(54) Title: HANSENULA POLYMORPHA MUTANT STRAINS WITH DEFECT IN OUTER CHAIN BIOSYNTHESIS AND THE PRODUCTION OF RECOMBINANT GLYCOPROTEINS USING THE SAME STRAINS

(57) Abstract: The present invention relates to polynucleotide containing Hansenula polymorpha Hpoch1 gene; polypeptide coded thereby; Hansenula polymorpha mutant wherein hyperglycosylation of glycoprotein id inhibited by the mutation of the Hansenula polymorpha HpOCH1, or Hansenula polymorpha natural mutant; recombinant Hansenula polymorpha strain expressing a foreign protein prepared by introducing a gene coding a foreign protein to the Hansenula polymorpha mutant or Hansenula polymorpha natural mutant; and a method for preparing a foreign protein comprising the steps of culturing said mutant under the condition that a foreign protein can be expressed, and isolating the foreign protein from the obtained culture broth.
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.
*Hansenula polymorpha* mutant strains with defect in outer chain biosynthesis and the production of recombinant glycoproteins using the same strains

**Field of the invention**

The present invention relates to *Hansenula polymorpha* mutant strains with a defect in the outer chain biosynthesis of glycoproteins and the production method of recombinant glycoproteins using these strains. More specifically, the present invention relates to the nucleic acid molecules containing *H. polymorpha* *HpOCH1* gene, the polypeptides encoded by it, and *H. polymorpha* artificial mutant strains or its natural mutant strains in which hyperglycosylation of glycoproteins is prevented. Furthermore, the present invention relates to recombinant *H. polymorpha* strains expressing a foreign protein produced by transformation with a gene encoding a foreign protein, and the production method of a foreign protein, which comprises cultivating the strains under conditions that allow them to express the foreign protein and isolating the expressed foreign protein from the cultures.

**Background of the invention**

In order to express a foreign protein recombinantly on a large scale, an optimal expression system should be selected to establish an efficient production system because amounts, solubility, locations and modifications etc. of expressed
proteins are dependent on host cell lines or features of desired proteins. For large-scale expression of proteins, various host systems including bacteria, yeasts, fungi, plants and animals have been developed. Among them, microbes have been wildly used to express recombinant proteins because of easy culture thus getting a high concentration of recombinant protein with a low-cost.

Yeast, microbes having features of the eukaryotic expression and secretion of proteins, are a suitable expression system to produce recombinant proteins of higher eukaryotes on a large-scale. In comparison to bacterial expression systems, yeast expression systems have a major advantage in that, as eukaryotic microbes, they have protein secretory organelles similar to those of higher eukaryotes. Therefore, the secretory proteins in yeast become biologically active through post-translational modifications such as digestion of secretory signal sequences, formation of disulfide bonds, glycosylation etc. Furthermore, the expressed recombinant proteins can be easily recovered and purified, since most yeast cells secrete only a small fraction of the proteins to the outside.

Recently, methylotrophic yeasts such as Hansenula polymorpha, Pichia pastoris and other non-conventional yeasts have been developed as alternative hosts, because they are able to replace the inherent disadvantages of the traditional yeast Saccharomyces cerevisiae as hosts for industrial production of desired proteins. The disadvantages of S. cerevisiae include instability of expression vectors in long-term fermentation, hyperglycosylation of glycoproteins, and low productivity of the expressed proteins in comparison to

Most proteins utilized for medical therapeutic purposes in humans are glycoproteins, which are modified by attachment of oligosaccharides via covalent bonds in a secretory pathway. An important issue in large-scale protein production in the field of biotechnology is the production of recombinant proteins modified by suitable glycosylation because the structures and classes of carbohydrates attached to the glycoproteins can greatly affect folding, secretion, stability, half-life in serum, and antibody inductivity of the proteins.

