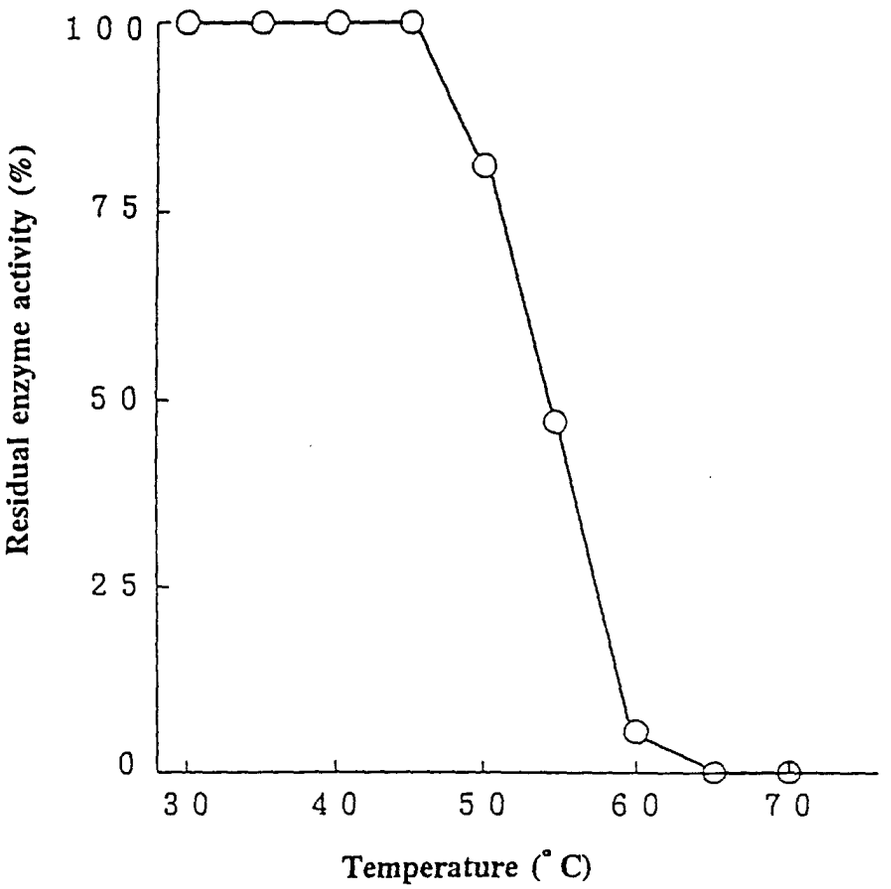

100
75
50
25
0
Residual enzyme activity (%)
30
40
50
60
70
Temperature (°C)

