



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/55, 9/16	A3	(11) International Publication Number: WO 99/67398 (43) International Publication Date: 29 December 1999 (29.12.99)
(21) International Application Number: PCT/US99/14106 (22) International Filing Date: 23 June 1999 (23.06.99) (30) Priority Data: 09/104,769 25 June 1998 (25.06.98) US (71) Applicant: CORNELL RESEARCH FOUNDATION, INC. [US/US]; Suite 105, 20 Thornwood Drive, Ithaca, NY 14850 (US). (72) Inventor: LEI, Xingen; 114 Lexington Drive W., Ithaca, NY 14850 (US). (74) Agents: GOLDMAN, Michael, L. et al.; Nixon, Hargrave, Devans & Doyle LLP, Clinton Square, P.O. Box 1051, Rochester, NY 14603 (US).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> (88) Date of publication of the international search report: 20 April 2000 (20.04.00)	
(54) Title: OVEREXPRESSION OF PHYTASE GENES IN YEAST SYSTEMS		
(57) Abstract The present invention relates to a method of producing a heterologous protein or polypeptide having phytase activity in a yeast system. The invention also provides proteins having phytase activity which have increased thermostability. Yeast strains which produce a heterologous phytase and the vectors used to produce the phytase are also provided.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

INTERNATIONAL SEARCH REPORT

International Application No
PC JS 99/14106

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/55 C12N9/16

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 6 C12N

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^o	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 20139 A (FINNFEEDS INT LTD (GB); MORGAN A.J.; HESSING M.; SLEIJSTER-SELIS H.E.) 14 May 1998 (1998-05-14)	1-8,11, 12,21, 22,30, 41-45
Y	abstract page 4, line 14-20 page 5, line 21-32 page 11, line 14-27; example 7 page 16 -page 18; claims ---	1,8
Y	WO 98 05785 A (BIOCEM (FR); INST NAT RECH AGRONOMIQUE (FR); MAUGENEST; LESCURE; PEREZ) 12 February 1998 (1998-02-12) abstract page 6, line 28-32 page 13, line 22-35 ---	1,8
	-/--	

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

^o 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 document 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 15 February 2000	Date of mailing of the international search report 02.03.00
---	--

Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040. Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Macchia, G
--	--------------------------------------

INTERNATIONAL SEARCH REPORT

International Application No
PC , US 99/14106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 420 358 A (GIST BROCADES NV (NL); VAN GORCOM; VAN HARTINGSVELDT; VAN PARIDON ET A) 3 April 1991 (1991-04-03)	1-7, 9-12, 21-23, 25-33, 41-47
Y	page 7; table 1	13,14, 34,35
	page 8, line 31-33 page 9, line 18-25 page 28; claims 22,24 figures 6,8	

X	EP 0 684 313 A (F. HOFFMANN-LA ROCHE AG (CH); VAN LOON A.; MITCHELL D.) 29 November 1995 (1995-11-29)	1-7,9, 11,12, 14,21, 22,30, 41-46
Y	abstract	13,14, 34,35
	page 2, line 47-52 page 5, line 43 -page 6, line 38 page 41; claim 18	

X	WEILIN SUN ET AL.: "Expression of Aspergillus niger Phytase in yeast Saccharomyces cerevisiae for poultry diet supplementation" POULTRY SCIENCE, vol. 76, no. suppl. 1, 1997, page 5 XP002118708 CHAMPAIGN, IL, US ISSN: 0032-5791 the whole document	1-7, 9-12, 21-23, 25-33, 41-47

X	YAO BIN ET AL.: "Recombinant Pichia pastoris overexpressing bioactive Phytase" SCIENCE IN CHINA SERIES C. LIFE SCIENCES, vol. 41, no. 3, June 1998 (1998-06), pages 330-336, XP002118576	1-7,9, 10,13, 14, 21-23, 25-31, 34,35, 41-47
	the whole document	

P,X	WYSS M. ET AL.: "Biophysical characterization of fungal phytases (myo-Inositol Hexakisphosphate Phosphohydrolases): molecular size, glycosylation pattern, and engineering of proteolytic resistance" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 2, February 1999 (1999-02), pages 359-366, XP002118709 WASHINGTON DC, US ISSN: 0099-2240 the whole document	1,2,5,6, 9-13, 21-23, 25,26, 29-34, 41-47