Wild type yeasts have some limits as an expression system. The recombinant glycoproteins expressed in *S. cerevisiae* have showed hypermannosylation resulting from adding over 40 mannose residues to the proteins and α 1,3-linked terminal mannose, which serves as an antigen in the human body (Romanos *et al.*, *Yeast* 8, 423-488, 1992). In contrast the recombinant proteins expressed in methylootrophic yeasts, *H. polymorpha* and *P. pastoris*, have been reported to contain the mannose outer chains that are shorter than those expressed in *S. cerevisiae* although they are still more hyperglycosylated than native proteins (Bretthauer and Castellino, *Biotechnol. Appl. Biochem.* 30, 193-200, 1999; Kang *et al.*, *Yeast* 14, 371-381, 1998). These methylootrophic yeasts are preferred over the wild type *S. cerevisiae* as a host system for medical therapeutic proteins because they do not produce the α 1,3-linked terminal mannose, which can evoke an immune response.
The core oligosaccharide is an intermediate of the biosynthesis pathway, which is found in all eukaryotes from yeasts to mammalian cells. However, the outer chains attached to the intermediate are differentially biosynthesized based on species of proteins, cells and animals. Researchers have actively pursued the development of a useful host system to produce recombinant glycoproteins, which closely resemble native proteins containing proper outer chains, by means of selecting mutant strains with defects in outer chain biosynthesis using an artificial mutant method or manipulating the gene related to the chain biosynthesis using molecular biological techniques.

In wild type S. cerevisiae, several strategies such as [3H]mannose suicide selection, sodium orthovanadate resistance and hygromycin B sensitivity are used to select the defective mutants of N-linked oligosaccharides biosynthesis (Herscovics and Orlean, FASEB 7, 540-550, 1999). Functional complementation experiments using these mutants led to the cloning of the OCH1 gene (Ngd29) playing an important role in the outer chain initiation (Nakanishi-Shindo et al., J. Biol. Chem. 268, 26338-26345, 1993), the MNN9 gene regulating the outer chain elongation and the MNN1 gene involved in attachment of the α 1,3-linked terminal mannose (Gopal and Ballou, Proc. Natl. Acad. Sci. USA 84, 8824, 1987). Those genes were targeted to make defective mutants by mutagenesis, which were then developed as a host cell to produce recombinant glycoproteins (Kniskern et al., Vaccine 12, 1021-1025, 1994; US Patent no. 5,798,226; US Patent no. 5,135,854).
Methylo trophic yeasts have recently been in the spotlight as a suitable host for recombinant protein expression over *S. cerevisiae*. However, a defective mutant of the N-linked oligosaccharide biosynthesis in methylo trophic yeasts has not yet been reported.

The goal of this invention was to develop a mutant using *H. polymorpha*, a methylo trophic yeast, which can produce recombinant glycoproteins that are suitable for use in the human body. This mutant was obtained by selection of a defective mutant in the glycosylation pathway or by mutation of the *OCH1* gene involved in the process. This defective mutant prevents hyperglycosylation of the outer chains and is a suitable host for recombinant glycoproteins attached with proper outer chains, which closely resemble the native proteins.

**Summary of the invention**

In order to develop a defective mutant of *H. polymorpha* for production of recombinant N-linked glycoproteins closely resembling those of human, we developed a method for selection of a defective mutant of the oligosaccharide chain biosynthesis. We used sensitivity of sodium orthovanadate to select a defective mutant of *H. polymorpha*, which exhibits more resistance against it. We also cloned the *OCH1* gene involved in initiation of the outer chain biosynthesis. The gene was mutated to make an *OCH1* deletion mutant (*Aoch1*) strain. This mutant strain is a suitable host, which provides techniques to produce
recombinant glycoproteins close to the structure of original proteins with proper outer oligosaccharide chains.

**Brief description of the drawings**

Figure 1 shows the difference in the resistance of *H. polymorpha* strains, against sodium orthovanadate.

Figure 2 shows the phenotype of the *H. polymorpha* mutant, DL42-15.

Yeast cells in a log phase were serially diluted 1 to 10, 5 μl was spotted onto the YPD plate and the cells were cultured for 2 days. A, YPD media containing 4 mM sodium orthovanadate; B, YPD media at 45°C; C, YPD media containing 0.3% sodium deoxycholate; D, YPD media at 37°C.

