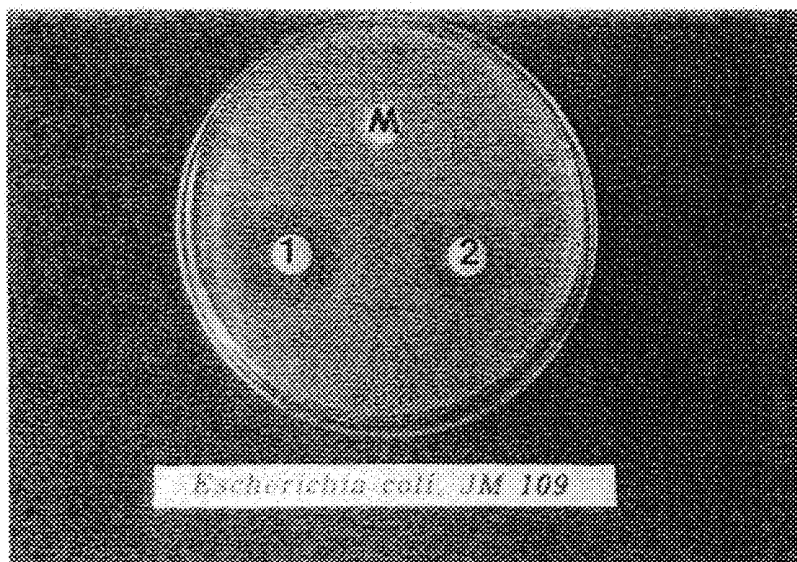




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(30) Priority Data: 1998/29351 15 July 1998 (15.07.98) KR 1999/29042 13 July 1999 (13.07.99) KR		(74) Agent: PARK, Jang, Won; Park, Kim & Partner, Jewoo Building, 4th floor, 200, Nonhyun-dong, Kangnam-ku, Seoul 135-010 (KR).	
(71) Applicant (for all designated States except US): SAMYANG GENEX CORPORATION [KR/KR]; 263, Younji-dong, Chongno-ku, Seoul 110-470 (KR).		(81) Designated States: AU, CA, CN, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(72) Inventors; and (75) Inventors/Applicants (for US only): SUNG, Chang, Keun [KR/KR]; Hanbit Apt., 111-1502, Eoeun-dong, Yusong-ku, Taejon 305-755 (KR). JOO, In, Sun [KR/KR]; Wonangmaeul, 101-1702, Kwanjeo-dong, Seo-ku, Taejon 302-758 (KR). WOO, Moon, Sook [KR/KR]; 302, 425-7, Goong-dong, Yusong-ku, Taejon 305-335 (KR). KIM, Sang, Kyu [KR/KR]; 15-46, Nae-dong, Seo-ku, Taejon 302-181 (KR). LEE, Jeong, Hyun [KR/KR]; Kumho Apt., 2-1201, Joongchon-dong, Joong-ku, Taejon 301-070 (KR). LEE, Keum, Soon [KR/KR]; 7/2, 146-2, Kyo-dong, Jechon-shi, Choongchungbook-do 390-070 (KR). KIM, Young, Ho [KR/KR]; Cheonggu-Narae Apt., 108-202, Junmin-dong, Yusong-ku, Taejon 305-390 (KR). HONG,		Published With international search report.	

(54) Title: MASS PRODUCTION METHOD OF LACTOFERRIN POLYPEPTIDE FROM YEAST AND USEFUL MICROORGANISM THEREOF



(57) Abstract

The present invention relates to a method of mass-producing lactoferrin polypeptide from yeast which is resistant to lactoferrin polypeptide. The present invention also provides *Pichia* strains, especially *Pichia pastoris* KCTC 0500BP that are resistant to lactoferrin polypeptide.

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MASS PRODUCTION METHOD OF LACTOFERRIN POLYPEPTIDE FROM YEAST AND USEFUL MICROORGANISM THEREOF

TECHNICAL FIELD AND BACKGROUND ART

5

Lactoferrin is a glycoprotein that has physiological and biological characteristics such as bactericidal, growth stimulatory, iron carrying activity, immune modulation, and specific reaction to membrane receptors.

The structure of human lactoferrin is composed of 681 amino acid residues.

10 There is an iron-binding site in between the domains of one of the two lobes. Lactoferrin is known to have immuno-stimulatory effect and antibacterial activity. Also, it is reported that the peptides or polypeptides derived from lactoferrin are superior to lactoferrin itself in antibacterial activity and stability (US patent 5304633; 5571697). Therefore, lactoferrin polypeptide is a good additive for infant formulas
15 and animal feed or drugs due to its bacteriostatic, cell growth stimulatory or inflammation inhibitory effects.

