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Screening of *Bacillus* sp. No. M-71 with High Alkaline Protease Productivity and Some Properties of the Enzyme

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Alkaline Protease 高 生産性 細菌의 選別 및 酵素 特性

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Abstract — A bacterial strain No. 71, which produced alkaline protease, was isolated from soil and identified to the genus *Bacillus*. With the successive mutation, a mutant strain No. M-71, having high alkaline protease productivity, was obtained from the parental strain No. 71. Alkaline protease productivity of mutant strain No. M-71 was about 50 times as much as that of the parental strain No. 71. The enzyme preparations showed strong activities toward casein, the optimum pH being 11.0 and the optimum temperature about 55℃. The enzymes was stable at pH values from 6 to 11 on 10 min incubation at 60℃ and retains its high activity even at a relatively low temperature. These results indicate that the alkaline protease from *Bacillus* sp. No. M-71 can be used with a detergent builder and thus it can be useful as a detergent additive. Also it can be used in the hydrolysis of soybean and fish protein.

Alkaline protease is widely used in the fields of, for example, a leather industry, a food industry, a fiber or textile industry, or pharmaceutical industry (1-4). Furthermore, market demand for enzymes as a detergent additive has recently increased. Alkaline protease exhibit a maximum activity at a pH of about 10 to about 11. Most of them exhibit a maximum activity at a relatively high temperature, especially around 60℃, but are inactive or less active at a relatively low temperature, especially around a room temperature (5). This means that the enzyme is not fully utilized as a detergent additive in Korea, where clothes are generally washed at room temperature. Therefore, the development of enzymes having a retained enzymatic activity at a relatively low temperature is needed due to the recent increased usage of chemical fiber clothes having a relatively low heat stability and also due to the recent energy-saving movement. Recently, some alkalophilic strains of *Bacillus* were isolated as producers of potential alkaline proteases for use in detergents (6-12).

Studies on protease of alkalophilic bacteria were summarized by Horikoshi *et al.* (13) and those of alkalophilic *Streptomyces* sp. were also reported by Yamamoto *et al.* (5). Therefore, in this work, we tried to screen an active alkaline protease producers, investigate the microbiological characters of isolated strain and describe some properties of crude preparations of the enzyme produced by isolated strain.

Key words: Alkaline protease, *Bacillus* sp. No. M-71 characterization
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Materials and Methods

Media

An screening medium comprised (w/v %) 1% glucose, 0.5% polypeptone, 0.5% Difco yeast extract, 0.1% K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$ and 1% Na_2CO_3 (pH 10.1). Na_2CO_3 was autoclaved separately and the media were solidified by the addition of 1.5% (w/v) agar when necessary.

Isolation of alkaline protease-producing bacteria

A number of alkaline protease-producing bacteria were isolated on screening medium containing 0.5% (w/v) non-fat milk powder. Each soil sample was suspended at the approximate ratio of 1g of moist sample to 5 m*l* of sterile water and then streaked onto the plates of screening described above. The colonies which produced clean, transparent zones on the agar plates were selected and the widths of the clear zones were measured. The isolates that formed rather large clear zones on the agar plates were selected.

Identification of the bacterial isolates

Cultural, morphological, physiological and biochemical characterization of the bacteria isolated was performed according to the methods recommended by Gordon *et al.* (14) and Suzuki *et al.* (15). The results were analyzed using "Bergey's Manual of Determinative Bacteriology" (16).

Enzyme production

Each isolate was fermented aerobically at 37℃ for 72 hr. After centrifugation (6000×g, for 10 min at 4℃), the supernatant was used to enzyme source.

Mutagenesis

Mutant No. M-71 were obtained from *Bacillus* sp. No. 71 as follows. 0.2 m*l* of a culture of *Bacillus* sp. No. 71 grown in nutrient broth (5 m*l*) at 37℃ for 14 hr was transferred to 20 m*l* of nutrient broth, followed by incubation at 37℃ for 2 hr. The logarithmic phase cells were collected by centrifugation and then suspended in 10 m*l* of 0.1 M Tris-maleate buffer (pH 6.0) containing 200 μg of N-methyl-N′-nitro-N-nitroso-guanidine (MNNG) per m*l*, in a 100-m*l* flask, followed by incubation at 37℃ for 30 min. After centrifugation of the culture broth, the treated cells were washed with the same buffer and plated on the screening medium containing 0.5% non fat-milk powder. After incubation for 2 days, colonies with a large clear transparent zone were selected.

Cultivation in jar fermenter

Mutant No. M-71 was precultured in a medium containing per liter of distilled water: 30g glucose, 10g soybean meal, 0.4g $CaCl_2$, 0.2g $MgCl_2$, 2g K_2HPO_4. The protease production medium contained per liter of distilled water: 60g glucose, 20g soybean meal, 0.4g $CaCl_2$, 0.2g $MgCl_2$, 2g K_2HPO_4. The seed cultures were incubated in a rotary shaker at 37℃ for 24 hr, the two percent of seed (v/v) was added aseptically to the production medium. Jar cultivation was continued under forced aeration at 0.5 vvm and stirring at 300 rpm. Cultivation was carried out at 37℃ for 70 hrs. Cell growth was measured spectrophotometrically at 625 nm (17). Total sugar was determined using the phenol-sulfuric acid method described by Dubois (18).

