J. Microbiol. Biotechnol. (2001), **11**(3), 508–514

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Cloning and Expression of a Yeast Cell Wall Hydrolase Gene (*ycl*) from Alkalophilic *Bacillus alcalophilus* subsp. YB380

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Received: February 15, 2001
Accepted: May 21, 2001

Abstract A structural gene (*ycl*) encoding novel yeast cell wall hydrolase, YCL, was cloned from alkalophilic *Bacillus alcalophilus* subsp. YB380 by PCR, and transformed into *E. coli* JM83. Based on the N-terminal and internal amino acid sequences of the enzyme, primers were designed for PCR. The positive clone that harbors 1.8 kb of the yeast cell wall hydrolase gene was selected by the colony hybridization method with a PCR fragment as a probe. According to the computer analysis, this gene contained a 400-base-paired N-terminal domain of the enzyme. Based on nucleotide homology of the cloned gene, a 850 bp fragment was amplified and the C-terminal domain of the enzyme was sequenced. With a combination of the two sequences, a full nucleotide sequence for YCL was obtained. This gene, *ycl*, consisted of 1,297 nucleotides with 27 amino acids of signal sequence, 83 redundant amino acids of pro-sequence, and 265 amino acids of the mature protein. This gene was then cloned into the pJH27 shuttle vector and transformed into the *Bacillus subtilis* DB104 to express the enzyme. It was confirmed that the expressed cell wall hydrolase that was produced by *Bacillus subtilis* DB104 was the same as that of the donor strain, by Western blot using polyclonal antibody (IgY) prepared from White Leghorn hen. Purified yeast cell wall hydrolase and expressed recombinant protein showed a single band at the same position in the Western blot analysis.

Key words: *Bacillus alcalophilus*, cell wall hydrolase, *Bacillus subtilis* DB104

Yeast is one of the most important organisms in biotechnology, particularly in the winery and baking industries. Yeast extract has been used for supplementary food and natural seasonings due to its high content of vitamins [17], inorganic salts [6, 8], some glucose tolerance factors [5, 9, 24], amino acids, proteins, and nucleic acids. It has been produced by the hydrolysis method, plasmolysis, autolysis, and enzymatic digestion. Among these autolysis is the most prevalent method in biotechnology, because it can produce good quality of yeast extract that contains much glutamic acid. On the other hand, this method takes a long time and has a low yield [19]. Hydrolysis is a rapid method to produce yeast extract but it also produces monosodium glutamate (MSG), monochloropropanol (MCP), and dichloropropanol (DCP), which are known to be possible carcinogens. Plasmolysis uses plasmolytic reagents such as NaCl, sucrose, ethanol, and isopropanol to dehydrate yeast cells. This is indeed a cheap and simple method, but it needs a high salt concentration. Enzymatic digestion using several enzymes of other microorganisms to hydrolyze the cell wall of yeast is one of the most effective ways to produce yeast extract. Enzymes used for production of yeast extract can also be used as additional amino acids and they do not have any effect on other components. Bacterial enzymes such as lysozyme have been used in research, elucidating the cell wall structure of the microorganisms [7], for food preservation because of their antimicrobial potentials [2, 3, 21], and in industrial microbiology such as biopolymer production [20]. They are also used for the isolation of the cytosolic fraction through a breakdown of the cell wall by treating with the lytic enzyme [12]. Several researches have been reported in regards to the cell wall hydrolases such as lysozyme [11], bacteriocin [22], and some bacterial proteases that have cell wall hydrolytic activities [14, 18].

Several models on yeast cell wall structure have been proposed [13]: Yeast cell wall consists of mannan, protein, chitin, and lipid, which make a network 0.1–0.4 μm thick; Yeast cell walls are composed of various specifically arranged polymers, including polysaccharides and proteins. *Bacillus circulans* was the first bacterium to be identified as the producer of extracellular enzymes that lysed yeast

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cell walls [29]. Several enzymes have been reported to have hydrolytic activity on yeast cell wall. They include glucanases [31] and proteinases [33]. We have previously reported characteristics of the microorganism which produced a novel yeast cell wall hydrolase, and purification and properties of the enzyme from alkalophilic *Bacillus alcalophilus* subsp. YB380, which was isolated from the soil [33]. In this report, we cloned the yeast cell wall hydrolase gene (*ycl*) from alkalophilic *B. alcalophilus* subsp. YB380. We also describe the nucleotide sequence of the hydrolase gene and the expression of the hydrolase in *Bacillus subtilis* DB104.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

E. coli JM83 was used for cloning of the cell wall hydrolase gene. *E. coli* JM83 and recombinant strains harboring the cell wall hydrolase gene were cultured in LB medium with or without 100 μg/ml ampicillin. *B. subtilis* DB 104 was used for the protoplast transformation.

