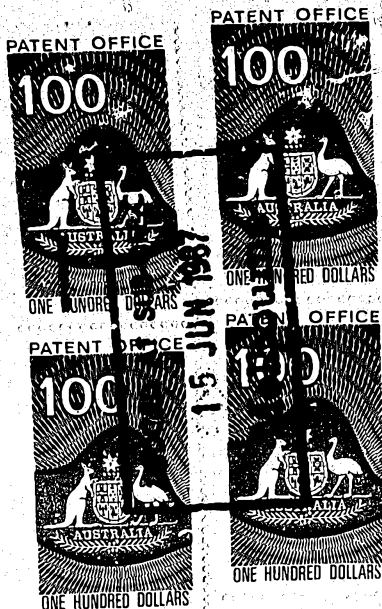


\$410

COMMONWEALTH OF AUSTRALIA

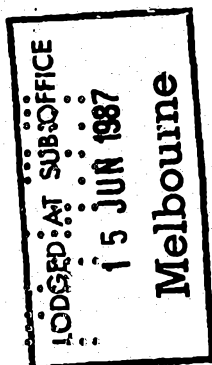
Patents Act 1952

612112



APPLICATION FOR A STANDARD PATENT

(Combined Form — Convention and Non-Convention)



X/We TRANSGENE S.A., a French body corporate, of 16 rue
Henri Regnault, 92400 Courbevoie, France,

hereby apply for the grant of a Standard Patent for an invention entitled BLOCK FOR...
THE EXPRESSION OF AN AMYLOGLUCOSIDASE ~~AMINOGLUCOSIDASE~~ IN A YEAST,
TRANSFORMED YEAST, ENZYME PREPARATION PROCESS AND
FERMENTATION PROCESS. Provisional Complete Specification.

Strike our part, 2.
for non-convention

2. This application is a convention application and is based on the application(s) for a patent or similar protection made —

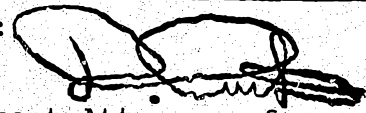
in FRANCE
on June 10, 1986 numbered 86 08387 and
on April 13, 1987 numbered 87 05207 and
on April 13, 1987 numbered 87 05208

3. My/Our address for service is: Care of COWIE, THOMSON & CARTER, Patent Attorneys, of 71 Queens Road, Melbourne, Victoria 3004, Australia.

DATED this 15th day of June 19 87

To The Commissioner of Patents
COMMONWEALTH OF AUSTRALIA

COWIE, THOMSON & CARTER.

by: 
Patent Attorneys for
TRANSGENE S.A.



COWIE, THOMSON & CARTER

Patent Attorneys
71 Queens Road, Melbourne,
Victoria, 3004, Australia

PATENT DECLARATION FORM
(CONVENTION OR NON-CONVENTION)

DECLARATION IN SUPPORT OF APPLICATION FOR A PATENT

Insert name of
applicant.In support of the application made by TRANSGENE S.A.Insert title of
invention.for a patent for an invention entitled: BLOCK FOR THE EXPRESSION OF AN AMINOGLUCOSIDASE IN
A YEAST, TRANSFORMED YEAST, ENZYME PREPARATION PROCESS AND FERMENTATION
PROCESSInsert full name(s)
and address(es) of
person(s) making
declaration. If
applicant a company,
person must be
authorised to make
declaration.I/We Etienne EISENMANN
c/o TRANSGENE S.A. 16 rue Henri Regnault, 92400 COURBEVOIE
FRANCEDelete alternatives
which do not apply

do solemnly and sincerely declare as follows:

- 1. (a) ~~I am/We are the applicant(s) for the patent.~~
- OR (b) I am authorized by the abovementioned applicant to make this declaration on its behalf.

- 2. (a) ~~I am/We are the actual inventor(s) of the invention.~~

Insert name(s) and
address(es) of actual
inventor(s).

- OR (b) Nathalie LABAT, 6 rue des Dentelles, 67000 STRASBOURG, FRANCE

- Martine NGUYEN-JULLERET, 32 Avenue du Général de Gaulle,
- 67000 STRASBOURG, FRANCE

- Gérard LOISON, 19 rue du Faubourg National, 67000 STRASBOURG,
- FRANCE

- Yves LEMOINE, 4 rue des Alisiers, 67100 STARBOURG-NEUDORF, FRANCE

Insert details of
entitlement to apply,
e.g. Applicant is
assignee of inventor(s)

I/we are the actual inventor(s) of the invention and the facts upon which the applicant(s) is/are entitled to make the application are as follows:—

The Applicant is the Assignee of the said actual inventors.

Delete 3 and 4 if
application non-
convention.
Otherwise insert
details of basic
application(s).

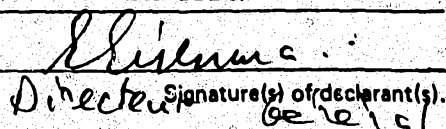
3. The basic application(s) as defined by Section 141 of the Act was/were made in the following country or countries on the following date(s) by the following applicant(s)
- | | | | | |
|----|---|----|-----------------|-------------|
| in | <u>FRANCE</u> | on | <u>JUNE 10</u> | <u>1986</u> |
| by | <u>TRANSGENE S.A. under number 86 08387</u> | | | |
| in | <u>FRANCE</u> | on | <u>APRIL 13</u> | <u>1987</u> |
| by | <u>TRANSGENE S.A. under number 87 05207</u> | | | |
| in | <u>FRANCE</u> | on | <u>APRIL 13</u> | <u>1987</u> |
| by | <u>TRANSGENE S.A. under number 87 05208</u> | | | |
| in | | on | <u>19</u> | |
| by | | | | |

4. The basic application(s) referred to in paragraph 3 of this Declaration was/were the first application(s) made in a Convention country in respect of the invention the subject of the application.

Place and date of
Signature.Declared at PARIS this 29 day of MAY 1987NO ATTESTATION
OR SEAL

(MANAGER)

Etienne EISENMANN



Signature(s) of declarant(s).

To: The Commissioner of Patents,
Australia

(12) PATENT ABRIDGMENT (11) Document No. AU-B-74435/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 612112

- (54) Title
EXPRESSION OF AMINOGLUCOSIDASE IN YEAST
- (51)⁴ International Patent Classification(s)
C07H 021/04 A21D 008/04 C12N 001/16 C12N 001/18
C12N 001/19 C12N 009/34 C12N 015/00 C12N 015/56
- (21) Application No. : **74435/87** (22) Application Date : **15.06.87**
- (30) Priority Data
- (31) Number (32) Date (33) Country
86 08387 10.06.86 FR FRANCE
87 05207 13.04.87 FR FRANCE
87 05208 13.04.87 FR FRANCE
- (43) Publication Date : **04.02.88**
- (44) Publication Date of Accepted Application : **04.07.91**
- (71) Applicant(s)
TRANSGENE S.A.
- (72) Inventor(s)
NATHALIE LABAT; GERARD LOISON; MARTINE NGUYEN-JUILLERET; YVES LEMOINE
- (74) Attorney or Agent
COWIE CARTER & HENDY , 71 Queens Road, MELBOURNE VIC 3004
- (56) Prior Art Documents
EP 163491
AU 59544/86 C12N 15/00
EP 126206
- (57) Claim

1. A block for the expression of a mature amyloglucosidase from *Aspargillus Niger* in a yeast, which block contains:

- (a) the amyloglucosidase gene devoid of all or part of its introns;
- (b) the DNA sequence containing the signals which provide for the transcription of the amyloglucosidase gene by yeast;
- (c) a coding sequence containing the signal required for the export of the mature amyloglucosidase into the extracellular medium, which coding sequence contains a secretion signal chosen from the alpha pheromone, the killer protein secretion system or a synthetic secretion sequence; and
- (d) a yeast termination sequence after the amyloglucosidase gene and wherein said yeast termination sequence is a transcription termination sequence.

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(10) 612112

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11. A transformed yeast strain containing an expression block as claimed in any one of claims 1 to 10.

18. The strain of Saccharomyces as claimed in any one of claims 11 to 13, which is transformed simultaneously by an expression block as claimed in any one of claims 1 to 10 and by an alpha-amylase expression block, and which simultaneously secretes alpha-amylase and amyloglucosidase.

25. A block for the expression of an amyloglucosidase in yeast and a transformed yeast strain containing said block substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.

26. A process for producing alpha-amyloglucosidase substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.

612112



PATENTS ACT 1952-1973

Form 10

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

Class:

Int. Cl:

Application Number:

Lodged:

Complete Specification—Lodged:

Accepted:

Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT

Name of Applicant: TRANSGENE S.A., a French body corporate, of 16 rue
Henri Regnault, 92400 Courbevoie, France.

Address of Applicant:

Actual Inventor:

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MARTINE NGUYEN-JUILLERET

GERARD LOISON

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Address for Service:

COWIE, THOMSON & CARTER

PATENT & TRADEMARK ATTORNEYS

71 QUEENS ROAD

MELBOURNE, 3004, AUSTRALIA



Complete Specification for the invention entitled:

AMYLOGLUKOSIDASE
BLOCK FOR THE EXPRESSION OF AN ~~AMINOGLUCOSIDASE~~ IN A
YEAST, TRANSFORMED YEAST, ENZYME PREPARATION PROCESS AND
FERMENTATION PROCESS

The following statement is a full description of this invention, including the best method of performing it known to me:—

- 1 -

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 160 mm in width, on tough white paper of good quality and it is to be inserted inside this form.

The present invention describes processes by means of which different strains of yeast of the genus Saccharomyces, including the industrial strains which do not have the capacity to degrade complex carbon-containing substrates such as starch or dextrans, may be caused to secrete a glucoamylase.

Starch, which is commonly used as a source of carbon in alcoholic fermentation, has to be hydrolyzed beforehand (either chemically or enzymatically) in a separate process in order to produce substrates which are usable for fermentative yeasts.

It would hence be desirable to construct, by genetic recombination, industrial strains which were capable of synthesizing one or more enzymes for degrading starch to substrates which are directly assimilable by these strains.

More especially, the objective of the present invention is to construct, by genetic recombination, a strain responsible for brewery fermentation capable of degrading the residual dextrans in the wort so as to produce beers having a low content of sugars.

Similarly, the enrichment of the wort with respect to fermentable sugars also enables the production of beers having a high alcohol content to be envisaged.

The use of a brewery strain which produces an enzyme responsible for the degradation of dextrans during alcoholic fermentation may prove to be a means of simplifying the brewing processes and making them profitable.

The preparation of beers having a low content of residual sugars can, in particular, be achieved by adding, at various stages of the brewing process, an Aspergillus niger glucoamylase (amylo-1,6-glucosidase). This glycoamylase catalyses the release of glucose residues from the non-reducing ends of starch or dextrans.

This enzyme plays a big part in the production of glucose syrups from starch and in the manufacture of other alcoholic drinks. This Aspergillus niger glucoamylase is accepted as an additive in the agri-foodstuffs industries.

Hence, in the first place, the subject of the present invention is the expression of Aspergillus niger NC122343

alpha-amyloglucosidase in a brewery strain.

Secondly, one of the advantageous features of the invention is that the transformed brewery strains possess the foreign DNA integrated in a yeast chromosome. When the foreign DNA is integrated in the chromosome according to the process described later, the property of producing alpha-amyloglucosidase is stable and the strain differs from the starting strain only in respect of this new character.

The stability of the information can also be achieved by constructing a DNA structure known as a minichromosome consisting of three main elements: telomere, centromere and origin of replication.

Another subject of the present invention relates, more especially, to a functional DNA block enabling amyloglucosidase to be secreted from yeast, this block containing the following features:

- . the amyloglucosidase gene devoid of all or part of its introns;
- . a DNA sequence containing the signals which provide for the transcription of the amyloglucosidase gene by yeast;
- . a coding sequence containing the signal required for the export of the mature amyloglucosidase into the extracellular medium; the choice was made of a secretion system specific to yeast, that of the alpha pheromone (1), but other systems could be used [for example, the killer protein system (2) or that of a synthetic signal sequence];
- . finally, the expression block may possess, after the amyloglucosidase gene, a yeast termination sequence, for example that of the phosphoglycerate kinase gene.

Preferably, the amyloglucosidase gene will be devoid of all of its introns (cDNA).

Among the elements of the sequence which provide for the transcription of the amyloglucosidase gene, promoters should be mentioned, in particular the PGK promoter.

This functional block can be integrated in an autonomously replicating plasmid containing, for example, an origin of replication in yeast, for example the origin of replication of the 2 μ plasmid.

When such a plasmid is used, it is advantageous that it should be a shuttle plasmid capable of replicating in a bacterium; for this purpose, it may contain elements for replication in a bacterium, for example E. coli, and a selection gene such as that for resistance to an antibiotic, for example Amp^r.

In general, the plasmid will preferably contain an effective gene for selection in yeasts, for example a gene complementing an auxotrophy, such as ura3+, or alternatively a gene conferring a dominant phenotype, for example resistance to G418.

When the expression block is integrated in the genome of the yeast strain, the integration will be performed in a site which is nonessential for the yeast, for example the MFalpha₁ gene.

The constitution of an integration vector is known and will be described in detail in the examples.

The invention also relates to the strains containing an expression block according to the invention, involving either a plasmid or a chromosomal integration.

Among the transformed strains, there should be mentioned, in particular, the strains of Saccharomyces, in particular S. cerevisiae and S. uvarum, in particular the industrial strains such as the brewery strains.

Finally, the invention relates to a process for preparing amyloglucosidase, in particular alpha-amyloglucosidase, wherein a strain according to the invention is fermented on a suitable culture medium and the enzyme is recovered in the extracellular medium.

The invention relates, finally, to a process for degrading starch or dextrans, wherein an industrial medium containing starch or dextrans is fermented.

Finally, the alpha-amyloglucosidase gene used is preferably that of Aspergillus niger.

Lastly, the combined action of amyloglucosidase and a liquefying alpha-amylase leads to a total synergy of degradation of starch to glucose residues.

Starting with industrial strains such as Saccharomyces,

in which the genetic information for an alpha-amyloglucosidase and also that for an endo-alpha-amylase (for example, of Bacillus licheniformis) are integrated together in the yeast chromosomes, the production of ethanol may be obtained using starch as a source of carbon.

Thus, the present invention relates to the introduction of the gene for B. licheniformis alpha-amylase and its expression in a strain of Saccharomyces producing alpha-amyloglucosidase.

The simultaneous expression of amyloglucosidase and of alpha-amylase should lead to a synergy of these two enzymatic activities in the degradation of starch or dextrans to glucose residues.

The present invention relates, more especially, to the simultaneous expression of alpha-amylase and amyloglucosidase in a polyploid strain of brewer's yeast, S. uvarum, in particular the strain 2285 TG2 AMGC, as well as to the use of these strains for preparing alpha-amylase.

Moreover, a most especially preferred application according to the invention relates to the transformation of particular strains of Saccharomyces, in particular the polyploid and prototrophic strains used in the bread-making industry; such a strain may be, for example, S. cerevisiae strain TGF1.

These polyploid and prototrophic strains have to be cotransformed with a vector as described above and a plasmid which confers a dominant phenotype.

One of the subjects of the present invention is, in effect, the use of alpha-amyloglucosidase, in particular of Aspergillus niger, during panary fermentation, since alpha-amyloglucosidase, which degrades starch directly to glucose, has a beneficial effect on panary fermentation since this sugar is directly assimilable by baker's yeast.

The addition of this enzyme enables the baking processes to be improved by making the dough drier to the touch and hence easier to process by machine, and above all enables the fermentative activity of the baker's yeast to be significantly enhanced and hence the fermentation times to be reduced

greatly.

Furthermore, this addition should be the source of innovation by enabling relatively sweet bread-type products to be developed, which is virtually impossible without adding an enzyme in a short baking process.

The present invention hence relates, in addition, to processes by means of which a bakery strain of the Saccharomyces cerevisiae type is caused to secrete an amyloglucosidase.

In particular, the invention relates to a panary fermentation process in which at least one of the strains used in the fermentation is a strain as described above.

The examples below are designed to illustrate other characteristics and advantages of the present invention and will be described with reference to the figures, wherein:

Figure 1 shows:

- a) the structure and the restriction map of the Aspergillus niger glucoamylase gene,
- b) the structure of plasmid pTG1831,
- c) the structure of plasmid pTG1830,

Figure 2 shows the sequence of the Aspergillus niger amyloglucosidase gene,

Figure 3 shows the structure of M13TG1839,

Figure 4 shows the structure and construction of M13TG1843,

Figure 5 shows the structure and construction of M13TG1837,

Figure 6 shows the structure and construction of M13TG1846,

Figure 7 shows the structure and construction of M13TG1848,

Figure 8 shows:

- a) the structure of plasmid pTG1848,
- b) the structure and construction of plasmid pTG1850,

Figure 9 shows the structure and construction of M13TG1857,

Figure 10 shows the structure of plasmid pTG1858,

Figure 11 shows the detection of the production of amyloglucosidase by the strain TGY2sp13 pTG1858 by means of the I_2/KI test,

Figure 12 shows the structure and construction of plasmid pTG1867,

Figure 13 shows the hybridization of the EcoRI restriction fragments of the genomic DNA of the strains TGY2sp13b AMGB

- and TGY2sp13b with the oligonucleotide TG529 corresponding to the 3' portion of the MFalpha₁ gene of S. cerevisiae,
Figure 14 shows the hybridization of the restriction fragments of the genomic DNA of the strains TGY2sp13b and 2285TG2 with either the oligonucleotide TG529 or the oligonucleotide TG170,
Figure 15 shows the hybridization of the EcoRI-digested genomic DNAs of strains TGF1-AMG1 and TGF1 with an oligonucleotide TG529 specific for the MFalpha₁ gene of Saccharomyces cerevisiae,
Figure 16 shows the hybridization of the BamHI-digested genomic DNAs of strains 2285 TG2 AMGC-AMY1 and 2285 TG2 AMGC with an oligonucleotide TG227 specific for the His3 gene of S. uvarum.