Preparation of enzymes

The crude enzymes were prepared by ammonium sulfate saturation at 75%. The precipitates were dissolved in 20 m*l* Tris-HCl buffer, pH 7.5, dialyzed overnight at 5℃ against the same buffer and then freeze dried.

Enzymatic assays

The protease activity was assayed by the casein-280 nm method (19) with some modification as follows: One m*l* of enzyme solution was mixed with 5 m*l* of 1.2% casein solution (pH 10.1). After 30 min incubation at 40℃, 5 m*l* of a TCA mixture (a solution containing 0.33 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid) was added to the incubation mixture and the mixture was kept at 40℃ for 20 min.

The precipitate formed was removed by filtration (filter paper, Whatman No. 2) and the absorbance of the filtrate was measured at 280 nm. The reading was corrected for the value of a blank in which the enzyme was mixed with the TCA mixture before the casein was added. One unit of enzyme activity was defined as the activity capable of increasing the value of absorbance by 1.0 under the above conditions.

Results and Discussion

Screening for alkaline protease-producing bacteria

First screening test; A total of 211 isolates were

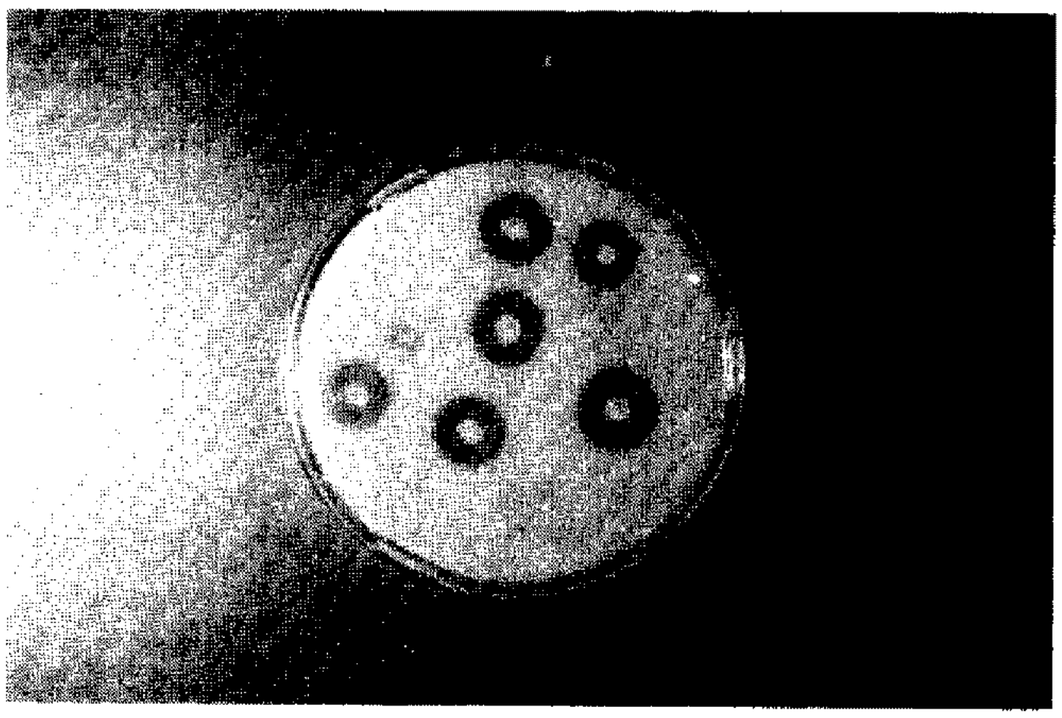

Fig. 1. Formation of clearing zone in the screening media contained 0.5% non-fat milk powder.