Lactoferrin polypeptides have been isolated mainly from milk serum. Recently, mass production of polypeptides by genetic engineering have been attempted. Ward and Piddington have produced 2 g/l quantity of recombinant
20 lactoferrin in *Aspergillus oryzae* using a glucoamylase promoter (Ward, P.P. et.al., *Biotechnology*, 13:498-503 (1995)). Qianwa reported that 1.5~2.0 mg/l of recombinant human lactoferrin was obtained by expressing it in the form of a fusion protein between a yeast invertase and human lactoferrin by using a chelatin promoter in *Saccharomyces cerevisiae* (Qianwa Liang et.al., *J. Agric. Food. Chem.*
25 41:1800-1907 (1989)). It was discussed that the recombinant lactoferrin synthesized from *S. cerevisiae* had additional sugar units, and the yield was not

reproducible. In the case of producing lactoferrin or its antibacterial peptide derivatives by using genetic engineering techniques, expressed lactoferrin or antibacterial peptide could slow down the growth of or even kill the host microorganism. To overcome this problem, it is reported that the antibacterial peptide was expressed in the form of a fusion peptide. In US patent 5571697, antibacterial polypeptide derived from lactoferrin was expressed as a fusion protein in *Aspergillus*.

BRIEF SUMMARY OF THE INVENTION

10 The present invention provides a method to mass-produce lactoferrin polypeptide from microorganisms.

The present invention provides a method to mass-produce lactoferrin polypeptide alone from microorganisms.

The present invention provides a novel germ strain, which is resistant
15 against lactoferrin polypeptide.

The present invention provides a germ strain that produces lactoferrin polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1 is a photograph showing the resistance of microorganisms against lactoferrin polypeptide of the present invention.

Figure 2 is a scheme of the preparation method of an expression vector used in the present invention.

Figure 3 is a photograph showing the resistance of the transformant by
25 expressing human lactoferrin polypeptide of the present invention against

lactoferrin polypeptide.

Figure 4A~4F are photographs showing the resistance of the transformant by expressing Korean cow lactoferrin polypeptide of the present invention against lactoferrin polypeptide.

5

DETAILED DESCRIPTION OF THE INVENTION

The present inventors have explored lactoferrin polypeptide resistant germ strains from samples of soybean sauce, soybean paste and wine obtained from Choongchung province, Korea. The inventors also screened and identified superior lactoferrin polypeptide resistant strains.

In the present application lactoferrin polypeptides means lactoferrin or all of the antibacterial peptides or polypeptides derived from lactoferrin unless otherwise specified. The origin of the lactoferrin is not limited, and the lactoferrin can be from human, cow, pig or other mammals.

Since the microorganism of the present invention is extremely resistant against lactoferrin polypeptide, the lactoferrin polypeptide can be produced as a fusion protein or a peptide itself by the microorganism of the present invention.

Also the microorganism of the present invention can be used in mass-producing not only lactoferrin polypeptide but also other antibacterial peptides that are difficult to mass-produce due to its activity to slow down the growth of or to kill the host cells.

The present invention also relates to the method of mass-producing the lactoferrin polypeptides from microorganisms composed of the following steps:

preparing a plasmid vector composed of a promoter sequence active in a yeast cell and a sequence that is connected to the promoter and which codes the

lactoferrin polypeptide;

transforming the lactoferrin polypeptide resistant yeast with the above vector; and

culturing the transformed yeast.

5 The yeast cell of the present invention can be selected from *Saccharomyces*, *Aspergillus*, *Pichia*, and *Candida*, and preferably *Pichia* genus cells.

In the preparation method of the present invention, the sequence coding lactoferrin polypeptide can be a sequence coding a fusion polypeptide fused with
10 another peptide or a sequence coding additional amino acid residues in addition to the lactoferrin polypeptide or a sequence coding the lactoferrin polypeptide only. An altered sequence can also be used if it codes the identical polypeptide as the lactoferrin polypeptides.

The present invention is also directed to the *Pichia pastoris* cell that
15 contains the vector having the lactoferrin polypeptide genes.

The restriction enzymes and other enzymes used in the Examples of the present invention were obtained from Boehringer Mannheim Biochemical Company. Oligonucleotide primer was obtained from Invitrogen, and the components of the media such as amino acids, yeast nitrogen base (YNB), yeast extracts, peptone
20 and glucose were obtained from Difco. Human lactoferrin and other reagents used in the protein electrophoresis were obtained from Sigma.

E. coli Top10F' and *Pichia pastoris* SJW-28 of the present invention were used in the transformation.

The compositions of the media in the present invention are as follows.

25 YM medium: yeast extracts 0.3 %, malt extract 0.3 %, peptone 0.5 %,

dextrose 1 %, agar 2 %.

YM-L medium: the medium that contains 1 mg of lactoferrin-pepsin hydrolysis product in 1 ml YM medium.

YPD liquid medium: yeast extract 1 %, peptone 2 %, dextrose 2 %, 5

The invention will be further illustrated by the following examples, but the invention is not limited to the examples given.

EXAMPLE 1. Selection of the host microorganism that is resistant against lactoferrin polypeptide

10 The lactoferrin polypeptide resistant microorganisms were selected primarily by smearing wine and soybean sauce samples in YM-L medium.

