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Isolation of A Moderately Alkaline Pullulanase-Producing *Bacillus* sp. S-1 and Enzyme Characterization

Moon-Jo Lee, Jae-Kyoung Shim, Jin-Woo Park*, Dong-Soo Kim** and Cheorl-Ho Kim†

Department of Biochemistry and Molecular Biology, College of Oriental Medicine, Dongguk University, Kyungju City, Kyungpook 780-714, Korea
**Department of Food Technology, Dongeui Technical Junior College, Pusan 614-053, Korea*
***Department of Food Science and Technology, Kyungsung University, Pusan 614-740, Korea*

Abstract

The moderately alkalophilic bacterium, identified as *Bacillus* sp. S-1, was isolated from soils and effectively secrete extracellular pullulanase. The isolate was moderately alkalophilic since enzyme production occurred at pHs from 6.0 to 10.0. Extracellular crude enzymes of the isolate gave maltotriose as the major product formed from soluble starch and pullulan hydrolysis. Compared to other alkalophilic microbes, this isolate secreted extremely high concentration (7.0 units/ml) of pullulanase. The purified pullulanase was moderately alkalophilic and thermoactive; optimal activity was detected at pH 8.0-10.0 and between 50-60℃. Even at pH 12.0, 10% of S-1 pullulanase activity remained and the strain had broad pH ranges and moderate thermo-stability for their enzyme activities. These results indicate that the new isolate have potential as producer of pullulanase for use in the starch industry.

Key words : *Alkalophilic bacteria, alkaline pullulanase, (Bacillus sp. S-1)*

Introduction

Enzymes of pullulanase (pullulan 6-glucanohydrase, EC 3.2.1.41), isoamylase (glycogen 6-glucanogydrolase, EC 3.2.1.68) and amylo-1,6-glucosidase (amylo-1,6-glucosidase/1,4-α-glucan: 1,4-α-glucan 4-α-glycosyltransferase, EC 3.2.1.33) are well known as debranching enzymes of oligo- and polysaccharides such as pullulan, glycogen, amylopectin and β-limited dextrin[1]. The most important industrial application of these enzymes is for production of glucose or maltose, when used in combination with glucoamylase or β-amylase, respectively. Recently, 6-O-α-maltosyl- and 6-O-α-maltotriosyl cyclodextrins are being produced in high yields by applying the condensation reaction or transfer reaction of the enzymes[2].

Pullulanase hydrolyzes the α-1,6-glucosidic linkages in pullulan and in starch[1]. Well known producers are *Klebsiella pneumoniae*[3], *Bacillus acidopullulyticus*[4] and *B. flavocaldrius*[5]. Pullulanases from *K. pneumoniae* and *B. acidopullulyticus* are used for saccharifying starch to produce glucose and maltose on an industrial scale[1]. Over the last decade, a variety of pullulanolytic enzymes with different substrate specificities have been characterized [6]. The enzymatic classification of pullulan-degrading enzymes has four groups based on substrate specificity

† Corresponding author

and products[7] : (1) pullulan hydrolase type I is the enzyme that attacks α-1,4-glycosidic linkages in pullulan forming panose (it was previously classified as neopullulanase[8]) ; (2) pullulan hydrolase type II attacks α-1,4-glycosidic linkages in pullulan forming isopanose (it was previously classified as isopullulanase[9]) ; (3) pullulanase type I specifically hydrolyzes the α-1,6-linkages in pullulan forming maltotriose ; (4) pullulanase type II attacks, in addition to α-1,6-linkages in pullulan, α-1,4-linkages in other polysaccharidies.

Recently, some thermostable pullulanases have been studied from extreme thermophilic microorganisms[7,10-12]. However, alkalophilic microorganisms, producing extracellular alkaline pullulanase, have been reported in only three studies[13-15]. For industrial application of pullulanase, it is desirable that enzymes have activity over alkaline and a broad pH range. We describe the isolation of two alkalophilic bacterium producing alkaline and broad pH range-pullulanase.

