

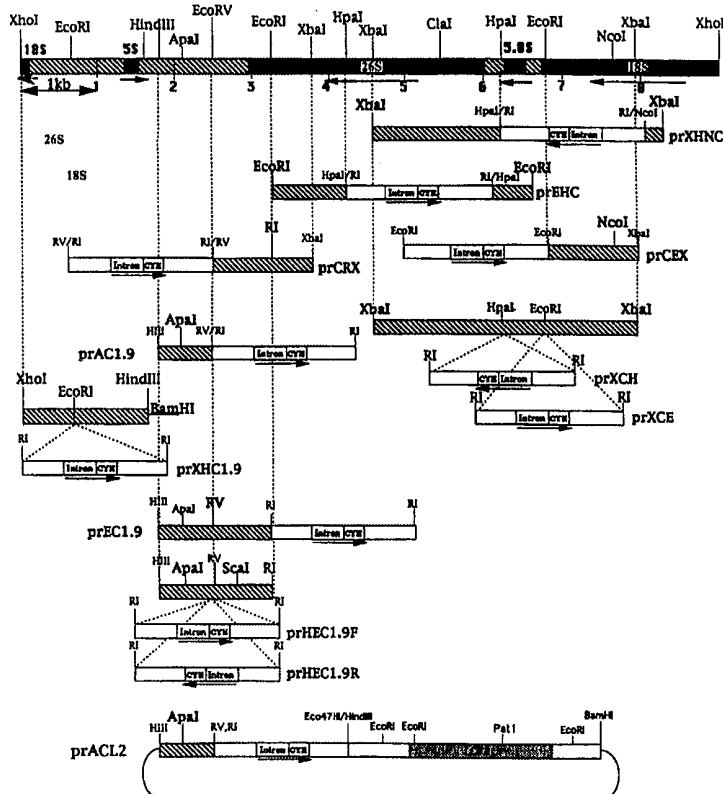
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| <p><b>(21) International Application Number:</b> PCT/KR98/00346</p> <p><b>(22) International Filing Date:</b> 31 October 1998 (31.10.98)</p> <p><b>(30) Priority Data:</b></p> <table border="0"> <tr> <td>1998/16309</td> <td>7 May 1998 (07.05.98)</td> <td>KR</td> </tr> <tr> <td>1998/16310</td> <td>7 May 1998 (07.05.98)</td> <td>KR</td> </tr> <tr> <td>1998/33969</td> <td>21 August 1998 (21.08.98)</td> <td>KR</td> </tr> </table> <p><b>(71) Applicants (for all designated States except US):</b> DOOSAN CORPORATION [KR/KR]; 39-3, Sungbok-ri, Sooji-eup, Yongin-city, Kyungki-do 449-840 (KR). KOREA INSTITUTE OF SCIENCE AND TECHNOLOGY [KR/KR]; 39-1, Hawolgok-dong, Songbuk-ku, Seoul 136-130 (KR).</p> |                           | 1998/16309                                                                                                                                                                                                        | 7 May 1998 (07.05.98) | KR | 1998/16310 | 7 May 1998 (07.05.98) | KR | 1998/33969 | 21 August 1998 (21.08.98) | KR | <p>ment, Kalma-dong, Seo-ku, Taejon 302-171 (KR). PARK, Chang, Seo [KR/KR]; No. 710-401, Jookong Apartment, Byulyang-dong, Kwachun-city, Kyungki-do 427-040 (KR).</p> <p><b>(74) Agents:</b> YOON, Dong, Yol et al.; Yoon &amp; Lee International Patent &amp; Law Firm, 648-23, Yoksam-dong, Kangnam-ku, Seoul 135-081 (KR).</p> <p><b>(81) Designated States:</b> CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> |
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(54) Title: PLASMID FOR GENE EXPRESSION IN *PICCHIA CIFERRII* AND TRANSFORMATION METHOD USING THE SAME

**(57) Abstract**

Expression cassettes for transforming *Pichia ciferrii* and their use are disclosed. The present invention relates to expression cassettes containing *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup> gene resistant to cycloheximide, and a desired gene. Expression cassette further comprising *Pichia ciferrii* GAPDH promoter gene which allows an increase in the expression of the desired gene also provided. Moreover, the present invention provides a process for producing tetraacetyl phytosphingosine using transformed *Pichia ciferrii* cells with the expression cassettes in a higher yield.



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## PLASMID FOR GENE EXPRESSION IN PICHIA CIFERRII AND TRANSFORMATION METHOD USING THE SAME

### BACKGROUND OF THE INVENTION

#### 1. FIELD OF THE INVENTION

5 The present invention relates to expression cassettes for transforming *Pichia ciferrii*. More particularly, it relates to expression cassettes containing *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup> gene resistant to cycloheximide, and a desired gene, and to uses thereof.

#### 10 2. DESCRIPTION OF THE PRIOR ARTS

*Pichia ciferrii* has been used to biologically desulfurize coals (Stevens *et al.*, USP 4,851,350), to produce D-alpha-amino acids (Takeichi *et al.*, USP 5,068,187), to produce (S)-1-phenyl-1,3-propandiol (Ajinomoto, JP 6-90789A) or to produce secondary alcohols by stereospecific ketone reduction (Merck, EP-300287). Further,  
15 it produces and secretes tetraacetyl phytosphingosine (TAPS) which is a precursor of ceramides (Barenholz *et al.*, Biochem. Biophys. Acta, 248, 458, 1971; *ibid*, 306, 341, 1973).

Phytosphingosines including TAPS, like ceramides, show an activity of surface skin-protection and of preventing excessive water-loss and dry out of the  
20 skin, facilitating their uses in cosmetics. They can be obtained from various microorganisms and easily converted to ceramides by N-acylation.

TAPS productions by wild type *Pichia ciferrii* ATCC 14091 and F-60-10 (NRRL 1301) are not satisfactory for commercial uses. To improve the production of TAPS in the strains of *Pichia ciferrii*, attempts to provide mutants which are  
25 capable of producing a higher level of TAPS have been made (Wickerham & Burton,

J. Bacteriol., 80, 484, 1960; USP 5,618,706). The present inventors also developed novel useful mutant (KFCC-10937) which allows a larger amount of TAPS production in a shorter time (KR 98-49305A).

*Pichia ciferrii* had been classified into genus *Hansenula* and is recently  
5 reclassified into genus *Pichia* by 5S-RNA analysis (Yamada *et al.*, *Biosci, Biotechnol. Biochem.*, 58, 1245, 1994). By this reason, the genetic study of the *Pichia* yeasts is not sufficient and transformation method of *Pichia ciferrii* has not been established.

The present invention provides plasmid prACL2 comprising *Pichia ciferrii*  
10 serine palmitoyl transferase gene and a transformed *Pichia ciferrii* cell which allows an improved production of TAPS.

The inventors found that the known transformation method for *Candida utilis* (Kondo *et al.*, J. Bacteriol., 177, 7171, 1995) can be modified and applied to the *Pichia ciferrii*. They cloned *Pichia ciferrii* ribosomal protein L41-coding gene to  
15 determine its nucleotide sequence and manipulated to give a resistance to cycloheximide, an antibiotic from yeasts, so as to be used as a selection marker. Thus recombinant gene may be linked to a plasmid which carry a desired gene to give an expression cassette which is useful to transform *Pichia ciferrii* in which the desired gene is expressed.

Moreover, the inventors succeeded in cloning of *Pichia ciferrii* GAPDH  
20 promoter gene and found that its insertion into the expression cassette allows an unexpected improvement of the expression level. In fact, they increased the production amount of TAPS by transforming the strain of *Pichia ciferrii* with the expression cassette carrying LCB2 gene as a desired gene and culturing the resulting  
25 transformed cells. LCB2 gene codes for palmitoyl transferase which is involved in the TAPS synthesis in the living body.

## SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to determine and use genetic information of *Pichia ciferrii* ribosomal protein L41 gene.

Another object of the present invention is to provide an expression cassette for transforming *Pichia ciferrii*, which comprises *Pichia ciferrii* ribosomal DNA, *Pichia ciferrii* L41 gene as a selection marker, and a desired gene. In one preferred embodiment of the present invention, the marker is a gene conferring resistance to an antibiotic cycloheximide.

Another object of the present invention is to provide a method for transforming *Pichia ciferrii* with a plasmid containing the expression cassette.

The present invention determines and uses genetic information of *Pichia ciferrii* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and GAPDH promoter gene.

The present invention provides an expression cassette for transforming *Pichia ciferrii*, which comprises *Pichia ciferrii* ribosomal DNA, *Pichia ciferrii* L41 gene as a selection marker, *Pichia ciferrii* GAPDH promoter gene and a desired gene. In one preferred embodiment of the present invention, the marker is a gene conferring resistance to an antibiotic cycloheximide.

The present invention further provides an expression cassette for transforming *Pichia ciferrii*, which comprises *Pichia ciferrii* ribosomal DNA, *Pichia ciferrii* L41 gene as a selection marker, *Pichia ciferrii* GAPDH promoter gene, a desired gene and *Pichia ciferrii* ribosomal DNA. In one preferred embodiment of the present invention, the marker is a gene conferring resistance to an antibiotic cycloheximide.

The present invention determines and uses genetic information of *Pichia ciferrii* serine palmitoyl transferase which is involved in TAPS synthesis.

The present invention provides plasmid prACL2 comprising an expression cassette having *Pichia ciferrii* serine palmitoyl transferase gene and a transformed

*Pichia ciferrii* cell with an improved production of TAPS.

The present invention further provides plasmid prACGL2 comprising an expression cassette having *Pichia ciferrii* serine palmitoyl transferase gene and a *Pichia ciferrii* transformant with an improved production of TAPS.

5 The present invention further provides plasmid prHECGL2 comprising an expression cassette having *Pichia ciferrii* serine palmitoyl transferase gene and a *Pichia ciferrii* transformant with an improved production of TAPS.

The present invention still further provides a method for producing TAPS by culturing the transformed *Pichia ciferrii* cells.

10

The objects mentioned above, other features and applications of the present invention would be much more apparent by those of ordinary skills in the art from the following explanation in detail.

#### BRIEF DESCRIPTION OF THE DRAWINGS

15 Figure 1 is a restriction map of various plasmids having *Pichia ciferrii* ribosomal DNA incorporated with L41 genes of *Pichia ciferrii* at different sites for each plasmid, in which

hatched boxes indicate ribosomal DNA fragments;

restriction enzymes in bold letters indicate the enzymes used for linearization

20 of the respective plasmids;

arrows indicate the transcription direction of the genes; and

plasmid prACL2 carries LCB2 gene coding for serine palmitoyl transferase from *Pichia ciferrii*.

Figure 2 is a restriction map of *Pichia ciferrii* LCB2 gene, and the orientation and  
25 the length of the arrows indicate the direction and degree of nucleotide sequencing,

respectively.

Figure 3 represents the construction and restriction map of plasmid prACL2.

Figure 4 is a restriction map of *Pichia ciferrii* GAPDH gene, and the orientation and the length of the arrows indicate the direction and degree of nucleotide sequencing, respectively.

Figure 5 represents the construction and restriction map of plasmid prACGL2.

Figure 6 represents the construction and restriction map of plasmid prHECGL2.

Figure 7 is results of Southern blot analysis carried out to measure the copy number of LCB2 gene transformed into host cell.

10

## DETAILED EXPLANATION OF THE INVENTION

Kondo *et al* describes a transformation method for *Candida utilis* in which an antibiotic resistant gene from yeast is used as a marker gene instead of the conventional bacterial one (Kondo et al., J. Bacteriol., 177, 7171, 1995). The present inventors made attempts to apply such an idea to *Pichia ciferrii*.

15 For this purpose, they cloned *Pichia ciferrii* ribosomal protein L41 gene and determined its nucleotide sequence. Further, they identified 56th amino acid which is responsible for the sensitivity to cycloheximide is proline and replaced it by glutamine to give cycloheximide-resistance to L41 protein. In the present invention, L41 gene is used as a selection marker.

### 20 1. Isolation of *Pichia ciferrii* ribosomal protein L41 gene

By following and modifying the method by Kondo *et al* (Kondo *et al.*, J. Bacteriol., 177, 7171, 1995), two primers CYH1 and CYH4 were synthesized.

CYH1 : 5'-CGC GTA GTT AAY GTN CCN AAR AC-3'

CYH4 : 5'-GCC TGG CCY TTY TGY TTY TTN TC-3'

The two primers are also represented as SEQ. ID. NO. 7 and SEQ. ID. NO. 8, respectively in SEQUENCE LISTING.

PCR was performed using the two primers to isolate *Pichia ciferrii* ribosomal protein L41 gene of about 300bp, which was labeled to be used as a probe.

5 Thus obtained probe was used to carry out Southern blot analysis for *Pichia ciferrii* genome DNA. A genome DNA digested with EcoRI gives a desired band at 1.9 kb. EcoRI-digested DNA fragments of 1.9 kb was collected and used to construct pCYH1.9 which is used for nucleotide sequencing. The results are shown in Figure 1. DNA sequence of *Pichia ciferrii* ATCC 14091 ribosomal protein L41 gene was  
10 submitted on March 7, 1998 to GenBank under accession number AF 053457. *Pichia ciferrii* ATCC 14091 ribosomal protein L41 gene has 737 base pairs including 419 bp intron. The putative amino acid sequence deduced from the nucleotide sequence shows a homology of 90% or more to those of other yeasts. It was also identified that the cycloheximide-sensitive amino acid is amino acid 56, proline.

15

## 2. Impartation of cycloheximide resistance to L41 gene : Construction of plasmid pCYH1.9<sup>r</sup> for use in the selection of transformed cell.

Site-directed mutagenesis was carried out to replace proline (aa 56) in L41 gene with glutamine in order to impart cycloheximide resistance to L41 gene. Thus  
20 obtained gene-manipulated gene is designated as 'plasmid CYH1.9<sup>r</sup>'.

Hereinafter, the L41 gene is indicated by an abbreviation of 'CYH' and the cycloheximide-resistant L41 gene is indicated by 'CYH<sup>r</sup>' throughout the description.

## 3. Isolation of *Pichia ciferrii* ribosomal DNA : Construction of plasmid prDX9.0 for 25 improving integration efficiency of desired gene into chromosome

In order to improve the efficiency of integration of the desired gene into the chromosomal DNA, ribosomal DNA was employed. Within the cell, several hundred copies of ribosomal DNA occur.

PCR primers were synthesized by using the partial nucleotide sequence of *Pichia ciferrii* ribosomal RNA (Yamada *et al.*, *Biosci. Biotechnol. Biochem.*, 58, 1245, 1994). PCR of *Pichia ciferrii* ATCC 14091 genome DNA was carried out by using the primers to isolate ribosomal DNA fragment of 6.0 kb. The fragment was used as a probe for Southern blot analysis to isolate *Pichia ciferrii* ATCC 14091 ribosomal DNA fragment of 9 kb, which is inserted into plasmid pBluescript KS+ to produce plasmid prDX9.0.

Partial sequence of the ribosomal DNA fragment (9 kb) was determined, and the location and orientation of 5S, 26S, 5.8S and 18S ribosomal protein genes are shown in Figure 1.

#### 4. Construction of recombinant plasmids

Ribosomal DNA was digested with various restriction enzymes and ligated to CYH<sup>r</sup> to give various plasmids as shown in Figure 1 which will be analyzed for transformation efficiency in regard with (1) the transcription directions of respective genes; (2) the arrangement of ribosomal DNA and CYH<sup>r</sup> gene; and (3) the kind of insertion site, ribosomal RNA structural gene or non-transcribed region.