Secondary verification of the antibacterial activity against lactoferrin polypeptide was performed by following the method by H. Wakabayashi (Hiroyuki Wakabayashi, et.al., J. Food Protection, 55(4):280-240.6 (1992)). In a medium 15 containing the primarily selected germs, a paper disk that was immersed in the lactoferrin hydrolysis product that was obtained by treating 1 ml of lactoferrin 1mg/ citrate buffer solution at pH 2 with pepsin (10 unit/ml) at 37 °C for 4 hours was placed. A formation of a clear zone was observed. The germ strains that had excellent resistance against lactoferrin polypeptide were selected (Figure 1) and 20 named as "SJW-28". The identification procedure using a PI-CHE kit and other microorganism identification methods, based on N.J.W. Kreger-Van Rij. 1987 were used. The yeasts: a taxonomic study third revised and enlarged edition, Elsevier, were used to identify it as *P. pastoris* or related germ strains. This cell line was deposited at the Korean Collection of Type Cultures (KCTC) in Korea Research 25 Institute of Bioscience and Biotechnology located at Yusung-ku Eoeun-dong,

Taejon, Korea on July 8, 1998, and the number KCTC0500BP was given.

Table 1

Property	SWJ-28
D-Arabinose	-
D-Ribose	-
D-Xylose	-
D-Galactose	-
L-Rhamnose	+
Maltose	-
Sucrose	-
Lactose	-
Melibiose	-
Cellobiose	-
Trehalose	+
Raffinose	-
Melitose	-
Methyl-D-Glucoside	-
D-Glucosamine	-
Inulin	-
Methanol	+
Ethanol	+
Erythritol	-
Inositol	-
D-Mannitol	+
D-Gluconic acid salt	-
Glycerin	+
Succinic acid	+
Citric acid	- or slowly glowing

Table 2

Property	SWJ-28
Optimum growth pH	pH 6
Optimum growth temperature	29 °C
Nitrate utilization	-
Fat decomposition	+
Gelatin liquefaction	+
Carotinoid production	-
Marked production of organic acid	+
Production of starch-like materials	-
Vitamin requirement	+
Malt or YM liquid medium	Medium becomes milky after one day culture at 29 °C. Growth is very slow at 37 °C.
Malt or YM agar medium	Milky colony was formed when cultured at 29 °C for one day. Surface is a little shiny and has minute hypha.
Spore production	Sexual sporulation. There is no attachment between Ascomycetes and grow well with matricytes. There are attachment between the cells. One or four hat-shaped spore are formed in the sporangium and detach as the cell grows.

EXAMPLE 2. Subcloning of human lactoferrin polypeptide

The expression vector containing the lactoferrin polypeptide genes was prepared as described in Figure 2. Primers A (forward; 35mer) and B (backward; 30 mer) identified below were used to obtain a lactoferrin polypeptide gene by performing polymerase chain reaction (PCR) with pRL100 (North Dakota State University, Dept. of Biochemistry, Molecular Biology Laboratory) containing a human lactoferrin gene. The primers A and B were designed to amplify the sequence containing disulfide bond of cysteine in the human lactoferrin base sequence.

Primer A 5'-GGAAGCTTAAAAGATACGTAAAATGCTTCCAATGG-3'

*Hind*III

*Sna*BI

Primer B 5'-GGGAATTCTCAAATCTCTTTATGCAGCTG

5

*Eco*RI

Lactoferrin polypeptide gene and amino acid sequences derived from pRL100

AAA	GC	TTC	CAA	TGG	CAA	AGG	AAT	ATG	AGA	AAA	GTG	CGT
K	C	F	Q	W	Q	R	N	M	R	K	V	R
GGC	CCT	CCT	GTC	AGC	GC	ATA	AAG	AGA				
G	P	P	V	S	C	I	K	R				

Finally amplified lactoferrin polypeptide gene and amino acid sequences

GTA	AAA	TGC	TTC	CAA	TGG	CAA	AGG	AAT	ATG	AGA	AAA	GTG
V	K	C	F	Q	W	Q	R	N	M	R	K	V
CGT	CCT	CCT	CCT	GTC	AGC							
R	P	P	P	V	S							

Derived from Primer A

10

TGC	ATA	AAG	AGA	TTT
C	I	K	R	F

Derived from Primer B

Expression vector pPIC9-LFP was obtained by treating the amplified lactoferrin polypeptide gene and pPIC9(Invitrogen) with *Sna*BI and *Eco*RI and by connecting them. pPIC9-LFP was transformed in *E. coli*, Top 10F'. The transformed colony was selected by obtaining the growing colonies after smearing in an ampicillin containing medium.