	-/--	

INTERNATIONAL SEARCH REPORT

International Application No

PCT/JS 99/14106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	HAN Y. ET AL.: "Expression of Aspergillus niger Phytase gene (phyA) in Saccharomyces cerevisiae" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 65, no. 5, May 1999 (1999-05), pages 1915-1918, XP002118710 WASHINGTON DC, US ISSN: 0099-2240 the whole document	1,2,4-6, 9-12, 21-23, 25,26, 29-33, 41-47
P,X	--- HAN Y. AND LEI X.G.: "Role of glycosylation in the functional expression of an Aspergillus niger Phytase (phyA) in Pichia pastoris" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 364, no. 1, 1 April 1999 (1999-04-01), pages 83-90, XP002118711 NEW YORK, US ISSN: 0003-9861 the whole document	1,2,4,5, 7,9,10, 13,14, 21-23, 25,26, 29-31, 34,35, 41-47
X	--- EP 0 699 762 A (MITSUI TOATSU CHEM INC (JP) MOCHIZUKI; TOKUDA; SUZUKI; SHIMADA; TAWAKI) 6 March 1996 (1996-03-06) page 5, line 1-15 page 7, line 6-22; figures 2-4 page 9, line 18-36; example 9 page 22 -page 23; claims	1-7,11, 12,21, 22,25-30
X	--- WO 97 35017 A (NOVO NORDISK BIOTECH INC. (US); BERKA R.M.; RAY M.W.; KLOTZ A.V.) 25 September 1997 (1997-09-25) page 15, line 10 -page 18, line 12 page 20, line 5 -page 24, line 7 page 43, line 23 -page 45, line 12; figures 6-8 page 58 -page 59; claims 38-40	1-7,9, 11,12, 14,21, 22, 25-30, 41-46
X	--- GB 2 316 082 A (FINNFEEDS INT LTD (GB); APAJALAHTI; HEIKKINEN; KEROVUO; LAURAEUS ET AL) 18 February 1998 (1998-02-18) abstract page 9, paragraph 4	1-7,11, 12,15, 21,22, 30,41-46
	--- -/--	

INTERNATIONAL SEARCH REPORT

International Application No

PC. JS 99/14106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 97 48812 A (DPT AGRICULTURE, AGRI-FOOD CANADA; CHENG; SELINGER; YANKE; BAE ET AL.) 24 December 1997 (1997-12-24)	1-7,11, 12,15, 21,22, 25,26, 29,30, 41-46
Y	abstract page 4, line 3,4	1,15-20, 30, 36-41, 46,48
	page 5, line 9-12 page 10, line 29 -page 11, line 26 page 14, line 28 -page 16, line 9 page 23, line 4-19; figures 4-6 page 30, line 10 -page 36, line 11 page 42 -page 43; tables 4,5	
X	--- DASSA J. ET AL.: "The complete nucleotide sequence of the Escherichia coli gene appA reveals significant homology between pH 2.5 acid phosphatase and glucose-1-phosphatase" JOURNAL OF BACTERIOLOGY, US, WASHINGTON, DC, vol. 172, no. 9, September 1990 (1990-09), pages 5497-5500, XP002911650 ISSN: 0021-9193 cited in the application	21,22, 24,49, 50,55
Y	the whole document	1,15-20, 30, 36-41, 46,48
X	--- OSTANIN K. ET AL.: "Overexpression, site-directed mutagenesis, and mechanism of Escherichia coli acid phosphatase" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 32, 15 November 1992 (1992-11-15), pages 22830-22836, XP002129674 cited in the application	21,22, 24,49, 50,55
Y	the whole document	1,15-20, 30, 36-41, 46,48, 56-58
	--- -/--	

INTERNATIONAL SEARCH REPORT

International Application No

PC JS 99/14106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GREINER R. ET AL.: "Purification and characterization of two Phytases from Escherichia coli" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 303, no. 1, 15 May 1993 (1993-05-15), pages 107-113, XP000876512	21,22,24
Y	abstract	56-58
A	page 109, right-hand column	
A	page 112 -page 113	16,36, 48-50
A	--- KONIETZNY U. AND GREINER R.: "Model system for developing detection methods for foods deriving from genetic engineering" JOURNAL OF FOOD COMPOSITION AND ANALYSIS, vol. 10, 1997, pages 28-35, XP000878550 abstract page 31, paragraph 2	16,36, 48-50
P,X	--- WO 99 08539 A (DIVERSA CORPORATION (US); KRETZ KEITH) 25 February 1999 (1999-02-25) page 21, line 13-20 page 24, line 13-24 figures 1,2	1,2,4-7, 11,12, 15-18, 21,22, 24-26, 30, 36-38, 41-46, 48-51, 53-58
P,X	--- RODRIGUEZ E. ET AL.: "Cloning, sequencing, and expression of an Escherichia coli acid phosphatase / phytase gene (appA2) isolated from pig colon" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 257, no. 1, 2 April 1999 (1999-04-02), pages 117-123, XP002129675 ACADEMIC PRESS INC., ORLANDO, FL, US ISSN: 0006-291X the whole document	1,2,4-7, 15,16, 19-22, 24,25, 30,36, 39-46, 48-51, 55,56,58
A	--- WODZINSKI R.J. AND ULLAH A.H.J.: "Phytase" ADVANCES IN APPLIED MICROBIOLOGY, vol. 42, 1996, pages 263-302, XP002118577 NEW YORK, NY, US	
	--- -/--	