Materials and Methods

Media and cultivation

Pure cultures were obtained by repeated serial dilution with a alkaline Basal Medium (10-fold stepwise). The bacteria were grown on alkaline Basal Medium composed of(w/v) 1% soluble starch (from Wako Pure Chemical. Co. Ltd., Tokyo, Japan), 1% pullulan (Mr about 65,000, Hayashibara Biochemical Lab., Okayama, Japan) and 1% dextran(Mr about 200,000, Sigma Co., St. Louis, USA), 0.5% Bacto peptone (Difco, Detroit, USA), 0.1% Bacto yeast extract(Difco), 0.1% K_2HPO_4, 0.1% NaCl, 0.1% $MnCl_2$, 0.2% $MgSO_4 \cdot 7H_2O$, 1% Na_2CO_3, 1ml(v/v) trace element solution. The trace element solution was comprised of (mg per liter of distilled water) : $FeSO_4 \cdot 7H_2O$, 1000 ; H_3BO_3, 300 ; $CoCl_2 \cdot 6H_2O$, 190 ; $ZnCl_2$,42 ; $NiCl_2 \cdot 6H_2O$, 24 ; $NaMoO_4 \cdot 2H_2O$, 18. Na_2CO_3 was separately added to the basal medium after autoclaving. Media were solidified by the addition of 2.0%(w/v) Bacto agar (Difco) for plates. For large scale production of pullulanase, bacterium were cultivated aerobically at 50℃ and pH 8.0 in a 5 liter stirred-tank fermentor (Korea Fermentator Co. LTD, Incheon, Korea) for 36 h. The liquid medium for enzyme production contained 1% soluble starch instead of pullulan.

Isolation of alkaline bacterium producing pullulanase

The soil samples (0.5 g) for screening were obtained and were suspended in 10 ml of sterile water and spread onto soluble starch, dextran, or pullulan-reactive red agar plates[15], and incubated at 50℃ for 3 days. Colonies that had formed a clear zone around their margins were picked, inoculated into a liquid medium (100 ml) in 500 ml flasks and cultured at 50℃ for 2 days with shaking on a reciprocal shaker. The pullulan-reactive red agar contained (w/v) 1.0% pullulan, 0.3% red-pullulan and 1.5% agar. These procedures were repeated several times to ensure the purity of the culture. Producers of alkaline pullulanase were selected after growth on basal medium containing pullulan as sole carbon source. From 28 candidates, *Bacillus* sp. was isolated as potent producers with higher pullulanase activities at pH 10.0.

Chracterization of the isolated strain.

Morphological and biochemical characteristics of S-1 was determined and identified by the methods of Gordon et al. (1973)[16] and Bergey's Manual of Determinative Bacteriology[17]. The mol % guanine plus cytosine of the DNA was determined using HPLC according to the method of Tamaoka and Komagata (1984)[18]. Unless stated otherwise, media used for identification were supplemented with 1% Na_2CO_3.

Purification of the pullulanase from culture medium.

The basal buffer used throughout all steps of the purification consisted of 20 mM Tris-HCl huffer (pH 8.5)

and purification was carried out at 4℃. Bacterium was cultivated aerobically on the basal medium containing 1 % soluble starch at 50℃ and pH 8.0 in a 5 liter stirred-tank fermentor (Korea Fermentator Co. LTD, Incheon, Korea) for 36 h. After 36 h aerobic cultivation, cells were harvested by centrifugation at 6000 g for 10 min. Supernatant per se was used for time-course production studies. The crude enzyme(s) in each culture supernatant (1000 ml) of the strain was concentrated by addition of solid ammonium sulfate (40−70% saturation) and dissolved in 57 ml of 20 mM Tris-HCl buffer (pH 8.5). The enzyme was dialysed against the same buffer overnight at 4℃ and stored at 4℃ (no significant loss of activity was seen even after 10 month's storage). The dialysed enzyme solutions were concentrated by ultracentrigugation (Diaflo PM30 membrane, Amicon Corp., Amicon, Md., USA), and fractionated by gel filtration chromatography on a column of a FPLC Sepharose 12 (1.2×36 cm, Pharmacia LKB Co., Upsala, Sweden) with the same buffer containing 100 mM NaCl at a flow rate of 0.5 ml/min. Total active fraction (52 ml) was pooled and lyophilized. Lyophilized enzyme (21.5 mg) was dissolved in 1 ml of 50 mM Tris-HCl buffer (pH 8.3). For the electrophoretic separation of the pullulanase, dissolved enzyme solutions were applied to 7.5% acrylamide gel and the active pullulanase bands, identified by pullulanase activity staining on the gel, were electroeluted. The resolved pullulanase was fairly pure (almost homogeneous and showing a single major band on native gels, Fig. 4), and were suitably diluted and used as the enzyme source for characterization studies with respect to optimum pH, pH stability, and temperature dependency.