EXAMPLE 3. Production of human lactoferrin polypeptide using SJW-28.

pPIC-LFP was transformed in SJW-28 by electrophoration using the method by Chang et.al. (Chang et.al., Guide to electrophoration and electrofusion. Academic Press, p501 (1992)). In other words, after SJW-28 was cultured in YPD liquid medium to OD₆₀₀ 1.3~1.5, pellet was obtained by centrifuging 500 ml of the culture medium. After the pellet was resuspended in 100 ml of YPD, it was treated with 20 mM and 25 mM of HEPES (N-[2-hydroxyethyl]piperazine-N'[2-ethanesulfonic acid], pH 8.0) and dithiothreitol, respectively and reacted at 30 °C for 15 minutes. After the solution was dissolved in 0.5 ml of 1M sorbitol, 40 µl was taken and added into a pre-chilled gap cuvette with 100 mg of pPIC9-LFP digested by *Sall*. After the mixture was reacted for 5 minutes in ice, electrophoration was performed at 1500 V and 25 mA. Immediately after the electrophoration, 750 µl of cold 1 M sorbitol was added, and 100 µl of the mixture was smeared in YM medium. Genomic DNA of the transformant that grows in YM medium was purified to finally select the strain in which lactoferrin polypeptide gene had been inserted, by performing PCR using the above mentioned primers A and B.

Lactoferrin polypeptide gene and amino acid sequences produced by the transformant

GAG	GCT	GAA	GCT	TAA	GTA	AAA	TGC	TTC	CAA	TGG	CAA	AGG
E	A	E	A	Y	V	K	C	F	Q	W	Q	R
AAT	ATG	AGA	AAA	GTG	CGT	GGC	CCT	CCT				
N	M	R	K	V	R	G	P	P				

Derived from pPIC9, Derived from Primer A

GTC AGC TGC ATA AAG AGA TTT
 V S C I K R F

Derived from Primer B

After finally selecting the transformant it was cultured to OD₆₀₀ 1.3~1.5 in PD medium, a paper disc that was immersed in the medium containing the transformant was placed on a LB agar medium evenly smeared with *E. coli* JB 109.

- 5 Antibacterial activity was identified by observing the formation of the clear zone (Figure 3; M: SJW-28 transformed with pPIC9, 1: transformant, 2: transformant).

EXAMPLE 4. Production of Korean cow lactoferrin polypeptide using SJW-28.

The following primers were prepared based on the known lactoferrin amino acid sequence.

10 Forward primer 5' – GGCTCGAGCTTGGACTGTGTCTGGCT – 3'

Xho I

Backward primer 5' – GGCTCGAGTTAATCMAGGGTCACAGCATC – 3'

Xho I

- A DNA fragment of about 220 bp containing a part of the Korean cow
 15 lactoferrin polypeptide was obtained by PCR using the Korean cow lactoferrin cDNA as a template. Expression vector pKLFC was obtained by treating the Korean cow lactoferrin polypeptide gene and pPIC9(Invitrogen) with *Xho*I, respectively and by connecting them. pKLFC was transformed in *E. coli*, Top 10F'.
 The transformed colony was selected by obtaining the growing colonies after
 20 smearing in an ampicillin containing medium. A plasmid containing the Korean cow lactoferrin polypeptide forwardly was selected by the treatment of restriction enzyme and PCR. The plasmid containing the Korean cow lactoferrin polypeptide in forward direction was isolated and this was found to have nearly similar

sequence to a part of the cow lactoferrin gene which as reported. The DNA sequences of the Korean cow lactoferrin polypeptide and the DNA sequences of the cow lactoferrin polypeptide were compared as below. The portions of different bases were marked with underline.

5

```

Cow:          CTTGGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTGATG
Korean Cow:   CTTGGACTGTGTCTGGCTGCCCCGAGGAAAAACGTTGATG
Cow:          GTGTACCATCTCCCAACCCGAGTGGTTCAAATGCCGCCGCC
Korean Cow:   GTGTACCATCTCCCGACCCGAGTGGTTCAAATGCCGCCGCC
10 Cow:        GATGGCAGTGGAGGATGAAGAAGCTGGGTGCTCCCTCTATC
Korean Cow:   GATGGCAGTGGAGGATGAAGAAGCTGGGTGCTCCCTCTATC
Cow:          ACCTGTGTGAGGAGGGCCTTTGCCTTGGAATGTATCCGGGC
Korean Cow:   ACCTGTGTGAGGAGGGCCTTTGCCTTGGAATGTATCCGGGC
Cow:          ATCGCGGAGAAAAAGGCGGATGCTGTGACCCTGGATGGT
15 Korean cow: ATCGCGGAGAAAAAGGCGGATGCTGTGACCCTTGATTAA
               (stop codon)

```

As described in Example 3, a plasmid containing the DNA of the Korean cow lactoferrin polypeptide in forward direction was transformed in *Pichia pastoris* SJW-28 by electrophoration. The strain inserted with the lactoferrin polypeptide gene was finally selected with PCR by using the aforementioned forward primer and backward primer.

After finally selecting the transformant it was cultured to OD₆₀₀ 1.3~1.5 in YPD medium, a paper disc that was immersed in the medium containing the transformant was placed on a LB agar medium evenly smeared with the test strains.

