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# Pullulanases of alkaline and broad pH range from a newly isolated alkalophilic *Bacillus* sp. S-1 and a *Micrococcus* sp. Y-1

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## SUMMARY

Two highly alkalophilic bacteria, and potent producers of alkaline pullulanase, were isolated from Korean soils. The two isolates, identified as *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1, grow on starch under alkaline conditions and effectively secrete extracellular pullulanases. The two isolates were extremely alkalophilic since bacterial growth and enzyme production occurred at pH values ranging from pH 6.0 to 12.0 for *Micrococcus* sp. Y-1 and pH 6.0 to 10.0 for *Bacillus* sp. S-1. Both strains secrete enzymes that possess amylolytic and pullulanolytic activities. Extracellular crude enzymes of both isolates gave maltotriose as the major product formed from soluble starch and pullulan hydrolysis. Compared to other alkalophilic microbes such as *Micrococcus* sp. (0.57 units $ml^{-1}$), *Bacillus* sp. KSM-1876 (0.56 units $ml^{-1}$) and *Bacillus* No. 202-1 (1.89 units $ml^{-1}$), these isolates secreted extremely high concentrations (7.0 units $ml^{-1}$ for *Bacillus* sp. S-1 and 7.6 units $ml^{-1}$ for *Micrococcus* sp. Y-1) of pullulanases in batch culture. The pullulanase activities from both strains were mostly found in the culture medium (85–90%). The extracellular enzymes of both bacteria were alkalophilic and moderately thermoactive; optimal activity was detected at pH 8.0–10.0 and between 50 and 60 °C. Even at pH 12.0, 65% of original Y-1 pullulanase activity and 10% of S-1 pullulanase activity remained. The two newly isolated strains had broad pH ranges and moderate thermostability for their enzyme activities. These results strongly indicate that these new bacterial isolates have potential as producers of pullulanases for use in the starch industry.

## INTRODUCTION

Debranching enzymes hydrolyze the α-1-6-glucosidic linkages of oligo- and polysaccharides such as pullulan, glycogen, amylopectin and β-limited dextrin. Enzymes such as pullulanase (pullulan 6-glucanohydrase, EC 3.2.1.41), isoamylase (glycogen 6-glucanohydrolase, 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 [14]. 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 [17].

Pullulanase hydrolyses the α-1,6-glucosidic linkages in pullulan and starch [14]. Well-known producers are *Klebsiella pneumoniae* [24], *Bacillus acidopullulyticus* [29] and *B. flavocaldrius* [33]. Pullulanases from *K. pneumoniae* and *B. acidopullulyticus* are used for saccharifying starch to produce glucose and maltose on an industrial scale [14]. Over the last decade, a variety of pullulanolytic enzymes with different substrate specificities have been characterized [31]. The enzymatic classification of pullulan-degrading enzymes has four groups based on substrate specificity and products [23]: (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 [18]; (2) pullulan hydrolase type II attacks α-1,4-glycosidic linkages in pullulan forming isopanose (it was previously classified as isopullulanase [28]); (3) pullulanase type I specifically hydrolyses 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 polysaccharides.

Recently, some thermostable pullulanases have been studied from extreme thermophilic microorganisms [8,9, 16,23]. However, alkalophilic microorganisms, producing extracellular alkaline pullulanase, have been reported in only three studies [2,13,21]. 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 bacteria producing alkaline and broad pH range pullulanases.

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## 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 ($M_r$ about 65000; Hayashibara Biochemical Lab., Okayama, Japan) and 1% dextran ($M_r$ about 200000; Sigma Co., St Louis, MO, USA), 0.5% Bacto peptone (Difco, Detroit, MI, 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% $Na_2CO_3$, 1 ml (v/v) trace element solution. The trace element solution comprised (mg per litre 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 pullulanases, bacteria were cultivated aerobically at 50 °C and pH 8.0 (for S-1 strain) and pH 10.0 (for Y-1 strain) in a 5-litre stirred-tank fermentor (Korea Fermentator Co. Ltd, Seoul, Korea) for 36 h. The liquid medium for enzyme production contained 1% soluble starch instead of pullulan.

*Isolation of alkaline bacteria producing pullulanase*

Yusung Hot Spring area is a large hot-spring region located near Taejon in the central area of Korea. The sediment soil samples (0.5 g) for screening were obtained from hot-spring sources in Yusung hot-spring park. The samples were suspended in 10 ml of sterile water and spread onto soluble starch, dextran, or pullulan-reactive red agar plates [2], and incubated at 50 °C 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 °C 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. and *Micrococcus* sp. were isolated as potent producers with higher pullulanase activities at pH 10.0.

