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(12) **United States Patent**  
**Contreras et al.**(10) **Patent No.:** **US 8,883,445 B2**(45) **Date of Patent:** **\*Nov. 11, 2014**(54) **PROTEIN GLYCOSYLATION  
MODIFICATION IN METHYLOTROPHIC  
YEAST**(75) Inventors: **Roland Contreras**, Merelbeke (BE);  
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Zwijnaarde (BE)(\*) Notice: Subject to any disclaimer, the term of this  
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U.S.C. 154(b) by 0 days.This patent is subject to a terminal dis-  
claimer.(21) Appl. No.: **11/827,998**(22) Filed: **Jul. 13, 2007**(65) **Prior Publication Data**

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**C12N 15/00** (2006.01)(52) **U.S. Cl.**CPC ..... **C12Y 302/01114** (2013.01); **C12N 9/1288**  
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See application file for complete search history.(56) **References Cited**

## U.S. PATENT DOCUMENTS

5,834,251 A 11/1998 Maras et al. .... 435/71.1  
7,029,872 B2 \* 4/2006 Gerngross ..... 435/69.1  
2002/0137134 A1 9/2002 Gerngross ..... 435/69.1

## FOREIGN PATENT DOCUMENTS

EP 1 211 310 A1 6/2002  
WO WO 92/09694 6/1992  
WO WO 96/21038 7/1996  
WO WO 02/00856 A2 1/2002  
WO WO 02/00879 A2 1/2002

## OTHER PUBLICATIONS

Branden et al., Introduction to Protein Structure, Garland Publishing  
Inc., New York, p. 247, 1991.\*  
Witkowski et al., Biochemistry 38:11643-11650, 1999.\*  
Seffernick et al., J. Bacteriol. 183(8):2405-2410, 2001.\*  
Kumar et al., PNAS 87:9948-9952, 1990.\*  
Maras et al., Journal of Biotechnology 77:255-263, 2000.\*  
Yoshida et al., Bioscience Biotechnology and Biochemistry  
62(2):309-315, 1998.\*  
Mucha et al., Glycobiology 11(9):769-778, 2001.\*  
Lal et al., J. Biol. Chem. 269(13):9872-9881, 1994.\*  
van Petegem et al., J. Mol. Biol. 312:157-165, 2001.\*  
Nilsson et al., Journal of Cell Science 109:1975-1989, 1996.\*  
Yoshida et al., Glycobiology 9(1):53-58, 1999.\*  
Schwientek et al., Journal of Biological Chemistry 271(7):3398-  
3405, 1996.\*  
Kornfeld et al., Annu. Rev. Biochem. 54:631-664, 1985.\*  
B.K. Choi, et al., "Use of Combinatorial Genetic Libraries to Human-  
ize N-Linked Glycosylation in the Yeast *Pichia pastoris*", PNAS, vol.  
100, No. 9, pp. 5022-5027, (2003).  
S.R. Hamilton, et al., "Production of Complex Human Glycoproteins  
in Yeast", Science, vol. 301, pp. 1244-1246, (2003).  
Maras, M., et al., "In vitro Conversion of the Carbohydrate Moiety of  
Fungal Glycoproteins to Mammalian-Type Oligosaccharides", Eur.  
J. Biochem. vol. 249, pp. 701-707 (1997).  
Martinet, W., et al., "Modification of the Protein Glycosylation Path-  
way in the Methylotrophic Yeast *Pichia pastoris*", Biotechnology  
Letters, vol. 20, No. 12, pp. 1171-1177 (1998).  
Martinet, W., et al., "Protection of Mice Against a Lethal Influenza  
Challenge by Immunization with Yeast-Derived Recombinant Influenza  
Neuraminidase", vol. 247, pp. 332-338 (1997), Eur. J. Biochem.  
Cereghino, G., et al., "New Selectable Marker/Auxotrophic Host  
Strain Combinations for Molecular Genetic Manipulation of *Pichia*  
*pastoris*", vol. 263, pp. 159-169 (2001).  
Callewaert, N. et al., "Use of HDEL-tagged *Trichoderma reesei*  
Mannosyl Oligosaccharide 1,2- $\alpha$ -D-mannosidase for N-glycan  
Engineering in *Pichia pastoris*" FEBS Letters (Aug. 17, 2001) pp.  
173-178, vol. 503, No. 2-3.

(Continued)

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## (57)

**ABSTRACT**The present invention provides genetically engineered strains  
of methylotrophic yeast including *Pichia* and especially  
*Pichia pastoris* capable of producing proteins with reduced or  
modified glycosylation. Methods of producing glycoproteins  
with reduced and/or modified glycosylation using such  
genetically engineered strains of *Pichia* are also provided.  
Vectors, which comprise coding sequences for  $\alpha$ -1,2-man-  
nosidase I, glucosidase II, GlcNAc-transferase I and mannosid-  
ase II or comprising OCH1 disrupting sequence, for trans-  
forming methylotrophic yeasts are contemplated by the  
present invention. Kit for providing the contemplated vectors  
are also included in this invention.**16 Claims, 35 Drawing Sheets**