Antibacterial activity was identified by observing the formation of the clear zone. The results were represented in Figure 4A ~ 4F wherein the antibacterial activities were tested against the following microorganisms: Figure 4A: *Streptococcus mutans* KCTC 3065, Figure 4B: *Pseudomonas aerogenosa* KCTC 2004, Figure 4C: 5 *Enterobacter aerogenos* KCTC 2190, Figure 4D: *Escherichia coli* JM109 KCTC 2427, Figure 4E: *Salmonella*, Figure 4F: *Escherichia coli* o157:H7. (Figure 4A ~ 4F: C: SJW-28; 3: transformant; 6: transformant).

It is possible to mass produce the lactoferrin polypeptide and other antibacterial peptides by the present invention.

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT
OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO: Samyang Genex Corp.
#263 Yeongji-dong, Chongno-ku, Seoul 110-725,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: <i>Pichia pastoris</i> SJW-28	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: KCTC 0500BP
II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION	
The microorganism identified under I above was accompanied by: ☒ a scientific description ☐ a proposed taxonomic designation (Mark with a cross where applicable)	
III. RECEIPT AND ACCEPTANCE	
This International Depositary Authority accepts the microorganism identified under I above, which was received by it on July 08 1998 .	
IV. RECEIPT OF REQUEST FOR CONVERSION	
The microorganism identified under I above was received by this International Depositary Authority on _____ and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on _____	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: Korea Research Institute of Bioscience and Biotechnology Korean Collection for Type Cultures Address: KCTC, KRIBB #52, Oun-dong, Yusong-ku, Taejeon 305-333, Republic of Korea	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):  Kyung Sook Bae, Curator Date: July 13 1998

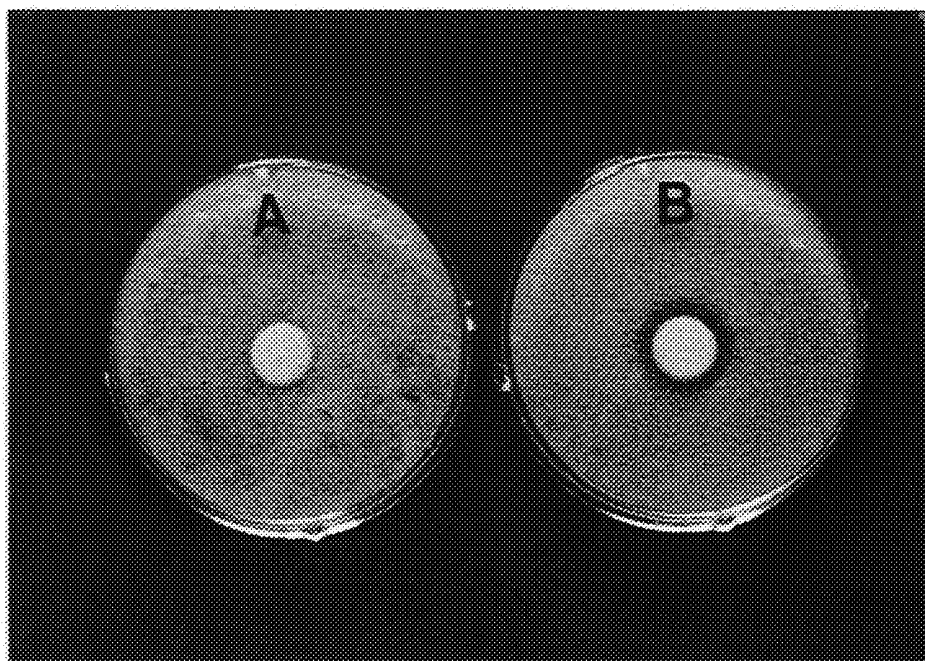
What is claimed is:

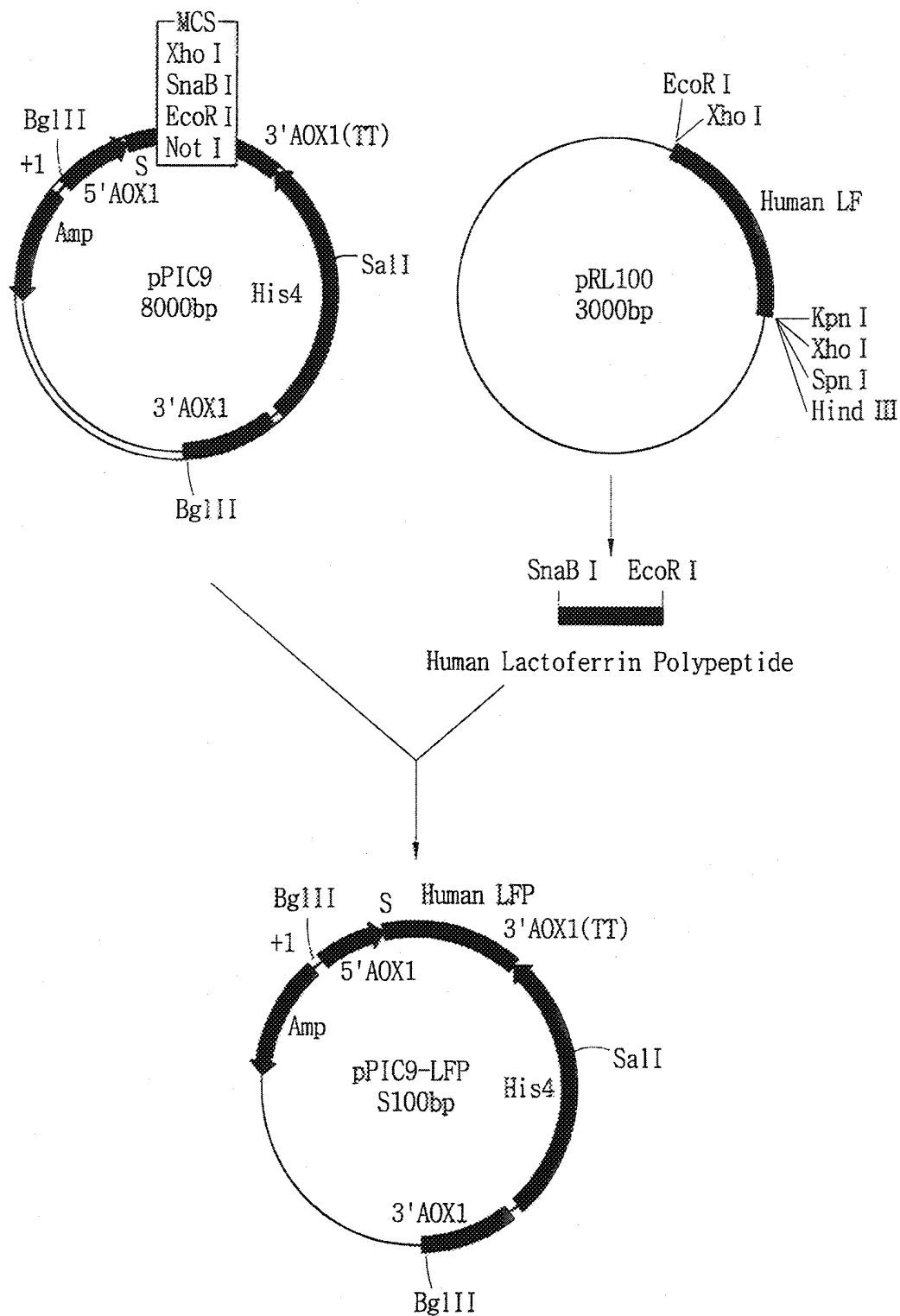
1. *Pichia pastoris* KCTC 0500BP.
2. A preparation method of lactoferrin polypeptides from microorganisms
5 comprises of the following steps;
 preparing a plasmid vector composed of a promoter sequence active in a
 yeast cell and a sequence that is connected to the promoter and which codes the
 lactoferrin polypeptides;
 transforming the yeast which is resistant against lactoferrin polypeptides
10 with above vector; and
 culturing the transformed yeast.
3. The method according to Claim 2 wherein the yeast cell is *Pichia* cell.
4. The method according to Claim 3 wherein the *Pichia* cell is KCTC 0500BP.
5. The method according to Claim 2 wherein the sequence coding lactoferrin
15 polypeptides is a sequence coding in the form of fusion peptide of lactoferrin or
 lactoferrin polypeptides.
6. A method according to the Claim 2 wherein the sequence coding lactoferrin
 polypeptides is a sequence coding lactoferrin polypeptides alone.

20

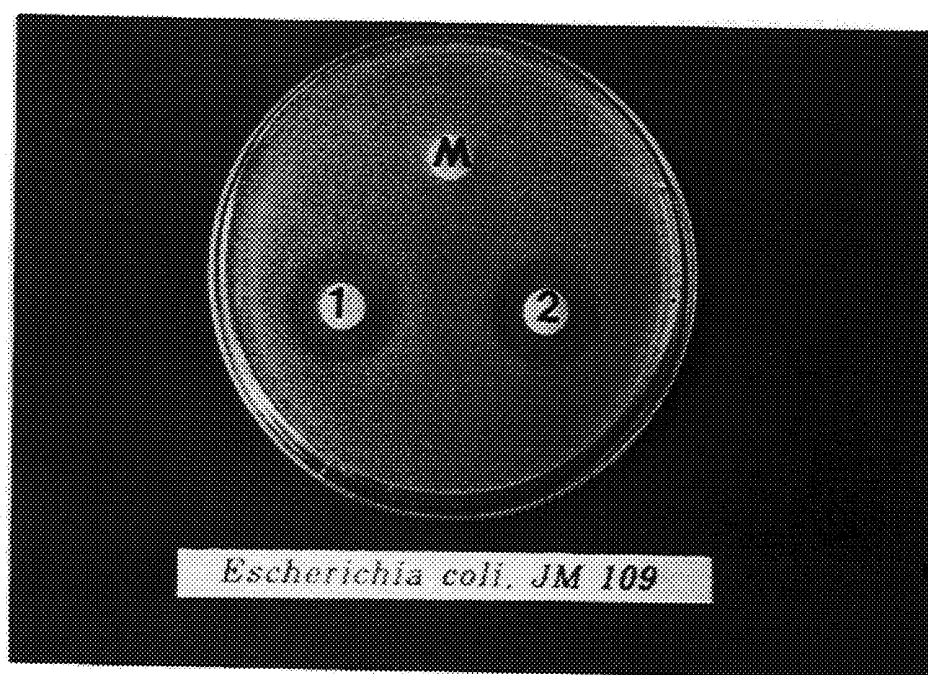
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^{1/6}
FIG. 1