Enzyme assay

Pullulanase and amylase activities were measured by determining the reducing sugar released from pullulan and soluble starch, respectively. The reaction mixture for each assay was the same as that described previously[19]. Enzymatic activities of amylase and pullulanase were measured in 50 mM glycine-NaOH buffer, pH 9.0 or 10.0, at 50℃. Suitably diluted enzyme (50 μl) was added to 150 μl substrate and incubated for 30 min. The reducing sugar liberated was quantified by following the 3,5-dinitrosalicylic acid (Merck, Darmstardt, FRG) method[20]. One unit of each enzyme activity was defined as the amount of enzyme which released 1 mol of reducing sugar, expressed as glucose per min under the reaction conditions.

Zymogram of activity staining

Zymogram of pullulanase activity in the slab gel was done essentially by the method of Ara et al.[15] with pullulan-reactive red agar plates as replica plates. The slab gel after native-PAGE was laid on top of a sheet of the replica plate and was left for 3 h at room temperature. The bands of protein that ware associated with pullulanase activity were seen as clear zones on the replica plate, which formed a brown background. An alternative direct staining method was developed using the starch-iodine interaction : after electrophoresis, the gel was soaked in 1% starch in 50 mM Tris-HCl buffer (pH 8.5) at 30℃ for 30 min, rinsed with water, and stained for pullulanase activity by spraying with an I_2 solution. Amylase activty showed a white zone on weak blue background while pullulanase activity showed strong blue band on the weak blue background since pullulanse hydrolyzes the α-1,6-glycosidic linkages in starch and thereby increases the blue color density with iodine[19]. The pullulanase activity showed a strong blue band because of its high α-1,6-glycosidic activity.

Preparation of cell-associated α-glucosidase and pullulanase

Crude cell-associated enzymes were obtained by the method of Suzuki and Brown[21] with a slight modification. Briefly, cells (50 g wet weight/11.2 l culture) collected by centrifugation at 4℃ for 30 min at 8,000 g

were disintegrated by mixing at 4℃ for 1 h with alumina and suspended in 50 mM Tris-HCl/2 mM EDTA/ 0.05% Tween 80/0.05% NaN_3 (pH 8.5 ; 5 ml/gram of cells), followed by centrifugation. The supernatant was assayed for the cell-associated α-glucosidase and pullulanase.

Fractionation of cellular enzyme components

Cell fractionation was performed by a slight modification of our previous method[22]. Briefly, cells from 50 ml of culture grown to mid-log phase (OD_{600}=2.0) were harvested and resuspended in 50 ml of 50 mM Tris-HCl (pH 8.0). The centrifuged supernatant was used as the enzyme source of the extracellular fraction. Resuspended cells were sonicated at 4℃ and debris was removed from the resulting mixture by centrigugation at 15,000 g for 30 min, and the supernatant was used as the intracellular enzyme source. The sum of intracellular and extracelluar

Table 1. Characteristics of isolate *Bacillus* sp. S-1

Characteristics		Characteristics	
Gram-staining	+	Growth and acid production from	
Motility	+	fructose	+
Cell form	rod	amygdalin	+
Spore form	rod	arabinose	−
Temp. optimum	45−55℃	cellobiose	+
Degradation of :		N-acetyl-D-glucosamine	+
gelatin	+	dextrin	+
starch	+	glycogen	+
cellulose	+	α-cyclodextrin	+
gas formation	+	β-cyclodextrin	+
Composition of bacterial		galactose	+
fatty acid	branched	gentiobiose	+
DAP of cell wall	meso	lactose	−
compostion of		lactulose	−
membrane quinone	MK-7	mannitol	−
GC content	60%	mannose	+
Milk reaction	nd	inulin	−
Growth and acid production from		raffinose	+
fucose	−	rhamnose	−
galacturonic acid	−	ribose	−
glucose	+	salicin	−
maltose	+	starch	+
maltotriose	+	sorbitol	−
melezitose	−	sucrose	+
melibiose	−	trehalose	+
palatinose	+	xylose	+

DAP, diamino pimeric acid ; nd, not determined; W, weak ; +, positive ; −, negative.

enzyme activities was expressed as total enzyme activity.

