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Isolation of Novel Alkalophilic *Bacillus alcalophilus* subsp. YB380 and the Characteristics of Its Yeast Cell Wall Hydrolase

YEO, IK-HYUN[1], SUK-KYUN HAN, JU-HYUN YU[1], AND DONG-HOON BAI*

Department of Food Engineering, Dankook University, Chunan, Chungnam 330-714, Korea
[1]Department of Biotechnology and Bioproduct Research Center, Yonsei University, Seoul 120-749, Korea

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Abstract An alkalophilic microorganism (strain YB380), which produces yeast cell wall hydrolase extracellulary, was isolated from Korean soil. The rod-shaped cells were 0.3~0.4 by 2~4 μm long, motile, aerobic, gram-positive, and spore-forming. The color of the colony was light yellow. The temperature range for growth at pH 9.0 was 25 to 45°C, with optimum growth at 35°C. The pH range for growth at 35°C was 8 to 11 with an optimum pH of 9.0. Therefore, the strain YB380 is an obligate alkalophile. The 16S rRNA of strain YB380 has a 99% sequence similarity with that of *Bacillus alcalophilus*. On the basis of physiological properties, cell wall fatty acid composition, and phylogenetic analysis, we propose that the isolated strain is *Bacillus alcalophilus*. The yeast cell wall hydrolase from *Bacillus alcalophilus* subsp. YB380 has been purified and partially characterized. The molecular weight was estimated to be 27,000 daltons with an optimum temperature and pH of 60°C and 9.0, respectively. The N-terminal amino acid sequence of the enzyme was analyzed as Gln-Thr-Val-Pro-Trp-Gly-Ile-Asn-Arg-Val.

Key words: Alkalophilic *Bacillus, Bacillus alcalophilus* subsp. YB380, yeast cell wall hydrolase, 16S rDNA

Since Fleming's 1922 discovery of lysozyme [14] from egg white, bacteria also have been known to produce several types of cell wall hydrolases. These include *N*-acetyl muramidase (lysozyme), *N*-acetylglucosaminidase, *N*-acetylmuramyl-L-alanine amidase, endopeptidase, and transglucosylase [10]. Many of these enzymes are bacteriolytic [26], and are believed to be involved in cell separation [9], the formation of helices [25], flagellation [8], and competence [1]. Bacteriolytic enzymes have been used in research elucidating the cell wall structure of microorganisms [10] and against microbial spoilage in food preservation because of their antimicrobial potential [3]. They are also used for the isolation of cytosolic fraction through breakdown of the cell wall by treatment with the lytic enzyme [13].

Yeast cells have been produced in large scale from various carbon sources and used in foods, feeds, and as yeast extract. However, the yeast cells are covered with indigestible cell walls composed of rigid mannoprotein connected with an inner glucan layer [17] and removal of them is necessary to improve digestibility or to extract the intracellular constituents. Up to now, considerable attention has been paid to the enzymatic degradation of yeast cells, and microorganisms capable of producing the yeast cell wall lytic enzymes have been found. Some of these microorganisms include *Rhizopus* sp. [32], *Basidiomycetes* [30], *Rhizoctonia* sp. [13], *Penicillium* sp. [2], and *Dicyma* sp. [5]. From soil collected from several areas in Korea, we have isolated a novel strain of alkalophilic bacterium, which produces a yeast cell wall hydrolase. In this paper, we describe the characteristics of the microorganism producing yeast cell wall hydrolase and the purification and properties of the enzyme from alkalophilic *Bacillus alcalophilus* subsp. YB 380.

MATERIALS AND METHODS

Screening of Microorganisms

Soils for the screening of alkalophilic bacteria were collected from several provincial areas of South Korea. Alkalophilic bacteria were grown on Horikoshi I agar medium (0.1% Glucose, 0.5% yeast extract, 0.5% Polypeptone, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% $NaHCO_3$, 0.2% KH_2PO_4, pH 9.0) [12] The microorganisms grown on Horikoshi agar medium were transferred to the Horikoshi I agar medium with 2% autoclaved and lyophilized *Saccharomyces cerevisiae* KCCM 11290. After

*Corresponding author
Phone: 82-417-550-3562; Fax: 82-417-554-4769;
E-mail: baidh@chollian.dacom.co.kr

incubation at 30°C for 24~48 h, microorganisms which had a clear zone around the colony were selected for primary screening. As a secondary screening procedure, 20 to 200 µl of each cell culture supernatant were applied to disc filter paper and placed on the surface of Horikoshi I agar plates with 2% heat inactivated and lyophilized yeast cells. After incubation for 1 h at 30°C, the sizes of the opaque haloes around the paper discs were measured. The cell wall lytic enzyme activity in the culture supernatant was also measured by the decrease of turbidity of the yeast cell suspension at $OD_{660\ nm}$.