The characteristics of the plasmid in Figure 1 are summarized in Table 1.

Table 1

| Plasmid | Characteristics                                                                                                                                   |
|---------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| prXHNC  | Ribosomal DNA (3.5 kb) obtained by XbaI digestion is treated with HpaI/NcoI to remove 1.6 kb ribosomal DNA and then ligated to CYH <sup>r</sup> . |
| prEHC   | Ribosomal DNA (3.8 kb) obtained by EcoRI digestion is treated with HpaI to remove 2 kb ribosomal DNA and then ligated to CYH <sup>r</sup> .       |
| prCEX   | CYH <sup>r</sup> is ligated to EcoRI site of 1.1 kb ribosomal DNA obtained by EcoRI/XbaI digestion                                                |

|           |                                                                                                       |
|-----------|-------------------------------------------------------------------------------------------------------|
| prCRX     | CYH <sup>r</sup> is ligated to EcoRV site of 1.3 kb ribosomal DNA obtained by EcoRV/XbaI digestion    |
| prXCH     | CYH <sup>r</sup> is ligated to HpaI site of 3.5 kb ribosomal DNA obtained by XbaI digestion           |
| prXCE     | CYH <sup>r</sup> is ligated to EcoRI site of 3.5 kb ribosomal DNA obtained by XbaI digestion          |
| prXHC1.9  | CYH <sup>r</sup> is ligated to EcoRI site of 1.6 kb ribosomal DNA obtained by XbaI/HindIII digestion  |
| prAC1.9   | CYH <sup>r</sup> is ligated to EcoRV site of 0.6 kb ribosomal DNA obtained by HindIII/EcoRV digestion |
| prEC1.9   | CYH <sup>r</sup> is ligated to EcoRI site of 1.4 kb ribosomal DNA obtained by HindIII/EcoRI digestion |
| prHEC1.9F | CYH <sup>r</sup> is ligated to EcoRV site of 1.4 kb ribosomal DNA obtained by HindIII/EcoRI digestion |
| prHEC1.9R | Same as prHEC1.9F except that the insertion orientation of CYH <sup>r</sup> is reversed               |

In the nomenclature of the plasmid, for example prAC, the letter 'p' indicates a plasmid, 'r' indicates that the plasmid has a ribosomal DNA, and 'C' means for 'CYH<sup>r</sup>'. The letters 'A' in prAC1.9 and 'E' in prEC1.9 indicate restriction enzymes used for liberalization by digesting the ribosomal DNA. In case of other plasmids, 'X' means XbaI, 'E' means EcoRI, 'R' means EcoRV, and 'H' means HindIII, respectively. Therefore, the plasmid containing these letters in their name comprise ribosomal DNA fragments digested with these respective restriction enzymes. Moreover, numerical number after C indicates the size (base pairs) of the L41 gene, while the letter 'F' or 'R' means the orientation of ribosomal DNA fragment and CYH<sup>r</sup>.

## 5. Transformation and selection of transformed cells

The method described by Klass and Peter (Klass & Peter, Curr. Genet., 25,

305, 1994) is followed. That is to say, cells of *Pichia ciferrii* ATCC 14091 grown in YEPD medium to OD<sub>600nm</sub> of 1.5 were collected by centrifugation and mixed with the plasmid. Electroporation was performed at 500V, 50 $\mu$ F and 800 $\Omega$  so as to transform the cells with the plasmid and subjected to growth in YEPD solid medium  
5 to which cycloheximide was added to select transformed cells containing cycloheximide-resistant L41 gene(CYH<sup>r</sup>).

The transformation efficiency is shown in Table 2 (Example 17). The data in Table 2 reveals that prHEC1.9F in which the non-transcribed region between 5S and 26S ribosomal RNA structural genes is used as an insertion site has the highest  
10 transformation efficiency. And, prHEC1.pR in which the transcription direction of 5S RNA gene is opposite to that of CYH<sup>r</sup> shows significantly decreased transformation efficiency.

#### 6. Analysis of transformed cells by Southern blot analysis

15 Genomic DNA was isolated from the transformed cells and the insertion patterns of CYH<sup>r</sup> were analyzed by Southern blot analysis. As a result thereof, it was found that 4-5 copies of CYH<sup>r</sup> were present in the chromosome of the transformed cells. This result indicates that the transformation of *Pichia ciferrii* with the expression cassette of the present invention optimizes the integration of the desired  
20 gene into the chromosome of transformed *Pichia ciferrii* cells.

#### 7. Construction of prACL2 for TAPS production; transformation; and the production of TAPS by cultivation of transformed cells

An expression plasmid was constructed from an expression cassette  
25 comprising LCB2 gene for producing serine palmitoyl transferase and transformed into the mutant *Pichia ciferrii* KFCC-10937 to evaluate the TAPS production. The desired gene, LCB2 gene codes for serine palmitoyl transferase, which is involved in TAPS synthesis, and the mutant KFCC-10937 was developed by the inventors (KR

98-49305A).

Serine palmitoyl transferase (3-ketosphinganine synthase, EC 2.3.1.50) is involved in the first step, which is the rate limiting step of the overall reaction, of the sphingolipids synthesis and forms 3-ketosphinganine having 18 carbon atoms by  
5 condensing serine and palmitoyl-CoA (Barenholz *et al.*, *Biochem. Biophys. Acta*, 248, 458, 1971; *ibid*, 306, 341, 1973). 3-ketosphinganine serves as one of long-chain compounds in animals and as a precursor for phytosphingosine in plants and fungi.

In the present invention, the TAPS production can be significantly improved by integrating multiful copies of LCB2 gene into the chromosome of *Pichia ciferrii*.  
10 The transformed *Pichia ciferrii* cell carrying multiful copies of LCB2 gene on its chromosome produces a large amount of TAPS in a shortened time.

#### 7-1. Isolation of LCB2 gene coding for serine palmitoyl transferase

For the purpose of cloning the gene coding for serine palmitoyl transferase from *Pichia ciferrii* ATCC 14091 genomic DNA, probes were prepared with  
15 reference to the subunits of the known serine palmitoyl transferase gene.

Serine palmitoyl transferase coding gene from *Saccharomyces cerevisiae* consists of two subunits, LCB1 and LCB2. LCB is abbreviation for 'long chain base.' (Nagiec *et al.*, *Proc. Natl. Aca. Sci. USA*, 91, 7899, 1994). DNA sequence of LCB2 genes from other organisms such as human, mouse, *Klebsiella lactis*, and  
20 *Schizosaccharomyces pombe* (Nagiec *et al.*, *Gene*, 177, 237, 1996; Hanada *et al.*, *J. Biol. Chem.*, 272, 32108, 1997; Weiss & Stoffel, *Eur. J. Biochem.*, 249, 239, 1997) are reported.

First, by referring the nucleotide sequence of LCB1 gene from *Saccharomyces cerevisiae* (Nagiec *et al.*, *Proc. Natl. Aca. Sci. USA*, 91, 7899, 1994), PstI fragment  
25 (1 kb) of LCB1 gene was used as probe for Southern blot analysis of genomic DNA of *Pichia ciferrii* ATCC 14091. In this analysis, no desired band was detected. On the other hand, the Southern blot analysis using SalI fragment (0.9 kb) of LCB2 gene

as probe gave DNA band of 12 kb LCB2 gene. This band was collected and inserted into plasmid pBluescript KS+ to give a library. By repeating the Southern blot analysis, ScaI/AflIII fragments (3.0 kb) were obtained and plasmid pL2SA was constructed (Figure 2). Nucleotide sequence of LCB2 gene was determined and is  
5 represented as SEQ. ID. NO. 3 together with its putative amino acid sequence (SEQ. ID. NO. 4) in SEQUENCE LISTING. The sequence was submitted on March 7, 1998 to GenBank under accession number AF053456.

*Pichia ciferrii* ATCC 14091 LCB2 gene comprises 1688 base pairs and has no intron. The putative amino acid sequence shows a high homology to that from  
10 *Saccharomyces cerevisiae*. Transmembrane helix region is present spanning the 55th-79th amino acids. The region containing lysine, which forms Schiff base together with pyridoxal phosphate, has identical amino acids sequence to those of *Saccharomyces cerevisiae*.

#### 7-2. Construction of plasmid prACL2

15 Plasmid pL2SA was treated with HindIII, Klenow and BamHI, in this order, to give a 3.0 kb fragment of LCB2 gene. This fragment was inserted to plasmid prHEC1.9F, which is treated with Eco47III and BamHI, to give plasmid prACL2.

Plasmid prACL2 has the *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41) and LCB2 gene, linked to each other, in this order. Plasmid prACL2 was deposited  
20 with Korea Collection of Type Cultures in Taejon on May 4, 1998 according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and given an accession number of KCTC-0468BP.

#### 7-3. Transformation

25 Plasmid prACL2 was transformed into the mutant *Pichia ciferrii* KFCC-10937 by following the procedure in the step 5 described above and transformed cells

having a high copy number of gene were selected.

#### 7-4. TAPS production by transformed cell cultivation

The transformed cells obtained in the step 7-3 above were cultivated in YGM optimum medium (glycerol 100 g/liter, yeast extract 2 g/liter, KNO<sub>3</sub> 3g/liter, 5 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g/liter, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.3 g/liter, NaCl 0.5 g/liter, CSL 3 g/liter and LS-300 1 g/liter) for 4(four) days to produce TAPS.

The transformed cells according to the present invention exhibited TAPS production at least 1.3 times greater than the parent strain KFCC-10937.

#### 10 8. Isolation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter gene from *Pichia ciferrii*

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential enzyme involved in glycolysis and converts glyceraldehyde-3-phosphate into 1,3-bis-phosphoglycerate. It is a constitutively expressed enzyme. Since GAPDH promoter 15 is hardly affected by carbon source, it attracts the researchers in gene manipulation filed (Kniskern *et al.*, *Gene*, 46, 135, 1986; Travis *et al.*, *J. Biol. Chem.*, 260, 4384, 1985; Hallewell *et al.*, *Biotechnol.*, 5, 363, 1987; Rosenberg *et al.*, *Method Enzymol.*, 185, 341, 1990; Waterham *et al.*, *Gene*, 16, 37, 1997).

Based on these studies, the present inventors cloned *Pichia ciferrii* GAPDH 20 promoter gene and evaluated whether the insertion of GAPDH promoter increases the expression of the desired genes.

#### 8-1. Isolation of GAPDH gene

GAPDH gene from *Saccharomyces cerevisiae* 2805 was used to clone GAPDH gene from the genomic DNA of *Pichia ciferrii* ATCC 14091. By using thus 25 cloned GAPDH gene, plasmid pGH2.2 was constructed (Figure 4). The nucleotide sequence of GAPDH gene of *Pichia ciferrii* ATCC 14091 was determined and is

represented as SEQ. ID. NO. 5 together with its putative amino acid sequence (SEQ. ID. NO. 6) in SEQUENCE LISTING. This sequence was submitted on March 7, 1998 to GenBank under accession number of AF053300.

*Pichia ciferrii* ATCC 14091 GAPDH gene comprises 1004 base pairs and has  
5 no intron. The nucleotide sequence and the putative amino acid sequence show 69.3% and 76.2% homology to that from *Saccharomyces cerevisiae*, respectively. This suggests that *Pichia ciferrii* ATCC 14091 GAPDH gene is new.

#### 8-2. Isolation of GAPDH promoter gene

PCR using plasmid pGH2.2 and primers (primer Nos. 3 and 4; SEQ. ID. NO.  
10 9 and SEQ. ID. NO. 10, respectively in SEQUENCE LISTING) was performed to isolate *Pichia ciferrii* GAPDH promoter gene (600 bp). This gene was inserted into EcoRV site of pT7-Blue T-vector to give plasmid pT7GH.

#### 8-3. Isolation of LCB2 gene free of its own promoter

LCB2 gene free of its own promoter was isolated from plasmid pL2SA by  
15 following the procedure in Figure 5. The gene has a size of 2.3 kb. It was inserted into BamHI site of pT7GH to give plasmid pGAL2.

#### 8-4. Construction of plasmid prACGL2

LCB2 gene of 2.9 kb was isolated from plasmid pGAL2 by following the  
procedure in Figure 5 and inserted into Eco47III/XbaI site of prHEC1.9F to give  
20 plasmid prACGL2.

Plasmid prACGL2 has the *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41), GAPDH promoter gene and LCB2 gene, linked to each other, in this order. Its restriction map is depicted in Figure 5. Plasmid prACGL2 was deposited with Korea Collection of Type Cultures in Taejon on June 25, 1998 according to the  
25 Budapest Treaty on the International Recognition of the Deposit of Microorganisms

for the Purposes of Patent Procedure and given an accession number of KCTC-0498BP.

#### 8-5. Construction of plasmid prHECGL2

When the host cell (e.g., strain of *Pichia ciferrii*) is transformed with plasmid prACGL2, the resulting transformed cell will carry genes from *Pichia ciferrii* as well as other undesired regions of the starting bacterial plasmid since the plasmid is treated with only one restriction enzyme for linearization prior to the transformation into the host cell. To avoid this, the present inventors add *Pichia ciferrii* ribosomal DNA fragment (800 bp) at the downstream of LCB2 gene of plasmid prACGL2. Thus obtained plasmid was designated as prHECGL2. (Figure 6)

Plasmid prHECGL2 has the *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41), GAPDH promoter gene, LCB2 and *Pichia ciferrii* ribosomal DNA fragment, operably linked to each other, in this order. Its restriction map is depicted in Figure 6. Plasmid prHECGL2 was deposited with Korea Collection of Type Cultures in Taejon on August 10, 1998 according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and given an accession number of KCTC-0511BP.

When the host cell is transformed with plasmid prHECGL2, the resulting transformed cell carry only genes from *Pichia ciferrii* by treating the plasmid by proper restriction enzymes to cut both of the *Pichia ciferrii* ribosomal DNAs. That is to say, when the plasmid prHECGL2 is transformed into *Pichia ciferrii*, the obtained transformed cell *Pichia ciferrii* will carry only endogenous genes.

#### 8-6. Transformation

Cells of *Pichia ciferrii* KFCC-10937 and plasmid prACL2, prACGL2 and prHECGL2 were mixed, respectively and electroporation was performed at voltage 500V, capacity 50 $\mu$ F and resistance 800 $\Omega$  so as to transform the cells with the

plasmid.

#### 8-7. TAPS production by transformed cell cultivation

The transformed cells obtained in the step 8-6 above were cultivated in YGM optimum medium (glycerol 100 g/liter, yeast extract 2 g/liter, KNO<sub>3</sub> 3g/liter, 5 (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g/liter, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.3 g/liter, NaCl 0.5 g/liter, CSL 3 g/liter and LS-300 1 g/liter) to produce TAPS.

The transformed cells with plasmid prACGL2 (KCTC-0498BP) or with plasmid prHECGL2 (KCTC-0511BP) according to the present invention exhibited TAPS production at least 2.1 times and at least 1.5 times greater than the parent strain 10 KFCC-10937 and than the transformed cell with plasmid prACL2 (KCTC-0468BP), respectively.

#### Free Texts in Sequence Listing

SEQ.ID.NO. 7 is an artificial sequence for PCR primer CYH1.

SEQ.ID.NO. 8 is an artificial sequence for PCR primer CYH4.

15 SEQ.ID.NO. 9 is an artificial sequence for PCR primer No. 3 which is used for isolation of GAPDH promoter gene.

SEQ.ID.NO. 10 is an artificial sequence for PCR primer No. 4 which is used for isolation of GAPDH promoter gene.