Analytical methods

Electrophoresis was carried out using the buffers of Davis (1964)[23]. The separation of the extracellular proteins (50 μg, 0.5 units) was performed in 2.5-mm-thick polyacrylamide gels. For the detection of protein band exibiting pullulanase and amylase activities, gels were soaked in 50 mM Tris-HCl buffer, pH 9.0 containing 1% soluble potato starch (Merck) for 30 min at 4℃. Gels were further incubated at 50℃ for 30 min and finally incubated in a solution containing 0.15% (w/v) iodine and 1.5% (w/v) potassium iodide until a clear zone or blue zone became visible. Oligosaccharide produced by the enzymatic action were examined by thin-layer chromatography (TLC), as previously described by Kim et al. (1992)[24]. Protein concentration was estimated by monitoring the absorbance at 280nm or by the method of Bradford (1976)[25] using a protein assay kit (Bio-Rad) with bovine serum albumin as the standard. Absorption spectra were recorded with a Kontron UV spectrophotometer equipped with an end-on photomultiplier. Prior to measurements, the cells were passed through a French press (Amicon, Silver Spring, Md. USA ; pressure=6.7×10^6 Pa). Pigments were extracted overnight at 4℃ with acetone.

Results

Isolation and identification of bacterium

As shown in Table 1, starch hydrolyzing strain had different characteristic. Although the inoculum medium used for the isolation of the strain had same pH 10, different bacteria with a variety of pH optima (pH 6.0−12.0) could be identified. The morphological and taxonomic characteristics of the isolate is summarized in Table 1. S-1 strain fermented cellulose. Densely grown cultures of S-1 were slightly white-yellow in color. The absorption spectra of the acetone-cell extracts revealed maxima at 415, 440 and 470 nm. (Fig. 1). The strain showed high GC contents 60%. This strain was compared to other pullulanase-producing microbes with high GC contents. Strain S-1 appears as a *Bacillus* sp. The isolates grew well over wide pH range (pH 6 to 11) and grew best between pH 8.0 to 10.0, a parameter used to define alkalophilic microorganism. Strain S-1 could grow well at temperatures up to 60℃. The pullulanase production of the isolate parallel growth through all growth stages.

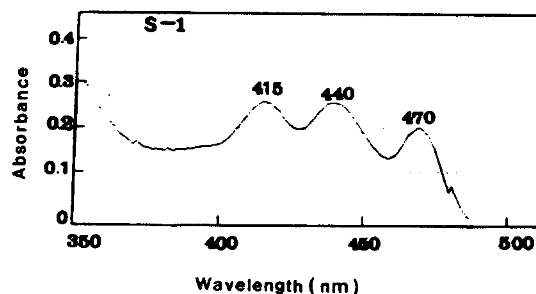

Fig. 1. Adsorption spectrum of acetone-extracts of S-1 cell grown aerobically.

Production of extracellular enzyme

The strain was cultivated in 5 l-fermentors under aerobic conditions using 1% soluble starch as carbon and energy source. Strain S-1 completely degraded 1.0% starch and the maximal optical density at 600nm was 3.3. Extracellular pullulanolytic and amylolytic activities paralled growth and reached their maximum after 36 h of growth in pH 6.0 and 8.0 for S-1 strain (Fig. 2). Unlike most pullulanase-producing bacteria[3−11], 85 to 90% of the enzyme was secreted into the culture fluid (Table 2) through all stages of growth and was not restricted to the end of the stationary phase (Fig. 2). Yields were high. Total pullulanolytic and amylolytic activities at pH 10 after 40 hrs of growth were 7.0 units/ml and 17.6 units/ml (Fig. 2 only for pullulanase production, data not shown for amylase production). This compares to 2.78 units/ml for *Klebsiella pneumoniae*[26].

Table 2. Amount and localization of the pullulanase produced by *Bacillus* sp. S-1.
The values represent units of pullulanase activity per ml of extract using conditions described in Materials and Methods. The cultures were grown in 50 ml Basal alkaline medium containing 1% soluble starchat pH 8.0 for 24 h. at 50℃ with vigorous shaking.

Strain	Extracellular fraction	Cellular fraction	Total[a] activity	E/T[b] (%)
Bacillus sp. S-1	6.62	1.20	7.82	84.7

[a]Sum of extracellular and cellular activities.
[b](Extracellular activity/Total activity)×100.