*Characterization of the isolated strains*

Morphological and biochemical characteristics of the isolates of S-1 and Y-1 were determined and identified by the methods of Gordon et al. [7] and *Bergey's Manual of Determinative Bacteriology* [15]. The mol % guanine plus cytosine of the DNA was determined using HPLC according to the method of Tamaoka and Komagata [35]. Unless stated otherwise, media used for identification were supplemented with 1% $Na_2CO_3$. The bacterial identification of the isolates was kindly carried out by Dr Yong-Ha Park, Korean Collection for Type Cultures, Genetic Engineering Research Institute, KIST, Korea.

*Partial purification of the extracellular pullulanases from two isolates*

The basal buffer used throughout all steps of the purification consisted of 20 mM Tris-HCl buffer (pH 8.5) and purification was carried out at 4 °C.

Bacteria were cultivated aerobically on the basal medium containing 1% soluble starch at 50 °C and pH 8.0 (for S-1 strain) and pH 10.0 (for Y-1 strain) in a 5-litre stirred-tank fermentor (Korea Fermentator Co. Ltd, Seoul, 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 both strains was concentrated by addition of solid ammonium sulphate (40–70% saturation) and dissolved in 57 ml (for S-1) and 72 ml (for Y-1) of 20 mM Tris-HCl buffer (pH 8.5). The enzyme was dialysed against the same buffer overnight at 4 °C and stored at 4 °C (no significant loss of activity was seen even after 10 month's storage). The dialysed enzyme solutions were concentrated by ultracentrifugation (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 $min^{-1}$. Total active fractions (52 ml of S-1 enzyme and 211 ml of Y-1 enzyme) were pooled and lyophilized. Lyophilized enzymes (21.5 mg for S-1 and 89 mg for Y-1) were dissolved in 1 ml of 50 mM Tris-HCl buffer (pH 8.3). For the electrophoretic separation of S-1 and Y-1 pullulanases, 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 pullulanases were 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.

*Enzymatic assays*

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 [10]. Enzymatic activities of amylase and pullulanase were measured in 50 mM glycine-NaOH buffer, pH 9.0 or 10.0, at 50 °C. 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 μmol of reducing sugar, expressed as glucose per min under the reaction conditions.

*Activity staining of S-1 and Y-1 pullulanase*

Activity staining of pullulanase in the slab gel was done essentially by the method of Ara et al. [2] 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 were 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 °C for 30 min, rinsed with water, and stained for pullulanase activity by spraying with an $I_2$ solution. Amylase activity showed a white zone on the weak blue background, while pullulanase activity showed strong blue band on the weak blue background since pullulanase hydrolyses the α-1,6-glycosidic linkages in starch and thereby increases the blue colour density with iodine [10]. S-1 pullulanase activity showed a strong blue band because of its high α-1,6-glycosidic activity. In contrast, Y-1 enzyme showed a white clear band since it has bifunctional activities of amylase and pullulanase.

*Preparation of cell-associated α-glucosidase and pullulanase*

Crude cell-associated enzymes were obtained by the method of Suzuki and Brown [32] with a slight modification. Briefly, cells (50 g wet weight 11.2-L culture$^{-1}$) collected by centrifugation at 4 °C for 30 min at 8000 *g* were disintegrated by mixing at 4 °C 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 g cells$^{-1}$), followed by centrifugation. The supernatant was assayed for the cell-associated α-glucosidase and pullulanase.

*Cell fractionation*

Cell fractionation was performed by a slight modification of our previous method [12]. 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) for S-1 strain and 50 mM glycine-NaOH (pH 10.0) for Y-1 strain. The centrifuged supernatant was used as the enzyme source of the extracellular fraction. Resuspended cells were sonicated at 4 °C and debris was removed from the resulting mixture by centrifugation at 15000 *g* for 30 min, and the supernatant was used as the intracellular enzyme source. The sum of intracellular and extracellular enzyme activities was expressed as total enzyme activity.