Identification of Microorganism

For the identification of bacteria, its biochemical and physiological characteristics were examined according to the "Biochemical Tests for Identification of Medical Bacteria" [21] and "Bergey's Manual of Determinative Bacteriology" [18]. The fatty acid composition of the cell wall was analyzed by the Microbial Identification System (MIDI Inc. U.S.A.)

Preparation of Chrosomal DNA and Analyses of 16S rDNA

Strains YB380 was cultured overnight in Horikoshi I broth in Teflon-stoppered serum bottles with shaking on a rotary shaker at 250 rpm at 30°C. Cells were harvested by centrifugation, and chrosomal DNA was extracted with a lysozyme-sodium dodecyl sulfate-proteinase K protocol based on that of Marmur *et al.* [23]. The full-length 16S rRNA gene was amplified from chromosomal DNA by polymerase chain reaction (PCR) with forward primer (Eubacterial 27F: 5'-AGAGTTTGATCMTGGCTCAG-3') and reverse primer (Universal 1492R: 5'-GGYTACCTTGTTACGACTT-3') [7, 20]. The 50 µl reaction mixture contained the following final concentrations or total amount: 10 µl template DNA (50 ng/µl), 5 µl 10× reaction buffer (100 mM Tris·HCl, 400 mM KCl, 500 µg/ml BSA, pH 8.3), 1.5 mM $MgCl_2$, 5 µl deoxynucleoside triphosphates (2.5 mM each), 100 pmol of each primer, and 2.5 U *Taq* polymerase. Amplification was carried out in a thermocycler (Perkin-Elmer, U.S.A.). All reagents were mixed and then heated to 94°C for 4 min. Thirty cycles of PCR were then run at 94°C for 2 min, 55°C for 1 min, and 72°C for 2 min followed by 72°C for 10 min. PCR products were visualized on a 0.8% agarose gel, the size was confirmed to be correct, and the band was excised and purified by a Bio101 gene cleaning kit (Bio rad, U.S.A.). The purified PCR products were then cloned into pGEM-T vector (Promega Co., U.S.A.) which is constructed with a 3'-terminal thymidine on each end of a blunt-end digestion product, thus improving the efficiency of ligation of PCR products into the vector by taking advantage of the non-template-dependent addition of a single deoxyadenosine to the 3' end of the PCR product by *Taq* polymerase. The clones with the full-length insert (approximately 1.5 kbp) were sequenced by using an ALF Red automated DNA sequencer (Pharmacia, Uppsala, Sweden) and M13 universal primers. The sequences of approximately 1510 nucleotide bases corresponding to the *Escherichia coli* 16S rDNA sequence from nucleotides 27 to 1508 were analyzed in both directions for strain YB380, and deposited to Genebank. The accession number is AF078812.

Phylogenetic Analyses

The 16S rDNA secondary structures of strains YB380 were constructed with templates published in the Ribosomal Database Project (RDP) [22] to aid in the identification of homologous sequence positions. Multiple sequence alignments were performed manually in the CLUSTAL W [33]. All reference sequences and the basic alignment were obtained from the Genbank. Only homologous sites at which the 16S rDNA sequences of strains YB380 could be aligned unambiguously with the reference sequences were included in a final data set of about 1500 nucleotides for further analyses. A neighbor-joining phylogenetic tree was constructed using the CLUSTAL W program [28]. The gene accession number of the microorganisms from GenBank used for phylogenetic analyses are as follows: *Bacillus thermoalkalophilus* DSM 6866, Z26931; *Bacillus thermoleovorans* JCM 7361, Z26923; *Aneurinibacillus aneurinolyticus* ATCC 12856, D78455; *Brevibacillus brevis* JCM 2503, D78457; *Bacillus psychrophilus* ATCC 23304, X60634; *Bacillus subtilis* NCDO 1769, X60646; *Paenibacillus polymyxa* NCDO 1774, X60632; *Bacillus megaterium* DSM 32, X60629; *Paenibacillus macerans* NCDO 1764, X60624; *Bacillus coagulans* NCDO 1761, X60614; *Bacillus cereus* NCDO 1771, X55060; *Bacillus pseudalcaliphilus* DSM8725, X76449; *Bacillus halmapalus* DSM8723, X76447; *Bacillus gibsonii* DSM8722, X76446; *Bacillus agaradhaerens* DSM8721, X76445; *Bacillus clarkii* DSM8720, X76444; *Bacillus halodurans* DSM8718, X76442; *Bacillus* sp. DSM8717, X76441; *Bacillus clausii* DSM8716, X76440; *Bacillus pseudofirmus* DSM8715, X76439; *Bacillus* sp. DSM8714, X76438; *Bacillus cohnii* DSM6307, X76437; *Bacillus acidocaldarius* DSM 446, X60742; *Bacillus alcalophilus* DSM485, X76436; *Sporolactobacillus inulinus* NRIC 1134, D16284; *Bacillus horti* K13, D87035; *Bacillus thermocloacae* DSM5250, Z26939; *Bacillus horikoshii* DSM8719, X76443; *Bacillus* sp. DSM8724, X76448; *Bacillus pallidus* DSM3670, Z26930.

