



US008354268B2

(12) **United States Patent**
Contreras et al.(10) **Patent No.:** **US 8,354,268 B2**
(45) **Date of Patent:** ***Jan. 15, 2013**(54) **PROTEIN GLYCOSYLATION
MODIFICATION IN METHYLOTROPHIC
YEAST**(75) Inventors: **Roland Contreras**, Merelbeke (BE);
Nico L. M. Callewaert, Lichtervelde
(BE); **Steven C. J. Geysens**,
Kruishoutem (BE)(73) Assignees: **VIB, VZW**, Zwijnaarde (BE); **Research
Corporation Technologies, Inc.**,
Tucson, AZ (US); **Universiteit Gent**,
Ghent (BE)(*) Notice: Subject to any disclaimer, the term of this
patent is extended or adjusted under 35
U.S.C. 154(b) by 975 days.This patent is subject to a terminal dis-
claimer.(21) Appl. No.: **10/672,484**(22) Filed: **Sep. 25, 2003**(65) **Prior Publication Data**

US 2004/0038381 A1 Feb. 26, 2004

Related U.S. Application Data(63) Continuation of application No. 09/896,594, filed on
Jun. 29, 2001, now Pat. No. 6,803,225.(60) Provisional application No. 60/215,676, filed on Jun.
30, 2000.(51) **Int. Cl.****C12N 1/14** (2006.01)**C12N 15/74** (2006.01)**C12P 21/04** (2006.01)**C12Q 1/00** (2006.01)(52) **U.S. Cl.** **435/254.23**; 435/483; 435/69.9(58) **Field of Classification Search** None
See application file for complete search history.(56) **References Cited**

U.S. PATENT DOCUMENTS

5,135,854 A 8/1992 MacKay et al.
5,705,616 A 1/1998 Lehle et al.
5,834,251 A 11/1998 Maras et al.
8,058,053 B2 * 11/2011 Contreras et al. 435/254.11
2002/0137134 A1 9/2002 Gerngross

FOREIGN PATENT DOCUMENTS

EP 0 314 096 A2 5/1989
EP 1 211 310 A1 6/2002
EP 1 297 172 B1 4/2003
JP 8-336387 12/1996
JP 9-261 1/1997
WO WO 91/05057 4/1991
WO WO 96/21038 7/1996
WO WO 02/00879 A2 1/2002
WO WO 2004/003205 A1 1/2004

OTHER PUBLICATIONS

Callewaert et al. Use of HDEL-tagged *Trichoderma reesei* mannosyl
oligosaccharide 1,2-alpha-D-mannosidase for N-glycan engineering
in *Pichia pastoris*. FEBS Letters 503:173-178, 2001.*Maras, M., et al. (2000) "Molecular Cloning and Enzymatic Charac-
terization of a *Trichoderma Reesei*, 1, 2—alpha-D-Mannosidase", *Journal
of Biotechnology* 77: 255-263.Brethauer, R. K., et al. (1999) "Glycosylation Biochem of *Pichia
pastoris*-derived Proteins", *Biotechnol. Appl Biochem* 30: 193-200.
Kukuruzinska, M. A., et al. (1987) "Protein Glycosylation in Yeast",
Ann. Rev. Biochem 56: 915-944.Chiba, Y., et al. (1998) "Production of Human Cpmpatible High
Mannse-Type (Man₅GlcNac₂) Sugar Chains in *Saccharomyces
cerevisiae*", *The Journal of Biological Chemistry* 273 (41): 26298-
26304.Maras, M., et al. (1999) In Vivo Synthesis of Complex N-Glycans by
Expression of Human N-Acetylglucosaminyltransferase I in the
Filamentous Fungus *Trichoderma reesei*, *FEBS Letters* 452: 365-
370.Nakanishi-Shindo, Y., et al. (1993) "Structure of the N-Linked
Oligosaccharides That Show the Complete Loss of alpha-1, 6-Polyman-
nose Outer Chain from och1, och1 mnn1, and och1 mnn1 alg3
Mutants of *Saccharomyces cerevisiae*", *The Journal of Biological
Chemistry* 268 (35): 26338-26345.Martinet, W., et al. (1998) "Modification of the Protein Glycosylation
Pathway in the Methylotrophic Yeast *Pichia pastoris*", *Biotechnoogy
Letters* 20(12):1171-1177.Maras, M., et al. (1997) "In Vitro Conversation of the Carbohydrate
Moiety of Fungal Glycoproteins to Mammalian-Type Oligosac-
charides", *Eur. J. Biochem.* 249:701-7707.Laroy, W., et al. (2000) "Cloning of *Trypanosoma cruzi* trans-
Sialidase and Expression in *Pichia pastoris*", *Protein Expression and
Purification* 20: 389-393.Inoue et al. Molecular cloning and nucleotide sequence of the 1,2-
alpha-D-mannosidase gene, msdS, from *Aspergillus saitoi* and
expression of the gene in yeast cells. *Biochim. Biophys. Acta*
1253:141-145, 1995.Herscovics et al. Isolation of a mouse Golgi mannosidase cDNA, a
member of a gene family conserved from yeast to mammals. *J. Biol.
Chem.* 269:9864-9871, 1994.Lal et al. Isolation and expression of murine and rabbit cDNAs
encoding an alpha 1,2-mannosidase involved in the processing of
asparagines-linked oligosaccharides. *J. Biol. Chem.* 269-9872-9881,
1995.Trombetta et al. Endoplasmic reticulum glucosidase II is composed
of a catalytic subunit, conserved from yeast to mammals, and a tightly
bound noncatalytic HDEL-containing subunit. *J. Biol. Chem.*
271:27509-27516, 1996.

(Continued)

Primary Examiner — Quang Nguyen(74) *Attorney, Agent, or Firm* — Scully, Scott, Murphy &
Presser, P.C.(57) **ABSTRACT**The present invention provides genetically engineered strains
of *Pichi* capable of producing proteins with reduced glyco-
sylation. In particular, the genetically engineered strains of
the present invention are capable of expressing either or both
of an alpha-1,2-mannosidase and glucosidase II. The genetically
engineered strains of the present invention can be further
modified such that the OCH1 gene is disrupted. Methods of
producing glycoproteins with reduced glycosylation using
such genetically engineered stains of *Pichia* are also pro-
vided.**21 Claims, 23 Drawing Sheets**