*Other analytical methods*

Electrophoresis was carried out using the buffers of Davis [6]. 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 bands exhibiting pullulanase

TABLE 1

Characteristics of isolates *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1

| Characteristic | S-1 | Y-1 | Characteristic | S-1 | Y-1 |
|---|---|---|---|---|---|
| Gram-staining | + | − | Growth and acid production from: | | |
| Motility | + | + | fructose | + | + |
| Cell form | rod | coccus | amygdalin | + | − |
| Spore form | rod | coccus | arabinose | − | + |
| Temp. optimum | 45–55 °C | 45–60 °C | cellobiose | + | + |
| Degradation of: | | | *N*-acetyl-D-glucosamine | + | − |
| gelatin | + | − | dextrin | + | + |
| starch | + | + | glycogen | + | + |
| cellulose | + | − | α-cyclodextrin | + | − |
| gas formation | + | + | β-cyclodextrin | + | − |
| Composition of bacterial fatty acid | branched | branched | galactose | − | + |
| DAP of cell wall | meso | nd | gentiobiose | + | − |
| Composition of membrane quinone | MK-7 | MK-7 | lactose | − | − |
| GC content | 60% | 72% | lactulose | − | − |
| Milk reaction | nd | curd | mannitol | − | − |
| Growth and acid production from: | | | mannose | + | + |
| fucose | − | − | inulin | − | − |
| galacturonic acid | − | − | raffinose | + | − |
| glucose | + | + | rhamnose | − | − |
| maltose | + | + | ribose | − | − |
| maltotriose | + | + | salicin | − | W |
| melezitose | − | − | starch | + | + |
| melibiose | − | − | sorbitol | − | − |
| palatinose | + | − | sucrose | + | + |
| | | | trehalose | + | + |
| | | | xylose | + | + |

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

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 °C. Gels were further incubated at 50 °C 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. [11]. Protein concentration was estimated by monitoring the absorbance at 280 nm or by the method of Bradford (1976) [4] 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 (Amincon, Silver Spring, MD, USA; pressure = $6.7 \times 10^6$ Pa). Pigments were extracted overnight at 4 °C with acetone.

## RESULTS

### *Bacterial identification*

As shown in Table 1, starch hydrolysing strains, from soils of the Yusung hot-spring area, Korea, had different characteristics. Although the inoculum medium used for the isolation of both strains had same pH value, pH 10, different bacteria with a variety of pH optima (pH 6.0–12.0) could be identified. The morphological and taxonomic characteristics of two isolates are summarized in Table 1. Y-1 strain was unable to utilize cellulose, while S-1 strain fermented cellulose. Densely grown cultures of S-1 were slightly white-yellow in colour. The absorption spectra of the acetone-cell extracts revealed maxima at 415, 440 and 470 nm. In contrast, Y-1 strain cultured in alkaline conditions (pH 8.0–12.0) was yellow in colour, and in acetone extracts, only one absorption maximum at 408 nm was detected thus confirming that the pigment was probably a cytochrome-like compound (Fig. 1). Both strains showed high guanine-cytosine (GC) contents—60% for strain S-1 and 72% for strain Y-1. These strains were compared to other pullulanase-producing microbes with high GC contents. Strain S-1 appears as a *Bacillus* sp., while strain Y-1 appears to be *Micrococcus* sp. The isolates grew well over a wide pH range (pH 6–11) and grew best between pH 8.0 and 10.0, a parameter used to define alkalophilic microorganism. They could grow well at temperatures up to 60 °C. The pullulanase production of the two isolates parallels growth through all growth stages.

### *Production of extracellular enzymes*

The strains were cultivated in 5-litre fermentors under aerobic conditions using 1% soluble starch as carbon and energy source. Both strains completely degraded 1.0% starch and the maximal optical density at 600 nm 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, and after 40 h of growth in pH 10 and 12 for Y-1 strain (Fig. 2). Unlike most pullulanase-producing bacteria [8,16,18,23,24,28,29,31,33], 85–90% of the enzymes were 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. For strain Y-1 the pullulanolytic and amylolytic activities were 7.6 units $ml^{-1}$ and 12.1 units $ml^{-1}$ after 20 h of growth, respectively, while for strain S-1 total pullulanolytic and amylolytic activities at pH 10 after 40 h of growth were 7.0 units $ml^{-1}$ and 17.6 units $ml^{-1}$, respectively (Fig. 2 only for pullulanase production, data not shown for amylase production). This compares to 2.78 units $ml^{-1}$ for *K. pneumoniae* [3].