Internal Amino Acid Sequencing

Lyophilized enzyme was dissolved in 70% formic acid to a 1% final concentration and mixed with an equal amount of 2% CNBr. After incubation at room temperature for 24 h in the dark, 10 volumes of distilled water was added to the reaction mixture to stop the reaction. The mixture was then dried under reduced pressure and suspended in a minimum amount of water. Digested protein was applied to SDS-PAGE and transferred onto the PVDF membrane. Protein bands were excised and subjected to the N-terminal amino acid sequencer. N-terminal amino acid sequencing was done by the method of Edman degradation [10] and analyzed using the Prosequencer 6600 (Milligen Co., U.S.A.).

Recombinant DNA Techniques

Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation, and other cloning-related techniques were used as described by Sambrook *et al.* [26]. Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.) and Boehringer Mannheim (Indianapolis, IN, U.S.A.). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Primers used for PCR and DNA sequencing were synthesized with a DNA synthesizer (Model 381A, Applied Biosystems, Foster City, CA, U.S.A.). Sequences of the primers used in this experiment are listed in Table 1.

Table 1. PCR primers used for this experiment.

Primers	Sequences
P1	5'-CAAACNGTNCCNTGGGGNAT-3'
P2	3'-TACCGNTGNGGNGTACANCG-5'
P3	5'-TTGAATTCTAGTTGGAGTAGGTCTCTT-3'
P4	5'-CCGGATCCTGAGGTAACGACAATGCAAA-3'
P5	5'-TTGTCGACAGATTACCAGCTGCGCAGGTTGG-3'
P6	5'-TTCTGCAGTAGTTGGAGTAGGTCTCTTTTGC-3'

Polymerase Chain Reaction

PCR was performed with a total volume of 100 μl, consisting of 0.8 μM of each primer, 2 to 5 μl of the template DNA solution (from 20 to 60 μg/ml), and sterile distilled water. The amplification reaction was performed in a model 480 thermal cycler (Perkin Elmer, U.S.A.) with the following cycling parameters: an initial denaturation at 95°C for 5 min, then 35 cycles consisting of 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min, followed by a final extension at 72°C for 10 min. Positive and negative controls were included in each PCR set and in each sample processing. The PCR products were then subjected to electrophoresis in a 1% agarose gel.

Colony Hybridization

Colonies grown on the LB plate that contained appropriate antibiotics were precooled at 4°C for 30 min and transferred to the nylon membrane disk by overlaying on the plate. The nylon membrane disk was sequentially incubated in C-1 (0.5 M NaOH, 1.5 M NaCl), C-2 (1 M Tris/HCl, pH 7.5, 1.5 M NaCl), and C-3 (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) buffers for 15 min and fixed under UV irradiation. The membrane disk was treated with 1 mg of proteinase K at 37°C for 1 h and cell debris was then removed with a water soaked filter paper. This membrane was used for Southern hybridization analysis [28].

DNA Sequencing Analysis

Nucleotide sequences were determined with the dideoxy-chain termination method, using the Fluoro-tagging automatic sequencing method. Samples were analyzed with an automated DNA sequencer (Model 310; Applied Biosystems, Foster City, CA, U.S.A.). The BLAST program (National Center for Biotechnology Information, U.S.A.) was used to find the protein coding regions.

Preparation of Protoplast and Transformation

Protoplast transformation was carried out by the method of Schaeffer [27] with slight modifications. *B. subtilis* DB104 was inoculated in a Bacto penassay broth (PAB) medium and incubated at 37°C overnight with shaking. Cells were harvested by centrifugation at 2,700 ×g for 10 min, when optical density of the broth reached 0.5 at 550 nm. The cell pellet was suspended in 5 ml of SMMP (equal mixture of 4× PAB and 2× SMM; 1 M sucrose, 0.04 M sodium maleate, 0.04 M $MgCl_2 \cdot 6H_2O$, pH 6.5) with 2 mg/ml of lysozyme and incubated at 37°C for 1 h. Protoplast formation was monitored by using a phase contrast microscope. Protoplasts

were harvested by centrifugation at 2,700 ×g for 15 min, washed two times with SMMP, and resuspended in 2 ml of SMMP for transformation.