INTERNATIONAL SEARCH REPORT

International Application No

PC., JS 99/14106

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>MINAMIGUCHI K.: "Secretive expression of the <i>Aspergillus aculeatus</i> Cellulase (FI-CMCase) by <i>Saccharomyces cerevisiae</i>"</p> <p>JOURNAL OF FERMENTATION AND BIOENGINEERING, vol. 79, no. 4, 1995, pages 363-366, XP002119099 SOCIETY OF FERMENTATION TECHNOLOGY, JP ISSN: 0922-338X</p> <p style="text-align: center;">-----</p>	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 99/14106

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 8-10, 13, 14, 23, 31-35, 47 all totally; 1-7, 11, 12, 21, 22, 25-30, 41-46 all partially

- 1.1. Claims: 8 totally; 1-7, 11, 12, 21, 22, 25-30, 41-45 all partially

A method of producing phytase in yeast comprising: providing a heterologous gene which encodes a protein or polypeptide with phytase activity and expressing said gene in a yeast strain. Said method wherein the heterologous gene is isolated from plant cells. Said method wherein the yeast strain is selected from the group consisting of *Saccharomyces*, namely *S.cerevisiae*, *Kluyveromyces*, *Torulasporea*, *Schizosaccharomyces*.

A protein or polypeptide produced by said method.

A yeast strain comprising a heterologous gene which encodes a protein or polypeptide with phytase activity.

A vector comprising a gene from a non-yeast organism which encodes a protein or polypeptide with phytase activity, said vector being functional in yeast.

- 1.2. Claims: 9, 10, 13, 14, 23, 31-35, 47 all totally; 1-7, 11, 12, 21, 22, 25-30, 41-46 all partially

As subject 1.1 but wherein the heterologous gene is isolated from fungal cells, as *phyA* of *Aspergillus niger*, and the group of the yeast strains comprises also a methylotropic yeast as *Pichia pastoris*.

2. Claims: 15-20, 24, 36-40, 48-58 all totally; 1-7, 11, 12, 21, 22, 25-30, 41-46 all partially

As subject 1.2 but wherein the heterologous gene is isolated from bacterial cells, as *appA* of *Escherichia coli*.

A method of producing a protein or polypeptide with phytase activity by means of the *appA* gene, expressed in a yeast strain or filamentous fungi.

A method of converting phytate to inositol and inorganic phosphorus by means of *appA*.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/14106

Patent document cited in search report	A	Publication date	Patent family member(s)	Publication date
WO 9820139	A	14-05-1998	GB 2319030	13-05-1998
			AU 7002198	29-05-1998
			BR 9712731	26-10-1999
			EP 0942993	22-09-1999
			PL 333097	08-11-1999

WO 9805785	A	12-02-1998	FR 2751987	06-02-1998
			AU 3944697	25-02-1998
			CA 2261913	12-02-1998
			EP 0938568	01-09-1999

EP 0420358	A	03-04-1991	EP 0779037	18-06-1997
			AT 180014	15-05-1999
			AU 636673	06-05-1993
			AU 6501190	28-04-1991
			BG 60108	15-10-1993
			CA 2042054	28-03-1991
			CN 1051058	01-05-1991
			DE 69033103	17-06-1999
			DE 69033103	09-12-1999
			DE 420358	12-10-1995
			ES 2072834	01-08-1995
			HU 215179	28-10-1998
			IL 95803	12-03-1999
			JP 4506007	22-10-1992
			KR 159782	16-11-1998
			LT 1527	26-06-1995
			LV 10310	20-10-1994
			WO 9105053	18-04-1991
			NO 303988	05-10-1998
			NZ 235478	26-03-1993
			PL 167790	30-11-1995
			PL 168470	29-02-1996
PT 95447	22-05-1991			
US 5436156	25-07-1995			
US 5863533	26-01-1999			

EP 0684313	A	29-11-1995	CN 1126243	10-07-1996
			JP 8056676	05-03-1996

EP 0699762	A	06-03-1996	AU 682606	09-10-1997
			AU 2484095	25-01-1996
			FI 953303	06-01-1996
			JP 8289782	05-11-1996
			US 5830732	03-11-1998
			US 5840561	24-11-1998

WO 9735017	A	25-09-1997	AU 2077197	10-10-1997
			AU 2539197	10-10-1997
			BR 9708209	27-07-1999
			CA 2248980	25-09-1997
			CN 1214081	14-04-1999
			EP 0904383	31-03-1999
			WO 9735016	25-09-1997
			US 5866118	02-02-1999

GB 2316082	A	18-02-1998	AU 4118197	06-03-1998
			CN 1228120	08-09-1999

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/14106

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2316082 A		WO 9806856 A EP 0920519 A PL 331587 A	19-02-1998 09-06-1999 19-07-1999
WO 9748812 A	24-12-1997	US 5939303 A US 5985605 A AU 3021697 A CA 2257101 A EP 0904385 A NO 985804 A PL 330532 A	17-08-1999 16-11-1999 07-01-1998 24-12-1997 31-03-1999 10-02-1999 24-05-1999
WO 9908539 A	25-02-1999	US 5876997 A AU 8904498 A	02-03-1999 08-03-1999