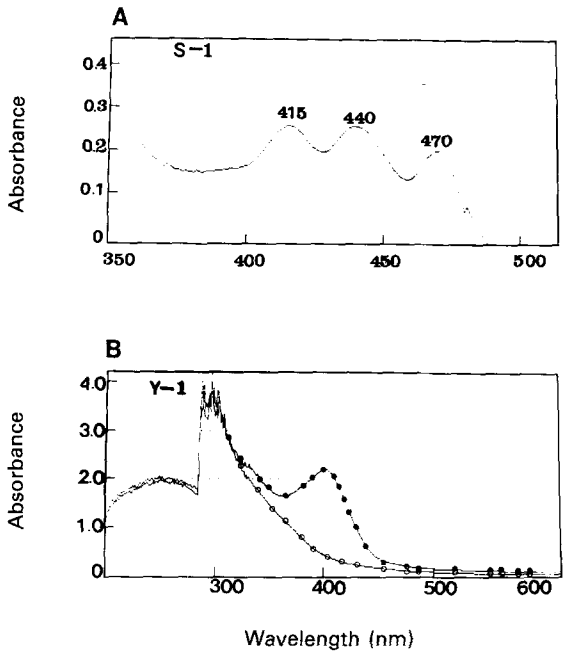


Fig. 1. Adsorption spectra of acetone extracts of S-1 and Y-1 cells grown aerobically. For experimental details see text. (A) Extracts of S-1 strain. (B) Extracts of Y-1 strain. –○–, grown in pH 6.0; –●–, grown in pH 12.0.

TABLE 2

Amount and localization of the pullulanase produced by *Bacillus* sp. S-1 and *Micrococcus* sp. Y-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 starch at pH 8.0 for strain S-1 and pH 10.0 for strain Y-1 for 24 h at 50 °C with vigorous shaking

| Strain | Extracellular fraction | Cellular fraction | Total activity[a] | E/T (%)[b] |
|---|---|---|---|---|
| *Bacillus* sp. S-1 | 6.62 | 1.20 | 7.64 | 86.7 |
| *Micrococcus* sp. Y-1 | 4.86 | 0.46 | 5.32 | 91.1 |

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

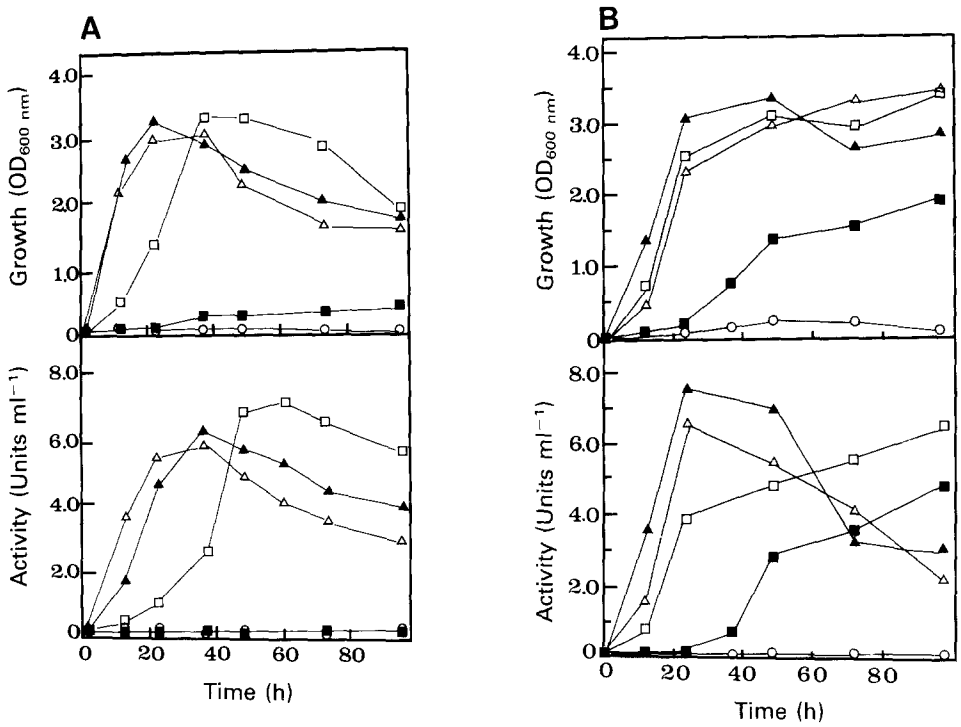


Fig. 2. Formation and secretion of extracellular pullalanases produced by strains of S-1 and Y-1 during the time course of starch degradation. Bacteria were cultivated under aerobic conditions with 1% starch at various pH values (pH 4 to pH 12) at 50 °C. 10-ml samples were taken, and cell growth and pullalanase activity were measured as described in Materials and Methods. (A) Strain S-1. (B) Strain Y-1. -■-, pH 12.0; -□-, pH 10.0; -△-, pH 6.0; -▲-, pH 8.0; -○-, pH 4.0.