One ml of the protoplast cell suspension was mixed with 1 ml of 2× SMM. Three ml of 40% PEG 6000 was added immediately after adding DNA. After incubation for 2 min at room temperature, 10 ml of SMMP was added and cells were harvested by centrifugation. One ml of SMMP was added and incubated at 37°C for 90 min with shaking. Cells were then plated on DM3 agar plate (0.5 M sodium succinate, 0.25% casamino acid, 0.5% yeast extract, 0.02 M K_2HPO_4, 0.01M KH_2PO_4, 0.6% glucose, 0.02 M $MgCl_2 \cdot 6H_2O$, 0.01% BSA, 0.8% agar, pH 7.3) containing 500 g/ml of kanamycin.

Preparation of Polyclonal Antibody

Polyclonal antibody (IgY) was prepared by the method of Akita *et al.* [1]. Two hundred μg/ml antigen (cell wall hydrolase) was mixed with the same amount of complete Freund's adjuvant and injected into a White Leghorn hen. Two weeks later, a booster injection was carried out with 100 μg/ml antigen mixed with the same amount of incomplete Freund's adjuvant. Seven days after the booster injection, antibody was purified from an egg. The egg yolk was isolated and washed with distilled water to remove albumin. This egg yolk was homogenized and diluted with distilled water, and 0.1 N of HCl was added to make pH 5.2. After 2 h incubation at 4°C, crude antibody was isolated by centrifugation at 10,000 ×g for 1 h, and was subjected to further purification by salt precipitation, alcohol precipitation, ultrafiltration, gel filtration, and ion exchange chromatography. This partially purified antibody was then further purified with the Immunopure plus (A) IgY purification kit (PIERCE Co., NY, U.S.A.).

Cell Wall Hydrolase Activity Assay

Yeast cell wall hydrolase activity was assayed by the method described previously [33]. Briefly, *Saccharomyces cerevisiae* KCCM 11290 cells were heated at 100°C for 20 min and then suspended in 50 mM Tris/HCl buffer (pH 9.0) to give an initial absorbance of 1.0 at 660 nm with a spectrophotometer (Shimazu UV 120-02). One-tenth ml of the enzyme solution was added to 2 ml of the cell suspension and the reaction mixture was incubated at 60°C for 10 min. The reduction of turbidity in the reaction mixture was measured at 660 nm. One unit of the cell wall hydrolase activity was defined as the amount of enzyme needed to decrease absorbance by 0.001 per minute.

Results and Discussion

Cloning of Cell Wall Hydrolase Gene

The DNA fragment that encodes the cell wall hydrolase (YCL) was amplified through PCR with primers which were designed based on the N-terminal and internal amino acid sequences of the enzyme. Purified enzyme was digested with CNBr and subjected to SDS-PAGE. The enzyme digested with CNBr showed two distinct bands of 14 kDa

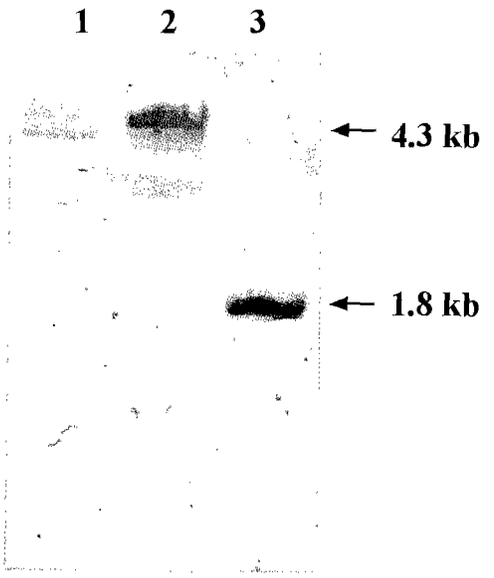

Fig. 1. Southern blot analysis of chromosomal DNA of *Bacillus alcalophilus* subsp. YB380 with 0.6 kb of PCR amplified product as a probe.
Lane 1: chromosomal DNA digested with *Bam*HI; Lane 2: chromosomal DNA digested with *Eco*RI; Lane 3: Chromosomal DNA digested with *Hind*III. Sizes of the DNAs are indicated by arrows.