Figure 3 shows the sequences of DNA and predicted amino acid of *H. polymorpha* OCH1 gene cloned in this study.

Figure 4 shows amino acid sequence alignment of the Och1p of *H. polymorpha* with homologues of other yeast strains. The numbers in parentheses represent homology of Och1p from other yeast strains versus Och1p of *H. polymorpha*. HpOch1p; *H. polymorpha* Och1 protein; ScOch1p, *S. cerevisiae* Och1 protein; ScHoc1p, *S. cerevisiae* Hoc1 protein; CaOch1p, *C. albicans* Och1 protein.
Figure 5 is an illustration showing the gene recombination and pop-out to induce the \textit{H. polymorpha OCH1} gene disruption.

Figure 6 shows the phenotype of the \textit{och1} defective mutant (\textit{Aoch1}) of \textit{H. polymorpha}. Yeast cells in a log phase were serially diluted 1 to 10, 5 \textmu l was spotted onto the YPD plate and the cells were cultured for 2 days. A, YDP media at 37 °C; B, YDP media at 45 °C; C, YPD media containing 40 \textmu g/ml of hygromycin B; D, YPD media containing 0.4% of sodium deoxycholate; E, YPD media containing 7 mg/ml of calcofluor white.

Figure 7 is a Western blot demonstrating the changes in the oligosaccharide formation of glucose oxidase expressed in the \textit{H. polymorpha} mutant, DL42-15, and the \textit{och1} defective strain (\textit{Aoch1}).

\textbf{Description of the preferred embodiment}

The present invention consists of selecting the naturally occurring sodium vanadate-resistant mutant strain, DL42-15, originated from \textit{H. polymorpha DL-1}; cloning the \textit{H. polymorpha OCH1} gene and analyzing the DNA sequence; disrupting the \textit{H. polymorpha OCH1} gene; testing for the glycosylation of the \textit{Aspergillus niger} glucose oxidase protein expressed in the sodium orthovanadate-resistant strain, DL42-15, and the defective mutant strain, \textit{Aoch1}. 

7
The invention describes engineering of the defective mutant, which was mutated in the outer chain biosynthesis of a methylotrophic yeast \textit{H. polymorpha} to prevent hyperglycosylation by subsequent attachment of mannose residues. This mutant is an ideal host for expression of human recombinant proteins because it produces glycoproteins with fewer outer chains that more closely resemble the native proteins and therefore do not initiate an immune response. The hyperglycosylation-inhibiting mutants originated from \textit{H. polymorpha} DL-1 were either a natural mutant selected by sodium orthovanadate or the mutant mutated in the \textit{OCH1} gene of \textit{H. polymorpha}.

The DNA sequence (nucleotide no. 1) of \textit{H. polymorpha OCH1} cloned in this study was deposited in GenBank (accession no. AF490971) and in the Korean Collection for Type Culture (KCTC) on May 29, 2002 (accession no. KCTC 10265BP). The sodium orthovanadate-resistant strain, DL42-15, and the \textit{OCH1} gene-mutated strain, \textit{\textit{\textalpha}och1}, of \textit{H. polymorpha} were also deposited in the KCTC on the same day (accession no. KCTC 10263BP and KCTC 10264BP, respectively).

This invention provides the DNA and amino acid sequences shown in Figure 3.

This invention provides the \textit{OCH1} gene mutant (\textit{\textalpha}och1), which inhibits hyperglycosylation of glycoproteins.

This invention provides this mutant yeast strain as an expression host to express genes encoding heterologous glycoproteins.
This invention provides the hyperglycosylation-inhibiting mutant yeast strain, DL42-15, deposited in KCTC (accession no. KCTC 10263 BP).

This invention provides this DL42-15 strain as an expression host to express genes encoding heterologous glycoproteins.

This invention provides suitable conditions for cell culture of these mutants as well as methods for the production and isolation of the recombinant proteins from the culture.

Methylo trophic yeasts such as *H. polymorpha* and *P. pastoris* have been extensively used for production of therapeutic recombinant proteins in medical and pharmaceutical industries.