*Mode of action of extracellular crude enzymes against soluble starch and pullulan*

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 (data not shown). For both strains, maltotriose was the major product formed from soluble starch hydrolysis, which was not converted to maltose or glucose (Fig. 3). Maltose was not attacked (data not shown). This indicates the lack of α-glucosidase or α-amylase-possessing α-glucosidase activity in the culture broth. Cell-associated fractions of both isolates were assayed to determine whether cell-associated α-glucosidases and pullulanases existed. Even though activities of cell-associated pullulanases were not detected, large amounts of α-glucosidase activities were detected in both strains (data not shown). These results indicated that the strains have a hydrolytic enzyme system for effective utilization of polymeric carbohydrates. Cell-associated α-glucosidases of both strains 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. Maltotriose and maltohexaose were formed from pullulan in Y-1 enzyme (Fig. 3). However, only maltotriose was shown through all stages of S-1 enzyme reaction. Thus, Y-1 enzyme clearly attacks the α-1,6-linkages in pullulan randomly forming maltotriose as the end product, while S-1 enzyme attacks the substrate with exosplitting action forming only maltotriose as the end product.

*Partial purification of extracellular enzymes from S-1 and Y-1 strains*

To characterize extracellular pullulanases from S-1 and Y-1 strains, the enzymes from culture broths were partially purified (Table 3). After ammonium sulphate fractionation (40–70%) as described in the Materials and Methods, the enzyme solutions were passed through a Sepharose 12 column (FPLC), and then separated using 7.5% native-polyacrylamide gel electrophoresis (PAGE). A large number of protein bands were detected in the fractionated supernatant of both strains. Electrophoretic analysis of the extracellular proteins from both strains revealed multiple bands with amylase activities. Extracellular pullulanase activities of S-1 and Y-1 strains were 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, and Y-1 pullulanase was stained on the gel with a white band on the blue background (Fig. 4). The molecular weight of S-1 pullulanase was estimated to be about 140 000 Da on SDS-gel and gel filtration chromatography, and Y-1 pullulanase to be about 500 000 on gel filtration of Sepharose 12, but it did not migrate on native-gel and SDS-gel,

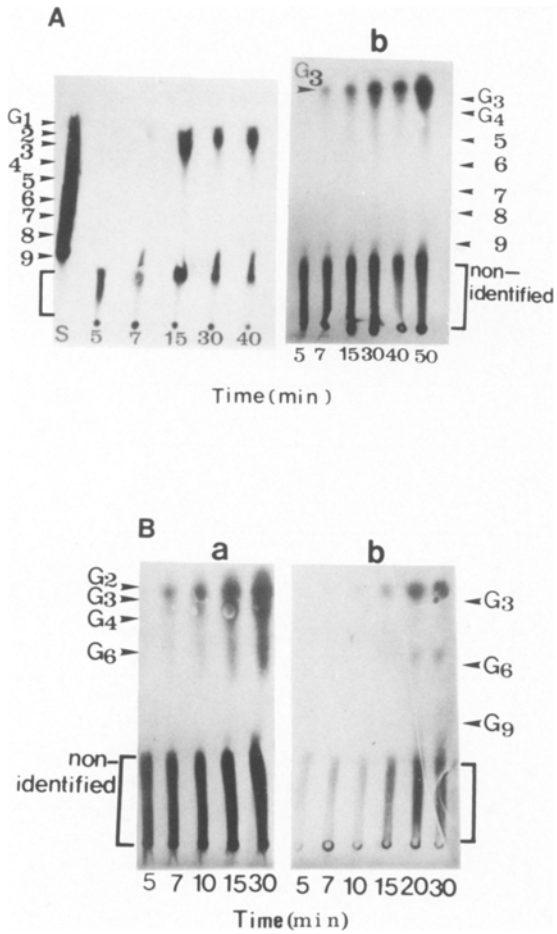


Fig. 3. TLC pattern of substrate hydrolysates of crude S-1 and Y-1 pullulanases. Enzyme solutions (0.5 ml) were incubated with 0.5 ml of 2% soluble starch or pullulan (pH 8.0 for S-1 enzyme and pH 10.0 for Y-1 enzyme) at 30 °C. 10-$\mu$l portions of the reaction mixture were withdrawn at intervals and subjected to TLC using Whatman silica plate K5F (Whatman Co., USA) with a developing solvent system of ethyl acetate : methyl alcohol : water (by vol. 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) Extracellular crude pullulanase of strain S-1. (B) Extracellular crude pullulanase of strain Y-1. (a) Soluble starch-hydrolysate. (b) Pullulan-hydrolysate.

indicating that Y-1 pullulanase may exist as a high-molecular weight protein with multienzyme activity.