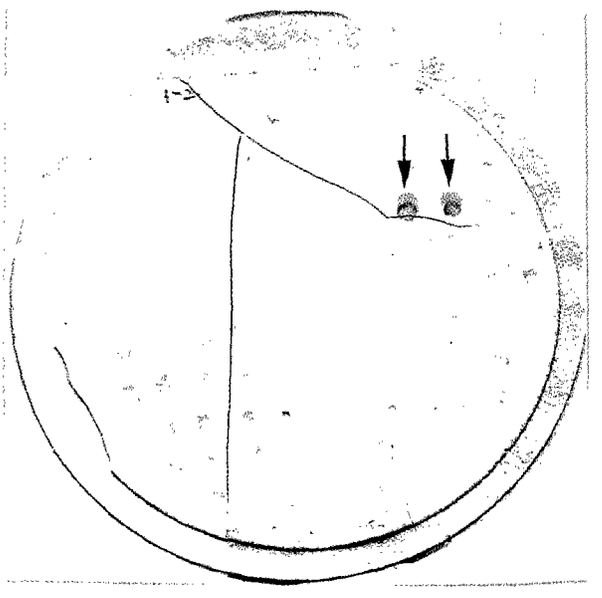

Fig. 2. Colony hybridization of a subgenomic library from the *Bacillus alcalophilus* subsp. YB380.
Positive clones are indicated by arrows. Duplicated colonies were blotted with NC membrane and hybridization was performed with a DIG-labeled probe.

and 6.0 kDa (data not shown). By Edman degradation, the amino-terminal amino acid sequence of the 14 kDa peptide was found to be identical to the sequence that was reported previously [33], but that of the 6.0 kDa peptide was quite different. Therefore, it can be inferred that the new sequence was an internal digested site of the enzyme. With those amino acid sequences of the enzyme, two degenerated primers (P1, P2) were designed, and a 600-base-paired PCR fragment was obtained through PCR. The size of this DNA fragment was similar to the size inferred from the CNBr digestion and SDS-PAGE analysis of the enzyme. This was used as a probe for Southern hybridization and colony hybridization for genomic library screening.

Chromosomal DNA of *B. alcalophilus* subsp. YB380 was partially digested with *Hin*dIII and the digest was applied to agarose gel electrophoresis. A 1.8 kb being fragment was identified as being positive through Southern hybridization with a probe that was generated through PCR (Fig. 1). This fragment was eluted and ligated into the pUC19 vector, and transformed into *E. coli* JM83. Two positive colonies were obtained through a process of colony hybridization (Fig. 2). Those colonies were transferred into a new plate, and plasmids