The term "hyperglycosylation-inhibiting" used in this study refers to reduction of the oligosaccharide chains attached to glycoproteins expressed in the mutants of the methylo trophic yeasts in comparison of those of the wild-type yeasts.

The term "glycoproteins" used in this study refers to proteins processing glycosylation on more than one residue of asparagine, serine or threonine of glycoproteins in *H. polymorpha*.

Possible glycoproteins that can be produced using these invented mutants include, but are not limited to, the *Aspergillus niger* glucose oxidase, the *S. cerevisiae* invertase, the HIV envelop protein, the influenza A virus hemagglutinin, the influenza neuraminidase, the bovine herpes type-1 virus glycoprotein D, the human angiotatin, erythropoietin, cytokine, human B7-1, B7-2, B-7 receptor CTLA-4, human tissue factors, human growth factors (e.g. blood
platelet-derived growth factor), tissue plasminogen activator, plasminogen activator inhibitor-1, eukinase, human lysosomal enzymes (e.g. α-galactosidase), plasminogen, thrombin, factor XIII and immune globulin. Those glycoproteins can be used for therapeutic medicine delivered by injection, oral or non-oral administration or other methods used in particular areas.

Glycoproteins produced in the mutants can be isolated and purified using general methods for protein isolation and purification. However, the specific methods employed depend on the property of the proteins to be isolated. These properties should be determined by the parties interested. In brief, cultured cells are collected, the secreted proteins are precipitated, and the proteins are isolated and purified according to a general method for protein isolation and purification using immune absorption, fractionation or chromatography.

The following examples explain the invention in detail, however, the claims are not limited to them.

<Experimental example 1>

Selection of the sodium orthovanadate-resistant mutant strain, DL42-15, of *H. polymorpha*

Even a low concentration (5 mM) of sodium orthovanadate generally inhibits the growth of yeast. Most *S. cerevisiae* vanadate-resistant mutant strains are mutants with mutations in genes involved in glycosylation processing.
in the Golgi (Kanik-Ennulat et al., Genetics 140; 933-943, 1995); Ucelletti et al. Res Microbiol 150:5-12, 1999). One of the most efficient methods for selection for oligosaccharide biosynthesis defective mutants is using sodium orthovanadate to select one with its resistance and this method has been extensively used in S. cerevisiae and Kluyveromyces lactis. However, this method cannot be used in the methylotrophic yeast P. pastoris because it itself is resistant to sodium orthovanadate (Martinet et al., Biotechnology Lett. 20, 1171-1177, 1999). In the case of another methylotrophic yeast H. polymorpha, CBS 4732 and NCYC 495 strains have also been reported that they can grow in the media containing 96 mM sodium orthovanadate (Mannazzu et al., FEMS Microbiol Lett. 147: 23-28, 1997; Mannazzu et al. Microbiology 144: 2589-2597, 1998).

The H. polymorpha DL1, used in this study to develop a expression host for production of recombinant proteins, showed a similar sensitivity to sodium orthovanadate to S. cerevisiae unlike CBS 4732 and NCYC 495 (Figure 1 and Table 1). The natural mutant cells of H. polymorpha DL1, which became resistant to the sodium orthovanadate, occurred at a frequency of 1 per10⁶ cells on the YPD media plate containing 4 mM sodium orthovanadate. showed that this mutation frequency is similar to that in the wild type S. cerevisiae (Table 1).

[Table 1]

Growth comparison of yeast strains grown on the YPD plates containing sodium orthovanadate.
### Yeast strains

<table>
<thead>
<tr>
<th>Yeast strains</th>
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<tr>
<td><em>S. cerevisiae</em> L3262 (WT)</td>
<td>±</td>
</tr>
<tr>
<td><em>S. cerevisiae</em> L3262 (mnn9)</td>
<td>++++</td>
</tr>
<tr>
<td><em>H. polymorpha</em> DL1 (WT)</td>
<td>±</td>
</tr>
<tr>
<td><em>H. polymorpha</em> CBS4732 (WT)</td>
<td>++++</td>
</tr>
</tbody>
</table>

* The results were obtained after culturing at 30°C (*S. cerevisiae*) or at 37°C (*H. polymorpha*) for 4 days.