SEQ.ID.NO. 11 is an artificial sequence for PCR primer CH-f.

20 SEQ.ID.NO. 12 is an artificial sequence for PCR primer CH-r.

SEQ.ID.NO. 13 is an artificial sequence for PCR primer 18R.

SEQ.ID.NO. 14 is an artificial sequence for PCR primer 26F.

SEQ.ID.NO. 15 is an artificial sequence for PCR primer L2f.

SEQ.ID.NO. 16 is an artificial sequence for PCR primer L2r.

25 SEQ.ID.NO. 17 is an artificial sequence for PCR primer No. 1 which is used

for isolation of GAPDH gene.

SEQ.ID.NO. 18 is an artificial sequence for PCR primer No. 2 which is used for isolation of GAPDH gene.

### Examples

5        The present invention will be described in more detail by way of various Examples, which should not be construed to limit the scope of the present invention.

#### Example 1 : Isolation of genomic DNA from *Pichia ciferrii* ATCC 14091.

Cells of *Pichia ciferrii* ATCC 14091 grown in YEPD medium (peptone 2%, yeast extract 1% and glucose 2%) by following the method described by Johnston  
10 (Johnston *et al.*, Yeast Genetics, molecular aspects, pp.107-123, IRL Press, 1988) were collected and suspended in SSEM solution (1M sorbitol, 100mM sodium citrate, 60mM EDTA, 100mM 2-mercaptoethanol). Novozyme was added to the suspension to a concentration of 0.1 mg/ml, and allowed to react at 37°C for 30 minutes to give protoplast. An equal volume of SDS-TE solution (2% SDS, 50mM  
15 EDTA in 1M Tris-Cl, pH 8.0) was added and allowed to react at 60°C for 10 minutes to disintegrate cells. To the supernatant obtained by centrifuging at 8,000 rpm for 15 minutes, a twice volume of ethanol was added. The resulting pellets were dissolved in TE solution containing RNase to extract *Pichia ciferrii* genomic DNA.

#### Example 2: Isolation of L41 gene from *Pichia ciferrii* ATCC 14091

20        Two following primers CYH1 and CYH4 were synthesized.

CYH1 : 5'-CGC GTA GTT AAY GTN CCN AAR AC-3'

CYH4 : 5'-GCC TGG CCY TTY TGY TTY TTN TC-3'

These two primers are also represented as SEQ. ID. NO. 7 and SEQ. ID. NO. 8, respectively in SEQUENCE LISTING.

PCR was performed using the two primers and *Pichia ciferrii* genomic DNA obtained in Example 1 to give L41 gene fragment of about 300 bp.

This fragment was labeled with DIG-labeling and detection kit (Boehringer Mannheim) by following the manufacturer's manual to give a probe.

5 Example 3: Sequencing of L41 gene

Genomic DNA of *Pichia ciferrii* obtained in Example 1 was digested with various restriction enzymes, and the digestion products were subjected to 0.9% agarose gel electrophoresis and transferred to Nytran<sup>R</sup> membrane (Schleicher & Schuell) followed by hybridization with a hybridization solution (5X SSC, 0.1% N-  
10 laurylsarcosine, 0.02% SDS, 2% blocking agent and 30% formaldehyde) using probes obtained in Example 2. The hybridization was carried out at 42°C for 6 hours according to the manufacturer's manual prepared by Boehringer Mannheims.

Antibodies coupled to alkaline phosphatase were introduced and BCIP and X-phosphate were added. Violet-stained band was observed.

15 Band is detected at 1.9 kb size of genomic DNA treated with EcoRI. This DNA fragment (1.9 kb) was collected and linked to EcoRI site of plasmid pBluescript KS+ (Stratagene) and transformed into *E. coli* DH5 $\alpha$  to establish a library.

This library was repeatedly subjected to Southern blot analysis to isolate 1.9 kb gene fragment containing L41 gene(plasmid pCYH1.9). Nucleotide sequencing  
20 was performed for this plasmid using Automatic sequencer Model 373A (Applied Biosystem). The result is represented as SEQ. ID. NO. 1 together with its putative amino acid sequence (SEQ. ID. NO. 2) in SEQUENCE LISTING. This sequence was submitted on March 7, 1998 to GenBank under accession number of AF053457.

L41 gene of *Pichia ciferrii* ATCC 14091 consists of 737 base pairs including  
25 419 bp intron. The amino acid residue 56 is proline.

Example 4: Construction of plasmid pCYH1.9<sup>r</sup> in which proline (aa 56) is replaced

by glutamine.

Two primers CH-f and CH-r were prepared:

Primer CH-f : GGT CAA ACC AAA CCA GTT TTC

Primer CH-r : ATG GAA AAC TTG TTT GGT TTG ACC

5        These two primers are also represented as SEQ .ID. NO. 11 and SEQ. ID. NO. 12, respectively in SEQUENCE LISTING.

Combinations of universal primer and primer CH-r, and of reverse primer and primer CH-f were employed for PCR using Pfu DNA polymerase and pCYH1.9 as a template to obtain two PCR products of 1.2 kb and 0.7 kb.

10        PCR was performed again using the two PCR products (1.2 kb and 0.7 kb), and universal and reverse primers to obtain plasmid pCYH1.9<sup>r</sup> in which proline (aa 56) is replaced by glutamine.

Example 5: Construction of plasmid prDX9.0 containing ribosomal DNA fragment

15        Two following primers 18R and 26F were synthesized.

Primer 18R : 5'-CAA TAA TTG CAA TGC TCT ATC CCC AGC ACG-3'

Primer 26F : 5'-GGA TAT GGA TTC TTC ACG GTA ACG TAA CTG-3'

These two primers are also represented as SEQ. ID. NO. 13 and SEQ. ID. NO. 14, respectively in SEQUENCE LISTING.

20        PCR was performed using the two primers and *Pichia ciferrii* genomic DNA obtained in Example 1 to give a PCR product of 6.0 kb. This PCR product was labeled in the same manner as in Example 2 to give a probe.

When the probe is subjected to Southern blot analysis with *Pichia ciferrii* genomic DNA, band is detected at 9 kb size of genomic DNA treated with XhoI. This  
25 DNA fragment (9 kb) was collected and inserted into plasmid pBluescript KS<sup>+</sup> (Stratagene) and its library was established. This library was repeatedly subjected to Southern blot analysis to isolate 9 kb gene fragment containing ribosomal DNA (plasmid prDX9.0).

Nucleotide sequencing was performed for this plasmid using Automatic sequencer Model 373A (Applied Biosystem). The results including the location and orientation of 26S, 18S, 5.8S and 5S ribosomal gene are shown in Figure 1.

Example 6: Construction of plasmid prXHNC

5 Plasmid prDX9.0 obtained in Example 5 was treated with XbaI to obtain 3.5 kb ribosomal DNA, which is then inserted into plasmid pBluescript KS+. The resulting recombinant plasmid is treated with HpaI/NcoI/Klenow to remove 1.6 kb ribosomal DNA and linked to 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, to obtain plasmid prXHNC.

10

Example 7: Construction of plasmid prEHC

Plasmid prDX9.0 obtained in Example 5 was treated with EcoRI to obtain 3.8 kb ribosomal DNA, which is then inserted into plasmid pBluescript KS+. The resulting recombinant plasmid is treated with HpaI to remove 2 kb ribosomal DNA  
15 and linked to 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, to obtain plasmid prEHC.

Example 8: Construction of plasmid prCEX

Plasmid prDX9.0 obtained in Example 5 was treated with EcoRI/XbaI to obtain 1.1 kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting  
20 plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, was linked to EcoRI site of the 1.1 kb ribosomal DNA to give plasmid prCEX.

Example 9: Construction of plasmid prCRX

Plasmid prDX9.0 obtained in Example 5 was treated with EcoRV/XbaI to obtain 1.3 kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting  
25 plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, was linked to EcoRV site of

the 1.3 kb ribosomal DNA to give plasmid prCRX.

Example 10: Construction of plasmid prXCH

Plasmid prDX9.0 obtained in Example 5 was treated with XbaI to obtain 3.5  
5 kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting plasmid  
pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, was linked to HpaI site of the 3.5 kb  
ribosomal DNA to give plasmid prXCH.

Example 11: Construction of plasmid prXCE

10 Plasmid prDX9.0 obtained in Example 5 was treated with XbaI to obtain 3.5  
kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting plasmid  
pCYH1.9<sup>r</sup> in Example 4 with EcoRI, was linked to EcoRI site of the 3.5 kb ribosomal  
DNA to give plasmid prXCE.

15 Example 12: Construction of plasmid prXHC1.9

Plasmid prDX9.0 obtained in Example 5 was treated with XhoI/HindIII to  
obtain 1.6 kb ribosomal DNA. A plasmid containing this 1.6 kb ribosomal DNA was  
digested with HindIII/Klenow to give ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was  
obtained by digesting plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI, was linked to  
20 EcoRI site of the HindIII/Klenow digestion product to give plasmid prXHC1.9.

Example 13: Construction of plasmid prAC1.9

Plasmid prDX9.0 obtained in Example 5 was treated with HindIII/EcoRV to  
obtain 0.6 kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting  
25 plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, was linked to EcoRV site of  
the 0.6 kb ribosomal DNA to give plasmid prAC1.9.

Example 14: Construction of plasmid prEC1.9

Plasmid prDX9.0 obtained in Example 5 was treated with HindIII/EcoRI to obtain 1.4 kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI, was linked to EcoRI site of the 1.4 kb  
5 ribosomal DNA to give plasmid prXCH.

Example 15: Construction of plasmid prHEC1.9F

Plasmid prDX9.0 obtained in Example 5 was treated with HindIII/EcoRI to obtain 1.4 kb ribosomal DNA. 1.9 kb CYH<sup>r</sup> gene, which was obtained by digesting  
10 plasmid pCYH1.9<sup>r</sup> in Example 4 with EcoRI/Klenow, was linked to EcoRV site of the 1.4 kb ribosomal DNA to give plasmid prHEC1.9F.

Example 16: Construction of plasmid prHEC1.9R

The procedure in Example 15 was repeated except that the insertion  
15 orientation of CYH<sup>r</sup> was opposite to that of Example 5 to obtain plasmid prHEC1.9R

Example 17: Transformation efficiency of plasmids.

The method described by Klass and Peter (Klass & Peter, Curr. Genet., 25, 305, 1994) was followed. That is to say, cells of *Pichia ciferrii* KFCC-10937 grown in YEPD medium (100 ml) to OD<sub>600nm</sub> of 1.5 were collected by centrifugation and  
20 dispersed in 50 mM phosphate buffer (pH 7.5; 40 ml), to which 25 mM DTT was added, at 37°C for 15 minutes. The mixture was washed twice with ice-cooled stabilization solution (100 ml; 270 mM sucrose, 10 mM Tris-Cl (pH 7.5), 1 mM MgCl<sub>2</sub>) and suspended in 1 ml of stabilization solution.

To the resulting suspension (50 microliters), 5 microliters of the solution of  
25 respective plasmids obtained in Examples 6 - 16 are added and allowed to stand on ice for 10 minutes. Then, the solution was transferred to 0.2 mm electroporation cuvettes (Bio-Rad). Electroporation was carried out using Gene-pulser II (Bio-Rad)

at 500V, 50 $\mu$ F and 800 $\Omega$ , and the electroporation product was suspended into 0.5 mL of stabilization solution. After adding 2 mL of YEPD medium, the cultivation was carried out at 25°C for 5 hours. Then the culture broth was plated on YEPD solid medium, to which 10  $\mu$ g/mL of cycloheximide was added, at 25°C for 4 - 5 days. The number of transformed cells was counted and transformation efficiency was shown in Table 2.

Table 2

| Plasmid   | No. of transformed cell per $\mu$ g | Plasmid  | No. of transformed cell per $\mu$ g |
|-----------|-------------------------------------|----------|-------------------------------------|
| prXHNC    | 276                                 | prEHC    | 250                                 |
| prCEX     | 194                                 | prCRX    | 226                                 |
| prXCH     | 314                                 | prXCE    | 134                                 |
| prAC1.9   | 1574                                | prXHC1.9 | 1287                                |
| prEC1.9   | 983                                 | prHC1.9F | 1760                                |
| prHEC1.9R | 54                                  |          |                                     |

The results in Table 2 reveals that the site of ribosomal DNA where CYH<sup>r</sup> gene is inserted and the transcription direction of CYH<sup>r</sup> gene are closely related with the transformation efficiency. Plasmids prAC1.9 and prHEC1.9F in which the non-transcribed region between 5S and 26S ribosomal RNA structural genes is used as an insertion site has the highest transformation efficiency. And, prHEC1.9R in which the transcription direction of 5S RNA gene is opposite to that of CYH<sup>r</sup> shows significantly decreased transformation efficiency.

Based on these results, plasmid prHEC1.9F was selected.

#### Example 18: Selection of transformation conditions

In order to establish the optimum conditions of transformation, linear prHEC1.9 obtained after treating with ApaI/ScaI was transformed by following the

procedure in Example 17 except that the voltage was changed to 500, 600 or 700V and the resistance to 100, 200, 300, 400, 500, 600, 700 or 800 $\Omega$  while keeping the capacity of 50 $\mu$ F. The results are shown in Table 3.

Table 3

| 5 | Voltage (V) | Resistance ( $\Omega$ ) | No. of transformed cell per $\mu$ g |
|---|-------------|-------------------------|-------------------------------------|
|   | 500         | 100                     | 0                                   |
|   |             | 200                     | 82                                  |
|   |             | 300                     | 1065                                |
|   |             | 400                     | 1770                                |
|   |             | 500                     | 2890                                |
|   |             | 600                     | 4250                                |
|   |             | 700                     | 6300                                |
|   |             | 800                     | 6850                                |
|   | 600         | 100                     | 0                                   |
|   |             | 200                     | 210                                 |
|   |             | 300                     | 1488                                |
|   |             | 400                     | 2644                                |
|   |             | 500                     | 5250                                |
|   |             | 600                     | 6750                                |
|   |             | 700                     | 6500                                |
|   |             | 800                     | 4740                                |
|   | 700         | 100                     | 18                                  |
|   |             | 200                     | 620                                 |
|   |             | 300                     | 2360                                |
|   |             | 400                     | 4400                                |
|   |             | 500                     | 3840                                |
|   |             | 600                     | 2640                                |

The results in Table 3 confirm that the optimum transformation conditions are capacity 50 $\mu$ F, voltage 500V and resistance 800 $\Omega$ .

Example 19: Analysis of transformed cell by Southern blot analysis

Each of transformed cells selected in Example 17 was inoculated into YEPD medium to which 5  $\mu$ g/mL of cycloheximide was added and subjected to cultivation while agitation at 25°C for 18 - 20 hours, followed by centrifugation to collect cell pellets. Thus obtained cells were placed in 1.5 mL tubes. Cells were suspended in 30 $\mu$ L of STES solution (0.5M NaCl, 0.01M EDTA, 1% SDS in 0.2M Tris-Cl, pH 7.6) and 0.8 volumes of glass beads (diameter 0.4 mm) were added thereto. The mixtures were stirred for 5 minutes, 200  $\mu$ L of TE buffer (1mM EDTA in 10mM Tris-Cl, pH 8.0) and 200  $\mu$ L of phenol/chloroform/isoamylalcohol (25:24:1) were added. The resulting mixtures were stirred for 2 minutes and centrifuged at 12,000 rpm. 2.5 Volumes of ethanol was added to the supernatant to precipitate genomic DNA and dried.