FIG. 7

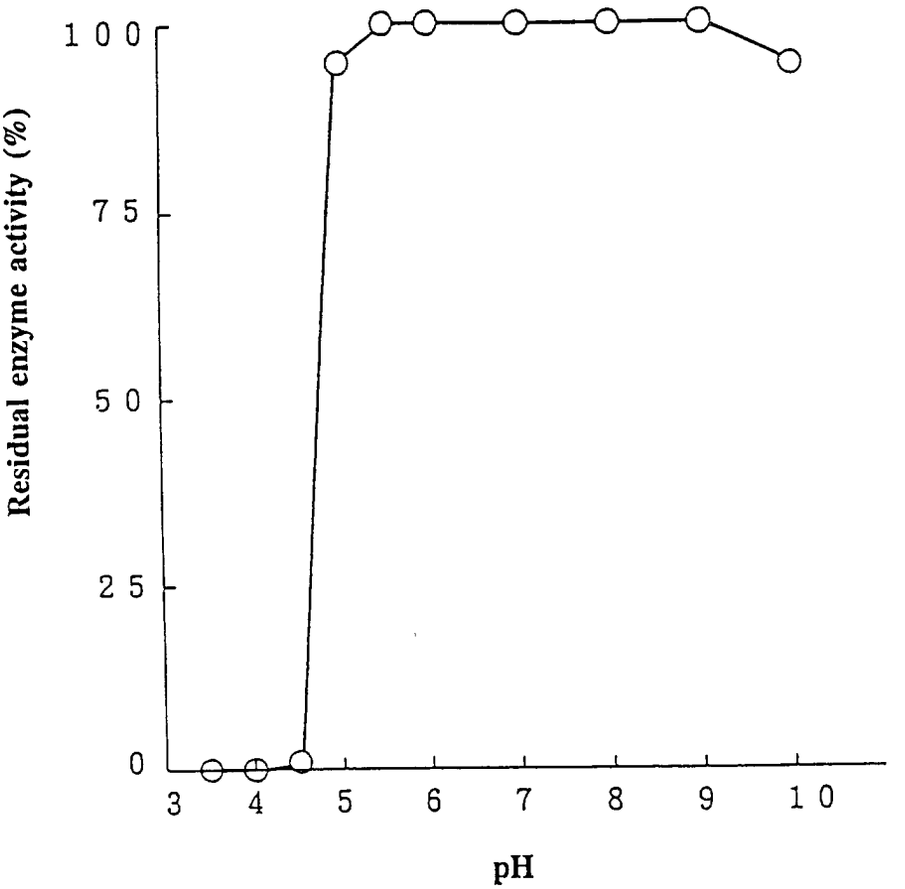


FIG. 8

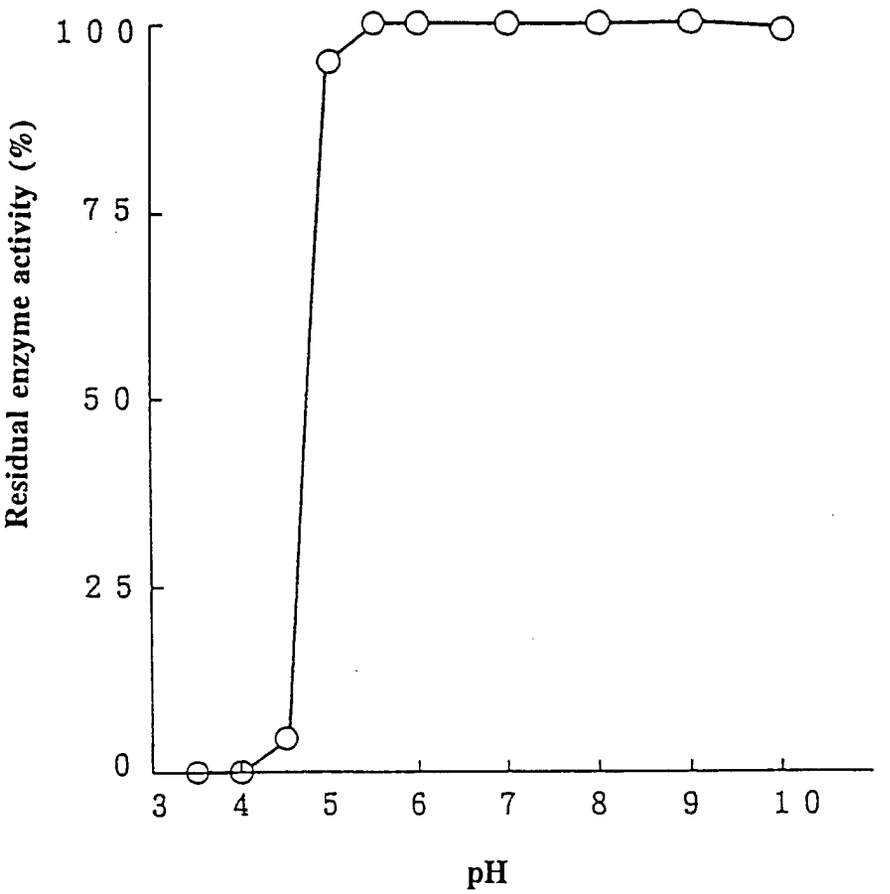


FIG. 9

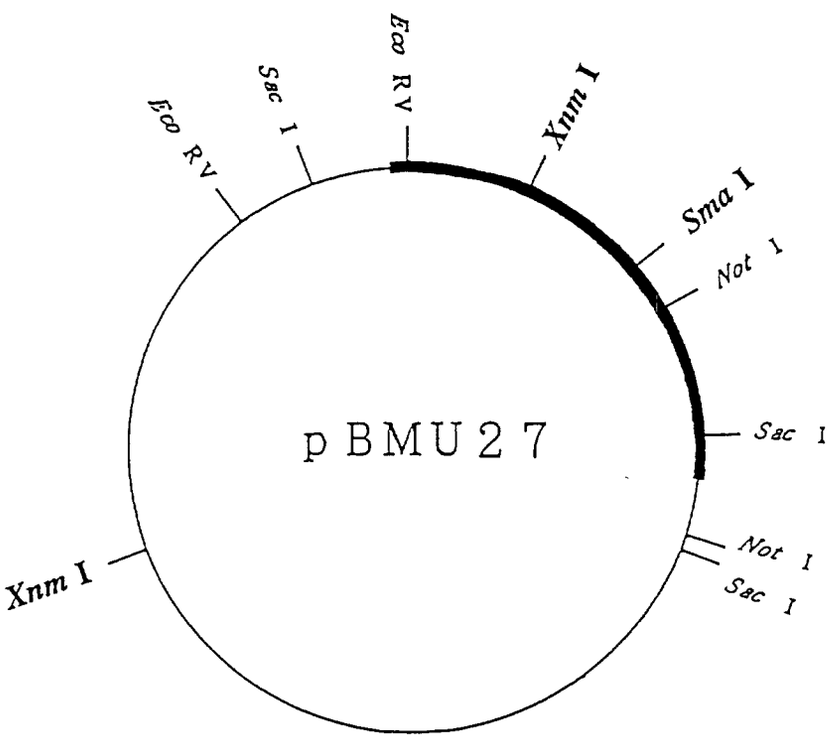
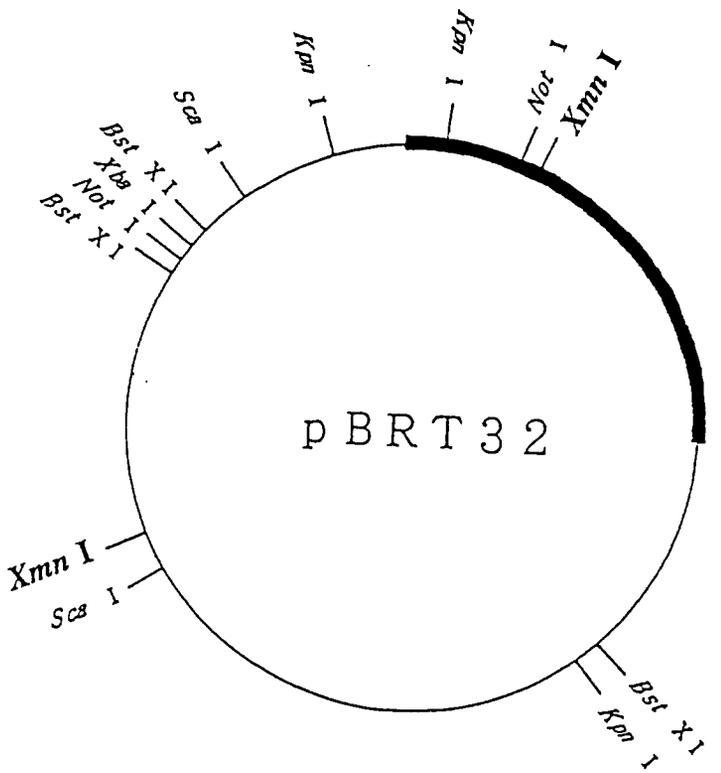


FIG. 10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Xmn I
Sca I
Bst X1
Kpn I
Kpn I
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Xmn I
Sca I
Bst X1
Xba I
Not I
Bst X1