Assays for Yeast Cell Wall Hydrolase Activity

Yeast cell wall hydrolase activity was determined by the method of Kiyoshi Hayashi [11] with modification. The *Saccharomyces cerevisiae* KCCM 11290 cells were heat treated 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). To 2 ml of this suspension was added 0.1 ml of enzyme solution 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 cell wall hydrolase activity was defined as the amount of the enzyme which caused a decrease of 0.001 absorbance per minute.

Protein Determination

Protein from the column was measured by A_{280}. Protein concentration was determined by the method of Bradford *et al.* [4], using bovine serum albumin as the standard.

SDS-polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method [19] with 12% polyacrylamide gel containing 0.1% SDS and Tris-glycine buffer (pH 8.5) at a constant current of 20 mA. After electrophoresis, the protein was stained with Coomassie Brilliant blue R-250.

Purification of Yeast Cell Wall Hydrolase

For enzyme purification, all procedures were performed at 4°C. The chromatographic procedure is described in the Result section below and in the figure legends.

RESULTS AND DISCUSSION

Screening of Microorganism Producing Yeast Cell Wall Lytic Enzyme

Among 235 alkalophilic microorganisms collected from Korean soil samples, 12 strains which showed yeast cell wall hydrolase activity were selected at the primary screening step. From among these 12 strains, strain YB 380 was chosen because it showed the highest lytic activity against cell wall of *Saccharomyces cerevisiae* KCCM 11290 with the least amount of culture supernatant in the secondary screening procedure. The lytic halo around strain YB380 is shown in Fig. 1.

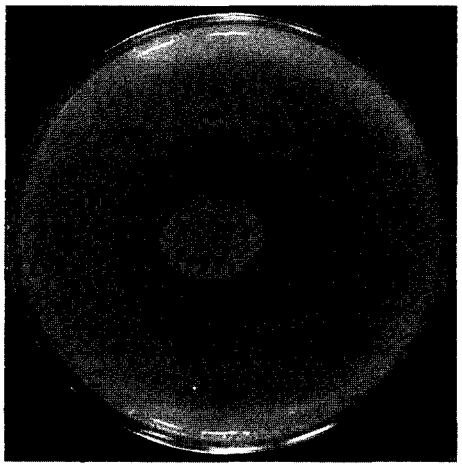

Fig. 1. Hydrolysis of *Saccharomyces cerevisiae* KCCM 11290 by *Bacillus alcalophilus* subsp. YB380 on agar plate.

Appearance and Physicochemical Characteristics of Strain YB380

Under scanning electron microscopy, the morphology of the strain YB380 was rod-shaped with cells 0.3~0.4 by 2~4 μm in size, but some of the cells were extraordinarily long, as shown in Fig. 2. It was motile, aerobic, Gram-positive, and spore forming. The color of the colony was light yellow and showed similar biochemical characteristics to *Bacillus subtilis*, as summarized in Table 1.

Fatty Acid Composition of the Cell

The major fatty acids of strain YB380 are iso-$C_{15:0}$ (57.2%) and anteiso-$C_{15:0}$ (15.4%). The rest of the fatty acids are straight saturated fatty acids $C_{14:0}$ (2.4%), $C_{15:0}$ (1.0%), $C_{16:0}$ (1.7%), and monounsaturated $C_{16:1}$ (8.2%) and branched saturated fatty acids iso-$C_{14:0}$ (3.7%), iso-$C_{16:0}$ (0.8%), iso-$C_{17:0}$ (5.6%), iso-$C_{17:1}$ (1.8%), and anteiso-$C_{17:0}$ (2.2%) (Table 2). It has been suggested that the branched fatty acid increases the fluidity of the membrane. Because the 16S rDNA sequence of strain YB380 shows 94% similarity with that of *Bacillus alcalophilus*, we compared the fatty acid composition of strain YB380 with that of *Bacillus alcalophilus*. As in *Bacillus alcalophilus* which is an obligate alkalophile, the branched 15-C fatty acids, iso-$C_{15:0}$ and anteiso-$C_{15:0}$ were the predominant fatty acids in strain