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attatgagccataacatgctttttttaatcatcaacttaacaaaaaagttaactattaatt      60
tacggatatttcccaatagctaaaaggtttcccaataccaaaagagttaaaatttttgtta     120
attttagattaccagctgcgcaggttggacatttt[aggagg]tataacgaattgaataag     180
                                                  M  N  K
aaaatggggaaaattgttgccggaacagcactaattatatcagtagcatttagttcatca     240
 K  M  G  K  I  V  A  G  T  A  L  I  I  S  V  A  F  S  S  S
atcgcacaagcagccgaggaagcgaaggaaaaatacctcattggctttaaggaacaagaa     300
 I  A  Q  A  A  E  E  A  K  E  K  Y  L  I  G  F  K  E  Q  E
gttatgtcccaatttgttgaccaaattgatggagatgagtattctatttcttcttctcaa     360
 V  M  S  Q  F  V  D  Q  I  D  G  D  E  Y  S  I  S  S  S  Q
gtggaagatgttgaaattgatctccttcatgaatttgattttattcctgttttatccgtt     420
 V  E  D  V  E  I  D  L  L  H  E  F  D  F  I  P  V  L  S  V
gaacttgacccacaagatgtagaagcattagagcttgaccctgccatctcctatatagag     480
 E  L  D  P  Q  D  V  E  A  L  E  L  D  P  A  I  S  Y  I  E
gaagatgctgaggtaacgacaatgcaaaccgttccatggggaattaaccgtgtacaagct     540
 E  D  A  E  V  T  T  [M] Q  T  V  P  W  G  I  N  R  V  Q  A
ccgattgctcaaagcagaggattcacaggtactggagttcgtgtggctgttttagacaca     600
 P  I  A  Q  S  R  G  F  T  G  T  G  V  R  V  A  V  L  D  T
gggatctcaaatcacgctgatttaagaatccgtggcggcgctagttttgtaccaggagag     660
 G  I  S  N  H  A  D  L  R  I  R  G  G  A  S  F  V  P  G  E
ccgaatattagtgatggaaacggtcatggtactcacgttgctggtacaattgcagcgtta     720
 P  N  I  S  D  G  N  G  H  G  T  H  V  A  G  T  I  A  A  L
aacaattcaattggtgtactcggcgtagcaccaaacgttgatttatatggggttaaagtg     780
 N  N  S  I  G  V  L  G  V  A  P  N  V  D  L  Y  G  V  K  V
ctaggagcaagtggctctgggtcaatcagtggtattgctcaagggttacaatgggctgca     840
 L  G  A  S  G  S  G  S  I  S  G  I  A  Q  G  L  Q  W  A  A
aataatggcatgcacattgcaaacatgagtttaggaagtagtgctgaatctgcaacaatg     900
 N  N  G  M  H  I  A  N  M  S  L  G  S  S  A  E  S  A  T  M
gaacaagctgttaaccaagcaacagcaagtggcgttcttgtagttgcggcttctggtaac     960
 E  Q  A  V  N  Q  A  T  A  S  G  V  L  V  V  A  A  S  G  N
tcaggtgcaggaaatgttggattcccagcacgctatgcaaatgcgatggcagtaggtgca    1020
 S  G  A  G  N  V  G  F  P  A  R  Y  A  N  A  M  A  V  G  A
acagatcaaaataacaaccgcgctagcttttctcagtacggagcaggtcttgacattgta    1080
 T  D  Q  N  N  N  R  A  S  F  S  Q  Y  G  A  G  L  D  I  V
gcaccaggtgttggtgtacaaagtacggttcctggtaatggatactcaagcttcaatggt    1140
 A  P  G  V  G  V  Q  S  T  V  P  G  N  G  Y  S  S  F  N  G
acgtctatggctacaccgcacgttgctggtgttgctgcgttagtgaagcaaaagaatcca    1200
 T  S  M  A  T  P  H  V  A  G  V  A  A  L  V  K  Q  K  N  P
tcttggtcaaatgttcaaattcgtaaccaccttaaaaatacggcaacaaacttaggaaat    1260
 S  W  S  N  V  Q  I  R  N  H  L  K  N  T  A  T  N  L  G  N
acaaatcaattcggaagtggtcttgttaacgcagaagcggcaacacgtaa              1309
 T  N  Q  F  G  S  G  L  V  N  A  E  A  A  T  R***
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Fig. 3. Nucleotide sequence of the gene coding yeast cell wall hydrolase.
Sequences found in CNBr cleaved fragments are shown in bold.

from those colonies were analyzed. Both plasmids were found to be identical to that harboring 1.8 kb heterogeneous DNA fragments. This plasmid was designated as pDBH1.

The nucleotide sequence of pDBH1 was analyzed with a double strand DNA sequencing method. Using the computer analysis of this nucleotide sequence, a 400-base-paired open reading frame (ORF) was found. This ORF showed high homology with several alkaline proteases of *Bacillus* sp. [30]. It was considered that this ORF contained only 400 bps of the enzyme gene with a truncated C-terminal, having, 99% homology with alkaline elastase [29]. For subsequent cloning of lytic enzyme gene through PCR, new primers (P3, P4) were designed based on the homology analysis between the cell wall lytic enzyme and alkaline elastase. An 850-base-paired PCR fragment was eluted and ligated with the pUC19 vector, as described above, to produce pDP-1, which was considered to have the C-terminal domain of the enzyme. By combining two sequences from pDBH1 and pDP-1, a full nucleotide sequence of the cell wall hydrolase gene was obtained (Fig. 3). This *ycl* gene encoding yeast cell wall hydrolase showed 90.2% homology to alkaline elastase. The deduced amino acid sequence of the enzyme contained the amino-terminal domain of purified enzyme (bold) [33]. This protein generates a mature protein through cleavage between Met-110 (boxed) and Gln-111. Generally, secretory proteins have signal sequences of about 30 amino acids including a positively-charged and highly hydrophobic domain at the N-terminus or in the middle of signal sequences [15, 23]. Translation initiation usually occurs at AUG, GUG, or UUG, preceded by the Shine-Dalgarno sequence (AGGAGG, boxed). According to this criteria and homology with alkaline elastase, YaB, a putative translation site, was investigated. Signal sequence of this gene starts at the 172nd nucleotide (TTG, bold) with 27 amino acid residues (underlined). Therefore, this gene which encoded cell wall hydrolase consisted of 1,297 nucleotides, starting with TTG which contained 27 amino acids of the signal sequence, 83 amino acids of the pro-sequence, and 265 amino acids of the mature protein. The molecular weight of this protein was estimated to be 26,600 Da, in agreement with a previously reported result [33].