2/6
FIG. 2

3/6
FIG. 3



4/6
FIG. 4A

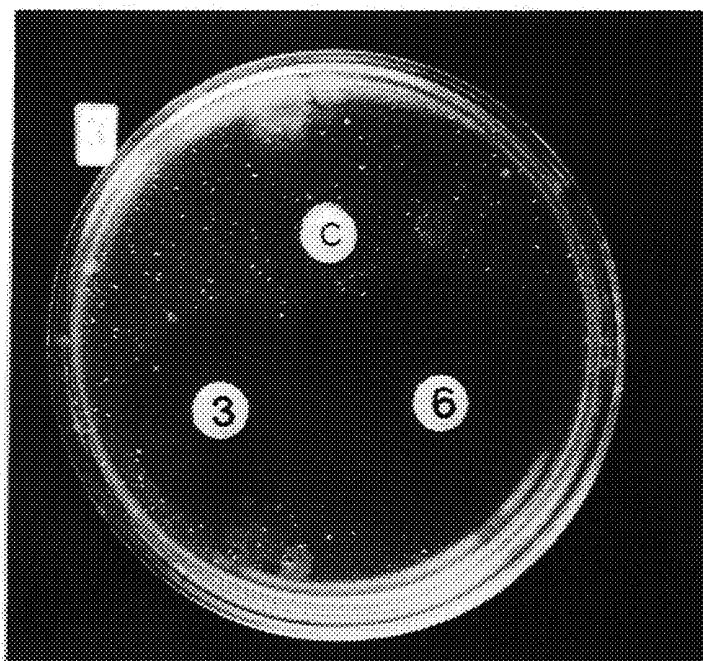
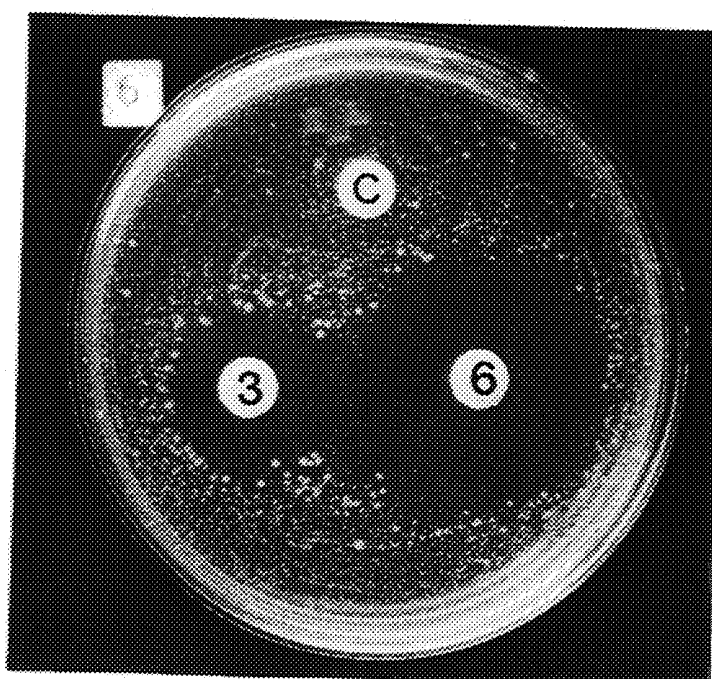