*Effect of pH and temperature on the isolated* Bacillus *sp. S-1 and* Micrococcus *sp. Y-1 pullulanases*

The pH optima of two pullulanases of *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1 were 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, while Y-1 pullulanase, had an optimum pH range from pH 8 to 11.5. Interestingly, these two enzymes showed activity over a broad pH range: from pH 6 to 10 for S-1 pullulanase and from pH 8 to 11.5 for Y-1 pullulanase (Fig. 5). These high pH optima are comparable to that of *Micrococcus* sp. 207 alkaline pullulanase (pH 9.0–9.7) [13]. The pH stability of the two enzymes was investigated; the two semipurified enzymes were most stable at pH 8–10, and exhibited broad stability with retention of more than 80% of maximum activity over the pH range 4–12 (data not shown).

Optimum temperatures for activities were determined at pH 9.0 (for S-1 pullulanase) and at pH 10.0 (for Y-1 pullulanase) (Fig. 6). The enzymes were most active at 40–50 °C for S-1 pullulanase and 55–60 °C for Y-1 pullulanase. However, at 70 °C more than 20–30% of maximum activities were observed. Moreover, Y-1 pullulanase activity showed 100% activity if incubated with 2 mM $MnCl_2$ even at 70 °C (Fig. 6). Thermal stability of the enzymes in 10 mM Tri-HCl buffer (pH 9.0) was measured. The enzymes were completely denatured at 80 °C, but only 14% (for S-1) and 42% (for Y-1) of the original activities were lost after 30 min incubation at 70 °C. The enzymes were highly stable at 60 °C and more than 80% of the initial activities remained after 24 h incubation at 60 °C (data not shown).

*Substrate specificity of S-1 and Y-1 pullulanases*

The semipurified enzymes, S-1 and Y-1 pullulanases, showed strong cleavage of $\alpha$-1,6-glucosidic linkages in pullulan, glycogen, amylopectin and $\beta$-limited dextran (Table 4). Y-1 pullulanase hydrolysed both $\alpha$-1,4-linkages of starch and $\alpha$-1,6-linkages of pullulan. It is suggested, therefore, that S-1 enzyme can be classified as pullulanase Type-I and that Y-1 is Type-II.

## DISCUSSION

The two aerobic alkalophilic strains that attack pullulan have been isolated. The strains differed in morphology, physiology and biochemical characteristics, and were assigned as *Bacillus* and *Micrococcus* species. The strains grew in neutral and highly alkaline conditions, and were pigmented (absorbing at 415, 440 and 470 nm for S-1 and at 408 nm for Y-1). Interestingly, the pigment of Y-1 strain was produced only when grown in alkaline conditions.

The enzyme systems of both newly isolated strains formed maltotriose as the major hydrolysis product rather than maltose or glucose from soluble starch and pullulan. It is very likely that an extracellular $\alpha$-glucosidase activity is not required by these isolates. However, $\alpha$-glucosidase activities were detected as cell-associated forms in both strains. It is suggested, therefore, that the strains can easily utilize polymeric carbon sources for survival. A difference between these new strains and all other aerobic (0.57 units $ml^{-1}$ for *Micrococcus* sp., 0.56 units $ml^{-1}$ for *Bacillus* sp. KSM-1876, and 1.9 units $ml^{-1}$ for *Bacilus* No. 202-1) [2,13,21] and anaerobic strains (1.9 units $ml^{-1}$ for *Thermoanaerobactor* strain B6A, 0.23 units $ml^{-1}$ for *Thermoanaerobactor brockii*

TABLE 3

Partial purification of extracellular S-1 and Y-1 pullulanases

| Purification step | *Bacillus* sp. S-1 | | | | | |
|---|---|---|---|---|---|---|
| | Protein (mg) | Amyl (U $ml^{-1}$)[a] | Pul (U $ml^{-1}$)[b] | Spec (U $mg^{-1}$)[c] | A/P[d] | Yield (%) |
| Crude enzyme | 1346 | 17.6 | 7.0 | 5.2 | 2.51 | 100 |
| Ammonium sulphate (40–70%) | 867 | 120 | 102 | 6.7 | 12.1 | 83 |
| FPLC on Sepharose 12 gel | 22 | 337 | 9.1 | 22 | 0.37 | 6.8 |
| Electroelution | 9 | 0.01 | 3.7 | 27 | 0 | 3.2 |
| Purification step | *Micrococcus* sp. Y-1 | | | | | |
| | Protein (mg) | Amyl (U $ml^{-1}$)[a] | Pul (U $ml^{-1}$)[b] | Spec (U $mg^{-1}$)[c] | A/P[d] | Yield (%) |
| Crude enzyme | 2235 | 12.1 | 7.6 | 3.4 | 1.59 | 100 |
| Ammonium sulphate (40–70%) | 906 | 112 | 64 | 5.1 | 1.89 | 60.8 |
| FPLC on Sepharose 12 gel | 89 | 4.02 | 5.3 | 12.6 | 0.26 | 14.8 |
| Electroelution | 16 | 0.52 | 0.7 | 21.7 | 0.74 | 4.6 |

[a] Amylase activity.
[b] Pullulanase activity.
[c] Specific activity.
[d] Pullulanse activity/amylase activity.