Transformation and Expression in *Bacillus subtilis*

Bacillus alcalophilus subsp. YB380, a donor strain of the cell wall hydrolase, was an alkalophilic strain, and its optimum pH for growth was 9.0. Furthermore, biological characteristics of this strain have not yet been determined. Therefore, *Bacillus subtilis*, which is generally regarded as safe (GRAS), was used for expression of this enzyme at a neutral pH. Bacilli can secrete many proteins and their cell walls consist of peptidoglycan and teichoic acid. Proteins expressed by recombinant *B. subtilis* are usually not contaminated with lipopolysaccharide endotoxins and they do not show any toxicity on animals or animal tissues [4].

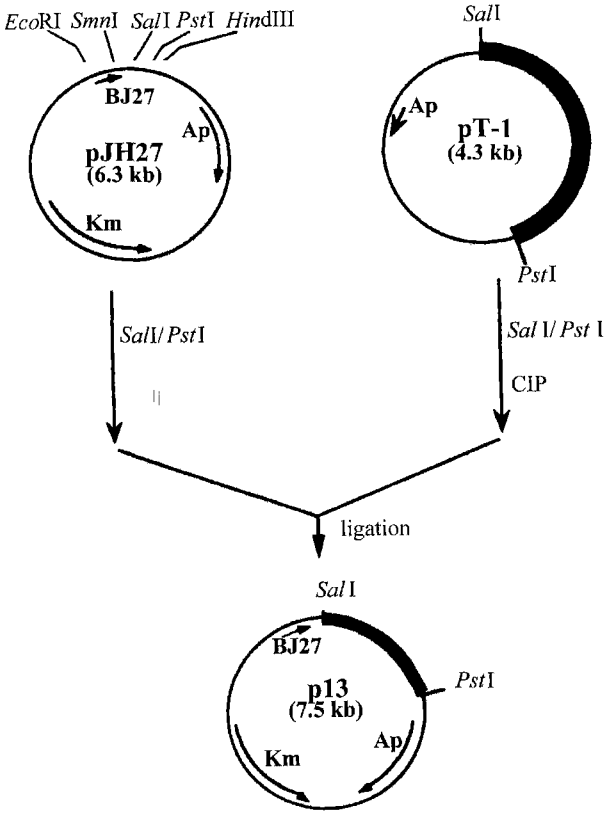

Fig. 4. Construction of expression vector, p13.
1.2 kb of the *SalI/PstI* fragment was eluted from pT-1 and inserted into the *Bacillus* expression vector, pJH27.

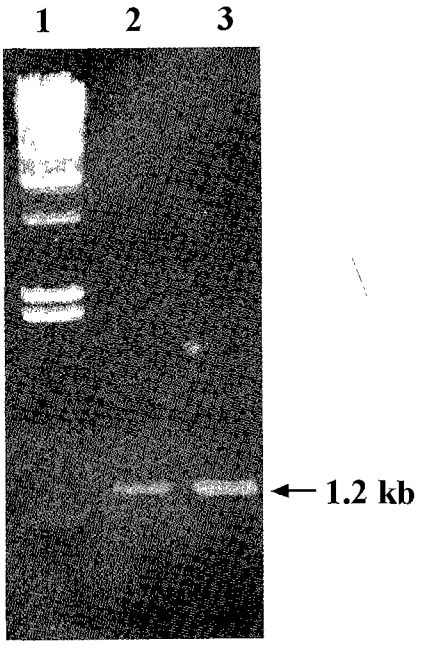

Fig. 5. Amplification of the whole sequence of the cell wall hydrolase gene.
Lane 1, marker; lane 2, 0.12 ng template DNA/μl; lane 3, 40.0 ng template DNA/μl.