All the defective mutants of oligosaccharide biosynthesis among the sodium orthovanadate-resistant mutants of *S. cerevisiae* have been shown to be more sensitive to antibiotics with a large molecular weight such as aminoglycoside, to synthetic detergents such as sodium deoxycholate, and to high temperature (Dean N., *Proc. Natl. Acad. Sci. USA* 92, 1287-1291, 1995). We selected 250 natural mutants from *H. polymorpha* DL1 showing more resistance to sodium orthovanadate, most (over 90%) of which were also resistant to hygromycin B. The selected mutants have been further tested on the media containing sodium orthovanadate at high temperature (45°C) to select the mutant colonies resistant to sodium orthovanadate but sensitive to high temperature. Finally, the mutants have been isolated and designated as *H. polymorpha* DL42-15 (Figure 2 and 3).

**<Experimental example 2>**

Cloning and DNA sequence analysis of the *H. polymorpha OCH1* gene
We analyzed the Random Sequenced Tags (RSTs) of the partial genomic analysis of *H. polymorpha* (Blandin *et al.*, *FEBS Lett.* 487, 76, 2000) and obtained the partial DNA sequences of genes showing homology with the genes involved in the oligosaccharide biosynthesis of *S. cerevisiae*. The predicted amino acid sequences deduced from the partial DNA sequences share homology with a region corresponding to the C-termini of *S. cerevisiae OCH1* (*ScOCH1*), which plays an important role in attachment of α1, 6-mannose in the beginning of the outer chain biosynthesis. *S. cerevisiae ScOCH1* also shares high homology to *S. cerevisiae HOCl* (*SchOCl*). A pair of primers designed based on the partial DNA sequences are 5'-CAATCAGACCGGCTGCTGAGGAGT-3' (nucleotide no. 3), 5'-ACATCAACGTGGAGAATCGGAGGCAC-3' (nucleotide no. 4). Using these primers, we amplified by PCR a 900 bp fragment from genomic DNA isolated from *H. polymorpha*.

We performed Southern blotting, probed with the 900 bp fragment, using the genomic DNAs digested with several restriction enzymes. In order to isolate the promoter region and full-length of the *H. polymorpha OCH1* gene, we gel-extracted the two fragments of 2.3 kb (digested with *BamHI*) and 5 kb (digested with *BglII*) corresponding to the signals of the Southern blot. Each fragment was then cloned into a cloning vector pBluescript KS+ (Stratagen Co.). The clones were sequenced in both strands.

The DNA sequence analysis revealed the clones include the promoter region of 1 kb and the open reading frame of 1.3 kb encoding a putative protein
with 435 amino acids (nucleotide no. 1, Figure 3). The predicted protein of *H. polymorpha* was designated as *HpOch1* (amino acid sequence no.2). This protein shares low homology (21-23%) to *ScOCH1* (accession no. YGL038C), *ScHOCl* (accession no. YJR075W) and *Candida albicans Och1* (accession no. AY064420) proteins. However, it contains a DXD motif, a possible activation site, and the transmembrane spanning region in the N-terminal found in the mannosyltransferase, a type II membrane protein (Figure 4).

<Experimental example 3>

Production and analysis of the *OCH1* gene-mutated strain (*Δoch1*) of *H. polymorpha*

In order to make the mutants where the *OCH1* gene was disrupted, two techniques, fusion PCR using the primers listed in Table 2 and *in vivo* DNA recombination, were used for the gene disruption (Oldenburg et al., *Nucleic Acid Res.* 25, 451, 1997). The regions corresponding to the N-terminal and the C-terminal of *URA3* and *OCH1* genes, respectively, were amplified by PCR. The fragment corresponding to the N-terminal of *HpOCH1* was then fused by fusion PCR to the fragment corresponding to the N-terminal of *URA3* while the fragment corresponding to the C-terminal of *HpOCH1* was fused to the fragment corresponding to the C-terminal of *URA3*. The fused DNA fragments were introduced into yeast cells to make recombination of the gene. Transformants
where the *HpOCH1* gene was disrupted were then selected (Figure 5). The mutants were first screened on the minimal media containing no uracil, selecting for the *URA3* marker. PCR was then performed on the genomic DNAs isolated from the mutants and the wild type to confirm the *HpOCH1* gene disruption. An *H. polymorpha* mutant Δ*och1*(*leu2 och1::URA3*) was selected based on analysis of the PCR products.