EXAMPLE 1

CLONING OF THE ASPERGILLUS NIGER ALPHA-AMYLOGLucosIDASE GENE IN PLASMID pBR322

1 µg of Aspergillus niger NCI22343 genomic DNA and 0.5 µg of plasmid pBR322 DNA were completely digested with EcoRI and SalI, and these DNAs were then covalently linked using T₄ DNA ligase. Escherichia coli strain 1106 was then transformed with the ligation products. The selection of the clones carrying an insert corresponding to the alpha-amyloglucosidase gene was carried out after transferring the recombinant clones to a nitrocellulose sheet according to the classical method (3). The recombinant clones were hybridized with a mixture of three oligonucleotides TG282, TG283, TG284, defined on the basis of the sequences already published (4).

TG282 5' ATAGTTAAAGGATGGGGATGAGGGC 3'

TG283 5' AGGACACGTACTACAACGGCAACCC 3'

TG284 5' CTGGGTGACTGGGAAACCAGCGACG 3'

Two positive clones were isolated and contain a different EcoRI-SalI fragment. One of the inserts (2.4 kb), which hybridizes with two of the three oligonucleotides (TG283, TG284), corresponds to the 5' portion of the amyloglucosidase gene. In contrast, the other insert (1 kb), which hybridizes with only one of the three oligonucleotides (TG282), corresponds to the 3' region of the Aspergillus niger

amyloglucosidase gene (Figure 2).

These new plasmids are referred to as pTG1830 and pTG1831 (Figure 1).

EXAMPLE 2

5 CLONING OF THE cDNA OF ASPERGILLUS NIGER ALPHA-AMYLOGLUCO- SIDASE INTO BACTERIOPHAGE LAMBDA

The sequence of the amyloglucosidase gene, as well as that of the cDNA of Aspergillus niger (BU-1) amyloglucosidase, has already been published (4a, b). Comparison of these two
10 sequences reveals the presence of 4 intron regions in the Aspergillus niger (BU-1) amyloglucosidase G1 gene (Figure 2).

With the exception of the intron D, which has a sequence comparable to the consensus sequence TATAAC, an essential element in the mechanism of excision of introns in yeast
15 (5), it appears that none of the other introns can be excised by yeast in vivo. Cloning of the cDNA of amyloglucosidase was hence undertaken.

Aspergillus niger NCI22343 messenger RNAs were purified from a culture of this fungus on rich medium containing
20 2% of starch as a carbon source, so as to produce a cDNA corresponding to amyloglucosidase by reverse transcription.

The extracted mRNAs were added to a reaction mixture containing a dT oligonucleotide as a transcription primer. The synthesis of the cDNA was carried out according to the
25 method described by Chang et al. (6). A cDNA library was prepared in bacteriophage lambda according to Le Bouc et al. (7). The bacteriophage lambda DNA was packaged in vitro in the phage particles, and the phages thereby formed were used to transduce the Escherichia coli indicator strain POP101.

The Aspergillus niger NCI22343 cDNA library was screened for amyloglucosidase, using as a probe an oligonucleotide TG433 defined on the basis of the published sequences
30 (4a):

TG433 5'GGTGTAGCCATTGTCAAGCAGCCACTGCCC 3'
35 corresponding to the sequence complementary to the sequence coding for amino acids 163 to 172 of the mature protein (Figure 2).

Five positive signals were detected. Two of the five

clones were confirmed by cross-hybridization with another oligonucleotide TG387 corresponding to the sequence complementary to the sequence coding for amino acids 226 to 236 of the mature protein (Figure 2):

5 TG387 5'CAGGACGAGCCGACGGCCGTCGCGA 3'

the size of the inserts in these two candidates being approximately 800 bp.

After analysis with restriction enzymes, one of the two fragments was recloned in an M13 derivative (M13mp8) in order to sequence it. This new vector is referred to as M13TG1839 (Figure 3). The sequence of the cDNA fragment of M13TG1839 coding for amino acids 62 to 319 of the mature protein proved to be identical to that already published (Figure 2).

15 EXAMPLE 3

CONSTRUCTION OF AN ALPHA-AMYLOGLucOSIDASE EXPRESSION VECTOR FOR YEAST S. CEREVISIAE

1) Reconstitution of the 3' portion of the Aspergillus niger amyloglucosidase gene

20 Plasmid pTG1830 DNA was digested with PstI and SalI so as to liberate a 1.2-kb fragment. Similarly, plasmid pTG1831 DNA was digested with EcoRI and SalI, liberating a 1-kb fragment. These two fragments were isolated and ligated with M13mp8 DNA which has been digested with PstI and EcoRI. A new vector, referred to as M13TG1843, is reconstituted (Figure 4). This vector M13TG1843 possesses the 3' part of the amyloglucosidase gene.

2) Reconstitution of the 5' portion of the region coding for the mature amyloglucosidase of Aspergillus Niger

a) Removal of the intron A by in vitro mutagenesis

30 In the first place, the BamHI-EcoRI fragment (2 kb) of pTG1830 containing the 5' portion of the amyloglucosidase gene was transferred into M13Tg127 phage DNA between the BamHI and EcoRI sites. This gave single-stranded M13TG1835 DNA, from which the in vitro mutagenesis was carried out. An oligonucleotide TG403 (30-mer), the sequence of which is complementary to those of the regions flanking the intron A, was used as a primer for the synthesis of the DNA, using the single

strand of M13TG1835 DNA as a template (Figure 5). The sequence of the oligonucleotide TG403 is as follows:

TG403 5' ACGGATAACCCGGACTACTTCTACACCT 3'

One picomole of single-stranded M13TG1835 DNA was hybridized with one picomole of oligonucleotide TG403 whose 5' ends are phosphorylated in a reaction mixture which contains 10 mM Tris pH 7.5, 10 mM MgCl₂, 50 mM NaCl. The mixture was heated to 100°C for 3 minutes and incubated for 1 hour at room temperature.

1.25 µl of each deoxynucleotide triphosphate at a concentration of 5 mM was added to the hybridization mixture in order to obtain a final concentration of 500 µM. 5 units of DNA polymerase I Klenow fragment were then added to one unit of T₄ DNA Ligase; the elongation reaction was continued at room temperature for two hours. After precipitation with ethanol, the ligation products obtained were digested with the restriction enzyme SacI. This enables the frequency of production of recombinant clones containing the mutated form to be increased. Competent cells of *E. coli* strain JM103 were transformed with the digestion mixture. The plaques derived from this transformation were analyzed by DNA/DNA hybridization, using as a probe the oligonucleotide TG403 depicted above, labelled with ³²P by kinase treatment according to the customary techniques (8). Several positive signals were observed. The DNA sequence of one of the clones is identical to that of M13TG1833 DNA without the intron A sequence. This new phage is referred to as M13TG1837 (Figure 5).

b) Construction of the vector M13TG1846

The vector M13TG1837 DNA was digested with BssHII and AvaII. The 380-bp BssHII-AvaII fragment containing the DNA sequence coding for the NH₂-terminal region of the mature amyloglucosidase of Aspergillus niger (amino acids 21 to 151) was isolated (Figure 2). Similarly, the 280-bp PstI-AvaII fragment of M13TG1839 was isolated. This fragment contains the cDNA sequence coding for amino acids 151 to 256. These two BssHII-AvaII, AvaII-PstI fragments, with two synthetic oligonucleotides TG453, TG454 designed to reconstitute the 5' end of the mature amyloglucosidase and which permit the

in-phase reading of the prepro sequence of the alpha pheromone followed by the mature sequence of amyloglucosidase (Figure 6), are ligated in M13mp8 which has been digested with HindIII and PstI. This new vector is referred to as M13TG1846 (Figure 6).

3) Construction of M13TG1848

In the first place, the HindIII-PstI fragment of M13TG1846 (670 bp) containing the sequence coding for amino acids 21 to 246 was isolated. M13TG1843 DNA was digested with PstI and EcoRV and the 1736-kb PstI-EcoRV fragment derived from this digestion was isolated and eluted from a gel. This fragment contains the 3' portion of the amyloglucosidase gene (presence of the intron D) coding for amino acids 246-640. The EcoRV site is situated downstream from the TGA stop codon (approximately 140 bp). The polyadenylation site is situated at approximately 70 bp upstream from the EcoRV site. These two fragments are ligated in M13TG131 which has been digested with HindIII and HindII. This new vector is referred to as M13TG1848 (Figure 7). This new vector was used as a source of amyloglucosidase coding sequence for the secretion vector described below, despite the presence of the last intron D.

4) Construction of an amyloglucosidase expression vector for yeast *S. cerevisiae*

The starting plasmid is pTG848 (identical to pTG849 described in French Patent Application No. 83/15,716, with the exception of the *ura3* gene whose orientation is inverse); it consists of the following fragments (Figure 8a):

- a) The approximately 3.3-kb EcoRI-HindIII fragment originating from plasmid pJDB207 (9). The HindIII site corresponds to coordinate 105 of the 2 μ plasmid B form, the EcoRI site to coordinate 2243. In this fragment, the 2 μ is in the B form (10). This fragment enables the plasmid to replicate autonomously in yeast.
- b) A HindIII fragment carrying the *URA3* gene (11) which enables the plasmid to be selected in *ura3⁻* strains on medium devoid of a pyrimidine source.
- c) The large EcoRI (coordinate 0)-SalI (coordinate 650)

fragment of pBR322 (12). In the PvuII site of this fragment, there has been inserted the 510-bp EcoRI-HindIII fragment corresponding to the end of the PGK gene (13), the ends of which fragment have been previously rendered blunt by the action of Klenow in the presence of the 4 deoxyribonucleotides. When joined to the PvuII end of pBR322, the flush EcoRI end of the PGK gene regenerates an EcoRI site.

d) The HindIII-SalI fragment (2.15 kb) of the PGK gene (13).

The BglII-HindIII fragment of M13TG1848 was isolated and eluted from gel. It was then ligated with the HindIII-BamHI fragment of M13TG889 containing the prepro sequence of the alpha pheromone in the BglII sites of plasmid pTG848. A new plasmid, referred to as pTG1850, was obtained (Figure 8b).

In summary, plasmid pTG1850 possesses:

- 1) a fragment of the 2 μ plasmid of S. cerevisiae which enables it to replicate in Saccharomyces;
- 2) the URA3⁺ of S. cerevisiae which enables the transformed cells to be selected from a ura3⁻ receptor strain on medium devoid of a pyrimidine source;
- 3) the origin of replication of plasmid pBR322 and the gene for resistance to ampicillin, which enable the plasmid to replicate and the transformants to be selected in E. coli;
- 4) the promoter and the terminator of the yeast PGK gene which provide, respectively, for the initiation and the stopping of transcription of the alpha-amyloglucosidase expression block;
- 5) a gene coding for a precursor of this alpha-amyloglucosidase, consisting of the prepro sequences of the alpha pheromone gene (MFalpha₁) of S. cerevisiae fused to the sequence coding for the mature portion of alpha-amyloglucosidase which still has its intron D.

5) Transformation of a laboratory strain with plasmid pTG1850 DNA

pTG1850 plasmid DNA was amplified in E. coli, purified on a cesium chloride gradient and used for transforming the strain TGY2sp13b, which is a haploid strain of S. cerevisiae having leucine and uracil as auxotrophic markers (Matalpha, ura3-251-373-328, Leu2 3-12). Following the techniques

described by Ito et al. (14), the *ura*⁺ transformants were tested for the expression of the *Aspergillus niger* amyloglucosidase gene. The technique is as follows: the *ura*⁺ clones were subcultured on solid medium containing 2% of starch.

- 5 After 48 hours' growth, 5 ml of aqueous iodine mixture (I₂/KI) were added to each dish. The starch in contact with the iodine (I₂) takes on a blue coloration. Yeasts which produce amyloglucosidase in the culture medium degrade the starch and this gives rise to a halo of decolorization around the colonies. None of the transformants secretes active amyloglucosidase.

6) Excision of the intron D by in vitro mutagenesis

To carry out this in vitro mutagenesis, an oligonucleotide TG496, the sequence of which is complementary to that of the regions flanking the intron D of phage M13TG1847 DNA, depicted in Figure 9, was hybridized. This oligonucleotide served as a primer for the in vitro synthesis of the strand complementary to the phage M13TG1847 genome. The sequence of the oligonucleotide TG496 is as follows:

20 TG496 5' TTCGTCTCTATTGTGGAAACTCACGCCGCA 3'.

The method used is identical to that used for the excision of the intron A. On the other hand, it was not possible to use any restriction enzyme to "sabotage" the non-mutated forms. Several positive signals were observed. The DNA of one of the positive clones was sequenced. The intron D was correctly deleted. This new phage is referred to as M13TG1857 (Figure 9).

7) Construction of plasmid pTG1858

In the first place, the 670-bp BamHI-SphI fragment of M13TG1857 was isolated and purified on gel. It was ligated with the large BamHI-SphI fragment of pTG1850. A new amyloglucosidase expression vector, referred to as pTG1858, was reconstituted. This vector possesses the region coding for the mature amyloglucosidase of *Aspergillus niger* (Figure 10).

EXAMPLE 4

TRANSFORMATION OF A LABORATORY STRAIN WITH pTG1858 DNA

pTG1858 DNA was used to transform a yeast TGY2sp13b

(Matalpha, ura3-251-373-328, leu 2-3-18) for ura⁺, following the techniques described (14); the presence of alpha-glucosidase was tested around the colonies as described above (Figure 11). As expected, it was found that all the cells transformed with plasmid pTG1858 secrete active amyloglucosidase. In addition, the growth of the yeast strains transformed with plasmid pTG1858 was compared with that of the same strain transformed with plasmid pTG848. The yeasts transformed with both plasmids grow on minimal medium containing 2% of starch as a source of carbon. However, the strain transformed with plasmid pTG1858 always shows better growth than the strain transformed with plasmid pTG848 on this medium.

EXAMPLE 5

CHARACTERIZATION OF AN AMYLOGLUCOSIDASE ACTIVITY IN THE YEAST CULTURE SUPERNATANTS

The amount of amyloglucosidase secreted by yeast strain TGY2sp13b containing plasmid pTG1858 was assayed. After two days' incubation in YNBG medium + 0.5% casamino acids at 30°C with stirring at 250 rpm, the culture of TGY2sp13b containing pTG1858 consumed all the glucose and was in stationary phase; the culture was arrested at an approximate cell density of 2.6×10^8 cells/mL. Measurement of the amount of amyloglucosidase present in the unconcentrated supernatant shows that 360 units of active amyloglucosidase per liter of supernatant were produced (one unit of amyloglucosidase activity corresponds to the release of one μ mol of glucose/min from 2% of Zulkowsky starch in 50 mM sodium acetate buffer, pH 4.3 at 55°C).

EXAMPLE 6

CONSTRUCTION OF AN INTEGRATION PLASMID FOR SACCHAROMYCES CEREVISIAE

For this purpose, an EcoRI fragment (1.7 kb) corresponding to the chromosomal region carrying the MFalpha₁ gene of S. cerevisiae (1) was cloned into the EcoRI site of a derivative of pUC8 (pTG1864). This new vector is referred to as pTG1865 (Figure 12).

pTG1865 DNA was digested with HindII, liberating a

small, approximately 0.5-kb fragment internal to the MFalpha₁ gene. At the HindII sites of pTG1865, there were inserted by blunt-end ligation the SmaI-BglII fragment of pTG1858 which includes the amyloglucosidase prepro precursor sequence under the control of the phosphoglycerate kinase gene promoter and the BglII-HindIII fragment of pTG830 which includes the PGK gene terminator sequence, after the action of Klenow polymerase on the HindIII site. This new vector is referred to as pTG1867 (Figure 12). Digestion of pTG1867 with EcoRI liberates a yeast DNA fragment (4 kb) in which the amyloglucosidase expression block has been integrated.

EXAMPLE 7

COTRANSFORMATION OF TGY2sp13b FOR THE PURPOSE OF OBTAINING YEASTS SACCHAROMYCES WHICH HAVE INTEGRATED THE AMYLOGLU- SIDASE EXPRESSION BLOCK

The presence, at the ends of a linear DNA fragment, of regions homologous with a region of the yeast genome favors recombination events between the chromosomal region and the linear fragment. This enables us to substitute the sequence of the linear fragment, which can contain foreign sequences, for the chromosomal sequence. It is hence also possible to obtain the integration of foreign sequences in the yeast chromosome.

The strain TGY2sp13b (Mata⁺ ura3-251-373-328, Leu 2-3-12) was hence cotransformed by the customary technique (14) with 2 µg of pFL1 DNA (15), conferring the ura⁺ phenotype, and at the same time with 10 µg of pUC1867 DNA which had previously been cut with EcoRI. Of the 300 ura⁺ clones obtained, those which had become MFalpha₁ were tested. In effect, the substitution at the MFalpha₁ locus by the linear DNA fragment leads to an inactivation of the MFalpha₁ gene, that is to say a loss in production of the alpha-factor of at least 75% (16). The MFalpha₁ phenotype is tested in the following manner: a lawn of FL100 Mata cells (5 x 10⁴ cells) is plated on a dish containing YNBG + 0.5% casamino acids. A clump of cells originating from the ura⁺ clones was deposited on this lawn. After 48 hours at room temperature, the presence of a halo of inhibition of growth of the Mata strain was

observed around the strains having the MFalpha₁ phenotype. This reflects the fact that the MFalpha₁ strain secretes sufficient alpha pheromone to inhibit the growth of the Mata strains. No halo of inhibition of growth was observed around the mfalpha₁ strains. Of the 300 ura⁺ clones, one mf-alpha₁ transformant, referred to as TGY2sp13b Ø AMGB, was obtained. This clone produces a halo of decolorization in the I₂/KI test after 5 days' growth at 30°C. This clone TGY2sp13b Ø AMGB hence produces active amyloglucosidase.