Therefore, expression and the optimization in *B. subtilis* are very important in biotechnology [25]. Shuttle vector pJH27 [16] was used to construct expression vector p13 (Fig. 4). The cell wall hydrolase gene encoded the signal sequence, and mature protein was amplified through PCR with chromosomal DNA of *B. alcalophilus* subsp. YB380 as a template (Fig. 5). This fragment was eluted and ligated into pGEM®-T vector (Promega, U.S.A.) to make pT-1. Primers (P5 and P6) used in PCR had *Sal*I or *Pst*I sites to provide corresponding restriction sites at the N-terminus and C-terminus of the protein, respectively. These restriction sites enabled to identify the orientation of the gene when cloned into a shuttle vector. A 1.2 kb *Sal*I and *Pst*I fragment of pT-1 was eluted and ligated into the corresponding sites of pJH27, which were located downstream of the BJ27 promoter. Plasmid p13 was then transformed into *B. subtilis* DB104 and this strain was designated as *Bacillus subtilis* JH15. Recombinant *B. subtilis* JH15 harboring p13 was cultured in LB medium containing 50 μg/ml of kanamycin, and enzyme activity of the culture supernatant was 0.5 unit/ml, which was 20 times more than the donor strain (data not shown). Only the culture supernatant of *B. subtilis* DB104 showed lytic activity. Expression of the cell wall hydrolase in *B. subtilis* DB104 was confirmed through Western blot analysis. *B. subtilis* DB104 (pJH27) was used as a negative control. By using polyclonal antibody (IgY) prepared with White Leghorn hen, the expressed recombinant protein from *B. subtilis* JH15 was shown to have a single band with the same molecular weight as that of the purified enzyme from donor strain, *B. alcalophilus* subsp. YB380 (Fig. 6).

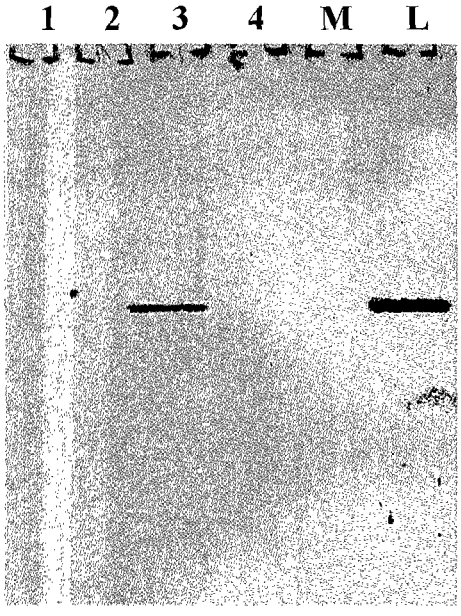

Fig. 6. Western blot of yeast cell wall lytic enzyme expressed in *B. subtilis*.

Lane 1, culture supernatant of *Bacillus subtilis* DB104 (pJH27); lane 2, cell extract of *Bacillus subtilis* DB104 (pJH27); lane 3, culture supernatant of *Bacillus subtilis* JH15 harboring p13; lane 4, cell extract of *Bacillus subtilis* JH15 harboring p13; M, molecular weight markers; L, purified cell wall hydrolase from *Bacillus alcalophilus* subsp. YB380.

In summary, a structural gene (*ycl*), encoding a novel cell wall hydrolase, YCL, was cloned from alkalophilic *B. alcalophilus* subsp. YB380 and transformed into *B. subtilis* DB104 to produce this enzyme. This gene had 90.2% of homology to the alkaline elastase gene, but repeated sequence analyses confirmed that this gene coded a new enzyme. Although this enzyme was originally produced and purified from the alkalophilic strain, it was successfully expressed in *B. subtilis* at a neutral pH. This was supported by previously reported results. This enzyme was stable at a pH range above pH 7.0 for 14 h [33]. Further research effort should be made to optimize the production.

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