Action pattern of the crude enzyme against substrates

In order to obtain detailed information of the enzymology, *in vitro* experiments using the extracellular culture broth of both strains were conducted. Culture broths were incubated with various glucans with α-1,4- as well as α-1,6-glycosidic linkages ; starch, pullulan, amylose and also maltose. Analysis of the products from amylose indicated random endosplitting attack to yield a range of oligosaccharides with different chain lengths, i.e. α-amylase activity. Maltotriose was the major product formed from soluble starch hydrolysis, which was not converted to maltose or glucose (Fig. 3). Maltose was not attacked. This indicates the lack of α-glucosidase or α-amylase-possessing α-glucosidase activity in the culture broth. Cell-associated fractions of the isolate was assayed to determine whether cell-associated α-glucosidases and pullulanases existed. Even though activities of cell-associated pullulanases were not detected, a large amounts of α-glucosidase activities were detected in the strain (data not shown). These results indicated that the strain has hydrolytic enzyme system for effective utilization of polymeric carbohydrates. Cell-associated α-glucosidases of the strain will be the subject of a separate publication. More rapid conversions were observed with pullulan as a substrate. Pullulan is made up of maltotriose units and the first and third glucose unit of each trimer is bound by α-1,6-linkages. Only maltotriose was shown through all stages of S-1 enzyme reaction. Thus, S-1 enzyme attacks the substrate with exosplitting action forming only maltotriose as the end product.

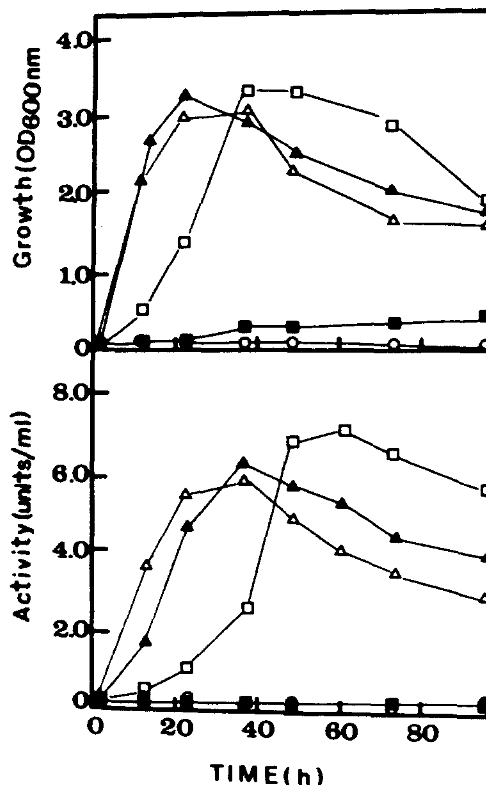

Fig. 2. Formation and secretion of extracellular pullulanase produced by S-1 during the time course of starch degradation.
Bacterium was cultivated under aerobic conditions with 1% starch at various pHs (pH 4 to pH 12) at 50℃. 10 ml samples were taken, and cell growth and pullulanase activity were measured as described in Materials and Methods. −■−, pH 12.0 ; −□−, pH 10.0 ; −△−, pH 6.0 ; −▲−, pH 8.0 ; −○−, pH 4.0.

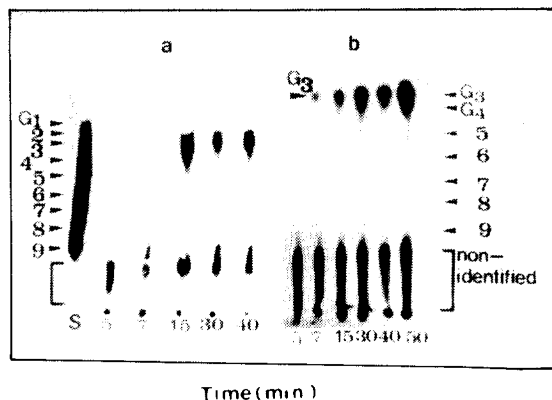

Fig. 3. TLC pattern of substrate hydrolysates of crude enzyme.

Enzyme solutions (0.5 ml) were incubated with 0.5 ml of 2% soluble starch or pullulan (pH 8.0) at 30℃. 10 μl portions of the reaction mixture were withdrawn at intervals and subjected to thin-layer chromatography using Whatman silica plate K5F (Whatman Co. USA) with a developing solvent system of ethyl acetate : methyl alchol : water (v/v/v, 43 : 23 : 34). S denotes a standard mixture containing a series of linear malto-oligosaccharides. Non-identified saccharides means unknown compounds in this analytical system. (a) Soluble starch-hydrolysate ; (b) Pullulan-hydrolysate.