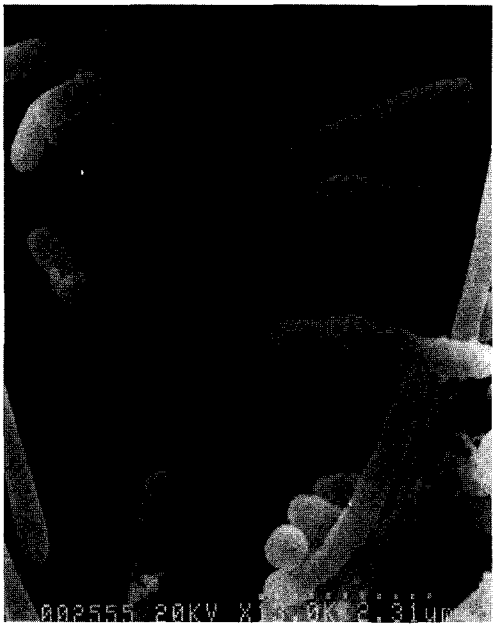

Fig. 2. Electron micrograph of *Bacillus alcalophilus* subsp. YB380. ×13,000.

Table 1. Biochemical and physiological properties of isolated strain YB380.

	Strain YB380	*Bacillus subtilis*[a]
Color of colonies	yellowish white	yellowish white
Form	rods	rods
Motility	+	+
Spore formation	+	+
Gram staining	+	+
Catalase	+	+
Oxidase	+	+
Indole production	-	-
Growth range	20~40°C	10~50°C
Hydrolysis of		
Casein	+	+
Gelatin	-	+
Starch	+	+
Growth in		
0% NaCl	+	+
2~7% NaCl	+	+
10% NaCl	+	-
15% NaCl	+	-
20% NaCl	-	-
Reduce NO_3 to NO_2	-	+

[a]Data of *Bacillus subtilis* is obtained from the Bergey's Manual of Systematic Bacteriology, volume 2.

Table 2. Fatty acid composition of total membrane lipid extract from *Bacillus* sp. YB380 and *B. alcalophilus*.

Fatty acid(s)[a]	% of fatty acid(s) in total membrane lipid extract of;	
	Bacillus sp. YB 380 (pH 10.2)	*Bacillus alcalophilus*[c] (pH 10.5)
Iso-$C_{12:0}$	ND[b]	2
Iso-$C_{14:0}$	3.7	1
n-$C_{14:0}$	2.4	1
Iso-$C_{15:0}$	57.2	21
Anteiso-$C_{15:0}$	15.4	29
n-$C_{15:0}$	1.0	ND
Iso-$C_{16:0}$	0.8	6
n-$C_{16:0}$	1.7	5
n-$C_{16:1}$	8.2	13
Iso-$C_{17:0}$	5.6	12
Anteiso-$C_{17:0}$	2.2	1
Iso-$C_{17:1}$	1.8	2
Anteiso-$C_{17:1}$	ND	2
n-$C_{18:0}$	ND	2
n-$C_{18:1}$	ND	1
n-$C_{18:2}$	ND	2

[a]Fatty acids are abbreviated such that the number of carbon atoms precedes the colon and the number of double bonds follows the colon. The prefixes anteiso and iso represent types of branched-chain structure.
[b]ND, not detected.
[c]Fatty acid composition of *Bacillus alcalophilus* obtained from reference 6.

YB380, but iso-$C_{15:0}$ outweighed anteiso-$C_{15:0}$ while the reverse was found in *Bacillus alcalophilus* [6]. The fatty acid composition of strain YB830 is similar to that of recently isolated *Bacillus horti* [33] which is a gram-negative alkalophilic strain.

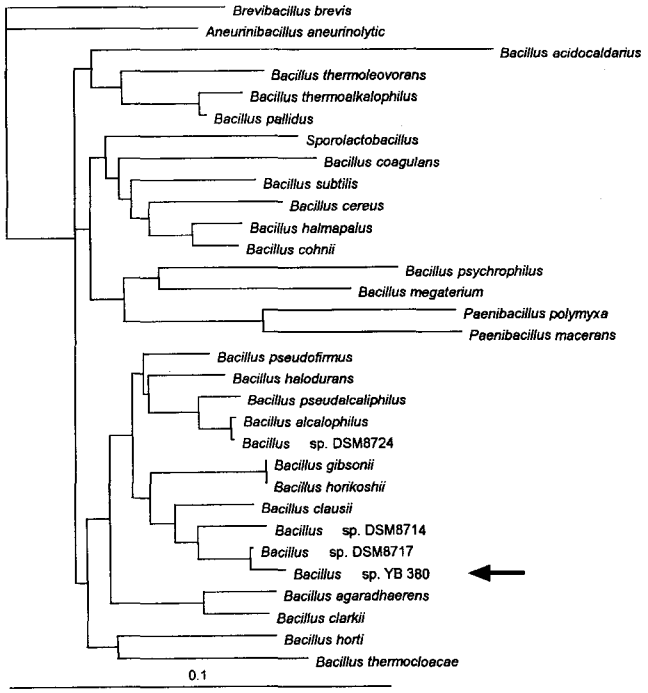

Fig. 3. Phylogenetic tree showing the relationship between the isolated alkalophilic *Bacillus* strain YB380 and other species belonging to the genus *Bacillus*.
Scale bar, 0.1 estimated substitution per nucleotide position.