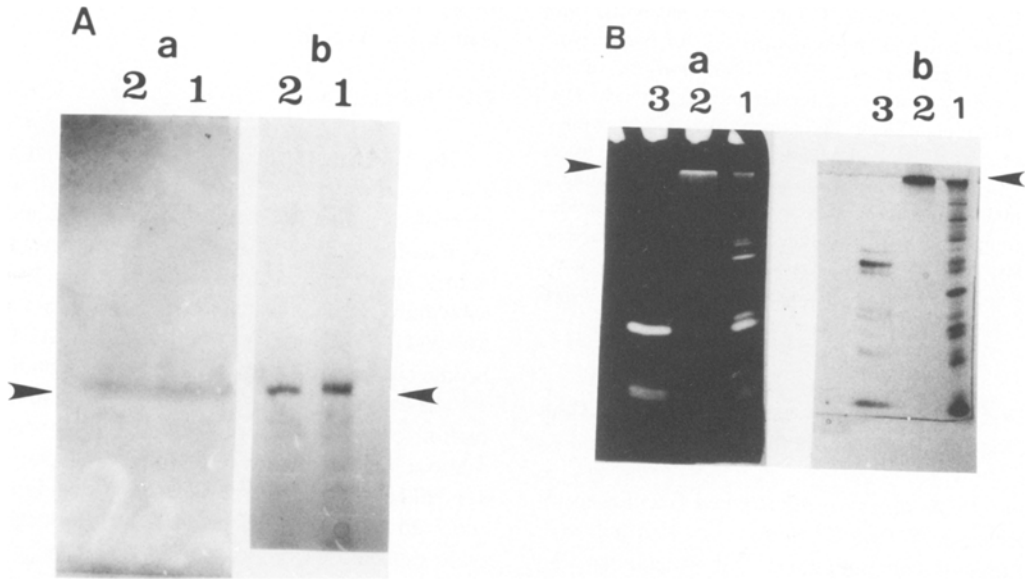


Fig. 4. Native-PAGE of the extracellular pullulanases produced by S-1 and Y-1 strains. (A) Partially purified S-1 pullulanase by Sepharose 12 gel filtration (lane 1) and electrophoretically isolated S-1 pullulanase (lane 2) were concentrated, and 300 $\mu$l of each sample (containing 30 $\mu$g of protein) were loaded onto the native gel (7.5%) and electrophoresed. (B) Culture supernatant of Y-1 strain (lane 1), partially purified Y-1 pullulanase (lane 2) and amylase fraction of Y-1 strain (lane 3) were concentrated, and electrophoresed. The gel was strained for pullulanase or amylase activity as described in Materials and methods. Arrowhead indicates the stained activities of amylase or pullulanase. (a) Enzyme activity staining with Iodine solution. (b) Protein staining with Coomassie Brilliant Blue R250.

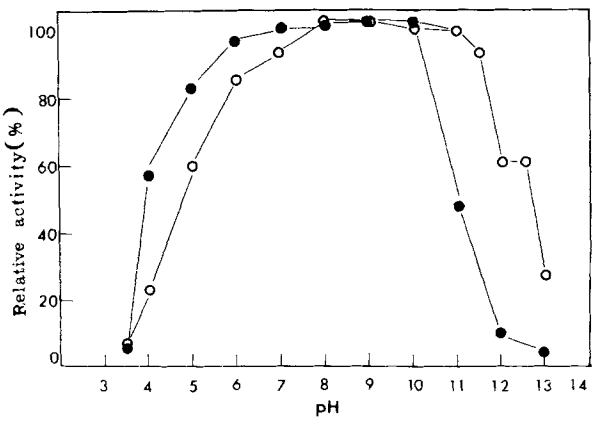


Fig. 5. Influence of pH on activity of extracellular pullulanases produced by strains of S-1 and Y-1. The following buffer systems were used: pH 3.0–5.5, 50 mM sodium acetate: pH 6.0–7.5, 50 mM sodium phosphate; pH 8.0–9.5, 50 mM Tris-HCl; pH 9.5–10.5, 50 mM glycine-NaOH; pH 11.0–13.0, 50 mM KCl-NaOH.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