Purification of extracellular pullulanase

To characterize extracellular pullulanases from S-1, the enzyme from culture broths was partially purified. After ammonium sulfate fractionation (40−70%) as described in Materials and Methods, the enzyme solutions were passed through a Sepharose 12 column (FPLC), and then separated using 7.5% native-polyacrylamide gel electrophoresis. A large number of protein bands were detected in the fractionated supernatant of strain S-1. Electrophoretic analysis of the extracellular proteins from both strains revealed multiple bands with amylase activities. Extracellular pullulanase activity of S-1 strain was directly stained on the gel with iodine solution after partial purification by electroeluting the objective bands. S-1 pullulanase activity showed a strong blue band (Fig. 4). The molecular weight of S-1 pullulanase was estimated to be about 140,000 Da on SDS-gel and gel filtration chromatography.

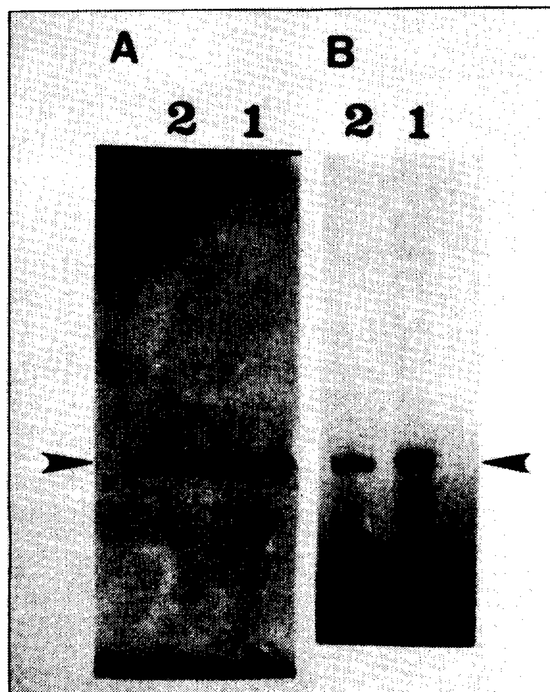

Fig. 4. Native-polyacrylamide gel electrophoresis of the extracellular pullulanase produced by S-1.

Partially purified S-1 pullulanase by Sepharose 12 gel filtration (lane 1) and electrophoretically isolated S-1 pullulanase (lane 2) were concentrated, and 300 μl of each sample (containing 30 μg of protein) were loaded onto the native gel (7.5%) and electrophoresised. The gel was stained for pullulanase or amylase activity as described in Materials and Methods. Arrow head indicates the stained activities of amylase or pullulanase. (A) enzyme activity staining with Iodine solution ; (B) protein staining with Coomassie Brilliant Blue R250.

Table 3. Partial purification of extracellular S-1 pullulanase

Purification step	*Bacillus* sp. S-1					
	Protein(mg)	Amyl[a](U/ml)	Pul[b](U/ml)	Spec[c](U/mg)	A/P[d]	Yield(%)
Crude enzyme	1346	17.6	7.0	5.2	2.51	100
Ammonium sulfate(40−70%)	867	120	102	6.7	1.18	83
FPLC on Sepharose12 gel	22	337	9.1	22	37	6.8
Electroelution	9	0.01	3.7	27	0	3.2

[a]Amylase activity
[b]Pullulanase activity
[c]Specific activity
[d]Amylase activity/Pullulanase activity

Enzymatic properties of pullulanase

The pH optima of pullulanases of *Bacillus* sp. S-1 was determined using 50 mM sodium acetate buffer (pH 3.5−5.5), 50 mM sodium phosphate buffer (pH 6.0−7.5), 50 mM Tris-HCl buffer (pH 8.0−9.5), 50 mM glycine-NaOH buffer (pH 9.5−10.5) and 50 mM KCl-NaOH (pH 11−13) after electrophoretic isolation of the pullulanase bands. Other conditions were the same for the standard assay. The S-1 pullulanase was most active at pH 8-10, and half the activity remained at pH 11.0. Interestingly, this enzyme showed activity over a broad pH range ; pH 6 to pH 10 for S-1 pullulanase (Fig. 5). These high pH optima are comparable to that of *Micrococcus* sp. 207 alkaline pullulanase (pH 9.0−9.7) (Kimura and Horikoshi, 1990)[13]. The pH stability of the enzyme was investigated. The semi-purified enzyme was most stable at pH 8−10, and exhibited broad stability with retention of more them 80% of maximum activity over the pH range 4−12 (data not shown). Optimum temperature for activity was determined at pH 9.0 (Fig. 6). The enzyme was most active at 40−50℃. However, at 70℃ more than 20−30% of maximum activity was observed. Thermal stability of the enzyme in 10 mM Tris-HCl buffer (pH 9.0) was measured. The enzyme was completely denatured at 80℃, but only 14% of the original activity was lost after 30 min incubation at 70℃. The enzyme was highly stable at 60 ℃ and more than 80% of the initial activity remained after 24 hrs incubation at 60℃ (data not shown).