Genomic DNA (2 - 3  $\mu$ g) was dissolved into 50  $\mu$ L of distilled water, treated with EcoRI, and subjected to electrophoresis on 0.8% agarose gel. Southern blot analysis was carried out by using L41 gene in Example 2 as a probe to detect bands. It was observed that 4 - 5 copies of L41 gene are carried on genomic DNA of all transformed cells.

Example 20 : Construction of plasmid prACL2

(1) Isolation of LCB2 gene from *Pichia ciferrii* ATCC 14091.

Cells of *Saccharomyces cerevisiae* grown in YEPD medium (peptone 2%, yeast extract 1% and glucose 2%) by following the method described by Johnston (Johnston *et al.*, Yeast Genetics, molecular aspects, pp.107-123, IRL Press, 1988) were collected and genomic DNA was isolated therefrom.

Two following primers L2f and L2r were synthesized.

Primer L2f : 5'-ATG AGT ACT CCT GCA AAC TA-3'

Primer L2r : 5'-TAA CAA AAT ACT TGT CGT CC-3'

These two primers are also represented as SEQ. ID. NO. 15 and SEQ. ID. NO. 16, respectively in SEQUENCE LISTING.

5        PCR was performed using the two primers and the *Saccharomyces cerevisiae* genomic DNA obtained in the above to give LCB2 gene fragment of about 1680 bp. The Sall fragment of 913bp was labeled with DIG-labeling and detection kit (Boehringer Mannheim) by following the manufacturer's manual to give a probe.

10        Genomic DNA of *Pichia ciferrii* obtained in Example 1 was digested with various restriction enzymes, BamHI, EcoRI, EcoRV, HindIII, PstI or Sall, and the digestion products were subjected to electrophoresis in TAE buffer and transferred to Nytran<sup>R</sup> membrane (Schleicher & Schuell) followed by Southern blot analysis.

15        The Southern blot analysis was carried out using a hybridization solution (5X SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 2% blocking agent and 30% formamide) at 42°C for 6 hours according to the manufacturer's manual prepared by Boehringer Mannheims.

Antibodies coupled to alkaline phosphatase were introduced and BCIP and X-phosphate were added. Violet color-stained bands were observed.

Band is detected at 12 kb size of genomic DNA treated with HindIII.

20        This DNA fragment(12kb) was collected and inserted into plasmid pBluescript KS+(Stratagene) and transformed into E. coli DH5 $\alpha$  to establish a library.

This library was repeatedly subjected to Southern blot analysis to isolate 3.0 kb SacI/AflIII fragment containing LCB2 gene(plasmid pL2SA).

25        Restriction map and sequencing of *Pichia ciferrii* LCB2 gene are shown in Figure 2. The nucleotide sequence is represented as SEQ. ID. NO. 3 together with its putative amino acid sequence (SEQ. ID. NO. 4) in SEQUENCE LISTING. The nucleotide sequence was submitted on March 7, 1998 to GenBank under accession number of AF053456.

LCB2 gene of *Pichia ciferrii* ATCC 14091 consists of 1688 base pairs without intron, and its putative amino acid sequence shows a high homology to that of *Saccharomyces cerevisiae*.

## (2) Construction of plasmid prACL2

5 Plasmid prHEC1.9F obtained in Example 15 was digested with Eco47III/BamHI to give a linear one. On the other hand, plasmid pL2SA obtained in (1) above was treated with HindIII, Klenow and BamHI, in this order, to give a 3.0 kb fragment of LCB2 gene. This 3.0 kb fragment was inserted to the above linear plasmid to give plasmid prACL2. (Figure 3)

10 Plasmid prACL2 has the *Pichia ciferrii* ribosomal DNA fragment, CYH'(L41) and LCB2 gene, linked to each other, in this order. Plasmid prACL2 was deposited with Korea Collection of Type Cultures in Taejon on May 4, 1998 according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and given an accession number of KCTC-  
15 0468BP.

## Example 21: Transformation

Plasmid prACL2 was linearized with ApaI and transformed into the mutant *Pichia ciferrii* KFCC-10937 by following the procedure in Example 17.  $10^2$  -  $10^3$  colonies per  $\mu$ g of plasmid were formed. Twelve largest colonies were selected.

20 Each of transformed cells was inoculated into YEPD medium to which  $5 \mu$ g/mL of cycloheximide was added and subjected to cultivation while agitation at 25°C for 18 - 20 hours, followed by centrifugation to collect cell pellets. Thus obtained cells were placed in 1.5 mL tubes. Cells were suspended in  $30 \mu$ L of STES solution (0.5M NaCl, 0.01M EDTA, 1% SDS in 0.2M Tris-Cl, pH 7.6) and 0.8 volumes of glass  
25 beads (diameter 0.4 mm) were added thereto. The mixtures were stirred for 5 minutes,  $200 \mu$ L of TE buffer (1mM EDTA in 10mM Tris-Cl, pH 8.0) and  $200 \mu$ L

of phenol/chloroform/isoamylalcohol (25:24:1) were added. The resulting mixtures were stirred for 2 minutes and centrifuged at 12,000 rpm. 2.5 Volumes of ethanol was added to the supernatant to precipitate genomic DNA and dried.

Genomic DNA (2 - 3  $\mu$ g) was dissolved into 50  $\mu$ L of distilled water, treated  
5 with EcoRI, and subjected to electrophoresis on 0.8% agarose gel. Southern blot analysis was carried out by using L41 gene in Example 2 as a probe to detect bands. It was observed that 5 - 10 copies of L41 gene are carried on genomic DNA of all transformed cells. One of them is picked up and named as 'Transformed cell 1.'

Comparative Example 1 : TAPS production by strain KFCC-10937 cultivation

10 The parent strain KFCC-10937 was cultivated in 100mL of YGM optimum medium (glycerol 100 g/liter, yeast extract 2 g/liter, KNO<sub>3</sub> 3g/liter, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 0.5 g/liter, MgSO<sub>4</sub>·7H<sub>2</sub>O 0.3 g/liter, NaCl 0.5 g/liter, CSL 3 g/liter and LS-300 1 g/liter) at 25°C and 250 rpm for 4 days. After standing at 25 C for 2 days, 4 volumes of mixed solvent of chloroform/methanol (1:1) was added to separate phases and extract  
15 TAPS.

TAPS was analyzed on HPLC using ELSD(Electron Light Scanning Detector). As solvent, a mixture of iso-octane and THF/formic acid (100:1.5) varying its ratio 9:1, 7:3 and then 9:1 was used.

Example 22 : TAPS production by Transformed cell 1

20 Transformed cell 1 obtained in Example 20 was cultivated in the same condition and manner as in Comparative Example 1 to produce TAPS.

TAPS productions in Comparative Example 1 and Example 22 are shown in Table 4.

Table 4

|                                    | KFCC-10937 | Transformed cell 1 |
|------------------------------------|------------|--------------------|
| Doubling Time (hr)                 | 1.5        | 1.5                |
| Biomass concentration (g/L)        | 41.6       | 43.6               |
| Amount of TAPS (mg/L)              | 5206       | 7444               |
| 5 TAPS specific yield (mg/gdw*)    | 125.1      | 170.7              |
| Volume productivity (mg TAPS/L/hr) | 54.2       | 77.5               |

\* gdw = dry weight (g)

The results in Table 4 affirmed that the transformation of *Pichia ciferrii* with  
 10 the expression cassette according to the present invention makes it possible to  
 maximize the integration of the desired gene onto the chromosome of the host.

#### Example 23: Preparation of probe for GAPDH gene cloning

Two following primers (Primer No. 1 and No. 2) were synthesized.

Primer 1 : 5'-ATG GTT AGA GTT GCT ATT AAC G-3'

15 Primer 2 : 5'-AAG CCT TGG CAA TGT GTT CAA-3'

These two primers also represented as SEQ. ID. NO. 17 and SEQ. ID. NO. 18,  
 respectively in SEQUENCE LISTING.

PCR was performed using the two primers to isolate 1 kb GAPDH gene from  
*Saccharomyces cerevisiae* 2805 (provided by courtesy of R.B.Wickner at NIH) . The  
 20 gene was inserted into EcoRV site of plasmid pT7-Blue T-vector (Novagen) to obtain  
 plasmid pT7-SGH.

Plasmid pT7-SGH was digested with XbaI and Sall to give 0.9 kb gene  
 fragment, which was labeled with DIG-labeling and detection kit (Boehringer  
 Mannheim) by following the manufacturer's manual to give a probe.

Example 24: Isolation of GAPDH gene from *Pichia ciferrii*

Genomic DNA of *Pichia ciferrii* obtained in Example 1 was digested with various restriction enzymes, BamHI, EcoRI, EcoRV, HindIII, PstI or Sall, and the digestion products were subjected to electrophoresis on 0.9% agarose gel and  
5 transferred to Nytran<sup>R</sup> membrane (Schleicher & Schuell) followed by Southern blot analysis using the probe obtained in Example 23.

The Southern blot analysis was carried out using a hybridization solution (5X SSC, 0.1% N-laurylsarcosine, 0.02% SDS, 2% blocking agent and 30% formamide) at 42°C for 6 hours according to the manufacturer's manual prepared by Boehringer  
10 Mannheim.

Antibodies coupled to alkaline phosphatase were introduced and BCIP and X-phosphate were added. Violet color-stained bands were observed at about 6 kb sized fragment of the genomic DNA treated with HindIII/EcoRI.

This DNA fragment (about 6 kb) was collected and inserted into plasmid  
15 pBluescript KS+ (Stratagene) and transformed into *E. coli* DH5 $\alpha$  to establish a library. This library was repeatedly subjected to Southern blot analysis to isolate 6.0 kb AflIII/HindIII fragment containing GAPDH gene(plasmid pGH2.2).

The restriction map and sequencing of GAPDH gene of *Pichia ciferrii* ATCC 14091 are shown in Figure 4. The nucleotide sequence is represented as SEQ. ID.  
20 NO. 5 together with its putative amino acid sequence (SEQ. ID. NO. 6) in SEQUENCE LISTING. This sequence was submitted on March 7, 1998 to GenBank under accession number of AF053300.

*Pichia ciferrii* ATCC 14091 GAPDH gene comprises 1004 base pairs and has no intron. The nucleotide sequence and the putative amino acid sequence show  
25 69.3% and 76.2% homology to that from *Saccharomyces cerevisiae*, respectively.

Example 25: Isolation of *Pichia ciferrii* GAPDH promoter gene

Two following primers (Primer No. 3 and No. 4) were synthesized.

Primer 3 : 5'-GAT ATC TAC ATA CAA TTG ACC CAT AG-3'

Primer 4 : 5'-GGA TCC TTA ATT ATT TGT TTG TTT-3'

These two primers are also represented as SEQ. ID. NO. 9 and SEQ. ID. NO. 10, respectively in SEQUENCE LISTING.

5        PCR using plasmid pGH2.2 obtained in Example 24 and the two primers (primer Nos. 3 and 4) was performed to isolate *Pichia ciferrii* GAPDH promoter gene (600 bp). This gene was inserted into EcoRV site of pT7-Blue T-vector to give plasmid pT7GH.

Example 26: Isolation of LCB2 gene free of its own promoter

10        Plasmid pL2SA obtained in Example 20 was treated with AflIII to give promoter-free LCB2 gene (2.3 kb). This 2.3 kb gene was treated with Klenow and inserted into plasmid pBluescript KS+ treated with BamHI/Klenow to give plasmid pL2B2.3. (Figure 5)

15        Plasmid pL2B2.3 was digested with BamHI to give 2.3 kb LCB2 gene, which was inserted to BamHI site of plasmid pT7GH in Example 4 to give pGAL2.

Example 27: Construction of expression cassette

20        Plasmid prHEC1.9F obtained in Example 15 was linearized by treating with Eco47III and XbaI. GAPDH promoter/LCB2 gene (2.9 kb) obtained by treating plasmid pGAL2 (Example 26) with EcoRV and XbaI was inserted to the linear plasmid prHEC1.9F to give prACGL2. (Figure 5)

25        Plasmid prACGL2 has the *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41), GAPDH promoter gene and LCB2 gene, linked to each other, in this order. Plasmid prACGL2 was deposited with Korea Collection of Type Cultures in Taejeon on June 25, 1998 according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and given an accession number of KCTC-0498BP.

Plasmid pCYH1.9<sup>r</sup> obtained in Example 4 was linearized by treating with Eco47III and XbaI. GAPDH promoter/LCB2 gene (2.9 kb) obtained by treating plasmid pGAL2 (Example 26) with EcoRV and XbaI was inserted to the linear plasmid pCYH1.9<sup>r</sup> to give pCHGL2. (Figure 6). This plasmid was treated with  
5 EcoRV/XbaI/Klenow to give 4.6 kb CYH<sup>r</sup>/GAPDH promoter/LCB2 gene which is then inserted to EcoRV site of 1.4 kb ribosomal DNA, which was obtained by treating plasmid prDX9.0 (Example 5) with HindIII/EcoRI, to give plasmid prHECGL2. (Figure 6)

Plasmid prHECGL2 has a structure that it contains additional *Pichia ciferrii*  
10 ribosomal DNA fragment (800 bp) linked at downstream of LCB2 gene of plasmid prACGL2. That is to say, plasmid prHECGL2 contains *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41), GAPDH promoter gene, LCB2 and *Pichia ciferrii* ribosomal DNA fragment, operatively linked to each other, in this order.

Plasmid prHECGL2 was deposited with Korea Collection of Type Cultures  
15 in Taejon on August 10, 1998 according to the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and given an accession number of KCTC-0511BP.

Plasmid prHECGL2 is distinguished from and advantageous over plasmid prACGL2 that the former introduces only *Pichia ciferrii* endogenous genes onto the  
20 chromosome of *Pichia ciferrii* transformed cell while the latter introduces *Pichia ciferrii* endogenous genes together with bacterial genes (originated from plasmid pBluescript KS+) when they are used to transform *Pichia ciferrii*.

#### Example 28: Transformation

Plasmid prACGL2 (Example 27) and prHECGL2 (Example 27) were  
25 linearized with ApaI and ApaI/ScaI, respectively. Then, these linear plasmids were transformed into *Pichia ciferrii* KFCC-10937 by following the procedure in Example 17. 10<sup>3</sup> colonies per  $\mu$ g of plasmid were formed. Four largest colonies were selected

for respective plasmid-transformed cells.

Each of transformed cells was inoculated into YEPD medium to which 5  $\mu\text{g/mL}$  of cycloheximide was added and subjected to cultivation while agitation at 25°C for 18 - 20 hours, followed by centrifugation to collect cell pellets. Thus obtained cells were placed in 1.5 mL tubes. Cells were suspended in 30  $\mu\text{L}$  of STES solution (0.5M NaCl, 0.01M EDTA, 1% SDS in 0.2M Tris-Cl, pH 7.6) and 0.8 volumes of glass beads (diameter 0.4 mm) were added thereto. The mixtures were stirred for 5 minutes, 200  $\mu\text{L}$  of TE buffer (1mM EDTA in 10mM Tris-Cl, pH 8.0) and 200  $\mu\text{L}$  of phenol/chloroform/isoamylalcohol (25:24:1) were added. The resulting mixtures were stirred for 2 minutes and centrifuged at 12,000 rpm. Two and half (2.5) volumes of ethanol was added to the supernatant to precipitate genomic DNA and dried.