16S rDNA Sequence Analyses

Strain YB380 possesses 16S rDNA sequences similar to alkalophilic *Bacillus*. Bootstrap analyses confirmed that strain YB380 clustered with alkalophilic bacteria (Fig. 3). The *Bacillus alcalophilus* fell into a closely related, coherent group, distinct from the other bacteria examined. Within the genus, the alkalophilic bacteria defined a distinct line of evolutionary descent, while the precise relationships among the other species remained unresolved because of low Bootstrap values. The full-length 16S rDNA sequences of strain YB380 consists of 1504 nucleotides. The levels of similarity of the strain YB380 sequences with alkalophilic *Bacillus* ranged from 91.0 to 94.0 %. The strain YB380 sequences were most identical to the sequences of *B. alcalophilus* (similarity, 94.0%). From fatty acid composition analysis of the cell wall and 16S rRNA sequence analysis, we propose that strain 4380 should be assigned to a species of *Bacillus alcalophilus*, and we named it as *Bacillus alcalophilus* subsp. YB380

Temperature, pH, and NaCl Effect on Cell Growth

The growth of strain YB380 occurred in the temperature range of 25 to 50°C with optimum growth occurring at 35°C (Fig. 4) at pH 9.0. The initial pH range of cell

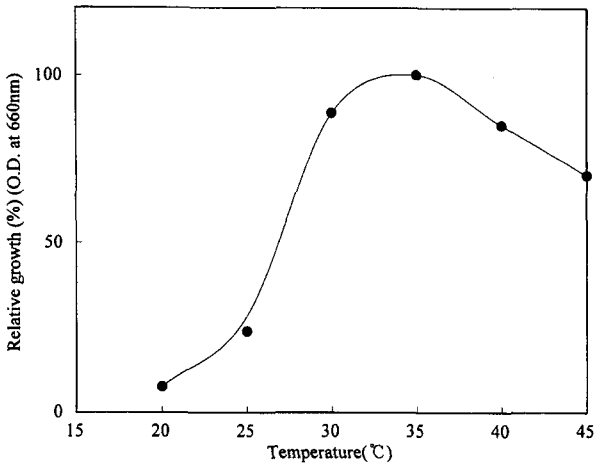

Fig. 4. Effect of temperature on growth of *Bacillus alcalophilus* subsp. YB380 in Horikoshi medium at pH 9.0.

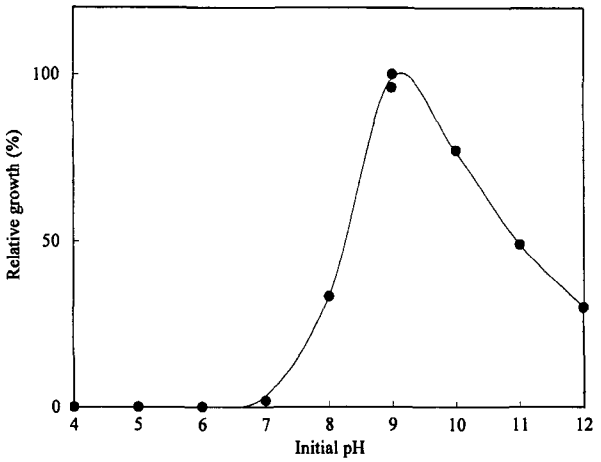

Fig. 5. Effect of initial pH on the growth of *Bacillus alcalophilus* subsp. YB380 in Horikoshi medium at 35°C.
pH 4.0 to 7.0, 50 mM Phosphate buffer; pH 7.0 to 9.0, 50 mM Tris-HCl buffer; pH 9.0 to 12.0, 50 mM carbonate buffer.

growth was measured from pH 7.0 to pH 12 with optimum pH at 9.0. Below pH 7.0, the growth of strain YB380 stopped, which indicates that it belongs to the obligate alkalophile *Bacillus* group of bacteria (Fig. 5). The strain was able to grow in the presence of up to 15% NaCl.