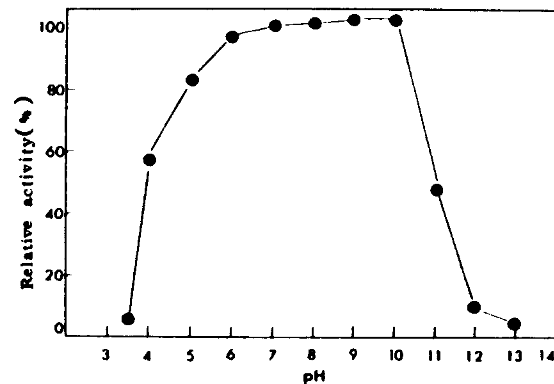

Fig. 5. Influence of pH on activity of extracellular pullulanase produced by strain of S-1.
The follwing buffer systems were used : pH 3.0 to 5.5, 50 mM sodium acetate ; pH 6.0 to pH 7.5, 50 mM sodium phosphate ; pH 8.0 to pH 9.5, 50 mM Tris-HCl ; pH 9.5 to pH 10.5, 50 mM glycine-NaOH ; pH 11.0 to 13.0, 50 mM KCl-NaOH. Other conditions were the same as for the standard assay. One hundred percent activity was taken as 100 mU.

The semi-purified enzyme, S-1 pullulanases, showed strong cleavage of α-1,6-glucosidic linkages in pullulan, glycogen, amylopectin and -limited dextran (Table 4).

It is suggested, therefore, that S-1 enzyme can be classified as pullulanase Type-I.

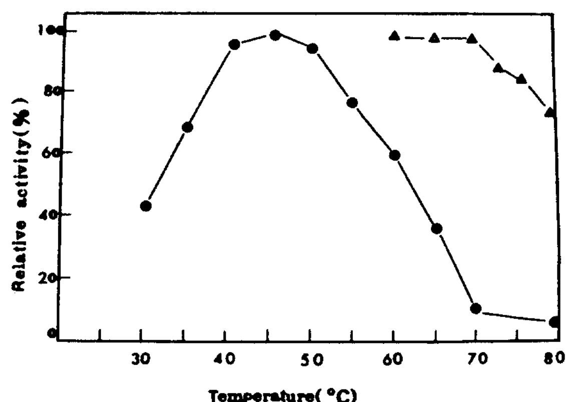

Fig. 6. Influence of temperature on activity of extracellular pullulanase produced by strain of S-1.
The enzyme activities at various temperatures were measured by the standard assay at pH 9.0 for S-1 pullulanase. -●-, S-1 pullulanase ; -△-, S-1 pullulanase with 2 mM $MnCl_2$ in reaction mixture.

Table 4. Substrate specificity of the pullulanase from Bacillus sp. S-1

Substrate	Relative activity(%)
pullulan	100
soluble starch	3.4
amylose	0
β-limited dextrin	18.3
oyster glycogen	6
amylopectin	31

Enzyme was prepared by electroelution of the band showing a pullulanase band from a native-PAGE gel. Enzyme assay was performed at 50℃ in glycine-NaOH buffer (pH 9.0) with the indicated substrate (1%, w/v). The activity of each preparation toward pullulan was taken as 100%.