Genomic DNA (2 - 3  $\mu\text{g}$ ) was dissolved into 50  $\mu\text{L}$  of distilled water, treated with HindIII, EcoRI or XbaI, and subjected to electrophoresis on 0.8% agarose gel. Southern blot analysis was carried out by using DIG-labeled L41 gene, DIG-labeled LCB2 gene or DIG-labeled GAPDH promoter gene as a probe to detect bands (Figure 7). It was observed that about 4 - 5 copies of the genes are carried on genomic DNA of all transformed cells. Each one of the respective prACGL2-transformed and prHECGL2-transformed cells is picked up and named as 'Transformed cell 2' and 'Transformed cell 3,' respectively.

#### Example 29 : TAPS production by Transformed cell 2

Transformed cell 2 obtained in Example 28 was cultivated in the same condition and manner as in Comparative Example 1 to produce TAPS. TAPS production is shown in Table 5.

Table 5

|                                    | Transformed cell 2 (prACGL2) |
|------------------------------------|------------------------------|
| Doubling Time (hr)                 | 1.5                          |
| Biomass concentration (g/L)        | 40.1                         |
| Amount of TAPS (mg/L)              | 10933                        |
| TAPS specific yield (mg/gdw*)      | 272.6                        |
| Volume productivity (mg TAPS/L/hr) | 113.9                        |

\* gdw = dry weight (g).

Example 30 and Comparative Example 2 : TAPS production by Transformed cell 3

Transformed cell 2 obtained in Example 28 or parent strain of *Pichia ciferrii* was cultivated in YMGL medium (yeast extract 3 g/L, malt extract 3 g/L, glycerol 30 g/L) under the same conditions as that in Comparative Example 1 to produce TAPS. TAPS productions are shown in Table 6.

Table 6

|                  | Parent Strain<br>(KFCC-10937) | Transformed cell 3<br>(prHECGL2) |
|------------------|-------------------------------|----------------------------------|
| TAPS in mg/mL    | 0.200                         | 0.420                            |
| TAPS in g/g cell | 0.012                         | 0.027                            |

The results in Table 6 reveal that the absolute production of TAPS in YMGL medium decreases when compared to that in YGM optimum medium, and that the TAPS production by transformed cell 3 is 2.1 times greater than that of the parent strain.

In summary, the present invention has following advantages:

- (1) The expression cassette of the present invention allows a maximized

integration of desired genes into chromosome of host *Pichia ciferrii* cells.

(2) Plasmid prACL2 according to the present invention has 0.6 kb *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41) and LCB2 gene coding for serine palmitoyl transferase, operatively linked to each other, in this order. It shows a good efficiency of transformation into host cells and the resulting transformed cells show at least 1.3 times greater TAPS production than the parent strain.

(3) Introduction of GAPDH promoter gene into the expression cassette according to the present invention allows a further increase in the expression of the desired gene in the transformed cell.

(4) Plasmid prACGL2 according to the present invention has 0.6 kb *Pichia ciferrii* ribosomal DNA fragment, CYH<sup>r</sup>(L41), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter gene and LCB2 gene, and plasmid prHECGL2 has further *Pichia ciferrii* ribosomal DNA fragment of 800 bp at the downstream of the LCB2 gene in plasmid prACGL2. These plasmids show an excellent transformation efficiency as well as show TAPS production at least 2.1 times greater than the parent strain KFCC-10937 due to an increased expression of LCB2 gene by action of GAPDH promoter.

Although preferred embodiments of the present invention have been described in detail herein above, it should be clearly understood that many variations and/or modifications of the basic inventive concepts herein taught which may appear to those skilled in the art will still fall within the spirit and scope of the present invention as defined in the appended claims.

## CLAIMS

1. A L41 gene represented as SEQ. ID. NO. 1 and coding for a *Pichia ciferrii* ribosomal protein.
2. An expression cassette for a desired gene in *Pichia ciferrii* comprising:
  - 5 (a) a *Pichia ciferrii* ribosomal DNA, which is operably linked to
  - (b) a CYH<sup>r</sup> gene which is responsible for resistance to cycloheximide and is operably linked to
  - (c) a desired structural gene.
3. An expression cassette according to claim 2, wherein said *Pichia ciferrii* ribosomal  
10 DNA is a non-transcribed region comprising an about 1.4 kb fragment of HindIII/EcoRI digestion product thereof.
4. An expression cassette according to claim 2, wherein said desired gene is a LCB2 gene coding for *Pichia ciferrii* serine palmitoyl transferase and represented as SEQ. ID. NO.3.
- 15 5. An expression cassette according to claim 3, wherein said desired gene is a LCB2 gene coding for *Pichia ciferrii* serine palmitoyl transferase and represented as SEQ. ID. NO.3.
6. A plasmid comprising the expression cassette according to claim 5 which is a plasmid prACL2 (KCTC-0468BP) having a restriction map in Figure 3.
- 20 7. A process for producing a protein coded by a desired gene comprising the step of transforming a *Pichia ciferrii* cell with a plasmid having the expression cassette of

claim 2.

8. A process according to claim 7, wherein *Pichia ciferrii* is transformed by an electroporation under conditions of a voltage of about 500V, a capacity of about 50 $\mu$ F and a resistance of about 800 $\Omega$ .

5 9. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 2.

10. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 3.

11. A *Pichia ciferrii* cell transformed with a vector comprising the expression  
10 cassette of claim 4.

12. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 5.

13. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 6.

15 14. A process for producing tetraacetyl phytospingosine comprising the steps of  
(a) cultivating the transformed *Pichia ciferrii* cell of claim 11 in a suitable cultivation medium; and  
(b) recovering tetraacetyl phytospingosine therefrom.

15. A process for producing tetraacetyl phytospingosine comprising the steps of  
20 (a) cultivating the transformed *Pichia ciferrii* cell of claim 12 in a suitable

cultivation medium; and

(b) recovering tetraacetyl phytospingosine therefrom.

16. A process for producing tetraacetyl phytospingosine comprising the steps of

(a) cultivating the transformed *Pichia ciferrii* cell of claim 13 in a suitable  
5 cultivation medium; and

(b) recovering tetraacetyl phytospingosine therefrom.

17. A LCB2 gene represented as SEQ. ID. NO. 3 and coding for *Pichia ciferrii* serine palmitoyl transferase.

18. A GAPDH gene represented as SEQ. ID. NO. 5 and coding for *Pichia ciferrii*  
10 glyceraldehyde-3-phosphate dehydrogenase

19. A promoter gene for the gene GAPDH of claim 18 and covering the sequence of nucleotide 1 to nucleotide 906.

20. An expression cassette for a desired gene in *Pichia ciferrii* comprising:

- (a) a *Pichia ciferrii* ribosomal DNA, which is operably linked to  
15 (b) a CYH<sup>r</sup> gene which is responsible for resistance to cycloheximide and is operably linked to  
(c) a GAPDH promoter gene of claim 19 operably linked to  
(d) a desired structural gene.

21. An expression cassette according to claim 20, wherein said *Pichia ciferrii*  
20 ribosomal DNA is a non-transcribed region comprising an about 1.4 kb fragment of HindIII/EcoRI digestion product thereof.

22. An expression cassette according to claim 20, wherein said desired gene is a LCB2 gene coding for *Pichia ciferrii* serine palmitoyl transferase and represented as SEQ. ID. NO.3.
23. An expression cassette according to claim 21, wherein said desired gene is a  
5 LCB2 gene coding for *Pichia ciferrii* serine palmitoyl transferase and represented as SEQ. ID. NO.3.
24. A plasmid comprising the expression cassette according to claim 23 which is a plasmid prACL2 (KCTC-0498BP) having a restriction map in Figure 5.
25. An expression cassette for a desired gene in *Pichia ciferrii* comprising:  
10 (a) a *Pichia ciferrii* ribosomal DNA, which is operably linked to  
(b) a CYH<sup>r</sup> gene which is responsible for resistance to cycloheximide and is operably linked to  
(c) a GAPDH promoter gene of claim 19 operably linked to  
(d) a desired structural gene operably linked to  
15 (e) an about 800 bp *Pichia ciferrii* ribosomal DNA fragment.
26. An expression cassette according to claim 25, wherein said *Pichia ciferrii* ribosomal DNA is a non-transcribed region comprising an about 1.4 kb fragment of HindIII/EcoRI digestion product thereof.
27. An expression cassette according to claim 25, wherein said desired gene is a  
20 LCB2 gene coding for *Pichia ciferrii* serine palmitoyl transferase and represented as SEQ. ID. NO.3.
28. An expression cassette according to claim 26, wherein said desired gene is a

LCB2 gene coding for *Pichia ciferrii* serine palmitoyl transferase and represented as SEQ. ID. NO.3.

29. A plasmid comprising the expression cassette according to claim 28 which is a plasmid prACL2 (KCTC-0511BP) having a restriction map in Figure 6.

5 30. A process for producing a protein coded by a desired gene comprising the steps of linearizing a plasmid having the expression cassette of claim 25 with *Apal* and *ScaI* and transforming a *Pichia ciferrii* cell with thus treated plasmid so that exogenous gene from organism origins other than *Pichia ciferrii* shall not be integrated onto *Pichia ciferrii* chromosome.

10 31. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 20.

32. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 21.

33. A *Pichia ciferrii* cell transformed with a vector comprising the expression  
15 cassette of claim 22.

34. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 23.

35. A *Pichia ciferrii* cell transformed with the plasmid of claim 24.

36. A *Pichia ciferrii* cell transformed with a vector comprising the expression  
20 cassette of claim 25.

37. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 26.

38. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 27.

5 39. A *Pichia ciferrii* cell transformed with a vector comprising the expression cassette of claim 28.

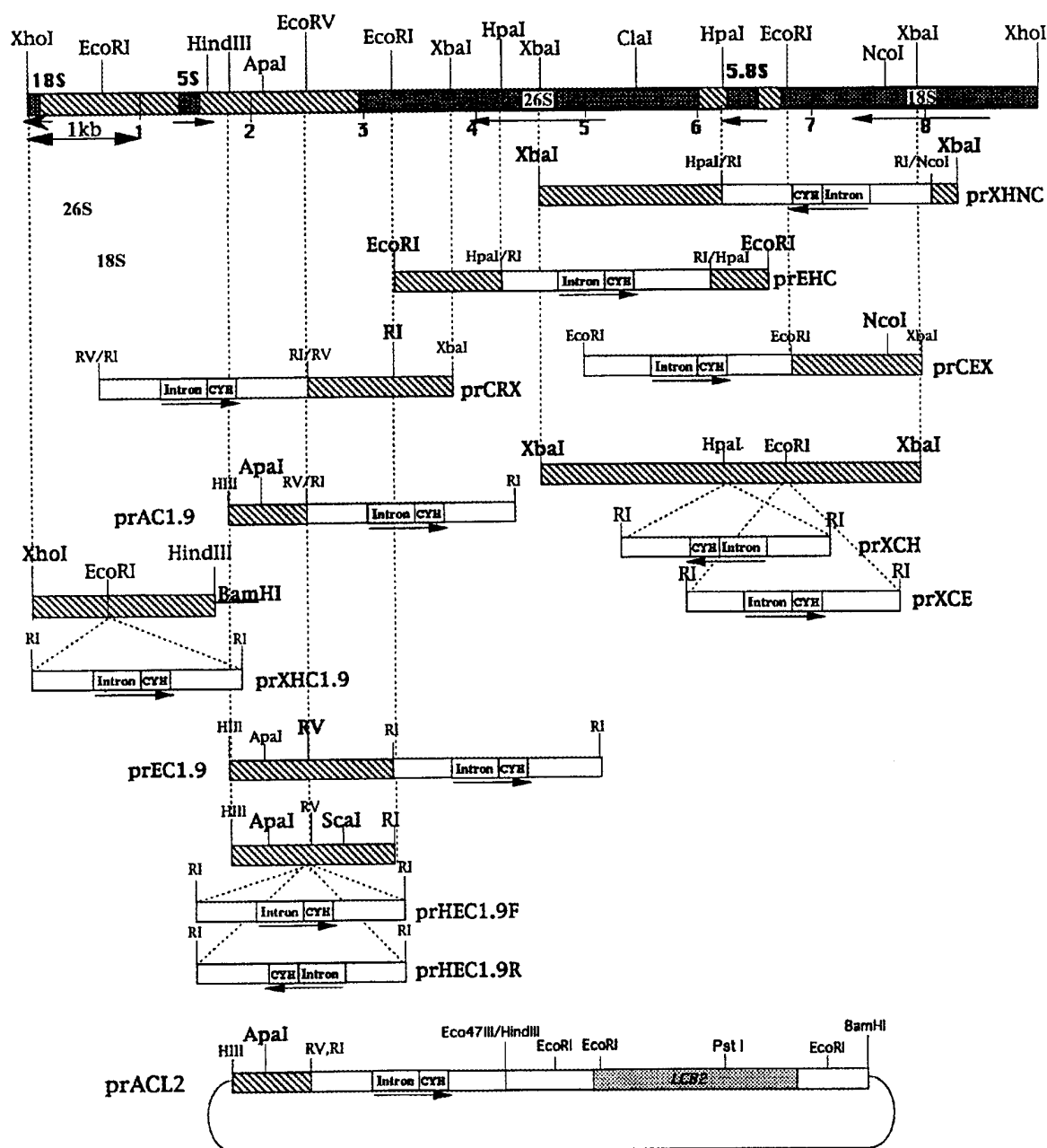
40. A *Pichia ciferrii* cell transformed with the plasmid of claim 29.

41. A process for producing tetraacetyl phytospingosine comprising the steps of  
(a) cultivating the transformed *Pichia ciferrii* cell of claim 35 in a suitable  
10 cultivation medium; and  
(b) recovering tetraacetyl phytospingosine therefrom.

42. A process for producing tetraacetyl phytospingosine comprising the steps of  
(a) cultivating the transformed *Pichia ciferrii* cell of claim 40 in a suitable  
cultivation medium; and  
15 (b) recovering tetraacetyl phytospingosine therefrom.