This clone expresses alpha-amyloglucosidase in stable fashion during several generations. Analysis of the genomic DNA of the strain TGY2sp13b Ø AMGB according to Southern reveals that the amyloglucosidase expression block is integrated in the genome at the MFalpha₁ locus (Figure 13) and that the integration of this foreign fragment has been carried out by an exchange process without integration of the vector pTG1864 sequence.

The amount of amyloglucosidase secreted by the yeast strain TGY2sp13b Ø AMGB and the strain TGY2sp13b containing plasmid pTG1858 was assayed.

After 2 hours' incubation at 30°C with stirring at 250 rpm, the strain TGY2sp13b Ø AMGB produces approximately 6- to 8-fold less active amyloglucosidase than the strain TGY2sp13b containing plasmid pTG1858.

EXAMPLE 8

INTEGRATION IN A POLYPLOID BREWERY STRAIN SACCHAROMYCES UVARUM (2285TG2)

The point at which the amyloglucosidase expression block is integrated in the genome of the brewery strain must be chosen in such a way as not to modify either the physiology or the characteristics of the strain 2285TG2. The choice was made, for the integration site, of the MFalpha₁ gene which is responsible for the synthesis of the alpha factor. A strain of sex type Mata shows no detectable inhibition of growth when placed in contact with a polyploid brewery strain. The alpha factor plays a part in the conjugation between two yeasts of opposite sex type. In strain 2285TG2, inactivation of the MFalpha₁ gene should not modify the physiology of

the strain. The presence of this gene in strain 2285TG2 was determined by analyzing the genomic DNA of this strain according to Southern (Figure 14). The MFalpha₁ gene present in the strain 2285TG2 appears to be broadly homologous with that of the S. cerevisiae strain. A single difference was detected in the region coding for the alpha factor situated between the two HindII sites (Figure 12). This region contains, depending on the strain, a variable number of copies of this alpha factor (17). The difference observed is probably due to the presence of at least one additional copy in the Saccharomyces uvarum strain. In consequence, the amyloglucosidase expression block was inserted into the HindII sites of the MFalpha₁ gene of S. cerevisiae bounding the region coding for the alpha factor.

Since the strain 2285TG2 is polyploid and hence has no auxotrophic marker, the cotransformation with pTG1867 is accomplished using a plasmid which confers a dominant phenotype. Resistance to G418 was used. This plasmid which was used is a derivative of plasmid pTG1861 described in French Patent No. 84/04,453. Plasmid pTG1825 differs from pTG1861 only in respect of the absence of an out-of-phase ATG which has been deleted by in vitro mutagenesis in order to permit better expression of the gene for resistance to G418.

Strain 2285TG2 was transformed with 1 µg of pTG1825 DNA and 10 µg of pTG1867 which has previously been digested with EcoRI, by the technique derived from Dickson (18). The following modification was carried out: approximately 15 hours after the transformation, an overlayer of complete medium (10 ml) containing geneticin, G418 (Difco), was added so that the final concentration of antibiotic was 300 µg/ml in the dish.

Of 300 G418-resistant clones, one clone, 2285TG2 AMGC, produces amyloglucosidase. This clone expresses alpha-amyloglucosidase in stable fashion during several generations.

Analysis of the genomic DNA of the strain 2285TG2 AMGC according to Southern reveals that the amyloglucosidase expression block is integrated in the genome at the MFalpha₁ locus. The amount of amyloglucosidase secreted by the brewery strain was

assayed. After 3 days' incubation in complete YPG medium at 30°C with stirring at 250 rpm, the 2285TG2 AMGC culture at an approximate cell density of 4×10^7 cells/ml produces 100 U of active alpha-amyloglucosidase per liter of supernatant.

5 EXAMPLE 9

The same experiment as described in Example 8 is carried out starting with a strain used in the bread-making industry.

10 Since this strain TGF1 is polyploid and prototrophic, a cotransformation has to be performed with pTG1867 and a plasmid conferring a dominant phenotype, plasmid pTG1825.

15 The strain TGF1 is transformed with the DNA of plasmids pTG1825 and pTG1867 which has previously been digested with EcoRI. Among the 500 clones resistant to G418 (300 µg/ml) obtained, one expresses amyloglucosidase in stable fashion during at least 50 generations; this clone is referred to as TGF1-AMG1.

20 Hybridization of the EcoRI-digested genomic DNAs of the strains TGF1-AMG1 and TGF1 with an oligonucleotide TG529 specific for the MFalpha₁ gene of Saccharomyces cerevisiae by Southern's technique reveals that the amyloglucosidase expression block is integrated in the genome at the MFalpha₁ locus (Figure 15) of TGF1-AMG1.

25 The amount of amyloglucosidase secreted by the strain TGF1-AMG1 was assayed. After three days' incubation in complete YPG medium at 30°C with stirring at 250 rpm, the TGF1-AMG1 culture produces 100 U of active alpha-amyloglucosidase per liter of supernatant of a culture at a cell density of 5×10^7 cells/ml.

30 EXAMPLE 10

INTEGRATION OF THE B. LICHENIFORMIS ALPHA-AMYLASE EXPRESSION BLOCK IN THE POLYPLOID STRAIN SACCHAROMYCES UVARUM 2285 TG2 AMGC

35 The strain 2285 TG2 AMGC was transformed with 1 µg of pTG1825 DNA and 10 µg of M13TG1817 which had previously been digested with BamHI, by the technique derived from Webster and Dickson (18).

By digestion with BamHI, the vector M13TG1817

Liberates a DNA fragment containing the His3 gene of Saccharomyces uvarum in which the cassette for expression of alpha-amylase has been inserted. The construction of the vector M13TG1817 has been described in French Patent No. 86/06,703.

- 5 Of 400 clones resistant to G418 (300 µg/ml), 4 clones produce alpha-amylase. These clones express alpha-amylase in stable fashion during 50 generations. Hybridization of the BamHI-digested genomic DNAs of the strains 2285 TG2 AMGC-AMY1 and 2285 TG2 AMGC with an oligonucleotide TG227 specific
- 10 for the His3 gene of S. uvarum by Southern's technique reveals that the alpha-amylase expression block is integrated in the genome at the His3 locus (Figure 16) of the strain 2285 TG2 AMGC-AMY1.

The following strains were deposited at the Collection Nationale de Cultures de Microorganismes (National Collection of Microorganism Cultures) of the Institut Pasteur, 28 rue du Docteur-Roux - 75724 PARIS, Cedex 15:

- Saccharomyces uvarum 2285 TG2 AMGC
on 6th June 1986 under No. I-568,
- 20 • Saccharomyces cerevisiae TGF1 AMG1
on 27th January 1987 under No. I-650,
- Saccharomyces uvarum 2285 TG2 AMGC-AMY1
on 27th January 1987 under No. I-649.

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The claims defining the invention are as follows:-

1. A block for the expression of a mature amyloglucosidase from ~~Aspergillus~~ ^{Aspergillus} Niger in a yeast, which block contains:
 - (a) the amyloglucosidase gene devoid of all or part of its introns;
 - (b) the DNA sequence containing the signals which provide for the transcription of the amyloglucosidase gene by yeast;
 - (c) a coding sequence containing the signal required for the export of the mature amyloglucosidase into the extracellular medium, which coding sequence contains a secretion signal chosen from the alpha pheromone, the killer protein secretion system or a synthetic secretion sequence; and
 - (d) a yeast termination sequence after the amyloglucosidase gene and wherein said yeast termination sequence is a transcription termination sequence.
2. The expression block as claimed in claim 1, wherein the DNA sequence which provides for the transcription of the gene contains the PGK gene promoter.
3. The expression block as claimed in claim 1 or claim 2, which is integrated in a plasmid containing an origin of replication in yeasts.
4. The expression block as claimed in claim 3, wherein the origin of replication in yeasts is the origin of replication of the 2 μ plasmid.
5. The expression block as claimed in claim 3 or claim 4, wherein the plasmid contains, in addition, a selection gene.
6. The expression block as claimed in claim 5, wherein the selection gene is a gene complementing an auxotrophy.
7. The expression block as claimed in claim 5, wherein the selection gene is a gene conferring a dominant phenotype.
8. The expression block as claimed in claim 7, wherein the dominant phenotype is resistant to G418.
9. The expression block as claimed in any one of claims 1 to 3, which is integrated in the genome of the yeast strain.
10. The expression block as claimed in claim 9, wherein the integration is performed in the MFalpha₁ gene.



11. A transformed yeast strain containing an expression block as claimed in any one of claims 1 to 10.
12. The strain as claimed in claim 11, which is a strain of Saccharomyces.
13. The strain as claimed in claim 12, which is a strain chosen from S. cerevisiae and S. uvarum.
14. The transformed strain as claimed in any one of claims 11 to 13, which is a polyploid and protrophic strain of Saccharomyces.
15. The strain as claimed in claim 14, which is a strain of Saccharomyces used in the bread-making industry.
16. The strain as claimed in claim 15, which is Saccharomyces cerevisiae strain TGF 1.
17. The strain as claimed in any one of claims 14 to 16, wherein the amyloglucosidase expression block is derived from plasmid pTG1867.
18. The strain of Saccharomyces as claimed in any one of claims 11 to 13, which is transformed simultaneously by an expression block as claimed in any one of claims 1 to 10 and by an alpha-amylase expression block, and which simultaneously secretes alpha-amylase and amyloglucosidase.
19. The strain as claimed in claim 18, which is a brewery strain of the Saccharomyces uvarum type.
20. The strain as claimed in claim 19, which is S. uvarum strain 2285 TG2 AMGC.
21. The strain as claimed in any one of claims 18 to 20, wherein the alpha-amylase expression block is derived from the vector M13TG1817.
22. A process for producing alpha-amyloglucosidase, wherein a strain as claimed in any one of claims 11 to 21 is fermented on a suitable culture medium and wherein the enzyme is recovered in the extracellular medium.
23. A process for panary fermentation, wherein at least one of the strains used in the fermentation is a strain as claimed in any one of claims 14 to 17.
24. A fermentation process, wherein at least one of the strains used in the fermentation is a strain as claimed in any one of claims 18 to 21.
25. A block for the expression of an amyloglucosidase in yeast and a transformed yeast strain containing said block substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.
26. A process for producing alpha-amyloglucosidase substantially as hereinbefore



described with reference to any one or more of the examples and/or drawings.

27. A process for panary fermentation substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.

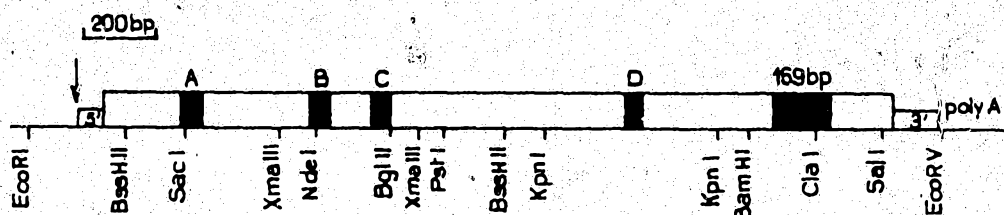
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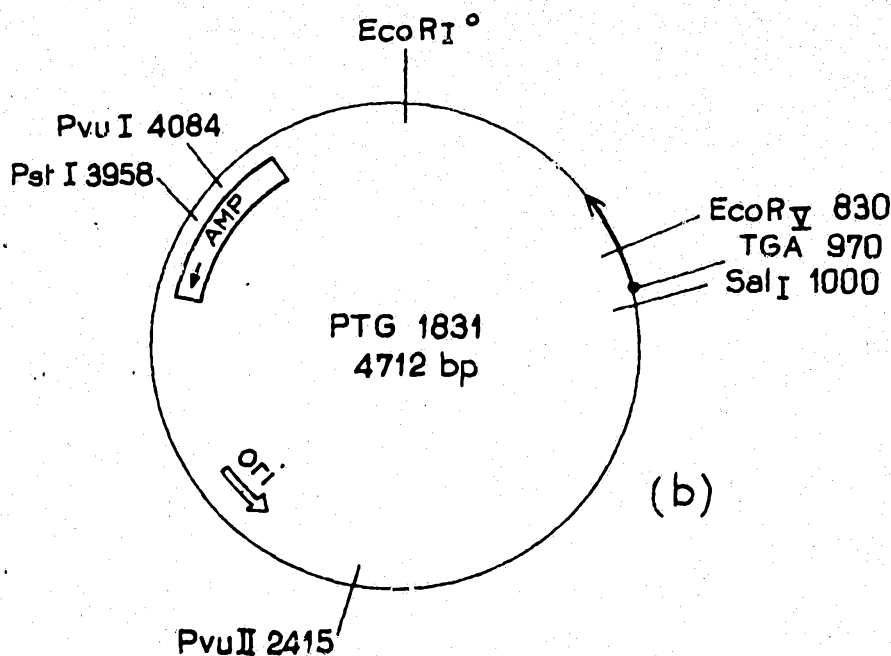


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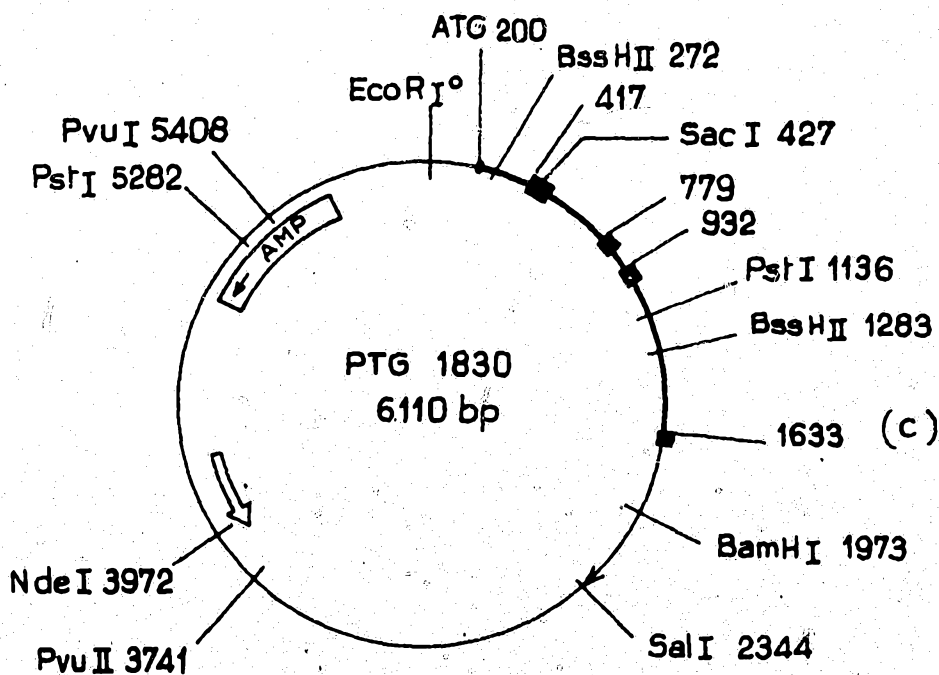
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(a)



(b)



(c)

FIG. 1

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FIG. 2

TTCTGTCGCCTAATGTCTCGTCCGTTACAAACTG

AAGAGCTTGAAGTGGCGAGATGTCTCTGCAGGAATTC
EcoRI

AAGCTAGATGCTAAGCGATATTGCAT GGCAATAT GTGTT

GATGCATGTGCTTCTTCCTTCAGCTTCCCCTCGTGCGAGTGA

GGTTTGGC TATAAAT TGAAGTGGTTGGTCGGGGGTTCT

CGTGAGGGGGCTGAAGTGCTTCCTCCCTTTTAGGCGCAAC

TGAGAGCCTGAGCTTCATCCCCAGCATCATTACACCTCAGCA

ATG TCG TTC CGA TCT CTA CTC GCC CTG AGC
Met Ser Phe Arg Ser Leu Leu Ala Leu Ser

GGC CTC GTC TGC ACA GGG TTG GCA AAT GTG
Gly Leu Val Cys Thr Gly Leu Ala Asn Val

ATT TCC AAG CGC GCG ACC TTG GAT TCA TGG
Ile Ser Lys Arg Ala Thr Leu Asp Ser Trp

TTG AGC AAC GAA GCG ACC GTG GCT CGT ACT
Leu Ser Asn Glu Ala Thr Val Ala Arg Thr

GCC ATC CTG AAT AAC ATC GGG GCG GAC GGT
Ala Ile Leu Asn Asn Ile Gly Ala Asp Gly

FIG. 2

GCT TGG GTG TCG GGC GCG GAC TCT GGC ATT⁶⁰
 Ala Trp Val Ser Gly Ala Asp Ser Gly Ile
 GTC GTT GCT AGT CCC AGC ACG GAT AAC CCG⁷⁰
 Val Val Ala Ser Pro Ser Thr Asp Asn Pro
 GAC T GTATGTTTCGAGCTCAGATTAGTATGAGTGTGTC.
 Asp IVS