Production of Enzyme

Yeast cell wall hydrolase was produced from a jar fermentor with 3 l working volume. 40 ml of the overnight grown seed culture was inoculated to culture media for enzyme production. Enzyme production was optimized with the following media composition: 1% soluble starch, 0.5% yeast extract, 0.5% corn steep liquor, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 1% $NaHCO_3$, pH 9.0. In particular, the substitution of 0.5% polypeptone in Horikoshi I medium with 0.5% corn steep liquor increased

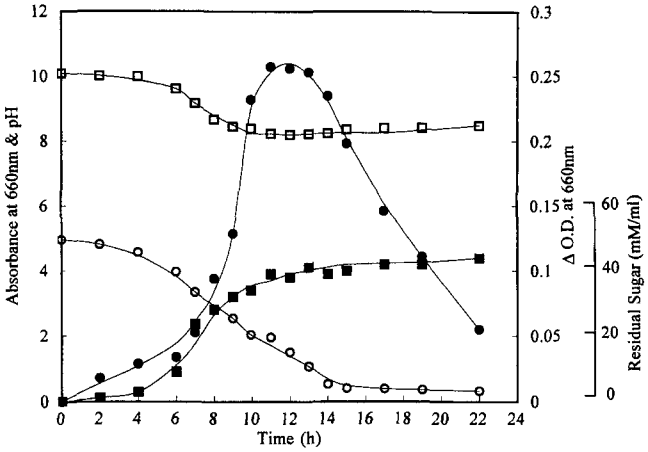

Fig. 6. Time course of cell growth, reducing sugar, pH, and the production of yeast cell wall hydrolase in 4 l jar fermentor.
The cultures were performed with a 3 l working volume. Symbols: (■) cell growth, (○) reducing sugar content, (□) pH of broth, (●) hydrolase activity.

the amount of enzyme two fold in the culture supernatant. The maximum amount of enzyme was obtained at 12 h of incubation as shown in Fig. 6. The pH of the culture supernatant was gradually decreased from an initial pH 9 and remained at pH 8 as with other alkalophilic bacteria.

Enzyme Purification

The purification procedure of yeast cell wall hydrolase consisted of ethanol precipitation and CM-sepharose column chromatography. Three volumes of cold ethanol was added to the culture broth immediately after the removal of bacterial cells by centrifugation. When the ethanol precipitated fraction of the culture supernatant was applied to DEAE-sepharose resin, most of the yeast cell wall hydrolase activities were detected from the unbound fraction. After overnight incubation of an ethanol mixture at 4°C, the protein fraction was pelleted by centrifugation at 12,000×g for 20 min. The pellet was redissolved to 1 l of 10 mM sodium phosphate buffer (pH 6.6) and applied to a CM-sepharose column (10× 30 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.6). The yeast cell wall hydrolase fraction was eluted with a linear gradient of NaCl from 0~0.5 M in the same buffer. As shown in Fig. 7, the enzyme fraction was eluted from the CM-sepharose column with 0.1 M of NaCl and it was found to be homogeneous as a single protein band when it was analyzed by 12% SDS-poly acrylamide gel electropheresis (Fig. 8, lane 4). The enzyme was purified about 12.9-fold with a 25.7% yield, and specific activity was 1030 U/mg of protein as shown in Table 3. When the enzyme from CM-sepharose column was purified with Sephadex G-75, it was eluted at around 27,000 daltons fraction, and there was no more increase in specific activity of yeast cell

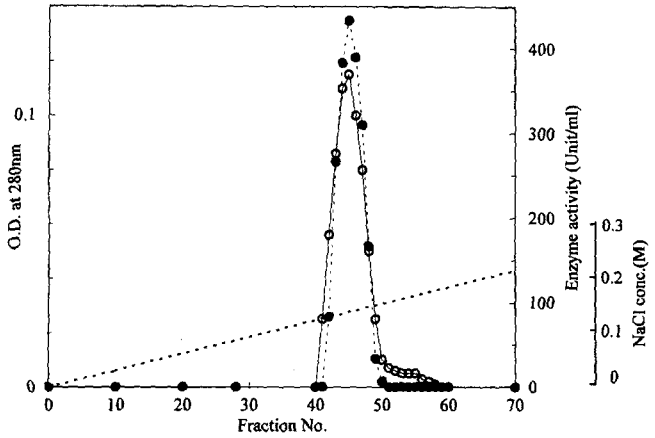

Fig. 7. Column chromatogram of yeast cell hydrolase from *Bacillus alcalophilus* subsp. YB380 on CM-Sepharose.

The column (300 ml) was equilibrated with 10 mM $NaHPO_4$ buffer (pH 6.6). Bound protein was eluted with 0~0.5 M NaCl gradient in 10 mM $NaHPO_4$. Symbols: ○, $OD_{280\,nm}$; ●, enzyme activity; ···, NaCl gradient.

wall hydrolase (data not shown). For the analysis of purified protein by SDS-PAGE, we denatured the protein sample with 5% trichloroacetic acid (TCA) prior to applying to the gel. Otherwise, the enzyme protein was successively degraded during the gel running because of its intrinsic protease activity. The molecular weight of purified enzyme was estimated as 27,000 daltons by SDS-PAGE. From these results, we concluded that the yeast cell wall hydrolase from *Bacillus alcalophilus* subsp. YB380 strain is composed of a monomeric subunit with a molecular weight of 27,000 daltons.