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FIG. 1



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FIG. 2

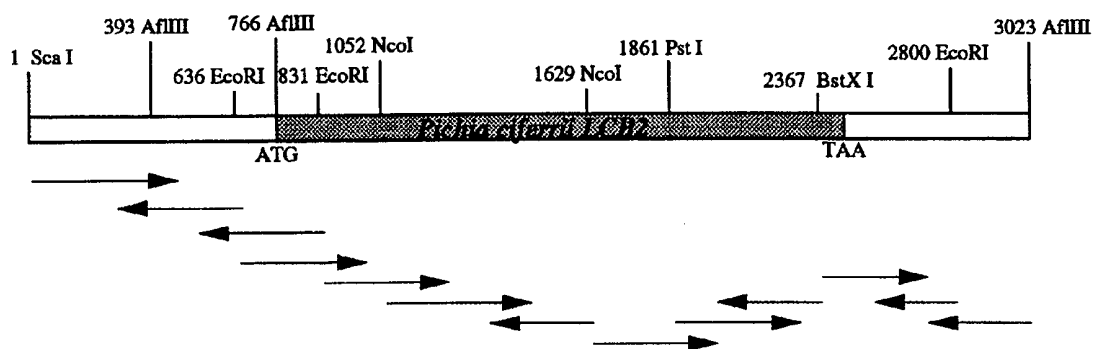


FIG. 3

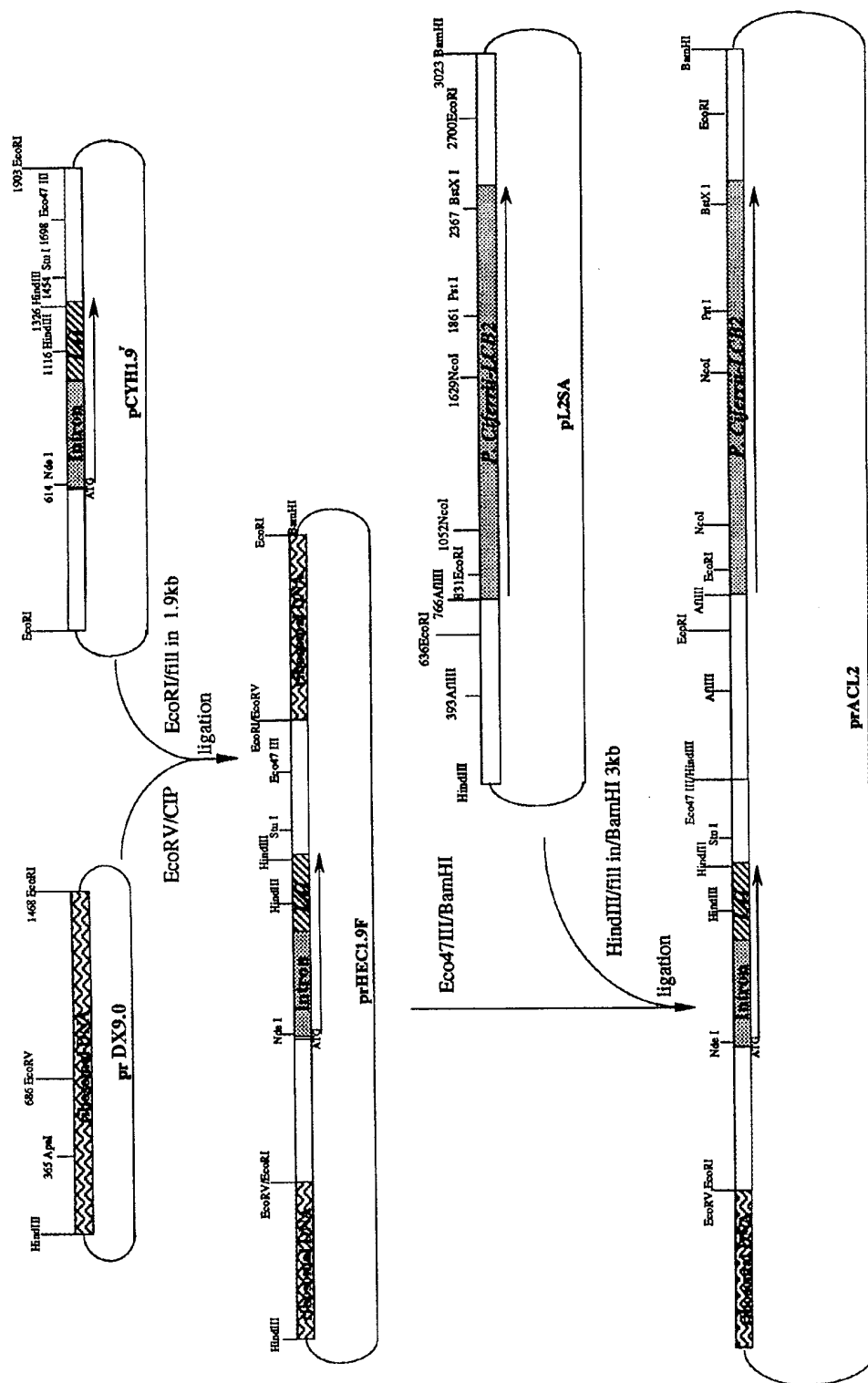
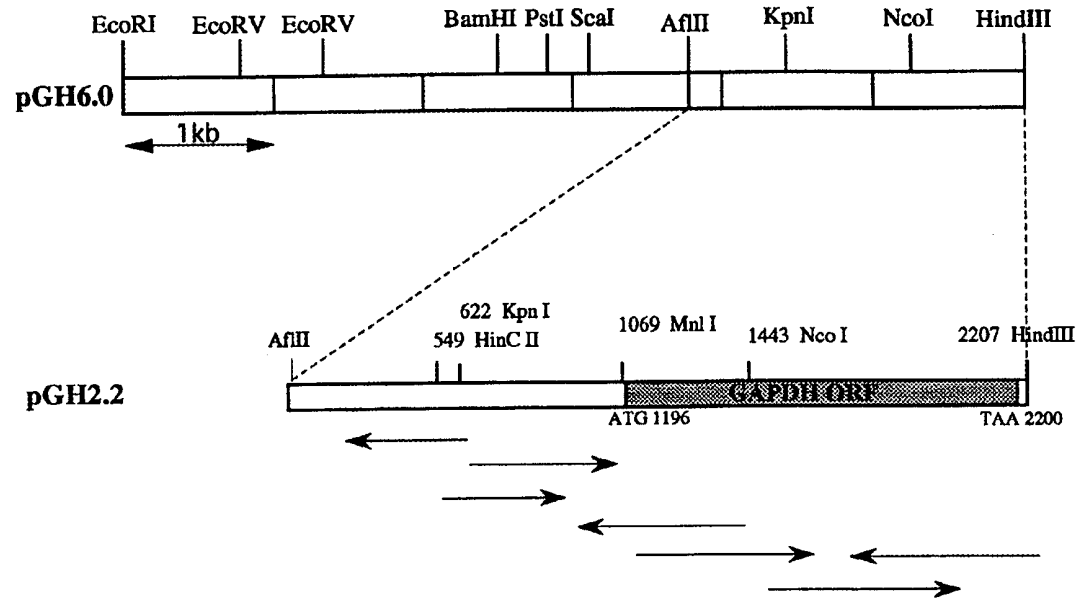


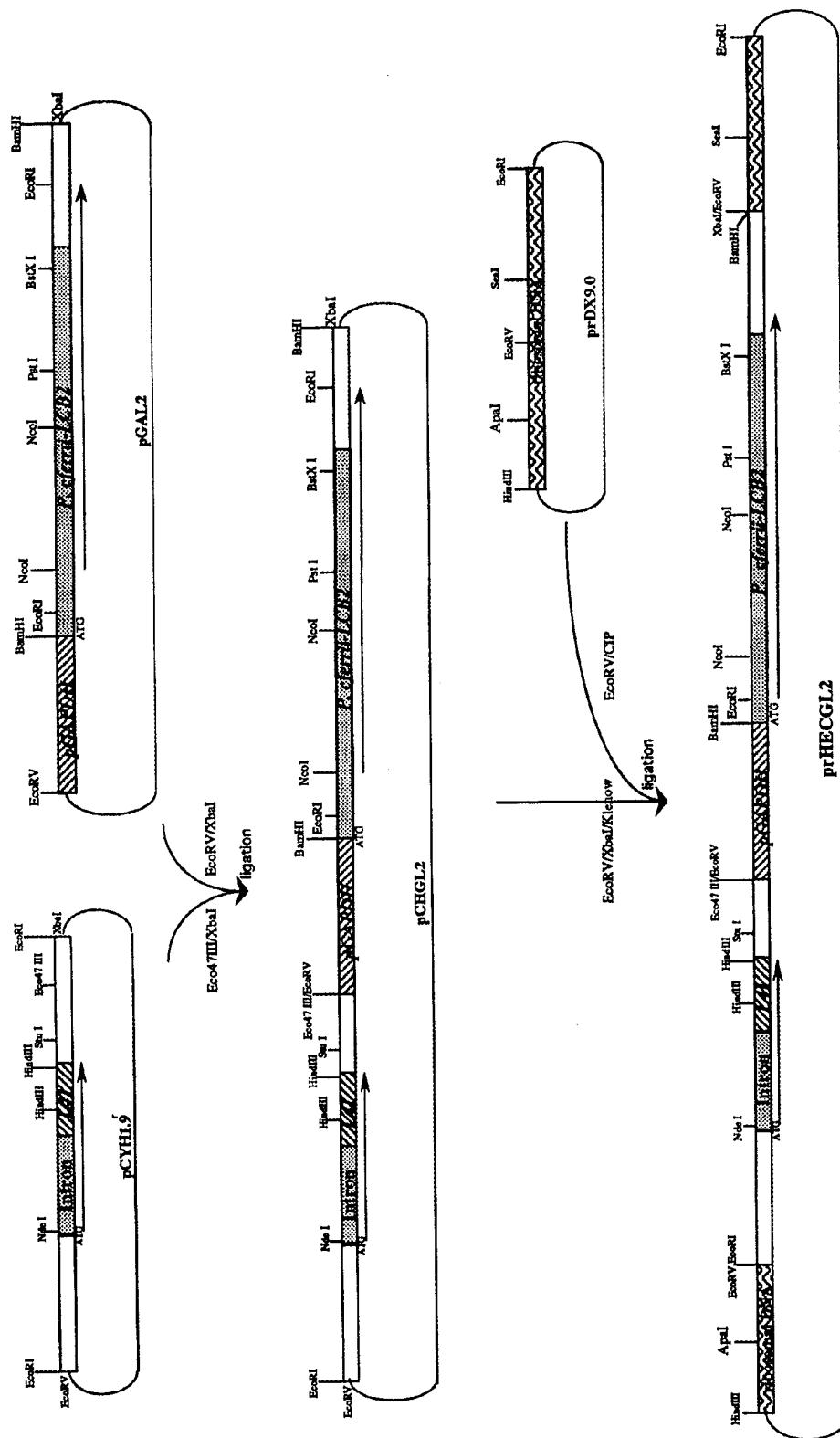
FIG. 4





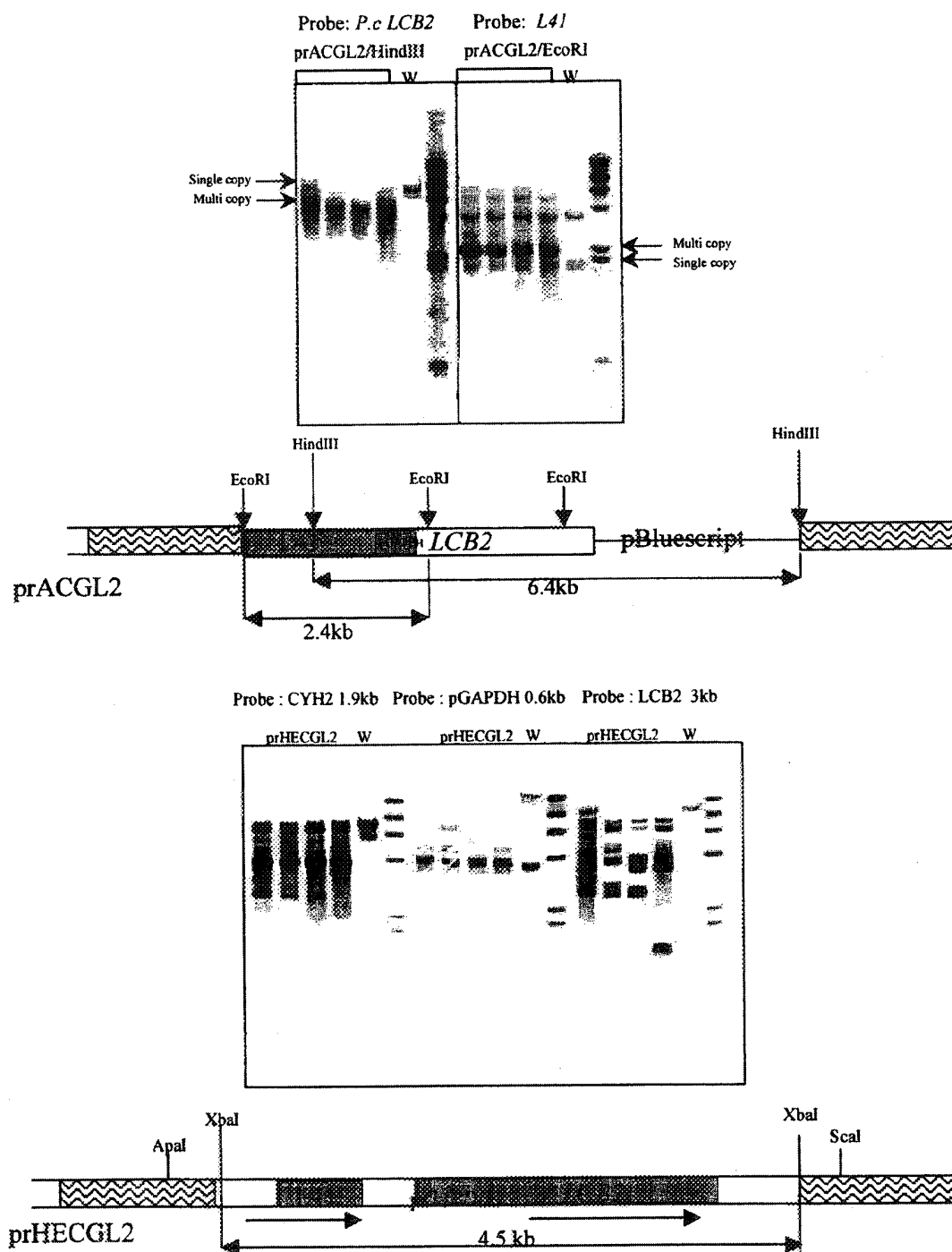
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FIG. 6



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FIG. 7



## SEQUENCE LISTING

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Korea Institute of Science and Technology

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transformation method using the same