Discussion

The aerobic alkalophilic strain that attack pullulan has been isolated. The strain differed in morphology, physiology and biochemical characteristics, and was assigned as *Bacillus* species. The strain grew in neutral and highly alkaline conditions, and was pigmented (absorbing at 415, 440 and 470nm). The enzyme system of the newly isolated strain S-1 formed maltotriose as the major hydrolysis product rather than maltose or glucose from soluble starch and pullulan. It is very likely that an extracellular α-glucosidase activity is not required by this isolate. However, α-glucosidase activity was detected as cell-associated forms in the strain. It is suggested, therefore, that the strain can easily utilize polymeric carbon sources for survival. A difference between the new strain and all other aerobic (0.57 units/ml for *Micrococcus* sp., 0.56 units/ml for *Bacillus* sp. KSM-1876, and 1.9 units/ml for *Bacillus* No. 202-1)[13,15,14] and anaerobic strains(1.9 units/ml for *Thermoanaerobactor* strain B 6A, 0.23 units/ml for *Thermoanaerobactor brockii*, and 1.4 units/ml for *Clostridium thermosulfurogens* EM1)[27,28,16] is their ability to secrete enormous amounts of pullulanases (up to 8.0 units/ml of extracellular enzymes). The strain produced only one kind of pullulanase in culture broth as determined by native-PAGE followed by enzyme staining. The electrophoretically separated pullulanases from the isolate had different modes of action : S-1 enzyme possessed only cleavage activity of α-1,6-glycosidic linkages in pullulan as pullulanase Type-I. Previously the only pullulanase that attack α-1,6-linkages in pullulan and branched polysaccharides (pullulanase type-I) were from *K. penumoniae*[3], *B. acidopullulyticus*[4], *B. flavocaldarius*[5], and *T. caldophilus*[29]. Pullulanase of strain S-1, is a fourth case of pullulanase type I, and is the first report of an alkaline pullulanase type I. In general, pullulanase debranches amylopectin, β-limited dextrins and soluble starch, but cannot act on amylose (Abdullah and French, 1970)[30]. However, it

has been shown that pullulanases from the anaerobic bacteria *Thermoanaerobium brockii*[28], *Thermoanaerobium* Tok B1[31] and *C. thermohydrosulfuricum*[32,33] differ from other pullulanases. These pullulanases degrade α-1,4-glycosidic linkages of starch to produce oligosaccharide mixtures, activities which seem to be distributed among a large number of anaerobic bacteria growing on starch. Probably this is a result of energy limitations, with anaerobes developing a more efficient enzyme system for microbial effectiveness. The pullulanase from aerobic bacterium, in hydrolysing different glycosidic linkages is unique. The question that now arises is whether the information of single protein band possessing the dual-enzymatic activity has a physiological significance in aerobic organisms. With respect to bifunctional properties of amylase and pullulanase produced by aerobic strains, only three studies are reported ; a pullulanase-amylase complex from *B. subtilis*[34], an α-amylase-like pullulanase from thermophilic *Bacillus* sp. 3183[35,36] and an amylase-pullulanase enzyme from *B. circulans* F-2[19]. The *B. subtilis* enzyme was found to be a multienzyme complex with a molecular mass of 450,000 dalton. In contrast, APE of *B. circulans* F-2 had a different active site responsible for amylase and pullulanase on a single polypeptide. The newly isolated Bacillus sp. S-1 described produce the extracellular alkaline pullulanases which exhibited maximum activity at 50−60℃ and at pH8.0−10.0. Most of other pullulanase reported are activated by $CaCl_2$, not by $MnCl_2$. Although mesophilic strains can produce pullulanase, these enzymes are not stable even above 45℃. The enzyme of the strain, therefore, could be valuable in fermentation processes since thermal properties and the wide pH ranges of activity and stability will give them enormous potential as a debranching enzyme in the starch-processing industry. The crude extracellular enzyme preparation of isolate contain pullulanase and amylase which can hydrolyze soluble starch into maltotriose as main product.

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초록 : 알칼리성 플루라나제를 생산하는 세균 *Bacillus* sp. S-1의 분리와 효소특성에 관한 연구

이문조 · 심재경 · 박진우* · 김동수** · 김철호†
(동국대학교 한의과대학 생화학교실, *동의공업전문대학 식품공업과, **경성대학교 공과대학 식품공학과)

전분의 효율적인 이용을 목적으로 토양으로부터 균체외 플루란아제효소를 대량분비하는 약알칼리성균주 *Bacillus* sp. S-1를 분리하였다. 본 균주의 풀루란아제효소 생산의 최적 pH는 6-10 사이였으며, 조효소의 가용성전분과 플루란에 대한 주요 반응최종산물은 말토트리오스로, 본 효소가 α-1,6-glycosidic결합에 특이적임을 알수 있었다. 본 균주는 지금까지 알려진 알칼리성균주들의 플루란아제생산성보다 월등히 높은 7.0 U/ml를 생산하였으며, 정제효소의 최적 pH와 온도는 각각 8.0-10.0와 50-60℃로서 알칼리성 및 호열성의 특성을 나타나었다. 또한 정제효소는 pH 12에서도 약 10%의 활성을 유지하며, 넓은 pH범위에서도 안정하였다. 이러한 결과들은 본 균주가 플루란아제 생산균주로서의 이용가능한 잠재력을 보유하고 있음을 시사하였다.