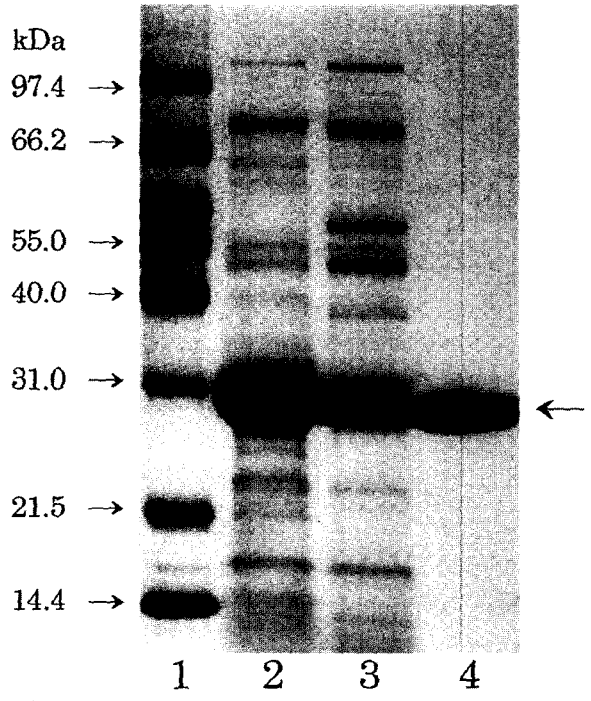

Fig. 8. Analysis of the enzyme purification procedure by SDS-Polyacrylamide gel electrophoresis.

Lane 1, molecular weight marker; Lane 2, culture broth; Lane 3, ethanol precipitate; Lane 4, CM-sepharose fraction. Gel concentration was 14% and yeast cell wall hydrolase is indicated by the arrow ←.

Table 3. Purification table of yeast cell wall hydrolase.

	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification folds
Culture broth	290	80.2	100.0	1.0
Ethanol precipitate	59	122.0	31.0	1.5
CM-agarose	5.81	1030.0	25.7	12.9

Effect of pH on Enzyme Activity and Stability

The effect of pH on enzyme activity and stability is shown in Fig. 9. The yeast cell wall hydrolase was most active at pH 9.0 which is similar to the pH profile of the cell growth. The enzyme showed its activity at an alkaline pH range above pH 7.0, but was mostly inactive in the acidic pH range. The pH effect on enzyme stability was investigated. Enzymes were incubated at a pH range from 2 to 13 at 4°C for 14 h and the remaining cell wall hydrolase activity was measured. The yeast cell wall hydrolase showed stable enzyme activity at a pH range between pH 6.0 and pH 13.0, but the enzyme stability decreased rapidly at the acidic pH range below pH 5.0.

Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on yeast cell wall hydrolase activity at pH 9.0 is shown in Fig. 10. The optimum temperature for the enzyme reaction was 60°C and 50% of the maximal activity was found at 30°C. The temperature

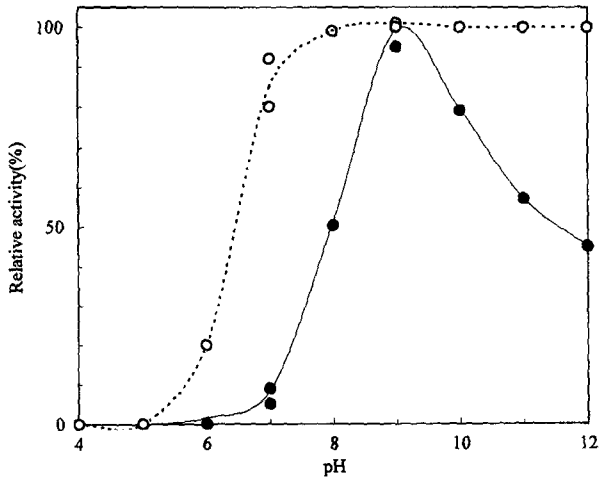

Fig. 9. Effect of pH on purified enzyme activity and stability.

pH 4.0 to 7.0, 50 mM phosphate buffer; pH 7.0 to 9.0, 50 mM Tris-HCl buffer; pH 9.0 to 12.0, 50 mM carbonate buffer. Symbols: ●, activity; ○, stability.