<130> PCT-981031

<140>

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<170> PatentIn Ver. 2.0

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| 25                                                              | 30  | 35  |      |
|-----------------------------------------------------------------|-----|-----|------|
| tca aga cag ggg aaa cca tta cct gat cca att gaa gat gaa cca cca |     |     | 920  |
| Ser Arg Gln Gly Lys Pro Leu Pro Asp Pro Ile Glu Asp Glu Pro Pro |     |     |      |
| 40                                                              | 45  | 50  |      |
| tat cat gtc ctt ttc atc act tat tta aac tat tta atc ttg att atc |     |     | 968  |
| Tyr His Val Leu Phe Ile Thr Tyr Leu Asn Tyr Leu Ile Leu Ile Ile |     |     |      |
| 55                                                              | 60  | 65  |      |
| gtt ggt cat att aaa gat ttc aca ggt att ctg ttc aac cca aaa aat |     |     | 1016 |
| Val Gly His Ile Lys Asp Phe Thr Gly Ile Leu Phe Asn Pro Lys Asn |     |     |      |
| 70                                                              | 75  | 80  |      |
| tac caa gat tta tta gaa caa aat ggc ctt gct cca tgg tat aat aaa |     |     | 1064 |
| Tyr Gln Asp Leu Leu Glu Gln Asn Gly Leu Ala Pro Trp Tyr Asn Lys |     |     |      |
| 85                                                              | 90  | 95  | 100  |
| ttt gaa agt ttt tat att cgt cgt atg aaa caa aaa att gat gat tgt |     |     | 1112 |
| Phe Glu Ser Phe Tyr Ile Arg Arg Met Lys Gln Lys Ile Asp Asp Cys |     |     |      |
| 105                                                             | 110 | 115 |      |
| ttt gca aga cca act tgt ggt gtc cca ggt aga tta atc act tgt att |     |     | 1160 |
| Phe Ala Arg Pro Thr Cys Gly Val Pro Gly Arg Leu Ile Thr Cys Ile |     |     |      |
| 120                                                             | 125 | 130 |      |
| gat cgt gat gct cat gat tat aat tca tat ttt agt tat cct ggt act |     |     | 1208 |
| Asp Arg Asp Ala His Asp Tyr Asn Ser Tyr Phe Ser Tyr Pro Gly Thr |     |     |      |
| 135                                                             | 140 | 145 |      |
| act tca act tgt tta aat tta tca tca tat aat tat ttg ggg ttt gca |     |     | 1256 |
| Thr Ser Thr Cys Leu Asn Leu Ser Ser Tyr Asn Tyr Leu Gly Phe Ala |     |     |      |
| 150                                                             | 155 | 160 |      |
| caa tct gaa ggg gca tgt act caa gcc gct tta gaa att ttg gat tat |     |     | 1304 |
| Gln Ser Glu Gly Ala Cys Thr Gln Ala Ala Leu Glu Ile Leu Asp Tyr |     |     |      |
| 165                                                             | 170 | 175 | 180  |
| tat ggt gtt ggt tct ggt ggt cca aga aat gtt att ggt act act gat |     |     | 1352 |
| Tyr Gly Val Gly Ser Gly Gly Pro Arg Asn Val Ile Gly Thr Thr Asp |     |     |      |
| 185                                                             | 190 | 195 |      |
| tta cat tta aaa act gaa aaa act ata gca aaa ttt att ggt aaa gat |     |     | 1400 |
| Leu His Leu Lys Thr Glu Lys Thr Ile Ala Lys Phe Ile Gly Lys Asp |     |     |      |
| 200                                                             | 205 | 210 |      |
| gat tca atc tta ttt tca atg ggg tat gca aca aat gca agt tta ttt |     |     | 1448 |
| Asp Ser Ile Leu Phe Ser Met Gly Tyr Ala Thr Asn Ala Ser Leu Phe |     |     |      |

| 215                                                             | 220 | 225 |      |
|-----------------------------------------------------------------|-----|-----|------|
| agt tct tta ttg gat aag aaa tca ctt gtt att tct gat gaa tta aat |     |     | 1496 |
| Ser Ser Leu Leu Asp Lys Lys Ser Leu Val Ile Ser Asp Glu Leu Asn |     |     |      |
| 230                                                             | 235 | 240 |      |
| cat gct tca att aga act ggt gtt aga tta tct ggt tct aca gtt aaa |     |     | 1544 |
| His Ala Ser Ile Arg Thr Gly Val Arg Leu Ser Gly Ser Thr Val Lys |     |     |      |
| 245                                                             | 250 | 255 | 260  |
| act ttc cct cat aat aat atg att gcc ttg gaa aaa att ctt aga gaa |     |     | 1592 |
| Thr Phe Pro His Asn Asn Met Ile Ala Leu Glu Lys Ile Leu Arg Glu |     |     |      |
| 265                                                             | 270 | 275 |      |
| caa att tct caa ggt caa cca aga tct cat cgt cca tgg aaa aaa atc |     |     | 1640 |
| Gln Ile Ser Gln Gly Gln Pro Arg Ser His Arg Pro Trp Lys Lys Ile |     |     |      |
| 280                                                             | 285 | 290 |      |
| att gtt gca gtt gaa ggg ctt tat tca atg gag ggt aca atg gca aat |     |     | 1688 |
| Ile Val Ala Val Glu Gly Leu Tyr Ser Met Glu Gly Thr Met Ala Asn |     |     |      |
| 295                                                             | 300 | 305 |      |
| tta cct gca tta att gaa tta aga aga aaa tat aaa ttt aat tta ttt |     |     | 1736 |
| Leu Pro Ala Leu Ile Glu Leu Arg Arg Lys Tyr Lys Phe Asn Leu Phe |     |     |      |
| 310                                                             | 315 | 320 |      |
| gtt gat gaa gct cat tca att ggt gct att ggt cca tca ggt cgt ggt |     |     | 1784 |
| Val Asp Glu Ala His Ser Ile Gly Ala Ile Gly Pro Ser Gly Arg Gly |     |     |      |
| 325                                                             | 330 | 335 | 340  |
| gtt tgt gat tat ttt ggt ata gat ccc tca aat gtt gat tta tta atg |     |     | 1832 |
| Val Cys Asp Tyr Phe Gly Ile Asp Pro Ser Asn Val Asp Leu Leu Met |     |     |      |
| 345                                                             | 350 | 355 |      |
| ggg act tta act aaa tca ttt ggt gct gca ggt ggt tat att gct ggt |     |     | 1880 |
| Gly Thr Leu Thr Lys Ser Phe Gly Ala Ala Gly Gly Tyr Ile Ala Gly |     |     |      |
| 360                                                             | 365 | 370 |      |
| tca caa caa att ata aat cgt tta aaa tta aat att aat tca caa aat |     |     | 1928 |
| Ser Gln Gln Ile Ile Asn Arg Leu Lys Leu Asn Ile Asn Ser Gln Asn |     |     |      |
| 375                                                             | 380 | 385 |      |
| tat gca gaa tct atc cct gca cct gtt ttg gca caa att att tct tcg |     |     | 1976 |
| Tyr Ala Glu Ser Ile Pro Ala Pro Val Leu Ala Gln Ile Ile Ser Ser |     |     |      |
| 390                                                             | 395 | 400 |      |
| tta aat atc atc tcg ggt gat tta aat cct ggt gaa ggt tcg gaa aga |     |     | 2024 |
| Leu Asn Ile Ile Ser Gly Asp Leu Asn Pro Gly Glu Gly Ser Glu Arg |     |     |      |

|                                                                     |      |     |     |  |
|---------------------------------------------------------------------|------|-----|-----|--|
| 405                                                                 | 410  | 415 | 420 |  |
| tta gaa aga att gct ttt aat tca cgt tat tta aga tta ggt tta caa     | 2072 |     |     |  |
| Leu Glu Arg Ile Ala Phe Asn Ser Arg Tyr Leu Arg Leu Gly Leu Gln     |      |     |     |  |
| 425                                                                 | 430  | 435 |     |  |
| aga tta ggt ttt atc gta tac gga gtt gat gat tca cca gtg atc cca     | 2120 |     |     |  |
| Arg Leu Gly Phe Ile Val Tyr Gly Val Asp Asp Ser Pro Val Ile Pro     |      |     |     |  |
| 440                                                                 | 445  | 450 |     |  |
| tta tta tta ttc gcc cca gcc aaa atg cca gca ttt tca cgt atg cta     | 2168 |     |     |  |
| Leu Leu Leu Phe Ala Pro Ala Lys Met Pro Ala Phe Ser Arg Met Leu     |      |     |     |  |
| 455                                                                 | 460  | 465 |     |  |
| tat caa gga aaa att tca gtt gtt gtt gtt gga tac ccg gca act cca     | 2216 |     |     |  |
| Tyr Gln Gly Lys Ile Ser Val Val Val Val Gly Tyr Pro Ala Thr Pro     |      |     |     |  |
| 470                                                                 | 475  | 480 |     |  |
| ctg act tca tca gga gtt cgt ctt ggt gtt cct gcatctt tta cca aag     | 2264 |     |     |  |
| Leu Thr Ser Ser Gly Val Arg Leu Gly Val Pro Ala Ser Leu Pro Lys     |      |     |     |  |
| 485                                                                 | 490  | 495 | 500 |  |
| gag gat atg gat tat ctt tta cgt cat tta tcc gag ttg ggt gat aaa     | 2312 |     |     |  |
| Glu Asp Met Asp Tyr Leu Leu Arg His Leu Ser Glu Leu Gly Asp Lys     |      |     |     |  |
| 505                                                                 | 510  | 515 |     |  |
| tta ttt tta aaa ttt agt tct ggt att gct ggt ggt tct tta gat ggt     | 2360 |     |     |  |
| Leu Phe Leu Lys Phe Ser Ser Gly Ile Ala Gly Gly Ser Leu Asp Gly     |      |     |     |  |
| 520                                                                 | 525  | 530 |     |  |
| tca cca cca aga tgg aat att gaa gat gtt ttg aaa gag act cca aag     | 2408 |     |     |  |
| Ser Pro Pro Arg Trp Asn Ile Glu Asp Val Leu Lys Glu Thr Pro Lys     |      |     |     |  |
| 535                                                                 | 540  | 545 |     |  |
| gat tgt aaa gaa tct aaa tat ttt att gca act gca aat aat tga         | 2453 |     |     |  |
| Asp Cys Lys Glu Ser Lys Tyr Phe Ile Ala Thr Ala Asn Asn             |      |     |     |  |
| 550                                                                 | 555  | 560 |     |  |
| ttaaataattg atttataatt ttaatagcta atttaataat ttaataataa tataacaacat | 2513 |     |     |  |
| ctatatgatc ttttttggtg taatttatag cttatcatct tatcatctta tataatcgga   | 2573 |     |     |  |
| atgttacaat gtaatagaaa agaatgaaaa aaaaccctta caatctgaaa aaaaagataa   | 2633 |     |     |  |
| agatcataaa tatgcaaatt attgtttaaa atatttcttt caaaccctta ttatgatctt   | 2693 |     |     |  |
| ttgaattcaa gtaattataa ttccttcttt cttgatcaaa ttatttccat tttttgtgta   | 2753 |     |     |  |

gttgatgata caaatttttc atttttgatc ttcatttttt tcaacattgc cataattttt 2813  
 tgaataccac caaaataaaa aataaaaaaa aaataaacac ggaaaaatta aaatcaaaac 2873  
 atttcaaaac attggttggtg aaaggtatac taaagatcag gttcagatat tagttattag 2933  
 gtattaccag gtttctcatc atagggttaag aattaggaga atacattact aggatctgat 2993  
 tgttgagttg ataattggtg gtagttacgt gttgctcatt tggatatagat ctcaattata 3053  
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 agaggttcat atcattacta cattgttgat tattacaagt ttgtgtggtt ttttgaattc 3173  
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 ttc 3296

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<211> 562

<212> PRT

<213> *Pichia ciferrii*

<400> 4

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Asp Lys Lys Gln Asn Glu Phe Gly Ala Leu Thr Ser Asn Glu Tyr Arg  
                   20                  25                  30

Tyr Lys Thr Ile Ser Arg Gln Gly Lys Pro Leu Pro Asp Pro Ile Glu  
           35                  40                  45

Asp Glu Pro Pro Tyr His Val Leu Phe Ile Thr Tyr Leu Asn Tyr Leu  
           50                  55                  60

Ile Leu Ile Ile Val Gly His Ile Lys Asp Phe Thr Gly Ile Leu Phe  
           65                  70                  75                  80

Asn Pro Lys Asn Tyr Gln Asp Leu Leu Glu Gln Asn Gly Leu Ala Pro  
                   85                  90                  95

Trp Tyr Asn Lys Phe Glu Ser Phe Tyr Ile Arg Arg Met Lys Gln Lys  
           100                  105                  110

Ile Asp Asp Cys Phe Ala Arg Pro Thr Cys Gly Val Pro Gly Arg Leu  
 115 120 125

Ile Thr Cys Ile Asp Arg Asp Ala His Asp Tyr Asn Ser Tyr Phe Ser  
 130 135 140

Tyr Pro Gly Thr Thr Ser Thr Cys Leu Asn Leu Ser Ser Tyr Asn Tyr  
 145 150 155 160

Leu Gly Phe Ala Gln Ser Glu Gly Ala Cys Thr Gln Ala Ala Leu Glu  
 165 170 175

Ile Leu Asp Tyr Tyr Gly Val Gly Ser Gly Gly Pro Arg Asn Val Ile  
 180 185 190

Gly Thr Thr Asp Leu His Leu Lys Thr Glu Lys Thr Ile Ala Lys Phe  
 195 200 205

Ile Gly Lys Asp Asp Ser Ile Leu Phe Ser Met Gly Tyr Ala Thr Asn  
 210 215 220

Ala Ser Leu Phe Ser Ser Leu Leu Asp Lys Lys Ser Leu Val Ile Ser  
 225 230 235 240

Asp Glu Leu Asn His Ala Ser Ile Arg Thr Gly Val Arg Leu Ser Gly  
 245 250 255

Ser Thr Val Lys Thr Phe Pro His Asn Asn Met Ile Ala Leu Glu Lys  
 260 265 270

Ile Leu Arg Glu Gln Ile Ser Gln Gly Gln Pro Arg Ser His Arg Pro  
 275 280 285

Trp Lys Lys Ile Ile Val Ala Val Glu Gly Leu Tyr Ser Met Glu Gly  
 290 295 300

Thr Met Ala Asn Leu Pro Ala Leu Ile Glu Leu Arg Arg Lys Tyr Lys  
 305 310 315 320

Phe Asn Leu Phe Val Asp Glu Ala His Ser Ile Gly Ala Ile Gly Pro  
 325 330 335

Ser Gly Arg Gly Val Cys Asp Tyr Phe Gly Ile Asp Pro Ser Asn Val  
 340 345 350

Asp Leu Leu Met Gly Thr Leu Thr Lys Ser Phe Gly Ala Ala Gly Gly  
 355 360 365

Tyr Ile Ala Gly Ser Gln Gln Ile Ile Asn Arg Leu Lys Leu Asn Ile  
 370 375 380

Asn Ser Gln Asn Tyr Ala Glu Ser Ile Pro Ala Pro Val Leu Ala Gln  
 385 390 395 400

Ile Ile Ser Ser Leu Asn Ile Ile Ser Gly Asp Leu Asn Pro Gly Glu  
 405 410 415

Gly Ser Glu Arg Leu Glu Arg Ile Ala Phe Asn Ser Arg Tyr Leu Arg  
 420 425 430

Leu Gly Leu Gln Arg Leu Gly Phe Ile Val Tyr Gly Val Asp Asp Ser  
 435 440 445

Pro Val Ile Pro Leu Leu Leu Phe Ala Pro Ala Lys Met Pro Ala Phe  
 450 455 460

Ser Arg Met Leu Tyr Gln Gly Lys Ile Ser Val Val Val Val Gly Tyr  
 465 470 475 480

Pro Ala Thr Pro Leu Thr Ser Ser Gly Val Arg Leu Gly Val Pro Ala  
 485 490 495

Ser Leu Pro Lys Glu Asp Met Asp Tyr Leu Leu Arg His Leu Ser Glu  
 500 505 510

Leu Gly Asp Lys Leu Phe Leu Lys Phe Ser Ser Gly Ile Ala Gly Gly  
 515 520 525

Ser Leu Asp Gly Ser Pro Pro Arg Trp Asn Ile Glu Asp Val Leu Lys  
 530 535 540

Glu Thr Pro Lys Asp Cys Lys Glu Ser Lys Tyr Phe Ile Ala Thr Ala  
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Asn Asn

<210> 5

<211> 1928

<212> DNA

<213> *Pichia ciferrii*

<220>

<221> CDS

<222> (907)..(1911)

&lt;220&gt;

&lt;221&gt; promoter

&lt;222&gt; (1)..(906)