ATTGATTGATTGATGCTGACTGGCGTGTCGTTTGTTGTAG
 A

AC TTC TAC ACC TGG ACT CGC GAC TCT GGT⁸⁰
 Tyr Phe Tyr Thr Trp Thr Arg Asp Ser Gly

CTC GTC CTC AAG ACC CTC GTC GAT CTC TTC⁹⁰
 Leu Val Leu Lys Thr Leu Val Asp Leu Phe

CGA AAT GGA GAT ACC AGT CTC CTC TCC ACC¹⁰⁰
 Arg Asn Gly Asp Thr Ser Leu Leu Ser Thr

ATT GAG AAC TAC ATC TCC GCC CAG GCA¹¹⁰
 Ile Glu Asn Tyr Ile Ser Ala Gln Ala

ATT GTC CAG GGT ATC AGT AAC CCC TCT GGT¹²⁰
 Ile Val Gln Gly Ile Ser Asn Pro Ser Gly

GAT CTG TCC AGC GGC GCT GGT CTC GGT GAA¹³⁰
 Asp Leu Ser Ser Gly Ala Gly Leu Gly Glu

FIG. 2

CCC AAG TTC AAT GTC GAT GAG ACT GCC TAC¹⁴⁰
 Pro Lys Phe Asn Val Asp Glu Thr Ala Tyr

ACT GGT TCT TGG GGA CGG CCG CAG CGA GAT¹⁵⁰
 Thr Gly Ser Trp Gly Arg Pro Gln Arg Asp

GGT CCG GCT CTG AGA GCA ACT GCT ATG ATC¹⁶⁰
 Gly Pro Ala Leu Arg Ala Thr Ala Met Ile

GGC TTC GGG CAG TGG CTG CTT GTATGTTCTCCAC
 Gly Phe Gly Gln Trp Leu Leu

CCCCTTGCGTCTGATCTGTGACATATGTAGCTGACTGGTCAG
 IVS B

GAC AAT¹⁷⁰ GGC TAC ACC AGC ACC GCA ACG GAC
 Asp Asn Gly Tyr Thr Ser Thr Ala Thr Asp
TG433

ATT GTT TGG CCC CTC GTT AGG AAC GAC CTG
 Ile Val Trp Pro Leu Val Arg Asn Asp Leu

TCG TAT¹⁹⁰ GTG GCT CAA TAC TGG AAC CAG ACA
 Ser Tyr Val Ala Gln Tyr Trp Asn Gln Thr

GGA TAT G GTGTGTTTGTGTTTATTTTAAATTTCCAAAGA
 Gly Tyr IVS C

TGCGCCAGCAGAGCTAACCCGCGATCGCAG²⁰⁰ AT CTC
 Asp Leu

FIG. 2

TGG	GAA	GAA	GTC	AAT	GGC	TCG	TCT	²¹⁰ TTC	TTT
Trp	Glu	Glu	Val	Asn	Gly	Ser	Ser	Phe	Phe
ACG	ATT	GCT	GTG	CAA	CAC	CGC	GCC	²²⁰ CTT	GTC
Thr	Ile	Ala	Val	Gln	His	Arg	Ala	Leu	Val
GAA	GGT	AGT	GCC	TTC	GCG	ACG	GCC	²³⁰ GTC	GGC
Glu	Gly	Ser	Ala	Phe	Ala	Thr	Ala	Val	Gly
TCG	TCC	TGC	TCC	TGG	TGT	GAT	TCT	²⁴⁰ CAG	GCA
Ser	Ser	Cys	Ser	Trp	Cys	Asp	Ser	Gln	Ala
CCC	GAA	ATT	CTC	TGC	TAC	CTG	CAG	²⁵⁰ TCC	TTC
Pro	Glu	Ile	Leu	Cys	Tyr	Leu	Gln	Ser	Phe
TGG	ACC	GGC	AGC	TTC	ATT	CTG	GCC	²⁶⁰ AAC	TTC
Trp	Thr	Gly	Ser	Phe	Ile	Leu	Ala	Asn	Phe
GAT	AGC	AGC	CGT	TCC	GGC	AAG	GAC	²⁷⁰ GCA	AAC
Asp	Ser	Ser	Arg	Ser	Gly	Lys	Asp	Ala	Asn
ACC	CTC	CTG	GGA	AGC	ATC	CAC	ACC	²⁸⁰ TTT	GAT
Thr	Leu	Leu	Gly	Ser	Ile	His	Thr	Phe	Asp
CCT	GAG	GCC	GCA	TGC	GAC	GAC	TCC	²⁹⁰ ACC	TTC
Pro	Glu	Ala	Ala	Cys	Asp	Asp	Ser	Thr	Phe
CAG	CCC	TGC	TCC	CCG	CGC	GCG	CTC	³⁰⁰ GCC	AAC
Gln	Pro	Cys	Ser	Pro	Arg	Ala	Leu	Ala	Asn
CAC	AAG	GAG	GTT	GTA	GAC	TCT	TTC	³¹⁰ CGC	TCA
His	Lys	Glu	Val	Val	Asp	Ser	Phe	Arg	Ser

TG387

ATC TAT ACC CTC AAC GAT GGT CTC AGT GAC³²⁰
 Ile Tyr Thr Leu Asn Asn Gly Leu Ser Asp

AGC GAG GCT GTT GCG GTG GGT CGG TAC CCT³³⁰
 Ser Glu Ala Val Ala Val Gly Arg Tyr Pro

GAG GAC ACG TAC TAC AAC GGC AAC CCG TGG³⁴⁰
 Glu Asp Thr Tyr Tyr Asn Gly Asn Pro Trp

TG283

TTC CTG TGC ACC TTG GCT GCC GCA GAG³⁵⁰ CAG
 Phe Leu Cys Thr Leu Ala Ala Ala Glu Gln

TTG TAC GAT GCT CTA TAC CAG TGG GAC³⁶⁰ AAG
 Leu Tyr Asp Ala Leu Tyr Gln Trp Asp Lys

CAG GGG TCG TTG GAG GTC ACA GAT GTG³⁷⁰ TCG
 Gln Gly Ser Leu Glu Val Thr Asp Val Ser

CTG GAC TTC TTC AAG GCA CTG TAC AGC³⁸⁰ GAT
 Leu Asp Phe Phe Lys Ala Leu Tyr Ser Asp

GCT GCT ACT GGC ACC TAC TCT TCG TCC³⁹⁰ AGT
 Ala Ala Thr Gly Thr Tyr Ser Ser Ser Ser

TCG ACT TAT AGT AGC ATT GTA GAT GCC⁴⁰⁰ GTG
 Ser Thr Tyr Ser Ser Ile Val Asp Ala Val

AAG ACT TTC GCC GAT GGC TTC GTC TCT ATT⁴¹⁰
 Lys Thr Phe Ala Asp Gly Phe Val Ser Ile

GTG GTAAGTCTACGCTAGACAAGCGCTCATGTTGACAG
Val IVS D

FIG. 2

AGGGTGCGTACTAACAGAAGTAG GAA ACT CAC GCC
Glu Thr His Ala

⁴²⁰
 GCA AGC AAC GGC TCC ATG TCC GAG CAA TAC
 Ala Ser Asn Gly Ser Met Ser Glu Gln Tyr
—TG496

⁴³⁰
 GAC AAG TCT GAT GGC GAG CAG CTT TCC GCT
 Asp Lys Ser Asp Gly Glu Gln Leu Ser Ala

⁴⁴⁰
 CGC GAC CTG ACC TGG TCT TAT GCT GCT CTG
 Arg Asp Leu Thr Trp Ser Tyr Ala Ala Leu

⁴⁵⁰
 CTG ACC GCC AAC AAC CGT CGT AAC TCC GTC
 Leu Thr Ala Asn Asn Arg Arg Asn Ser Val

⁴⁶⁰
 GTG CCT GCT TCT TGG GGC GAG ACC TCT GCC
 Val Pro Ala Ser Trp Gly Glu Thr Ser Ala

⁴⁷⁰
 AGC AGC GTG CCC GGC ACC TGT GCG GCC ACA
 Ser Ser Val Pro Gly Thr Cys Ala Ala Thr

⁴⁸⁰
 TCT GCC ATT GGT ACC TAC AGC AGT GTG ACT
 Ser Ala Ile Gly Thr Tyr Ser Ser Val Thr

⁴⁹⁰
 GTC ACC TCG TGG CCG AGT ATC GTG GCT ACT
 Val Thr Ser Trp Pro Ser Ile Val Ala Thr

⁵⁰⁰
 GGC GGC ACC ACT ACG ACG GCT ACC CCC ACT
 Gly Gly Thr Thr Thr Thr Ala Thr Pro Thr

FIG. 2

510
GGA TCC GGC AGC GTG ACC TCG ACC AGC AAG
Gly Ser Gly Ser Val Thr Ser Thr Ser Lys

520
ACC ACC GCG ACT GCT AGC AAG ACC AGC ACC
Thr Thr Ala Thr Ala Ser Lys Thr Ser Thr

530
A GT ACG TCA TCA ACC TCC TGT ACC ACT CCC
Ser Thr Ser Ser Thr Ser Cys Thr Thr Pro

540
ACC GCC GTG GCT GTG ACT TTC GAT CTG ACA
Thr Ala Val Ala Val Thr Phe Asp Leu Thr

550
GCT ACC ACC ACC TAC GGC GAG AAC ATC TAC
Ala Thr Thr Thr Tyr Gly Glu Asn Ile Tyr

560
CTG GTC GGA TCG ATC TCT CAG CTG GGT GAC
Leu Val Gly Ser Ile Ser Gln Leu Gly Asp

570
TGG GAA ACC AGC GAC GGC ATA GCT CTG AGT
Trp Glu Thr Ser Asp Gly Ile Ala Leu Ser
TG284

580
GCT GAC AAG TAC ACT TCC AGC GAC CCG CTC
Ala Asp Lys Tyr Thr Ser Ser Asp Pro Leu

590
TGG TAT GTC ACT GTG ACT CTG CCG GCT GGT
Trp Tyr Val Thr Val Thr Leu Pro Ala Gly

600
GAG TCG TTT GAG TAC AAG TTT ATC CGC
Glu Ser Phe Glu Tyr Lys Phe Ile Arg

FIG. 2

⁶¹⁰
 ATT GAG AGC GAT GAC TCC GTG GAG TGG GAG
 Ile Glu Ser Asp Asp Ser Val Glu Trp Glu

⁶²⁰
 AGT GAT CCC AAC CGA GAA TAC ACC GTT CCT
 Ser Asp Pro Asn Arg Glu Tyr Thr Val Pro

⁶³⁰
 CAG GCG TGC GGA ACG TCG ACC GCG ACG GTG
 Gln Ala Cys Gly Thr Ser Thr Ala Thr Val

⁶⁴⁰
 ACT GAC ACC TGG CGG TGA CAATCAATCCATTTCGCT
 Thr Asp Thr Trp Arg Stop

ATAGTTAAAGGATGGGGATGAGGGCAATTGGTTATATGATCATG
TG282

TATGTAGTGGGTGTGCATAATAGTAGTGAAATGGAAGCCA

AGTCATGTGATTGTAATCGA[↓]CCGACGGAATTGAGGATATC
 EcoRV

CGGAAATACAGACACCGGG

FIG. 2

ATT GAG AGC GAT ⁶¹⁰GAC TCC GTG GAG TGG GAG
Ile Glu Ser Asp Asp Ser Val Glu Trp Glu

AGT GAT CCC AAC ⁶²⁰CGA GAA TAC ACC GTT CCT
Ser Asp Pro Asn Arg Glu Tyr Thr Val Pro

CAG GCG TGC GGA ⁶³⁰ACG TCG ACC GCG ACG GTG
Gln Ala Cys Gly Thr Ser Thr Ala Thr Val

ACT GAC ACC TGG ⁶⁴⁰CGG TGA CAATCAATCCATTTCGCT
Thr Asp Thr Trp Arg Stop

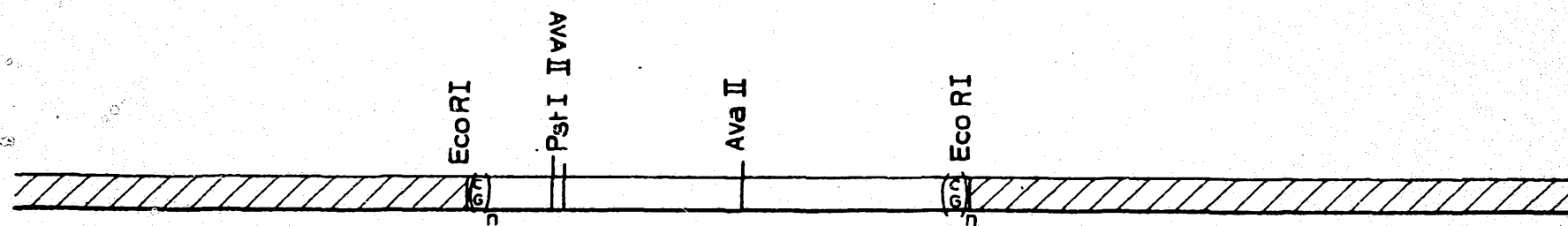
ATAGTTAAAGGATGGGGATGAGGGCAATTGGTTATATGATCATG
TG282

TATGTAGTGGGTGTGCATAAGTAGTAGTGAAATGGAAGCCA

AGTCATGTGATTGTAATCGA [↓]CCGACGGAATTGAG GATATC
EcoRV

CGGAAATACAGACACCGGG

13 13 13 13



M13mp 1839

 M13mp 8
 CDNA

FIG. 3

15 0 07 74438

PTG 1831

EcoRI et SalI

M13Tg 1843 :

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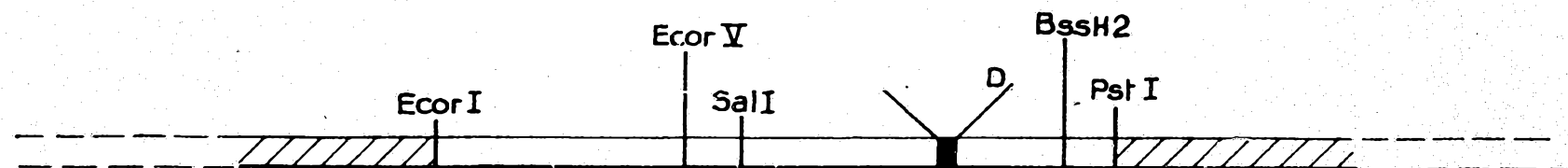
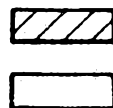
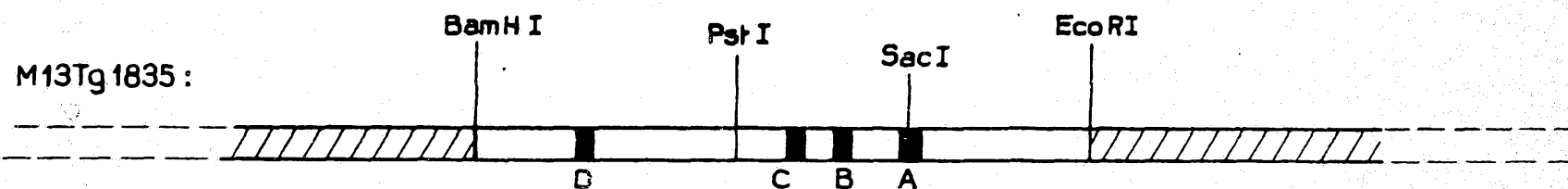


FIG.4



15 00 7435



5' - ACGGATAACCCGGACTACTTCTACACCT - 3'
 3'GGGTCGTGCCTATTGGGCCTGATGAAGATGTGGACCTGAG... ..5'

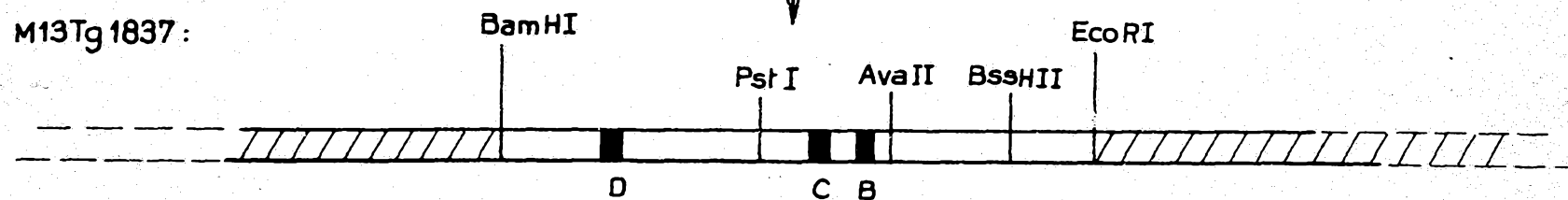
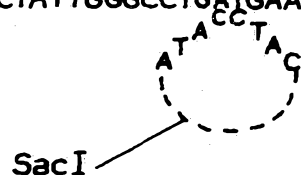


FIG. 5

15 6 87 74435

M13Tg 1837

M13Tg 1839



Tg 453:
Tg 454:

Ala	Ser	Leu	Asp	Lys
A	GCT	TCT	TTG	GAT
		AGA	AAC	CTA
			TTC	GCG

BssHII

Hind III

Hind III et Pst I



M13Tg 1846:

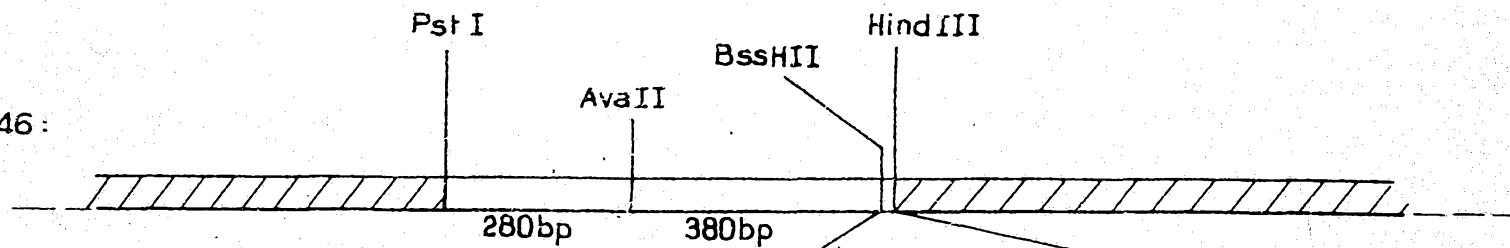


FIG. 6

13 00 7435

M13Tg 1846

Hind III et Pst I

M13Tg 1843

246 à 640

Hind III et Hind II

M13Tg 1848 :

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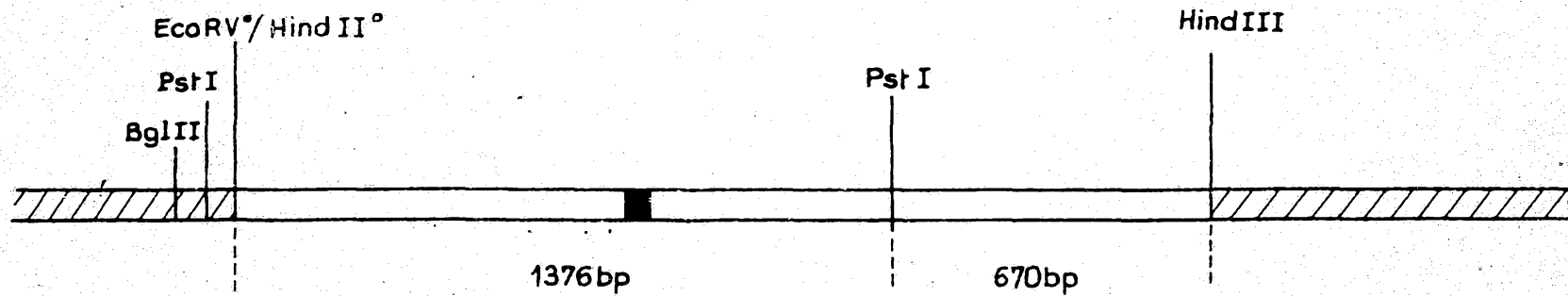


FIG. 7

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ptG 848

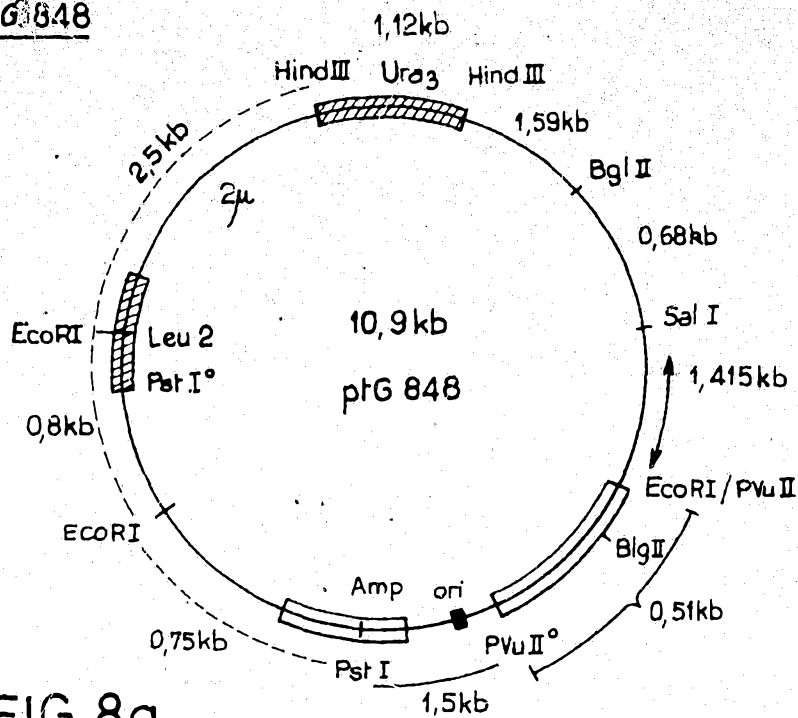


FIG.8a

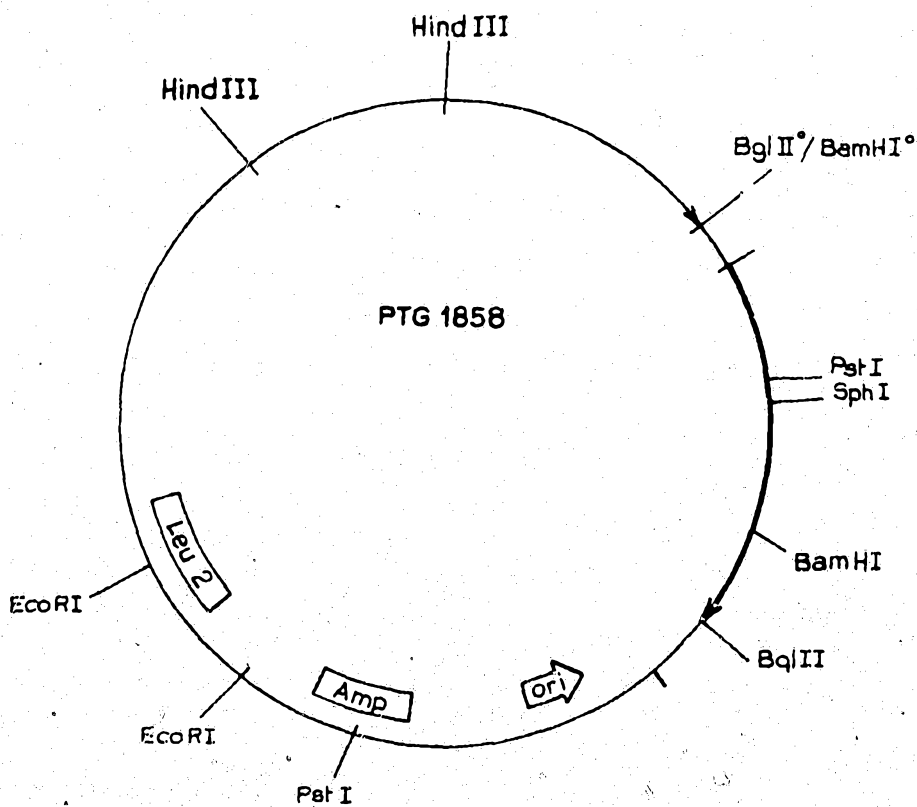


FIG.10

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+ M13Tg 889

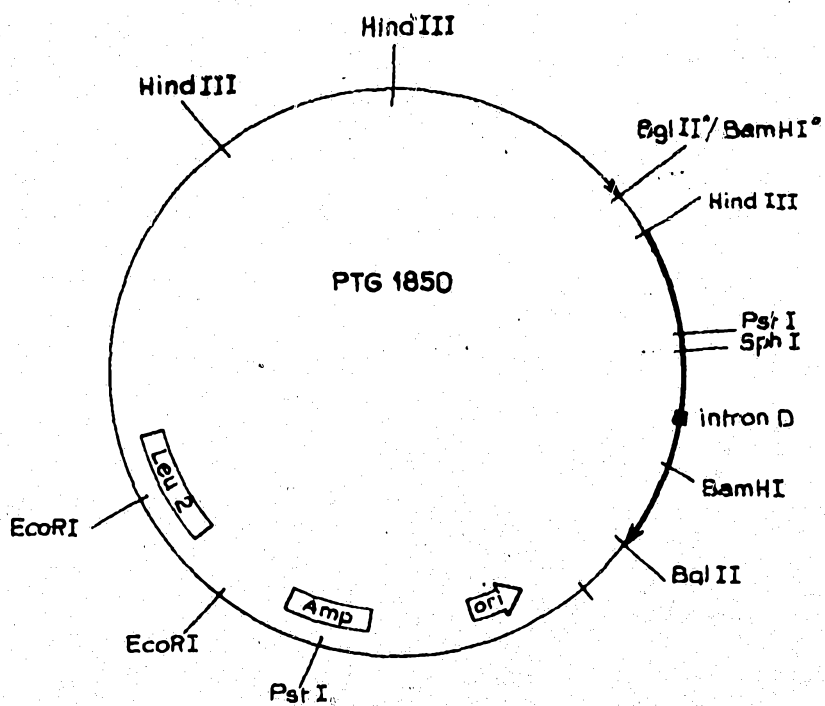
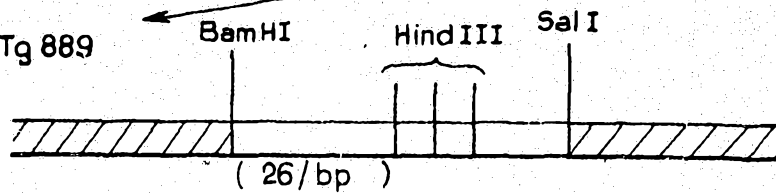
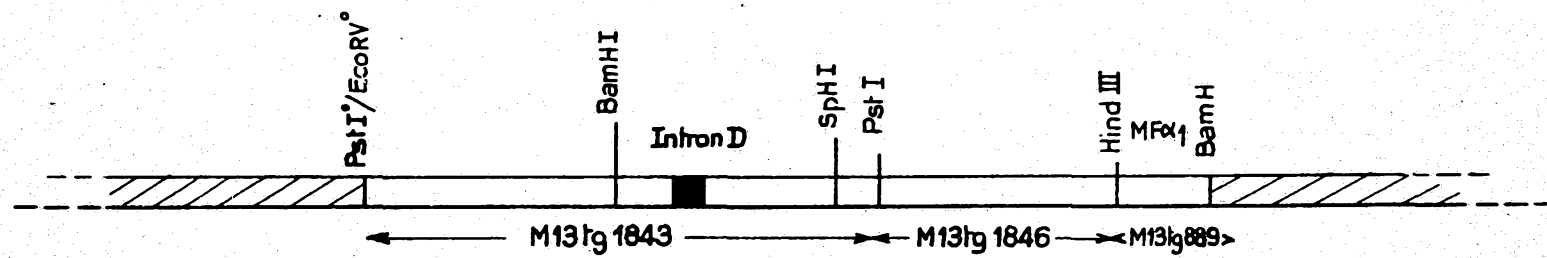


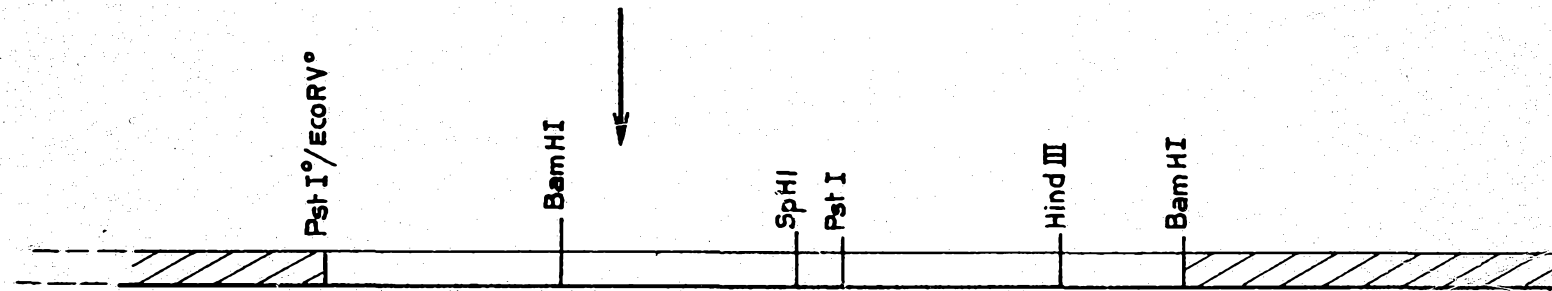
FIG. 8b

15 00 7433



M13lg 1847

5' TTCGTCTCTATTGTGGAAACTCACGCCGCA 3'
CCG AAGCAGAGATAACACCTTTGAGTGCGGCGT TCGTTG



M13lg 1857

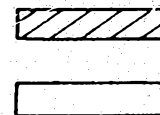


FIG. 9

15 0 0 7 7 4 3 3

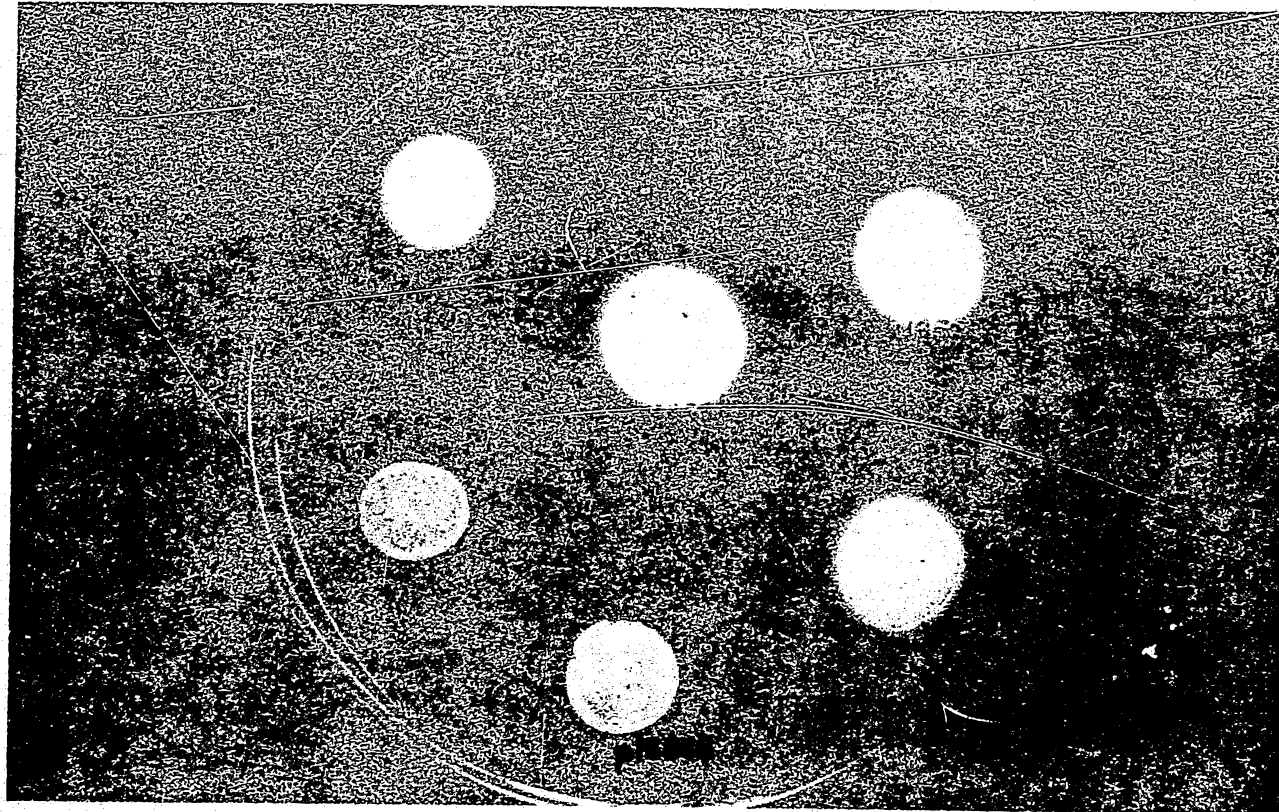
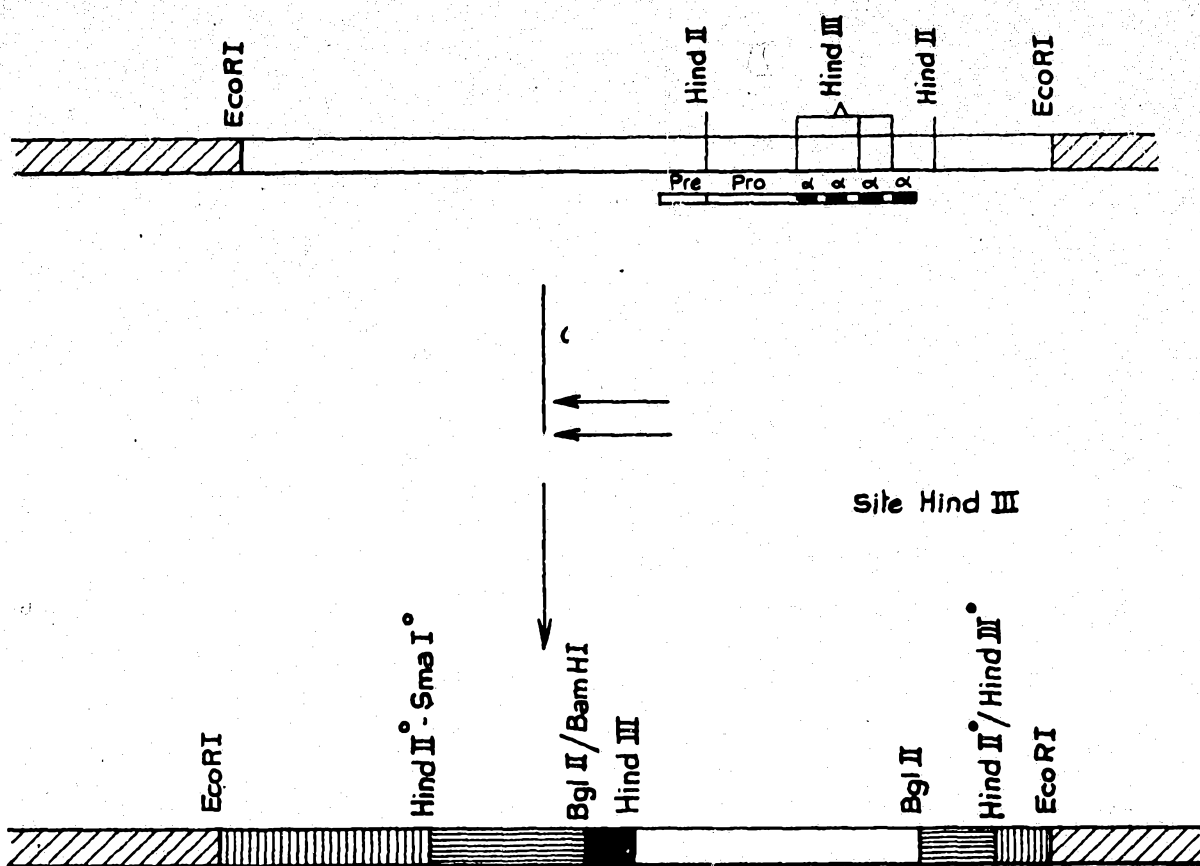