FIG. 4B



5/6
FIG. 4C

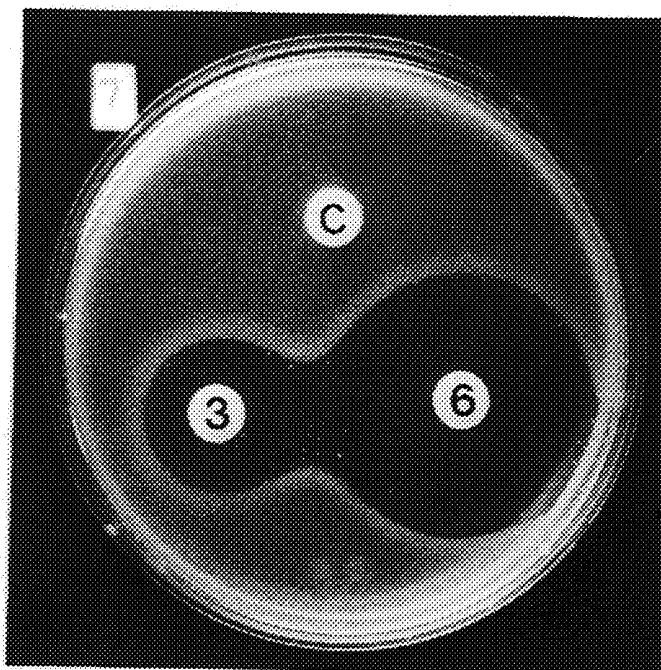


FIG. 4D



6/6
FIG. 4E

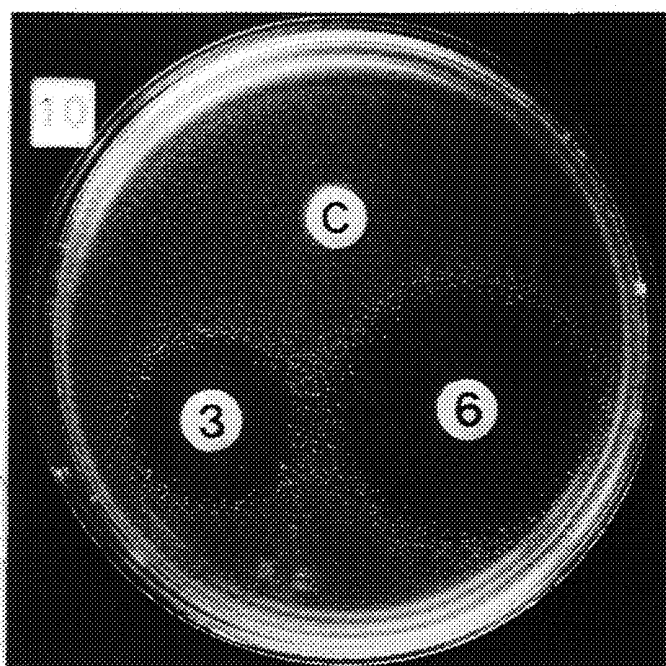
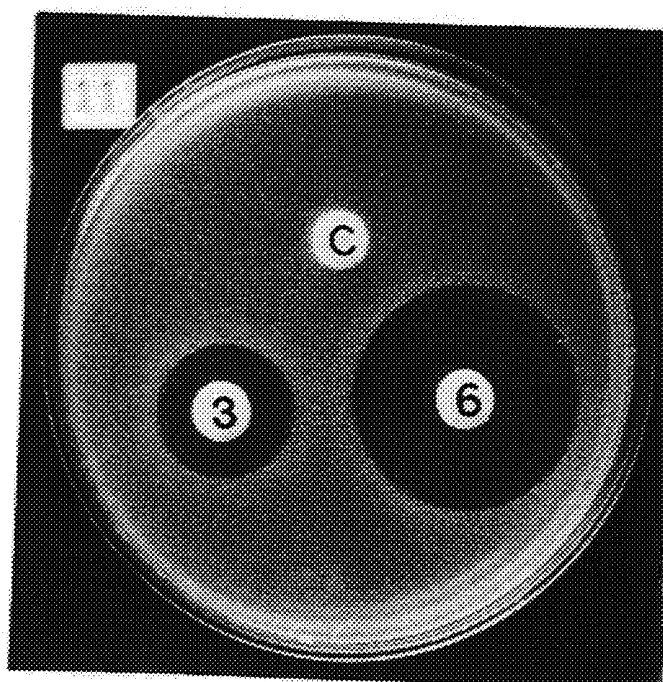


FIG. 4F



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 99/00373

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁷: C 12 N 1/16, 15/63, 15/12, (C 12 N 1/16; 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)

IPC⁷: C 12 N 1/16, 15/63, 15/12

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; CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Yim et al. Expression of human lactoferrin in Pichia pastoris Han'guk Sikp'um Yongyang Kwahak Hoechi 1997, 26(4), 669-674 (Korean) Columbus, Ohio, USA: Chemical abstract, Vol. 127, No. 26, 29 December 1997, page 96, left column, the abstract No. 355745y.	2, 3
A	Liang et al. Expression and characterization of human lactoferrin in yeast saccharomyces cerevisiae, I. Agric. Food Chem. 1993, 41(10), 1800-7 (Eng) Columbus, Ohio, USA: Chemical abstract Vol. 119, No. 23, 06 December 1993 page 175, left column, the abstract No. 242 250 Z.	2
A	WO 91 13 982 A1 (FERRODYNAMICS, INC.), 19 September 1991 (19.09.91), example 1; claims 1, 8 - 11, 38, 39.	2, 3
A	WO 93 22 348 A1 (BAYLOR COLLEGE OF MEDICINE), 11 November 1993 (11.11.93), claims 1, 3, 7, 8, 10, 11, 13, 16 -18, 21. ---	2

☐ 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

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„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

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„&“ document member of the same patent family

Date of the actual completion of the international search

6 October 1999 (06.10.99)

Date of mailing of the international search report

05 November 1999 (05.11.99)

Name and mailing address of the ISA/AT
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INTERNATIONAL SEARCH REPORT

Information on patent family members

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