&lt;400&gt; 5

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gttgataatt tataattgat aatgatttaa acttttgatt ataaattgat taaaacccat 60
gtcatctagt atataataac aatgaccata actataataa ttagtaccca tcaattacta 120
ataccataac atactatacc atataccata atatactata gtctatacgt ctataccata 180
tattataaca ttgtatttcc taatcaaact ttcattctgat gttttatcaa cttttgtaaa 240
ctttgatcaa ctgtttgtct gtttggtcaac tgatgattgg aaccacatga ttatatgcaa 300
agcaataaaa caacaaacaa caacaacttt acccagtggt acctacatac aattgaccca 360
tagtaaaacta ataactcata acacaataaa gcatctgac ttaatgtact actaaatact 420
aaactgatga taattacttt tgttgtaatg tttgtcatca ctttgatcat cactgagcatc 480
accggaccgt taattaccaa caatctcaat tgtacaacat agtggttaaaa caggataact 540
tgatgattat atgtgatatt aagttcaaac aagtaccaat aaatagataa ttaatagctc 600
tataatatat catttaattg aattaatatc aatagttgtt gtttaattat ccctagtttt 660
ctgggttaaag ttacaccatc agatggttca ccaccaatgt tgttcaaacc atttccactc 720
aactgacgtt tccagaacat caccctgaaa aaaaaaatt catcacacat tggggagaaat 780
tggggaggat tgtatataag gagtggaat tcgctaatat ttttataatt ctaactcact 840
tgttttaatt caacatcagt attttataat acaaaaacaa acaaacaac aaataattaa 900

ttaaca atg gct atc aca gtt ggt att aac ggt ttc ggt cgt att ggt      948
      Met Ala Ile Thr Val Gly Ile Asn Gly Phe Gly Arg Ile Gly
            1             5             10

cgt tta gtc cta aga att gct ctt tca aga aaa gat att caa att gtt      996
Arg Leu Val Leu Arg Ile Ala Leu Ser Arg Lys Asp Ile Gln Ile Val
      15             20             25             30

gca att aat gat cca ttc att gca cca gaa tat gct tca tat atg ttt      1044
Ala Ile Asn Asp Pro Phe Ile Ala Pro Glu Tyr Ala Ser Tyr Met Phe
            35             40             45

aaa tat gat tct act cat ggt cgt tat tca ggt gaa gtt tct cat gaa      1092

```

|                                                                 |      |
|-----------------------------------------------------------------|------|
| Lys Tyr Asp Ser Thr His Gly Arg Tyr Ser Gly Glu Val Ser His Glu |      |
| 50 55 60                                                        |      |
| ggt gaa aac att gtt att gat ggt aaa aaa atc aga gtt tat caa gaa | 1140 |
| Gly Glu Asn Ile Val Ile Asp Gly Lys Lys Ile Arg Val Tyr Gln Glu |      |
| 65 70 75                                                        |      |
| cgt gat cca gtt aat atc cca tgg ggt aaa gat ggt gtt gat tat gtt | 1188 |
| Arg Asp Pro Val Asn Ile Pro Trp Gly Lys Asp Gly Val Asp Tyr Val |      |
| 80 85 90                                                        |      |
| att gat tca act ggt gtt ttt aaa gaa tta gat tct gct caa aaa cat | 1236 |
| Ile Asp Ser Thr Gly Val Phe Lys Glu Leu Asp Ser Ala Gln Lys His |      |
| 95 100 105 110                                                  |      |
| att gat gcc ggt gct aaa aaa gtt gtt att act gct cca tca tca act | 1284 |
| Ile Asp Ala Gly Ala Lys Lys Val Val Ile Thr Ala Pro Ser Ser Thr |      |
| 115 120 125                                                     |      |
| gct cca atg ttt gtt gtt ggt gtt aat gaa gat aaa tat act cca gat | 1332 |
| Ala Pro Met Phe Val Val Gly Val Asn Glu Asp Lys Tyr Thr Pro Asp |      |
| 130 135 140                                                     |      |
| tta aac att att tca aat gct tca tgt aca aca aat tgt tta gct cca | 1380 |
| Leu Asn Ile Ile Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro |      |
| 145 150 155                                                     |      |
| tta gct aaa att att aac aat aaa ttt ggt att gaa gaa ggt tta atg | 1428 |
| Leu Ala Lys Ile Ile Asn Asn Lys Phe Gly Ile Glu Glu Gly Leu Met |      |
| 160 165 170                                                     |      |
| act act gtt cat tca att act gct act caa aaa act gtt gat ggt cca | 1476 |
| Thr Thr Val His Ser Ile Thr Ala Thr Gln Lys Thr Val Asp Gly Pro |      |
| 175 180 185 190                                                 |      |
| tct cat aaa gat tgg aga agt ggt cct act gct tca ggt aat att att | 1524 |
| Ser His Lys Asp Trp Arg Ser Gly Pro Thr Ala Ser Gly Asn Ile Ile |      |
| 195 200 205                                                     |      |
| cca tca tca act ggt gct gct aaa gct gtt ggt aaa gtt att cca gaa | 1572 |
| Pro Ser Ser Thr Gly Ala Ala Lys Ala Val Gly Lys Val Ile Pro Glu |      |
| 210 215 220                                                     |      |
| ttg gct ggt aaa tta act ggt atg tct tta aga gtt cca act gtt gat | 1620 |
| Leu Ala Gly Lys Leu Thr Gly Met Ser Leu Arg Val Pro Thr Val Asp |      |
| 225 230 235                                                     |      |
| ggt tca gtt gtt gat tta act gtt aaa tta tta aaa gat gcc act tat | 1668 |

Val Ser Val Val Asp Leu Thr Val Lys Leu Leu Lys Asp Ala Thr Tyr  
 240 245 250  
  
 gat gaa att aaa gct gct gtt aaa gaa gct gct gaa ggt cca tta aaa 1716  
 Asp Glu Ile Lys Ala Ala Val Lys Glu Ala Ala Glu Gly Pro Leu Lys  
 255 260 265 270  
  
 ggt gtt gtt ggt tat act gaa gat caa gtt gtt tct tca gat ttc tta 1764  
 Gly Val Val Gly Tyr Thr Glu Asp Gln Val Val Ser Ser Asp Phe Leu  
 275 280 285  
  
 act gat aac aga tca tca att ttt gat gct gaa gct ggt att tgg tta 1812  
 Thr Asp Asn Arg Ser Ser Ile Phe Asp Ala Glu Ala Gly Ile Trp Leu  
 290 295 300  
  
 tca cca aga ttt gtt aaa tta att gct tgg tat gat aat gaa tat ggt 1860  
 Ser Pro Arg Phe Val Lys Leu Ile Ala Trp Tyr Asp Asn Glu Tyr Gly  
 305 310 315  
  
 tac tct acc aga gtt gtt gat tta tta gaa tac gtt gct tca aag aac 1908  
 Tyr Ser Thr Arg Val Val Asp Leu Leu Glu Tyr Val Ala Ser Lys Asn  
 320 325 330  
  
 taa gagtagaacg aaagctt 1928  
  
 335

&lt;210&gt; 6

&lt;211&gt; 334

&lt;212&gt; PRT

&lt;213&gt; Pichia ciferrii

&lt;400&gt; 6

Met Ala Ile Thr Val Gly Ile Asn Gly Phe Gly Arg Ile Gly Arg Leu  
 1 5 10 15  
  
 Val Leu Arg Ile Ala Leu Ser Arg Lys Asp Ile Gln Ile Val Ala Ile  
 20 25 30  
  
 Asn Asp Pro Phe Ile Ala Pro Glu Tyr Ala Ser Tyr Met Phe Lys Tyr  
 35 40 45  
  
 Asp Ser Thr His Gly Arg Tyr Ser Gly Glu Val Ser His Glu Gly Glu  
 50 55 60  
  
 Asn Ile Val Ile Asp Gly Lys Lys Ile Arg Val Tyr Gln Glu Arg Asp  
 65 70 75 80

Pro Val Asn Ile Pro Trp Gly Lys Asp Gly Val Asp Tyr Val Ile Asp  
                             85                            90                            95

Ser Thr Gly Val Phe Lys Glu Leu Asp Ser Ala Gln Lys His Ile Asp  
                             100                            105                            110

Ala Gly Ala Lys Lys Val Val Ile Thr Ala Pro Ser Ser Thr Ala Pro  
                             115                            120                            125

Met Phe Val Val Gly Val Asn Glu Asp Lys Tyr Thr Pro Asp Leu Asn  
                             130                            135                            140

Ile Ile Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Leu Ala  
 145                            150                            155                            160

Lys Ile Ile Asn Asn Lys Phe Gly Ile Glu Glu Gly Leu Met Thr Thr  
                             165                            170                            175

Val His Ser Ile Thr Ala Thr Gln Lys Thr Val Asp Gly Pro Ser His  
                             180                            185                            190

Lys Asp Trp Arg Ser Gly Pro Thr Ala Ser Gly Asn Ile Ile Pro Ser  
                             195                            200                            205

Ser Thr Gly Ala Ala Lys Ala Val Gly Lys Val Ile Pro Glu Leu Ala  
                             210                            215                            220

Gly Lys Leu Thr Gly Met Ser Leu Arg Val Pro Thr Val Asp Val Ser  
 225                            230                            235                            240

Val Val Asp Leu Thr Val Lys Leu Leu Lys Asp Ala Thr Tyr Asp Glu  
                             245                            250                            255

Ile Lys Ala Ala Val Lys Glu Ala Ala Glu Gly Pro Leu Lys Gly Val  
                             260                            265                            270

Val Gly Tyr Thr Glu Asp Gln Val Val Ser Ser Asp Phe Leu Thr Asp  
                             275                            280                            285

Asn Arg Ser Ser Ile Phe Asp Ala Glu Ala Gly Ile Trp Leu Ser Pro  
                             290                            295                            300

Arg Phe Val Lys Leu Ile Ala Trp Tyr Asp Asn Glu Tyr Gly Tyr Ser  
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<210> 7  
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<212> DNA  
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<220>  
<223> Description of Artificial Sequence: PCR primer  
CYH1

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23

<210> 8  
<211> 23  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: PCR primer  
CYH4

<400> 8  
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23

<210> 9  
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<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: PCR primer No.  
3 which is used for isolation of GAPDH promoter  
gene

<400> 9  
gatatctaca tacaattgac ccatag

26

<210> 10  
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<212> DNA  
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<220>

<223> Description of Artificial Sequence: PCR primer No.  
4 which is used for isolation of GAPDH promoter  
gene

<400> 10  
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<210> 11  
<211> 21  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: PCR primer  
CH-f

<400> 11  
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<210> 12  
<211> 24  
<212> DNA  
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<223> Description of Artificial Sequence: PCR primer  
CH-r

<400> 12  
atggaaaact tgtttggttt gacc 24

<210> 13  
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<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: PCR primer 18R

<400> 13  
caataattgc aatgctctat ccccagcacg 30

<210> 14  
<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer  
26F

<400> 14

ggatatggat tcttcacggt aacgtaactg

30

<210> 15

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer  
L2f

<400> 15

atgagtactc ctgcaaacta

20

<210> 16

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer L2r

<400> 16

taacaaaata cttgtcgtcc

20

<210> 17

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer No.  
1 which is used for isolation of GADPH gene

<400> 17

atggttagag ttgctattaa cg

22

<210> 18

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: PCR primer  
No. 2 which is used for isolation of GADPH gene

<400> 18

aagccttggc aatgtgttca a

21

Applicant's or agent's  
file reference

PC - 981031

International application No

PCT/K 98/00346

INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
OR OTHER BIOLOGICAL MATERIAL

REC'D 17 NOV. 1998

WIPO PCT

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description  
on page 11, line 19~23

B. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☒

Name of depositary institution Korea Research Institute of Bioscience and  
Biotechnology Korean Collection for Type Cultures.

Address of depositary institution (including postal code and country)

KCTC, KRIBB #52 Oum-Long, Yusong-Ku,  
Taejeon 305-600, Republic of Korea

Date of deposit

May 4 1998.

Accession Number

KCTC 0468BP

C. ADDITIONAL INDICATIONS (leave blank if not applicable)

This information is continued on an additional sheet ☐

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession  
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on page 13 ~ 14, line 23 ~ 2

B. IDENTIFICATION OF DEPOSIT

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Biotechnology Korean Collection for Type Cultures.

Address of depositary institution (including postal code and country)

KCTC, KRIBB #52 Oum-Long, Yusong-Ku,  
Taejon 305-600, Republic of Korea

Date of deposit

June 25 1998

Accession Number

KCTC 0498 BP

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PC - 981031

International application No.

PCT/K. 98/00346

INDICATIONS RELATING TO DEPOSITED MICROORGANISM  
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(PCT Rule 13bis)

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|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------|
| A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page <u>14</u> , line <u>14 ~ 17</u> .  |                                        |
| B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></span>                            |                                        |
| Name of depositary institution <u>Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures.</u>                                        |                                        |
| Address of depositary institution (including postal code and country)<br><u>KCTC, KRIBB #52 Oum-Long, Yusong-Ku,</u><br><u>Taejon 305-600, Republic of Korea</u>           |                                        |
| Date of deposit<br><u>August 10 1998</u>                                                                                                                                   | Accession Number<br><u>KCTC 0511BP</u> |
| C. ADDITIONAL INDICATIONS (leave blank if not applicable) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span> |                                        |
|                                                                                                                                                                            |                                        |
| D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)                                                                 |                                        |
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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 98/00346

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>6</sup>: C 12 N 15/31, 1/19; C 12 P 13/00 // ( C 12 N 1/19; C 12 R 1:84 )

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>6</sup>: C 12 N 15/31, 1/19; C 12 P 13/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages     | Relevant to claim No.          |
|-----------|----------------------------------------------------------------------------------------|--------------------------------|
| A         | EP 0 688 871 A2 (QUEST INTERNATIONAL B.V.)<br>27 December 1995 (27.12.95), abstract.   | 1,2,7,14-18,20,<br>25,30,41,42 |
| A         | WO 94/10 131 A1 (QUEST INTERNATIONAL B.V. et al.)<br>11 May 1994 (11.05.94), abstract. | 1,2,7,14-18,20,<br>25,30,41,42 |
|           | ----                                                                                   |                                |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

02 February 1999 (02.02.99)

Date of mailing of the international search report

15 February 1999 (15.02.99)

Name and mailing address of the ISA/

Austrian Patent Office

Kohlmarkt 8-10; A-1014 Vienna

Facsimile No. 1/53424/535

Authorized officer

Wolf

Telephone No. 1/53424/436

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR 98/00346

| In Recherchenbericht<br>angeführtes Patentdokument<br>Patent document cited<br>in search report<br>Document de brevet cité<br>dans le rapport de recherche | Datum der<br>Veröffentlichung<br>Publication<br>date<br>Date de<br>publication | Mitglied(er) der<br>Patentfamilie<br>Patent family<br>member(s)<br>Membre(s) de la<br>famille de brevets                                                                                                                               | Datum der<br>Veröffentlichung<br>Publication<br>date<br>Date de<br>publication                                                                                       |
|------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| EP A2 688871                                                                                                                                               | 27-12-95                                                                       | EP A3 688871<br>JP A2 8168392<br>US A 5618706                                                                                                                                                                                          | 08-07-98<br>02-07-96<br>08-04-97                                                                                                                                     |
| WO A1 9410131                                                                                                                                              | 11-05-94                                                                       | AU A1 53738/94<br>AU B2 680841<br>DE C0 69320980<br>DE T2 69320980<br>EP A1 667853<br>EP B1 667853<br>ES T3 2123066<br>GB A0 9223001<br>JP T2 8502961<br>NZ A 257361<br>US A 5627056<br>ZA A 9308141<br>GB A0 9316629<br>GB A0 9306973 | 24-05-94<br>14-08-97<br>15-10-98<br>04-02-99<br>23-08-95<br>09-09-98<br>01-01-99<br>16-12-92<br>02-04-96<br>26-11-96<br>06-05-97<br>02-05-95<br>29-09-93<br>26-05-93 |