FIG. 11

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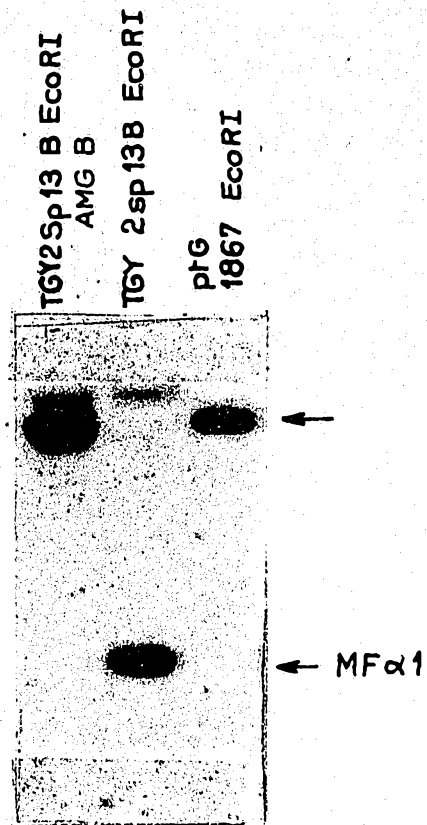
12 / 16



ptG 1867

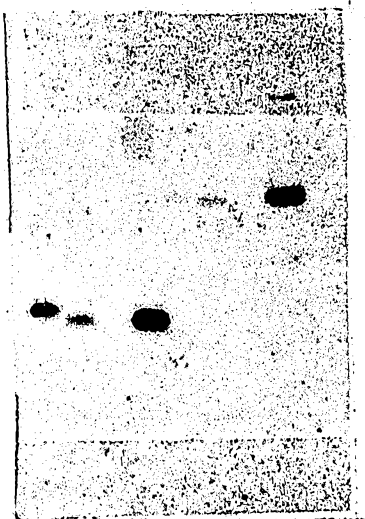
FIG. 12

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FIG_13

R285 TG2 Hind II
 TGY2 Sp13 B Hind II
 pTG 1867 Hind II
 2285 TG2 EcoRI
 TGY 2 Sp13 EcoRI
 pTG 1867 EcoRI



TG 170

pTG 1867 EcoRI
 Hind II
 2285 TG EcoRI
 Hind II
 TGY 2Sp13 B
 EcoRI - Hind II

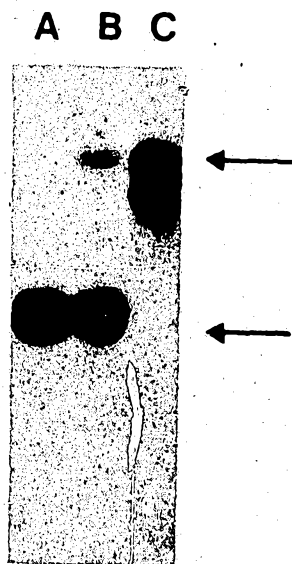


TG 529

FIG. 14

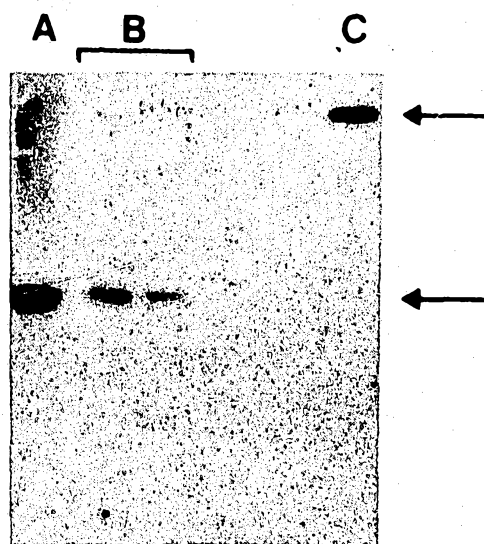
15 / 16

FIG_15

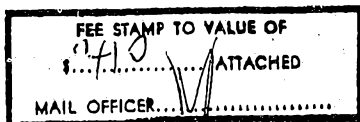
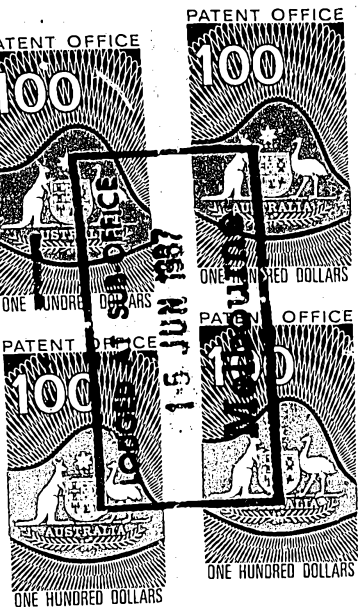


A TGF1
B TGF1-AMG1 Eco RI
C pTG 1867 Eco RI

FIG. 16



A 2285 TG2 AMGC
B 2285 TG2 AMGC-AMY1
C M13TG1817

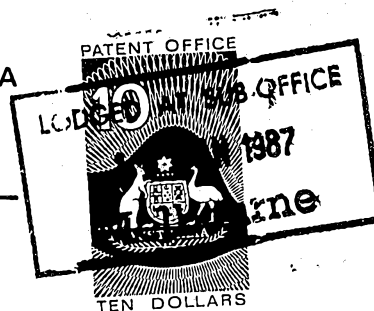


\$410

COMMONWEALTH OF AUSTRALIA

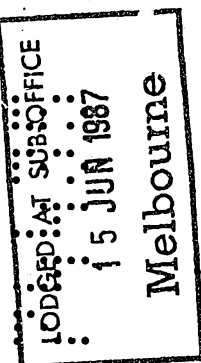
Patents Act 1952

612112



APPLICATION FOR A STANDARD PATENT

(Combined Form — Convention and Non-Convention)



X/We TRANSGENE S.A., a French body corporate, of 16 rue
Henri Regnault, 92400 Courbevoie, France,

hereby apply for the grant of a Standard Patent for an invention entitled BLOCK FOR ...
THE EXPRESSION OF AN AMINOGLUCOSIDASE IN A YEAST,
TRANSFORMED YEAST, ENZYME PREPARATION PROCESS AND
FERMENTATION PROCESS.
which is described in the accompanying ~~Provisional~~ Complete Specification.

2. This application is a convention application and is based on the application(s) for a
patent or similar protection made —

in FRANCE
on June 10, 1986, numbered 86 08387, and
on April 13, 1987, numbered 87 05207, and
on April 13, 1987, numbered 87 05208

3. My/Our address for service is: Care of COWIE, THOMSON & CARTER, Patent
Attorneys, of 71 Queens Road, Melbourne, Victoria 3004, Australia.

DATED this 15th day of June 19 87

To The Commissioner of Patents
COMMONWEALTH OF AUSTRALIA

COWIE, THOMSON & CARTER.

by:

Patent Attorneys for
TRANSGENE S.A.

COWIE, THOMSON & CARTER

Patent Attorneys
71 Queens Road, Melbourne,
Victoria, 3004, Australia

PATENT DECLARATION FORM
(CONVENTION OR NON-CONVENTION)

DECLARATION IN SUPPORT OF APPLICATION FOR A PATENT

Insert name of
applicant.In support of the application made by TRANSGENE S.A.
16 rue Henri Regnault, 92400 COURBEVOIE, FRANCEInsert title of
invention.for a patent for an invention entitled: BLOCK FOR THE EXPRESSION OF AN AMINOGLUCOSIDASE IN
YEAST, TRANSFORMED YEAST, ENZYME PREPARATION PROCESS AND FERMENTATION
PROCESSInsert full name(s)
and address(es) of
person(s) making
declaration. If
applicant a company,
person must be
authorised to make
declaration.I/We Etienne EISENMANN
c/o TRANSGENE S.A. 16 rue Henri Regnault, 92400 COURBEVOIE
FRANCEDelete alternatives
which do not apply

do solemnly and sincerely declare as follows:

- 1. (a) ~~I am/We are the applicant(s) for the patent.~~
- OR (b) I am authorized by the abovementioned applicant to make this declaration on its behalf.

- 2. (a) ~~I am/We are the actual inventor(s) of the invention.~~

Insert name(s) and
address(es) of actual
inventor(s).

~~OR~~ (b) Naïmalé LABAT, 6 rue des Dentelles, 67000 STRASBOURG, FRANCE
Martine NGUYEN JUILLE IT, 32 Avenue du Général de Gaulle,
67000 STRASBOURG, FRANCE
Gérard LOISON, 19 rue du Faubourg National, 67000 STRASBOURG,
FRANCE
Yves LEMCINE, 4 rue des Alisiers, 67100 STARBOURG-NEUDORF, FRANCE

Insert details of
entitlement to apply,
e.g. Applicant is
assignee of inventor(s)is/are the actual inventor(s) of the invention and the facts upon which the applicant(s) is/are entitled
to make the application are as follows:—The Applicant is the Assignee of the said actual inventors.Delete 3 and 4 if
application non-
convention.
Otherwise insert
details of basic
application(s).

3. The basic application(s) as defined by Section 141 of the Act ~~was/were~~ made in the follow-
ing country or countries on the following date(s) by the following applicant(s)
in FRANCE on JUNE 10 1986
by TRANSGENE S.A. under number 86 08387
in FRANCE on APRIL 13 1987
by TRANSGENE S.A. under number 87 05207
in FRANCE on APRIL 13 1987
by TRANSGENE S.A. under number 87 05208
in FRANCE on APRIL 13 1987
by TRANSGENE S.A. under number 87 05208
in FRANCE on APRIL 13 1987
by TRANSGENE S.A. under number 87 05208
in FRANCE on APRIL 13 1987
by TRANSGENE S.A. under number 87 05208

4. The basic application(s) referred to in paragraph 3 of this Declaration ~~was/were~~ the first
application(s) made in a Convention country in respect of the invention the subject of the
application.

Place and date of
Signature.Declared at PARIS this 29 day of MAY 1987NO ATTESTATION
OR SEAL(MANAGER) Etienne EISENMANNDirector Signature(s) of declarant(s).
Etienne EISENMANNTo: The Commissioner of Patents,
Australia

(12) PATENT ABRIDGMENT (11) Document No. AU-B-74435/87
(19) AUSTRALIAN PATENT OFFICE (10) Acceptance No. 612112

- (54) Title
EXPRESSION OF AMINOGLUCOSIDASE IN YEAST
- International Patent Classification(s)
(51)⁴ C07H 021/04 A21D 008/04 C12N 001/16 C12N 001/18
C12N 001/19 C12N 009/34 C12N 015/00 C12N 015/56
- (21) Application No. : 74435/87 (22) Application Date : 15.06.87
- (30) Priority Data
- | | | |
|-------------|-----------|--------------|
| (31) Number | (32) Date | (33) Country |
| 86 08387 | 10.06.86 | FR FRANCE |
| 87 05207 | 13.04.87 | FR FRANCE |
| 87 05208 | 13.04.87 | FR FRANCE |
- (43) Publication Date : 04.02.88
- (44) Publication Date of Accepted Application : 04.07.91
- (71) Applicant(s)
TRANSGENE S.A.
- (72) Inventor(s)
NATHALIE LABAT; GERARD LOISON; MARTINE NGUYEN-JUILLERET; YVES LEMOINE
- (74) Attorney or Agent
COWIE CARTER & HENDY , 71 Queens Road, MELBOURNE VIC 3004
- (56) Prior Art Documents
EP 163491
AU 59544/86 C12N 15/00
EP 126206
- (57) Claim

1. A block for the expression of a mature amyloglucosidase from *Aspargillus Niger* in a yeast, which block contains:

- (a) the amyloglucosidase gene devoid of all or part of its introns;
- (b) the DNA sequence containing the signals which provide for the transcription of the amyloglucosidase gene by yeast;
- (c) a coding sequence containing the signal required for the export of the mature amyloglucosidase into the extracellular medium, which coding sequence contains a secretion signal chosen from the alpha pheromone, the killer protein secretion system or a synthetic secretion sequence; and
- (d) a yeast termination sequence after the amyloglucosidase gene and wherein said yeast termination sequence is a transcription termination sequence.

(11) AU-B-74435/87
(10) 612112

-2-

11. A transformed yeast strain containing an expression block as claimed in any one of claims 1 to 10.

18. The strain of Saccharomyces as claimed in any one of claims 11 to 13, which is transformed simultaneously by an expression block as claimed in any one of claims 1 to 10 and by an alpha-amylase expression block, and which simultaneously secretes alpha-amylase and amyloglucosidase.

25. A block for the expression of an amyloglucosidase in yeast and a transformed yeast strain containing said block substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.

26. A process for producing alpha-amyloglucosidase substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.

612112



PATENTS ACT 1952-1973

Form 10

COMPLETE SPECIFICATION

(ORIGINAL)

FOR OFFICE USE

Class:

Int. Cl:

Application Number:

Lodged:

Complete Specification—Lodged:

Accepted:

Published:

Priority:

Related Art:

TO BE COMPLETED BY APPLICANT

Name of Applicant: TRANSGENE S.A., a French body corporate, of 16 rue
Henri Regnault, 92400 Courbevoie, France.

Address of Applicant:

Actual Inventor: NATHALIE LABAT MARTINE NGUYEN-JUILLERET
GERARD LOISON YVES LEMOINE

COWIE, THOMSON & CARTER
PATENT & TRADEMARK ATTORNEYS
71 QUEENS ROAD
MELBOURNE, 3004, AUSTRALIA

Address for Service:

Complete Specification for the invention entitled:

BLOCK FOR THE EXPRESSION OF AN AMINOGLUCOSIDASE IN A
YEAST, TRANSFORMED YEAST, ENZYME PREPARATION PROCESS AND
FERMENTATION PROCESS

The following statement is a full description of this invention, including the best method of performing it known
to me:—

- 1 -

*Note: The description is to be typed in double spacing, pica type face, in an area not exceeding 250 mm in depth and 160 mm in width,
on tough white paper of good quality and it is to be inserted inside this form.

The present invention describes processes by means of which different strains of yeast of the genus Saccharomyces, including the industrial strains which do not have the capacity to degrade complex carbon-containing substrates such as starch or dextrans, may be caused to secrete a glucoamylase.

Starch, which is commonly used as a source of carbon in alcoholic fermentation, has to be hydrolyzed beforehand (either chemically or enzymatically) in a separate process in order to produce substrates which are usable for fermentative yeasts.

It would hence be desirable to construct, by genetic recombination, industrial strains which were capable of synthesizing one or more enzymes for degrading starch to substrates which are directly assimilable by these strains.

More especially, the objective of the present invention is to construct, by genetic recombination, a strain responsible for brewery fermentation capable of degrading the residual dextrans in the wort so as to produce beers having a low content of sugars.

Similarly, the enrichment of the wort with respect to fermentable sugars also enables the production of beers having a high alcohol content to be envisaged.

The use of a brewery strain which produces an enzyme responsible for the degradation of dextrans during alcoholic fermentation may prove to be a means of simplifying the brewing processes and making them profitable.

The preparation of beers having a low content of residual sugars can, in particular, be achieved by adding, at various stages of the brewing process, an Aspergillus niger glucoamylase (amylo-1,6-glucosidase). This glycoamylase catalyses the release of glucose residues from the non-reducing ends of starch or dextrans.

This enzyme plays a big part in the production of glucose syrups from starch and in the manufacture of other alcoholic drinks. This Aspergillus niger glucoamylase is accepted as an additive in the agri-foodstuffs industries.

Hence, in the first place, the subject of the present invention is the expression of Aspergillus niger NC122343

alpha-amyloglucosidase in a brewery strain.

Secondly, one of the advantageous features of the invention is that the transformed brewery strains possess the foreign DNA integrated in a yeast chromosome. When the foreign DNA is integrated in the chromosome according to the process described later, the property of producing alpha-amyloglucosidase is stable and the strain differs from the starting strain only in respect of this new character.