The selected mutant strain Δ*och1* grows more slowly than the wild type; it is more sensitive to a high temperature of 45°C and to hygromycin B; its growth is inhibited by addition of sodium orthovanadate and calcofluor white (Figure 6).

All these properties are common in the defective mutant strains of the outer chain biosynthesis in yeasts, suggesting the mutant strain Δ*och1* has a defect in the biosynthesis.

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**[Table 2]**

Primers used in this study for PCR to disrupt the *HpDCH1* gene

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<th>Name</th>
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<td>ACATCAACGTGGAGAACTGG</td>
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</tr>
<tr>
<td>OCH1 N-A</td>
<td>AGCTCGGTACCCGGGGATCTCTGTCTGTCCACACAC</td>
<td>6</td>
</tr>
<tr>
<td>OCH1 C-S</td>
<td>GCACATCCCCCTTTCGCCAGCCCATACACTCCCTTACTAGG</td>
<td>7</td>
</tr>
<tr>
<td>OCH1 C-A</td>
<td>CAATCAGACCCCGGTCTGTGCAGGAGT</td>
<td>8</td>
</tr>
<tr>
<td>URA3 N-S</td>
<td>GGATCCCGGAGTACCGAGCT</td>
<td>9</td>
</tr>
<tr>
<td>URA3 N-A</td>
<td>CACCGGTAGCTAATGATCCC</td>
<td>10</td>
</tr>
<tr>
<td>URA3</td>
<td>CGAACATCCAGTGCGGCG</td>
<td>11</td>
</tr>
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<Experimental example 4>

Analysis of the recombinant glycoproteins expressed and isolated from the mutant strains, DL42-15 and \( \Delta ochl \), of \( H. \) polymorpha.

In order to examine the glycosylation defect on a recombinant glycoprotein expressed in the mutant strains, DL42-15 and \( \Delta ochl \), described in experimental example 1 and 3 respectively, we expressed the glucose oxidase (GOD) of an \textit{Aspergillus niger} glycoprotein in these mutants. The GOD protein contains the 8 potential sites for the N-linked glycosylation (Frederick \textit{et al.}, \textit{J. Biol. Chem.} 265, 3793, 1990).

In order to express the GOD in the mutant yeast strains, we constructed a GOD expression vector, pDLMOX-GOD using the pDLMOX-Hir vector (Kang \textit{et al.} \textit{Yeast} 14, 371, 1998)). The DNA fragment containing the hirudin gene was first removed from the pDLMOX-Hir vector and the GOD gene fused to the fragment corresponding to the secretory signal of the \( \alpha \)-amylase at the N-terminal was then replaced in the vector (Kim S. Y. Ph. D. Dissertations, Yonsei University, Korea, 2001). The resultant vector pDLMOX-GOD was introduced into the two mutant strains, DL42-15 and \( \Delta ochl \) as well as the wild type strain,
and they were cultured on the YPM media (1% yeast extract, 2% peptone, 2% methanol) to express the GOD proteins.

The GOD proteins expressed and secreted were isolated and purified for Western blot analysis. The proteins were run on a polyacrylamide gel, transferred to a nitrocellulose membrane, and blotted using a GOD antibody. Figure 7A shows that the GOD proteins of the mutant strains, DL42-15 and Δochl, have a smaller molecular weight than that of the wild type, suggesting the proteins expressed and secreted in the mutants are less hyperglycosylated, or in other words, hyperglycosylation is inhibited in the mutant strains. To confirm the blotting result, we treated all the proteins with endoglycosidase H enzyme to digest the oligosaccharide chains attached on the proteins, and repeated the blot. Figure 7B shows that all the proteins have the same molecular weight on the blot, suggesting they are all the same proteins. These results demonstrate that the proteins expressed and secreted in the mutant cells were smaller than the one expressed and secreted in wild type cells due to less hyperglycosylation on the proteins. Therefore, the mutant strains, DL42-15 and Δochl, unlike the wild type, are suitable host cells to produce the human glycoproteins, in which the hyperglycosylation of the proteins will be inhibited, resulting in a closer resemblance to native human proteins.