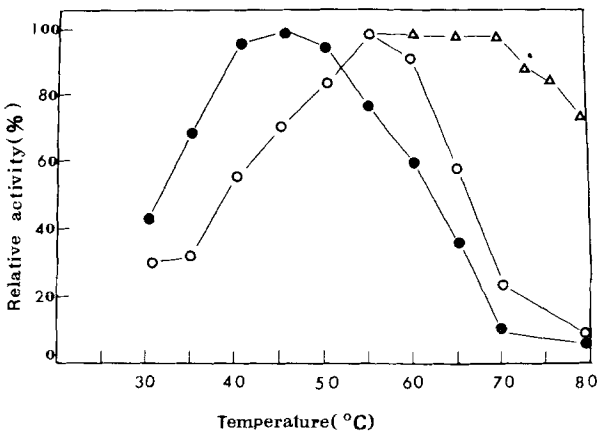


Fig. 6. Influence of temperature on activity of extracellular pullulanases produced by strains of S-1 and Y-1. The enzyme activities at various temperatures were measured by the standard assay at pH 9.0 for S-1 pullulanase and pH 10.0 for Y-1 pullulanase. –○–, Y-1 pullulanase; –●–, S-1 pullulanase; –△– S-1 pullulanase with 2 mM $MnCl_2$ in reaction mixture.

and 1.4 units $ml^{-1}$ for *Clostridium thermosulfurogens* EM1) [5,7,25] is their ability to secrete enormous amounts of pullulanases (up to 8.0 units $ml^{-1}$ of extracellular enzymes). Each 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 two isolates 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 pullulanases that attack α-1,6-linkages in pullulan and branched polysaccharides (pullulanase Type-I) were from *K. pneumoniae* [24], *B. acidopullulyticus* [29] and *B. flavocaldarius* [33]. Pullulanase of strain S-1 is a fourth case

TABLE 4

Substrate specificities of two pullulanases from *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1

| Substrate | Relative activity (%) | |
|---|---|---|
| | S-1 pullulanase | Y-1 pullulanase |
| Pullulan | 100 | 100 |
| Soluble starch | 3.4 | 64 |
| Amylose | 0 | 62 |
| β-Limited dextrin | 18.3 | 34 |
| Oyster glycogen | 6 | 44 |
| Amylopectin | 31 | 38 |

Each enzyme was prepared by electroelution of the bands showing a pullulanase band from a native-PAGE gel. Each enzyme assay was performed at 50 °C in glycine-NaOH buffer (pH 9.0 for S-1 and pH 10.0 for Y-1) with the indicated substrate (1%, w/v). The activity of each preparation toward pullulan was taken as 100%.

of pullulanase type I, and is the first report of an alkaline pullulanase type I.

The Y-1 enzyme showed two activities towards α-1,4-glycosidic linkages in starch or amylose and α-1,6-glycosidic linkages in pullulan, and is classified as pullulanase Type-II. In general, pullulanase debranches amylopectin, β-limited dextrins and soluble starch, but cannot act on amylose [1]. However, it has been shown that pullulanases from the anaerobic bacteria *Thermoanaerobium brockii* [5], *Thermoanaerobium* Tok B1 [22] and *Clostridium thermohydrosulfuricum* [19,27] 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 (ALP) from thermophilic *Bacillus* sp. 3183 [26,30] and an amylase-pullulanase enzyme (APE) from *B. circulans* F-2 [10]. The *B. subtilis* enzyme was found to be a multienzyme complex with a molecular mass of 450000 Da. In contrast, APE of *B. circulans* F-2 had a different active site responsible for amylase and pullulanase on a single polypeptide. Therefore, to elucidate whether Y-1 pullulanase has a single active site or not, requires further molecular characterization.

The newly isolated *Bacillus* sp. S-1 and *Micrococcus* sp. Y-1 described produce the extracellular alkaline pullulanases which exhibited maximum activity at 50–60 °C and at pH

8.0–10.0. Y-1 pullulanase activity was not denatured even at 70 °C in the presence of 2 mM $MnCl_2$, indicating that $MnCl_2$ can stabilize the enzyme activity. Most of the 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 °C. Both enzymes of the two strains, 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 preparations of two isolates contain pullulanase and amylase which can hydrolyse soluble starch into maltotriose as the main product. The characteristics of the purified pullulanases and their secretion will be reported in separate papers.

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