The stability of the information can also be achieved by constructing a DNA structure known as a minichromosome consisting of three main elements: telomere, centromere and origin of replication.

Another subject of the present invention relates, more especially, to a functional DNA block enabling amyloglucosidase to be secreted from yeast, this block containing the following features:

- . the amyloglucosidase gene devoid of all or part of its introns;
- . a DNA sequence containing the signals which provide for the transcription of the amyloglucosidase gene by yeast;
- . a coding sequence containing the signal required for the export of the mature amyloglucosidase into the extracellular medium; the choice was made of a secretion system specific to yeast, that of the alpha pheromone (1), but other systems could be used [for example, the killer protein system (2) or that of a synthetic signal sequence];
- . finally, the expression block may possess, after the amyloglucosidase gene, a yeast termination sequence, for example that of the phosphoglycerate kinase gene.

Preferably, the amyloglucosidase gene will be devoid of all of its introns (cDNA).

Among the elements of the sequence which provide for the transcription of the amyloglucosidase gene, promoters should be mentioned, in particular the PGK promoter.

This functional block can be integrated in an autonomously replicating plasmid containing, for example, an origin of replication in yeast, for example the origin of replication of the 2 μ plasmid.

When such a plasmid is used, it is advantageous that it should be a shuttle plasmid capable of replicating in a bacterium; for this purpose, it may contain elements for replication in a bacterium, for example E. coli, and a selection gene such as that for resistance to an antibiotic, for example Amp^r.

In general, the plasmid will preferably contain an effective gene for selection in yeasts, for example a gene complementing an auxotrophy, such as ura3+, or alternatively a gene conferring a dominant phenotype, for example resistance to G418.

When the expression block is integrated in the genome of the yeast strain, the integration will be performed in a site which is nonessential for the yeast, for example the MFalpha₁ gene.

The constitution of an integration vector is known and will be described in detail in the examples.

The invention also relates to the strains containing an expression block according to the invention, involving either a plasmid or a chromosomal integration.

Among the transformed strains, there should be mentioned, in particular, the strains of Saccharomyces, in particular S. cerevisiae and S. uvarum, in particular the industrial strains such as the brewery strains.

Finally, the invention relates to a process for preparing amyloglucosidase, in particular alpha-amyloglucosidase, wherein a strain according to the invention is fermented on a suitable culture medium and the enzyme is recovered in the extracellular medium.

The invention relates, finally, to a process for degrading starch or dextrans, wherein an industrial medium containing starch or dextrans is fermented.

Finally, the alpha-amyloglucosidase gene used is preferably that of Aspergillus niger.

Lastly, the combined action of amyloglucosidase and a liquefying alpha-amylase leads to a total synergy of degradation of starch to glucose residues.

Starting with industrial strains such as Saccharomyces,

in which the genetic information for an alpha-amyloglucosidase and also that for an endo-alpha-amylase (for example, of Bacillus licheniformis) are integrated together in the yeast chromosomes, the production of ethanol may be obtained using starch as a source of carbon.

Thus, the present invention relates to the introduction of the gene for B. licheniformis alpha-amylase and its expression in a strain of Saccharomyces producing alpha-amyloglucosidase.

The simultaneous expression of amyloglucosidase and of alpha-amylase should lead to a synergy of these two enzymatic activities in the degradation of starch or dextrans to glucose residues.

The present invention relates, more especially, to the simultaneous expression of alpha-amylase and amyloglucosidase in a polyploid strain of brewer's yeast, S. uvarum, in particular the strain 2285 TG2 AMGC, as well as to the use of these strains for preparing alpha-amylase.

Moreover, a most especially preferred application according to the invention relates to the transformation of particular strains of Saccharomyces, in particular the polyploid and prototrophic strains used in the bread-making industry; such a strain may be, for example, S. cerevisiae strain TGF1.

These polyploid and prototrophic strains have to be cotransformed with a vector as described above and a plasmid which confers a dominant phenotype.

One of the subjects of the present invention is, in effect, the use of alpha-amyloglucosidase, in particular of Aspergillus niger, during panary fermentation, since alpha-amyloglucosidase, which degrades starch directly to glucose, has a beneficial effect on panary fermentation since this sugar is directly assimilable by baker's yeast.

The addition of this enzyme enables the baking processes to be improved by making the dough drier to the touch and hence easier to process by machine, and above all enables the fermentative activity of the baker's yeast to be significantly enhanced and hence the fermentation times to be reduced

greatly.

Furthermore, this addition should be the source of innovation by enabling relatively sweet bread-type products to be developed, which is virtually impossible without adding an enzyme in a short baking process.

The present invention hence relates, in addition, to processes by means of which a bakery strain of the Saccharomyces cerevisiae type is caused to secrete an amyloglucosidase.

In particular, the invention relates to a panary fermentation process in which at least one of the strains used in the fermentation is a strain as described above.

The examples below are designed to illustrate other characteristics and advantages of the present invention and will be described with reference to the figures, wherein:

- 15 . Figure 1 shows:
 - a) the structure and the restriction map of the Aspergillus niger glucoamylase gene,
 - b) the structure of plasmid pTG1831,
 - c) the structure of plasmid pTG1830,
- 20 . Figure 2 shows the sequence of the Aspergillus niger amyloglucosidase gene,
- . Figure 3 shows the structure of M13TG1839,
- . Figure 4 shows the structure and construction of M13TG1843,
- . Figure 5 shows the structure and construction of M13TG1837,
- 25 . Figure 6 shows the structure and construction of M13TG1846,
- . Figure 7 shows the structure and construction of M13TG1848,
- . Figure 8 shows:
 - a) the structure of plasmid pTG1848,
 - b) the structure and construction of plasmid pTG1850,
- 30 . Figure 9 shows the structure and construction of M13TG1857,
- . Figure 10 shows the structure of plasmid pTG1858,
- . Figure 11 shows the detection of the production of amyloglucosidase by the strain TGY2sp13 pTG1858 by means of the I_2 /KI test,
- 35 . Figure 12 shows the structure and construction of plasmid pTG1867,
- . Figure 13 shows the hybridization of the EcoRI restriction fragments of the genomic DNA of the strains TGY2sp13b AMGB

and TGY2sp13b with the oligonucleotide TG529 corresponding to the 3' portion of the MFalpha₁ gene of S. cerevisiae,

Figure 14 shows the hybridization of the restriction fragments of the genomic DNA of the strains TGY2sp13b and 2285TG2 with either the oligonucleotide TG529 or the oligonucleotide TG170,

Figure 15 shows the hybridization of the EcoRI-digested genomic DNAs of strains TGF1-AMG1 and TGF1 with an oligonucleotide TG529 specific for the MFalpha₁ gene of Saccharomyces cerevisiae,

Figure 16 shows the hybridization of the BamHI-digested genomic DNAs of strains 2285 TG2 AMGC-AMY1 and 2285 TG2 AMGC with an oligonucleotide TG227 specific for the His3 gene of S. uvarum.

EXAMPLE 1

CLONING OF THE ASPERGILLUS NIGER ALPHA-AMYLOGLucosIDASE GENE IN PLASMID pBR322

1 µg of Aspergillus niger NCI22343 genomic DNA and 0.5 µg of plasmid pBR322 DNA were completely digested with EcoRI and SalI, and these DNAs were then covalently linked using T₄ DNA ligase. Escherichia coli strain 1106 was then transformed with the ligation products. The selection of the clones carrying an insert corresponding to the alpha-amyloglucosidase gene was carried out after transferring the recombinant clones to a nitrocellulose sheet according to the classical method (3). The recombinant clones were hybridized with a mixture of three oligonucleotides TG282, TG283, TG284, defined on the basis of the sequences already published (4).

TG282 5' ATAGTTAAAGGATGGGGATGAGGGC 3'

TG283 5' AGGACACGTACTACAACGGCAACCC 3'

TG284 5' CTGGGTGACTGGGAAACCAGCGACG 3'

Two positive clones were isolated and contain a different EcoRI-SalI fragment. One of the inserts (2.4 kb), which hybridizes with two of the three oligonucleotides (TG283, TG284), corresponds to the 5' portion of the amyloglucosidase gene. In contrast, the other insert (1 kb), which hybridizes with only one of the three oligonucleotides (TG282), corresponds to the 3' region of the Aspergillus niger

amyloglucosidase gene (Figure 2).

These new plasmids are referred to as pTG1830 and pTG1831 (Figure 1).

EXAMPLE 2

5 CLONING OF THE cDNA OF ASPERGILLUS NIGER ALPHA-AMYLOGLUCO- SIDASE INTO BACTERIOPHAGE LAMBDA

The sequence of the amyloglucosidase gene, as well as that of the cDNA of Aspergillus niger (BU-1) amyloglucosidase, has already been published (4a, b). Comparison of these two
10 sequences reveals the presence of 4 intron regions in the Aspergillus niger (BU-1) amyloglucosidase G1 gene (Figure 2).

With the exception of the intron D, which has a sequence comparable to the consensus sequence TATAAC, an essential element in the mechanism of excision of introns in yeast
15 (5), it appears that none of the other introns can be excised by yeast in vivo. Cloning of the cDNA of amyloglucosidase was hence undertaken.

Aspergillus niger NCI22343 messenger RNAs were purified from a culture of this fungus on rich medium containing
20 2% of starch as a carbon source, so as to produce a cDNA corresponding to amyloglucosidase by reverse transcription.

The extracted mRNAs were added to a reaction mixture containing a dT oligonucleotide as a transcription primer. The synthesis of the cDNA was carried out according to the
25 method described by Chang et al. (6). A cDNA library was prepared in bacteriophage lambda according to Le Bouc et al. (7). The bacteriophage lambda DNA was packaged in vitro in the phage particles, and the phages thereby formed were used to transduce the Escherichia coli indicator strain POP101.

The Aspergillus niger NCI22343 cDNA library was screened for amyloglucosidase, using as a probe an oligonucleotide TG433 defined on the basis of the published sequences
30 (4a):

TG433 5'GGTGTAGCCATTGTCAAGCAGCCACTGCCC 3'

35 corresponding to the sequence complementary to the sequence coding for amino acids 163 to 172 of the mature protein (Figure 2).

Five positive signals were detected. Two of the five

clones were confirmed by cross-hybridization with another oligonucleotide TG387 corresponding to the sequence complementary to the sequence coding for amino acids 226 to 236 of the mature protein (Figure 2):

5 TG387 5'CAGGACGAGCCGACGGCCGTCGCGA 3'

the size of the inserts in these two candidates being approximately 800 bp.

After analysis with restriction enzymes, one of the two fragments was recloned in an M13 derivative (M13mp8) in order to sequence it. This new vector is referred to as M13TG1839 (Figure 3). The sequence of the cDNA fragment of M13TG1839 coding for amino acids 62 to 319 of the mature protein proved to be identical to that already published (Figure 2).

15 EXAMPLE 3

CONSTRUCTION OF AN ALPHA-AMYLOGLucosIDASE EXPRESSION VECTOR FOR YEAST S. CEREVISIAE

1) Reconstitution of the 3' portion of the *Aspergillus niger* amyloglucosidase gene

20 Plasmid pTG1830 DNA was digested with PstI and SalI so as to liberate a 1.2-kb fragment. Similarly, plasmid pTG1831 DNA was digested with EcoRI and SalI, liberating a 1-kb fragment. These two fragments were isolated and ligated with M13mp8 DNA which has been digested with PstI and EcoRI. A new vector, referred to as M13TG1843, is reconstituted (Figure 4). This vector M13TG1843 possesses the 3' part of the amyloglucosidase gene.

2) Reconstitution of the 5' portion of the region coding for the mature amyloglucosidase of *Aspergillus Niger*

a) Removal of the intron A by in vitro mutagenesis

30 In the first place, the BamHI-EcoRI fragment (2 kb) of pTG1830 containing the 5' portion of the amyloglucosidase gene was transferred into M13Tg127 phage DNA between the BamHI and EcoRI sites. This gave single-stranded M13TG1835 DNA, from which the in vitro mutagenesis was carried out. An oligonucleotide TG403 (30-mer), the sequence of which is complementary to those of the regions flanking the intron A, was used as a primer for the synthesis of the DNA, using the single

strand of M13TG1835 DNA as a template (Figure 5). The sequence of the oligonucleotide TG403 is as follows:

TG403 5' ACGGATAACCCGGACTACTTCTACACCT 3'

One picomole of single-stranded M13TG1835 DNA was hybridized with one picomole of oligonucleotide TG403 whose 5' ends are phosphorylated in a reaction mixture which contains 10 mM Tris pH 7.5, 10 mM MgCl₂, 50 mM NaCl. The mixture was heated to 100°C for 3 minutes and incubated for 1 hour at room temperature.

1.25 µl of each deoxynucleotide triphosphate at a concentration of 5 mM was added to the hybridization mixture in order to obtain a final concentration of 500 µM. 5 units of DNA polymerase I Klenow fragment were then added to one unit of T₄ DNA ligase; the elongation reaction was continued at room temperature for two hours. After precipitation with ethanol, the ligation products obtained were digested with the restriction enzyme SacI. This enables the frequency of production of recombinant clones containing the mutated form to be increased. Competent cells of *E. coli* strain JM103 were transformed with the digestion mixture. The plaques derived from this transformation were analyzed by DNA/DNA hybridization, using as a probe the oligonucleotide TG403 depicted above, labelled with ³²P by kinase treatment according to the customary techniques (8). Several positive signals were observed. The DNA sequence of one of the clones is identical to that of M13TG1833 DNA without the intron A sequence. This new phage is referred to as M13TG1837 (Figure 5).

b) Construction of the vector M13TG1846

The vector M13TG1837 DNA was digested with BssHII and AvaII. The 380-bp BssHII-AvaII fragment containing the DNA sequence coding for the NH₂-terminal region of the mature amyloglucosidase of *Aspergillus niger* (amino acids 21 to 151) was isolated (Figure 2). Similarly, the 280-bp PstI-AvaII fragment of M13TG1839 was isolated. This fragment contains the cDNA sequence coding for amino acids 151 to 256. These two BssHII-AvaII, AvaII-PstI fragments, with two synthetic oligonucleotides TG453, TG454 designed to reconstitute the 5' end of the mature amyloglucosidase and which permit the

in-phase reading of the prepro sequence of the alpha pheromone followed by the mature sequence of amyloglucosidase (Figure 6), are ligated in M13mp8 which has been digested with HindIII and PstI. This new vector is referred to as M13TG1846 (Figure 6).

3) Construction of M13TG1848

In the first place, the HindIII-PstI fragment of M13TG1846 (670 bp) containing the sequence coding for amino acids 21 to 246 was isolated. M13TG1843 DNA was digested with PstI and EcoRV and the 1736-kb PstI-EcoRV fragment derived from this digestion was isolated and eluted from a gel. This fragment contains the 3' portion of the amyloglucosidase gene (presence of the intron D) coding for amino acids 246-640. The EcoRV site is situated downstream from the TGA stop codon (approximately 140 bp). The polyadenylation site is situated at approximately 70 bp upstream from the EcoRV site. These two fragments are ligated in M13TG131 which has been digested with HindIII and HindII. This new vector is referred to as M13TG1848 (Figure 7). This new vector was used as a source of amyloglucosidase coding sequence for the secretion vector described below, despite the presence of the last intron D.

4) Construction of an amyloglucosidase expression vector for yeast *S. cerevisiae*

The starting plasmid is pTG848 (identical to pTG849 described in French Patent Application No. 83/15,716, with the exception of the *ura3* gene whose orientation is inverse); it consists of the following fragments (Figure 8a):

- a) The approximately 3.3-kb EcoRI-HindIII fragment originating from plasmid pJDB207 (9). The HindIII site corresponds to coordinate 105 of the 2 μ plasmid B form, the EcoRI site to coordinate 2243. In this fragment, the 2 μ is in the B form (10). This fragment enables the plasmid to replicate autonomously in yeast.
- b) A HindIII fragment carrying the URA3 gene (11) which enables the plasmid to be selected in *ura3*⁻ strains on medium devoid of a pyrimidine source.
- c) The large EcoRI (coordinate 0)-SalI (coordinate 650)

fragment of pBR322 (12). In the PvuII site of this fragment, there has been inserted the 510-bp EcoRI-HindIII fragment corresponding to the end of the PGK gene (13), the ends of which fragment have been previously rendered blunt by the action of Klenow in the presence of the 4 deoxyribonucleotides. When joined to the PvuII end of pBR322, the flush EcoRI end of the PGK gene regenerates an EcoRI site.

d) The HindIII-SalI fragment (2.15 kb) of the PGK gene (13).

The BglII-HindIII fragment of M13TG1848 was isolated and eluted from gel. It was then ligated with the HindIII-BamHI fragment of M13TG889 containing the prepro sequence of the alpha pheromone in the BglII sites of plasmid pTG848. A new plasmid, referred to as pTG1850, was obtained (Figure 8b).

In summary, plasmid pTG1850 possesses:

- 1) a fragment of the 2 μ plasmid of S. cerevisiae which enables it to replicate in Saccharomyces;
- 2) the URA3⁺ of S. cerevisiae which enables the transformed cells to be selected from a ura3⁻ receptor strain on medium devoid of a pyrimidine source;
- 3) the origin of replication of plasmid pBR322 and the gene for resistance to ampicillin, which enable the plasmid to replicate and the transformants to be selected in E. coli;
- 4) the promoter and the terminator of the yeast PGK gene which provide, respectively, for the initiation and the stopping of transcription of the alpha-amyloglucosidase expression block;
- 5) a gene coding for a precursor of this alpha-amyloglucosidase, consisting of the prepro sequences of the alpha pheromone gene (MFalpha₁) of S. cerevisiae fused to the sequence coding for the mature portion of alpha-amyloglucosidase which still has its intron D.
- 5) Transformation of a laboratory strain with plasmid pTG1850 DNA

pTG1850 plasmid DNA was amplified in E. coli, purified on a cesium chloride gradient and used for transforming the strain TGY2sp13b, which is a haploid strain of S. cerevisiae having leucine and uracil as auxotrophic markers (Matalpha, ura3-251-373-328, leu2 3-12). Following the techniques

described by Ito et al. (14), the *ura*⁺ transformants were tested for the expression of the Aspergillus niger amyloglucosidase gene. The technique is as follows: the *ura*⁺ clones were subcultured on solid medium containing 2% of starch.