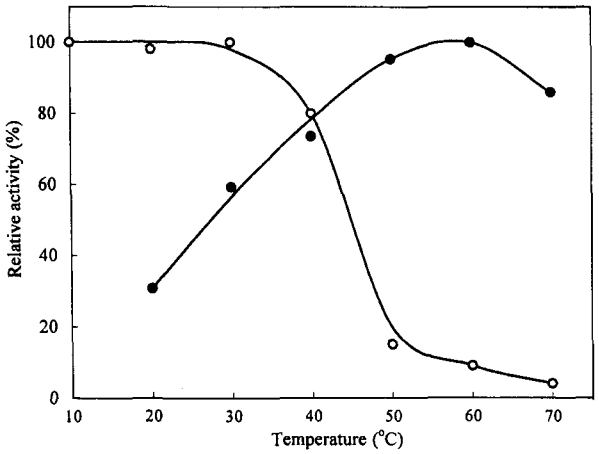

Fig. 10. Effect of temperature on purified enzyme activity and stability at pH 9.0.
Symbols: ●, activity; ○, stability.

effect on enzyme stability was also investigated. The enzyme was incubated at each temperature for 1 h and the remaining activity was measured. It showed that above 40°C, the enzyme activity greatly decreased. Because the yeast cell wall hydrolase has endopeptidase activity and the autolysis of the enzyme during incubation was observed (data not shown), we speculated that the instability of the enzyme at above 40°C is partly because of the autolysis of the enzyme during the incubation time. The cell wall hydrolysis of the enzyme was also measured at 40°C, 50°C, and 60°C for 3 h (Fig. 11). At 40°C, the enzyme hydrolysed the yeast cell wall linearly for one hour. As the reaction temperature increased and reaction time increased, the enzyme reaction rate gradually decreased. At 60°C, the linear hydrolysis was shown only for 10 min. Because Ca^{2+} increased the enzyme stability of alkaline protease YaB [29] which has an N-

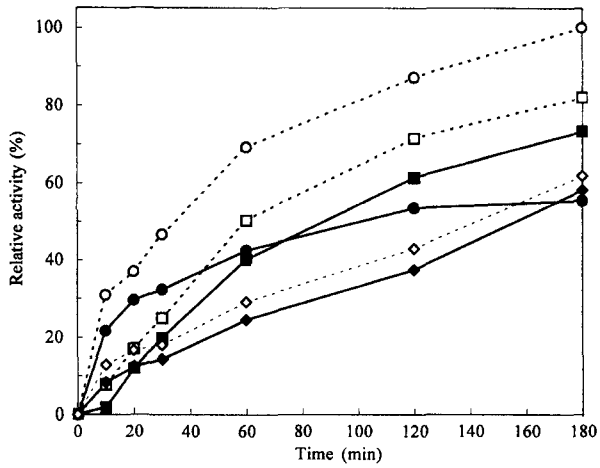

Fig. 11. Time course of yeast cell wall hydrolysis by purified enzyme with 5 mM Ca^{2+} (···) and without Ca^{2+} (—).
Symbols: (◆, ◇), 40°C; (■, □), 50°C; (●, ○), 60°C.

Table 4. N-terminal amino acid sequence of yeast cell wall hydrolase from the isolated strain and comparison with related proteins.

		Reference
Bacillus alcalophilus subsp. YB380	QTVPWGINRV	this work
Alkaline elastase YaB	QTVPWGINRV	15
Alkaline protease	AQSVPWGISRV	27
Subtilisin BPN	AQSVPYGVSQI	24

terminal sequence identity with the yeast cell wall hydrolase, we examined the effect of Ca^{2+} on yeast cell wall hydrolysis. When 5 mmol of Ca^{2+} was introduced into the reaction mixture, the linear hydrolysis was extended to 1 h of reaction. From this result, we concluded that the decrease of enzyme activity above 40°C was not because of autolysis but because of its instability, and introducing Ca^{2+} into the enzyme reaction could improve the heat stability of this enzyme.

N-terminal Amino Acid Sequence of Purified Enzyme

The purified yeast cell wall hydrolase was separated by 14% SDS-PAGE and western transferred to polyvinylidine difluoride (PVDF) membrane. After staining the blotted membrane with Coomassie Brilliant blue R-250, the protein band corresponding to MW 27,000 daltons was excised with a razor blade and subjected to N-terminal amino acid sequencing using gas phase sequenator. (Model 476A, Applied Biosystems Inc., U.S.A.). As shown in Table 4, the N-terminal amino acid was analyzed as Gln-Thr-Val-Pro-Trp-Gly-Ile-Asn-Arg-Val. Comparison of the N-terminal amino acid sequence with other sequences revealed complete sequence identity with the N-terminal amino acid sequence of elastase YaB [15, 29] and high sequence homology with the N-terminal amino acid sequence of subtilisin [24, 27] from alkalophilic *Bacillus* sp. Those proteases are known as serine proteases and were consequently completely inactivated in the presence of 1 mM of phenylmethylsulfonyl fluoride (PMSF). But the hydrolase activity of strain YB380 was inactivated about 50% with 5 mM of PMSF and 10 mmol of PMSF was needed for complete inactivation (data not shown). The difference of optimum pH between isolated cell wall hydrolase (pH 9.0) and elastase YaB (pH 11.75) reveals that the isolated cell wall hydrolase might be the new alkaline protease which hydrolyzes the peptide bond of the yeast cell wall.

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