Possible application of the invention to industries
The *H. polymorpha* mutants, DL42-15 and Δoch1, are able to be used as host cells to produce recombinant glycoproteins, which will express and secrete the proteins containing proper outer oligosaccharide chains closely resembling the native proteins because the hyperglycosylation of the proteins is inhibited in the mutants cells. These mutants will be useful in the medical therapeutic industry because *H. polymorpha* yeast cells has been broadly used to produce medical therapeutic recombinant proteins on a large scale.
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Date of deposit 29/05/2002

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The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only
☐ This sheet was received with the international application
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For international Bureau use only
☐ This sheet was received by the International Bureau on:
Authorized officer
What Is Claimed Is:

1. A nucleic acid molecule comprising the DNA sequence shown in Figure 3.

2. The nucleic acid molecule according to claim 1, wherein the nucleic acid molecule is *Hansenula polymorpha* HpOCH1 gene (KCTC 10265BP).

3. A polypeptide comprising the amino acid sequence shown in Figure 3.

4. *H. polymorpha* mutant strains which prevent the hyperglycosylation of glycoproteins by mutation of the HpOCH1 gene.

5. A *H. polymorpha* mutant strain Δoch1 (KCTC 10264BP) according to claim 4, wherein the HpOCH1 gene is disrupted.

6. A recombinant *H. polymorpha* strain expressing a foreign protein, wherein the recombinant strain is produced by introducing the gene encoding the foreign protein into *H. polymorpha* strain according to claim 4.

7. A recombinant *H. polymorpha* strain according to claim 6 expressing a foreign protein, wherein the recombinant strain is produced by introducing the gene encoding the foreign protein into the disrupted mutant strain, Δoch1.

9. A recombinant *H. polymorpha* strain expressing a foreign protein, wherein the recombinant strain is produced by introducing the gene encoding the foreign protein into *H. polymorpha* mutant strain, DL-42-15 according to claim 8.

10. A method for producing a foreign protein, wherein the method comprises cultivating the recombinant strains according to any one of claims 6, 7, and 9 under conditions that allow the strains to express the foreign protein and isolating the expressed foreign protein from the cultures.

11. The method according to claim 10, wherein hyperglycosylation of a foreign protein is prevented.
FIG. 1

A16 (CBS)  
8V (CBS)  
HPB1 (CBS)  
DL1  

YPD  

4mM Sodium orthovanadate
FIG. 2

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FIG. 5

1st PCR

2nd PCR (fusion PCR)

Anneal & extension (without primers)

Anneal & extension (with primers)

In vivo DNA recombination

(In genome)
FIG. 6

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Korea Research Institute of Bioscience and Biotechnology

Hansemula polymorpha mutant strains with defect in outer chain biosynthesis and the production of recombinant glycoproteins using the same strains

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Kopantem 1.7

1

2462

DNA

Hansemula polymorpha

sig_peptide

(650)..(852)

initiation codon

5'UTR

(1)..<(49)

3'UTR

(2158)..<(2462)

terminator

(2156)..<(2157)

termination codon

CDS

(850)..<(2154)

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC7  C12N 15/31

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

B. FIELDS SEARCHED

Maximum documentation searched (classification system followed by classification symbols)

IPC7 C12N 15/31

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)

BLAST, PubMed, Delphion "Hansenula", "mannosyltransferase", "Och"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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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 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
03 SEPTEMBER 2003 (03.09.2003)

Date of mailing of the international search report
04 SEPTEMBER 2003 (04.09.2003)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Form PC1/ISA/210 (second sheet) (July 1998)

Authorized officer

KWON, Oh Sig

Telephone No. 82-42-481-5773
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