5 After 48 hours' growth, 5 ml of aqueous iodine mixture (I₂/KI) were added to each dish. The starch in contact with the iodine (I₂) takes on a blue coloration. Yeasts which produce amyloglucosidase in the culture medium degrade the starch and this gives rise to a halo of decolorization around the colonies. None of the transformants secretes active amyloglucosidase.

6) Excision of the intron D by in vitro mutagenesis

To carry out this in vitro mutagenesis, an oligonucleotide TG496, the sequence of which is complementary to that of the regions flanking the intron D of phage M13TG1847 DNA, depicted in Figure 9, was hybridized. This oligonucleotide served as a primer for the in vitro synthesis of the strand complementary to the phage M13TG1847 genome. The sequence of the oligonucleotide TG496 is as follows:

20 TG496 5' TTCGTCTCTATTGTGGAACTCACGCCGCA 3'.

The method used is identical to that used for the excision of the intron A. On the other hand, it was not possible to use any restriction enzyme to "sabotage" the non-mutated forms. Several positive signals were observed. The DNA of one of the positive clones was sequenced. The intron D was correctly deleted. This new phage is referred to as M13TG1857 (Figure 9).

7) Construction of plasmid pTG1858

In the first place, the 670-bp BamHI-SphI fragment of M13TG1857 was isolated and purified on gel. It was ligated with the large BamHI-SphI fragment of pTG1850. A new amyloglucosidase expression vector, referred to as pTG1858, was reconstituted. This vector possesses the region coding for the mature amyloglucosidase of Aspergillus niger (Figure 10).

EXAMPLE 4

TRANSFORMATION OF A LABORATORY STRAIN WITH pTG1858 DNA

pTG1858 DNA was used to transform a yeast TGY2sp13b

(Matalpha, ura3-251-373-328, leu 2-3-18) for ura⁺, following the techniques described (14); the presence of alpha-glucosidase was tested around the colonies as described above (Figure 11). As expected, it was found that all the cells transformed with plasmid pTG1858 secrete active amyloglucosidase. In addition, the growth of the yeast strains transformed with plasmid pTG1858 was compared with that of the same strain transformed with plasmid pTG848. The yeasts transformed with both plasmids grow on minimal medium containing 2% of starch as a source of carbon. However, the strain transformed with plasmid pTG1858 always shows better growth than the strain transformed with plasmid pTG848 on this medium.

EXAMPLE 5

CHARACTERIZATION OF AN AMYLOGLUCOSIDASE ACTIVITY IN THE YEAST CULTURE SUPERNATANTS

The amount of amyloglucosidase secreted by yeast strain TGY2sp13b containing plasmid pTG1858 was assayed. After two days' incubation in YNBG medium + 0.5% casamino acids at 30°C with stirring at 250 rpm, the culture of TGY2sp13b containing pTG1858 consumed all the glucose and was in stationary phase; the culture was arrested at an approximate cell density of 2.6×10^8 cells/ml. Measurement of the amount of amyloglucosidase present in the unconcentrated supernatant shows that 360 units of active amyloglucosidase per liter of supernatant were produced (one unit of amyloglucosidase activity corresponds to the release of one μ mol of glucose/min from 2% of Zulkowsky starch in 50 mM sodium acetate buffer, pH 4.3 at 55°C).

EXAMPLE 6

CONSTRUCTION OF AN INTEGRATION PLASMID FOR SACCHAROMYCES CEREVISIAE

For this purpose, an EcoRI fragment (1.7 kb) corresponding to the chromosomal region carrying the MFalpha₁ gene of *S. cerevisiae* (1) was cloned into the EcoRI site of a derivative of pUC8 (pTG1864). This new vector is referred to as pTG1865 (Figure 12).

pTG1865 DNA was digested with HindII, liberating a

small, approximately 0.5-kb fragment internal to the MFalpha₁ gene. At the HindII sites of pTG1865, there were inserted by blunt-end ligation the SmaI-BglII fragment of pTG1858 which includes the amyloglucosidase prepro precursor sequence under
5 the control of the phosphoglycerate kinase gene promoter and the BglII-HindIII fragment of pTG830 which includes the PGK gene terminator sequence, after the action of Klenow polymerase on the HindIII site. This new vector is referred to as pTG1867 (Figure 12). Digestion of pTG1867 with EcoRI liberates a yeast DNA fragment (4 kb) in which the amyloglucosidase expression block has been integrated.
10

EXAMPLE 7

COTRANSFORMATION OF TGY2sp13b FOR THE PURPOSE OF OBTAINING YEASTS SACCHAROMYCES WHICH HAVE INTEGRATED THE AMYLOGLUCO-SIDASE EXPRESSION BLOCK

15

The presence, at the ends of a linear DNA fragment, of regions homologous with a region of the yeast genome favors recombination events between the chromosomal region and the linear fragment. This enables us to substitute the sequence
20 of the linear fragment, which can contain foreign sequences, for the chromosomal sequence. It is hence also possible to obtain the integration of foreign sequences in the yeast chromosome.

The strain TGY2sp13b (Matalpha ura3-251-373-328, Leu 2-3-12) was hence cotransformed by the customary technique (14) with 2 µg of pFL1 DNA (15), conferring the ura⁺ phenotype, and at the same time with 10 µg of pUC1867 DNA which had previously been cut with EcoRI. Of the 300 ura⁺ clones obtained, those which had become MFalpha₁ were tested. In effect, the
30 substitution at the MFalpha₁ locus by the linear DNA fragment leads to an inactivation of the MFalpha₁ gene, that is to say a loss in production of the alpha factor of at least 75% (16). The MFalpha₁ phenotype is tested in the following manner: a lawn of FL100 Mata cells (5 x 10⁴ cells) is plated
35 on a dish containing YNBG + 0.5% casamino acids. A clump of cells originating from the ura⁺ clones was deposited on this lawn. After 48 hours at room temperature, the presence of a halo of inhibition of growth of the Mata strain was

observed around the strains having the MFalpha₁ phenotype. This reflects the fact that the MFalpha₁ strain secretes sufficient alpha pheromone to inhibit the growth of the Mata strains. No halo of inhibition of growth was observed
5 around the mfalpha₁ strains. Of the 300 ura⁺ clones, one mf-alpha₁ transformant, referred to as TGY2sp13b Ø AMGB, was obtained. This clone produces a halo of decolorization in the I₂/KI test after 5 days' growth at 30°C. This clone TGY2sp13b Ø AMGB hence produces active amyloglucosidase.

10 This clone expresses alpha-amyloglucosidase in stable fashion during several generations. Analysis of the genomic DNA of the strain TGY2sp13b Ø AMGB according to Southern reveals that the amyloglucosidase expression block is integrated in the genome at the MFalpha₁ locus (Figure 13) and
15 that the integration of this foreign fragment has been carried out by an exchange process without integration of the vector pTG1864 sequence.

The amount of amyloglucosidase secreted by the yeast strain TGY2sp13b Ø AMGB and the strain TGY2sp13b containing
20 plasmid pTG1858 was assayed.

After 2 hours' incubation at 30°C with stirring at 250 rpm, the strain TGY2sp13b Ø AMGB produces approximately 6- to 8-fold less active amyloglucosidase than the strain TGY2sp13b containing plasmid pTG1858.

25 EXAMPLE 8

INTEGRATION IN A POLYPLOID BREWERY STRAIN SACCHAROMYCES UVARUM (2285TG2)

The point at which the amyloglucosidase expression block is integrated in the genome of the brewery strain must
30 be chosen in such a way as not to modify either the physiology or the characteristics of the strain 2285TG2. The choice was made, for the integration site, of the MFalpha₁ gene which is responsible for the synthesis of the alpha factor. A strain of sex type Mata shows no detectable inhibition of
35 growth when placed in contact with a polyploid brewery strain. The alpha factor plays a part in the conjugation between two yeasts of opposite sex type. In strain 2285TG2, inactivation of the MFalpha₁ gene should not modify the physiology of

the strain. The presence of this gene in strain 2285TG2 was determined by analyzing the genomic DNA of this strain according to Southern (Figure 14). The MFalpha₁ gene present in the strain 2285TG2 appears to be broadly homologous with that of the S. cerevisiae strain. A single difference was detected in the region coding for the alpha factor situated between the two HindII sites (Figure 12). This region contains, depending on the strain, a variable number of copies of this alpha factor (17). The difference observed is probably due to the presence of at least one additional copy in the Saccharomyces uvarum strain. In consequence, the amyloglucosidase expression block was inserted into the HindII sites of the MFalpha₁ gene of S. cerevisiae bounding the region coding for the alpha factor.

Since the strain 2285TG2 is polyploid and hence has no auxotrophic marker, the cotransformation with pTG1867 is accomplished using a plasmid which confers a dominant phenotype. Resistance to G418 was used. This plasmid which was used is a derivative of plasmid pTG1861 described in French Patent No. 84/04,453. Plasmid pTG1825 differs from pTG1861 only in respect of the absence of an out-of-phase ATG which has been deleted by in vitro mutagenesis in order to permit better expression of the gene for resistance to G418.

Strain 2285TG2 was transformed with 1 µg of pTG1825 DNA and 10 µg of pTG1867 which has previously been digested with EcoRI, by the technique derived from Dickson (18). The following modification was carried out: approximately 15 hours after the transformation, an overlayer of complete medium (10 ml) containing geneticin, G418 (Difco), was added so that the final concentration of antibiotic was 300 µg/ml in the dish.

Of 300 G418-resistant clones, one clone, 2285TG2 AMGC, produces amyloglucosidase. This clone expresses alpha-amyloglucosidase in stable fashion during several generations.

Analysis of the genomic DNA of the strain 2285TG2 AMGC according to Southern reveals that the amyloglucosidase expression block is integrated in the genome at the MFalpha₁ locus. The amount of amyloglucosidase secreted by the brewery strain was

assayed. After 3 days' incubation in complete YPG medium at 30°C with stirring at 250 rpm, the 2285TG2 AMGC culture at an approximate cell density of 4×10^7 cells/ml produces 100 U of active alpha-amyloglucosidase per liter of supernatant.

5 EXAMPLE 9

The same experiment as described in Example 8 is carried out starting with a strain used in the bread-making industry.

10 Since this strain TGF1 is polyploid and prototrophic, a cotransformation has to be performed with pTG1867 and a plasmid conferring a dominant phenotype, plasmid pTG1825.

15 The strain TGF1 is transformed with the DNA of plasmids pTG1825 and pTG1867 which has previously been digested with EcoRI. Among the 500 clones resistant to G418 (300 µg/ml) obtained, one expresses amyloglucosidase in stable fashion during at least 50 generations; this clone is referred to as TGF1-AMG1.

20 Hybridization of the EcoRI-digested genomic DNAs of the strains TGF1-AMG1 and TGF1 with an oligonucleotide TG529 specific for the MFalpha₁ gene of Saccharomyces cerevisiae by Southern's technique reveals that the amyloglucosidase expression block is integrated in the genome at the MFalpha₁ locus (Figure 15) of TGF1-AMG1.

25 The amount of amyloglucosidase secreted by the strain TGF1-AMG1 was assayed. After three days' incubation in complete YPG medium at 30°C with stirring at 250 rpm, the TGF1-AMG1 culture produces 100 U of active alpha-amyloglucosidase per liter of supernatant of a culture at a cell density of 5×10^7 cells/ml.

30 EXAMPLE 10

INTEGRATION OF THE B. LICHENIFORMIS ALPHA-AMYLASE EXPRESSION BLOCK IN THE POLYPLOID STRAIN SACCHAROMYCES UVARUM 2285 TG2 AMGC

35 The strain 2285 TG2 AMGC was transformed with 1 µg of pTG1825 DNA and 10 µg of M13TG1817 which had previously been digested with BamHI, by the technique derived from Webster and Dickson (18).

By digestion with BamHI, the vector M13TG1817

Liberates a DNA fragment containing the His3 gene of Saccharomyces uvarum in which the cassette for expression of alpha-amylase has been inserted. The construction of the vector M13TG1817 has been described in French Patent No. 86/06,703.

- 5 Of 400 clones resistant to G418 (300 µg/ml), 4 clones produce alpha-amylase. These clones express alpha-amylase in stable fashion during 50 generations. Hybridization of the BamHI-digested genomic DNAs of the strains 2285 TG2 AMGC-AMY1 and 2285 TG2 AMGC with an oligonucleotide TG227 specific
- 10 for the His3 gene of S. uvarum by Southern's technique reveals that the alpha-amylase expression block is integrated in the genome at the His3 locus (Figure 16) of the strain 2285 TG2 AMGC-AMY1.

The following strains were deposited at the Collection Nationale de Cultures de Microorganismes (National Collection of Microorganism Cultures) of the Institut Pasteur, 28 rue du Docteur-Roux - 75724 PARIS, Cedex 15:

- 15 . Saccharomyces uvarum 2285 TG2 AMGC
 on 6th June 1986 under No. I-568,
- 20 . Saccharomyces cerevisiae TGF1 AMG1
 on 27th January 1987 under No. I-65',
- . Saccharomyces uvarum 2285 TG2 AMGC-AMY1
 on 27th January 1987 under No. I-649.

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The claims defining the invention are as follows:-

1. A block for the expression of a mature amyloglucosidase from ~~Aspergillus~~ ^{Aspergillus} ~~Niger~~ in a yeast, which block contains:
 - (a) the amyloglucosidase gene devoid of all or part of its introns;
 - (b) the DNA sequence containing the signals which provide for the transcription of the amyloglucosidase gene by yeast;
 - (c) a coding sequence containing the signal required for the export of the mature amyloglucosidase into the extracellular medium, which coding sequence contains a secretion signal chosen from the alpha pheromone, the killer protein secretion system or a synthetic secretion sequence; and
 - (d) a yeast termination sequence after the amyloglucosidase gene and wherein said yeast termination sequence is a transcription termination sequence.
2. The expression block as claimed in claim 1, wherein the DNA sequence which provides for the transcription of the gene contains the PGK gene promoter.
3. The expression block as claimed in claim 1 or claim 2, which is integrated in a plasmid containing an origin of replication in yeasts.
4. The expression block as claimed in claim 3, wherein the origin of replication in yeasts is the origin of replication of the 2 μ plasmid.
5. The expression block as claimed in claim 3 or claim 4, wherein the plasmid contains, in addition, a selection gene.
6. The expression block as claimed in claim 5, wherein the selection gene is a gene complementing an auxotrophy.
7. The expression block as claimed in claim 5, wherein the selection gene is a gene conferring a dominant phenotype.
8. The expression block as claimed in claim 7, wherein the dominant phenotype is resistant to G418.
9. The expression block as claimed in any one of claims 1 to 3, which is integrated in the genome of the yeast strain.
10. The expression block as claimed in claim 9, wherein the integration is performed in the MFalpha₁ gene.



11. A transformed yeast strain containing an expression block as claimed in any one of claims 1 to 10.
12. The strain as claimed in claim 11, which is a strain of Saccharomyces.
13. The strain as claimed in claim 12, which is a strain chosen from S. cerevisiae and S. uvarum.
14. The transformed strain as claimed in any one of claims 11 to 13, which is a polyploid and protrophic strain of Saccharomyces.
15. The strain as claimed in claim 14, which is a strain of Saccharomyces used in the bread-making industry.
16. The strain as claimed in claim 15, which is Saccharomyces cerevisiae strain TGF 1.
17. The strain as claimed in any one of claims 14 to 16, wherein the amyloglucosidase expression block is derived from plasmid pTG1867.
18. The strain of Saccharomyces as claimed in any one of claims 11 to 13, which is transformed simultaneously by an expression block as claimed in any one of claims 1 to 10 and by an alpha-amylase expression block, and which simultaneously secretes alpha-amylase and amyloglucosidase.
19. The strain as claimed in claim 18, which is a brewery strain of the Saccharomyces uvarum type.
20. The strain as claimed in claim 19, which is S. uvarum strain 2285 TG2 AMGC.
21. The strain as claimed in any one of claims 18 to 20, wherein the alpha-amylase expression block is derived from the vector M13TG1817.
22. A process for producing alpha-amyloglucosidase, wherein a strain as claimed in any one of claims 11 to 21 is fermented on a suitable culture medium and wherein the enzyme is recovered in the extracellular medium.
23. A process for panary fermentation, wherein at least one of the strains used in the fermentation is a strain as claimed in any one of claims 14 to 17.
24. A fermentation process, wherein at least one of the strains used in the fermentation is a strain as claimed in any one of claims 18 to 21.
25. A block for the expression of an amyloglucosidase in yeast and a transformed yeast strain containing said block substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.
26. A process for producing alpha-amyloglucosidase substantially as hereinbefore



described with reference to any one or more of the examples and/or drawings.

27. A process for panary fermentation substantially as hereinbefore described with reference to any one or more of the examples and/or drawings.

DATED this 22nd day of April, 1991.

TRANSGENE S.A.



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