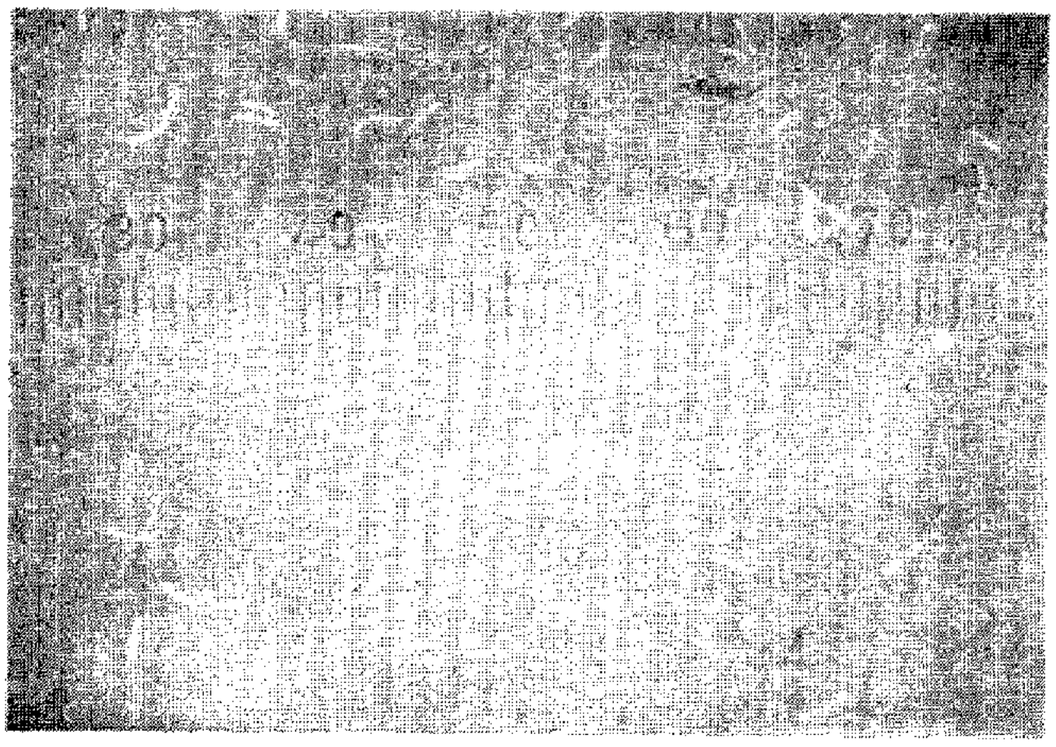

Fig. 2. The phase contrast micrograph of the alkaline protease producing strain, *Bacillus* sp. M-71.
*One scale indicates 1.0 μm

obtained from 30 soil samples. Among the collection of 211 isolates, twenty isolates with rather large clear zones were selected (Fig. 1).

Second screening test; After cultivation of these 20 isolates in liquid screening medium, alkaline protease activity was determined. The crude enzyme of isolated strain No. 71 exhibited an extremely high enzyme activity, the activity being about 4~5 fold in comparison with other selected strains. Therfore, we used this strian for further studies.

Identification of the isolated strain No. 71

The isolated bacterial strain No. 71 was an aerobic, spore-forming, gram positive, motile and rod-shaped bacterium, and should belong to the genus *Bacillus*. The morphological, cultural and physiological characteristics of the strain No. 71 are summarized in Table 1 (Fig. 2). The isolate grew well over the wide pH range of 7 to 11 and grew best at pH 8.5, i.e., this strain was alkalophilic.

This strain could grow at temperatures not exceeding 43℃ and could utilize citrate, inositol and sorbitol, but could not utilize lactose, ribose or D-fructose.

Selection of mutants

Four typical mutants representing different stages of induction were obtained by successive induction processes. From Table 2, it can be seen that mutant No. M-71 produced 50 times as much alkaline protease as the parental strain, No. 71.

Production of alkaline protease

Bacillus sp. No. M-71 obtained with successive mutation was cultivated in 30*l* Jar fermenter. As shown in Fig. 3, protease production has reached maximum level at 65 hr of cultivation and pH profiles showed decrease down to 6.5 at 20 hr and turned rapidly upward thereafter.

Effect of pH on the activity and stability of enzyme

The effect of pH on the alkaline protease activities were examined in buffers of various pH. As shown in Fig. 4, maximum activities were observed at about pH 11.0.

The residual alkaline protease activities of the crude enzyme preparations were measured at pH 11.0 after incubation of the enzymes at 60℃ for 10 min in buffers of various pH. As shown in Fig. 5, the crude enzyme was most stable at pH 9~11. In this pH region, the original activity of the crude enzyme was completely retained. Furthermore, 80% or more of the remaining activity was exhibited at a pH of 5 to 11.5.

Optimum temperature and thermal stability

The optimum temperature was determined by varying the incubation temperature. As shown in Fig. 6, the crude enzyme was active at a temperature of 35 to 60℃ and at a pH of 11.0. The optimum temperature was 50 to 55℃. The crude enzyme was

Table 1. Microbiological properties of the isolated strain No. M-71

Morphology							
Form	Rods						
Size (μm)	0.6−0.8×1.5−3.0						
Motility	+						
Spore (μm)	0.7−1.2×1.2−1.4						
Spore shape	Ellipsoidal						
Spore position	Central						
Gram staining	Positive						
Characterization of cultures					pH 7.0*	pH 10.1	
Peptone broth					±	++	
Nutrient broth					±	+	
Nutrient broth containing 7% NaCl					+	++	
Meat extract-peptone agar slant					±	++	
Glucose-peptone broth					±	++	
Casein-meat extract-peptone agar slant					±	++	
Starch-yeast extract broth					−	+	
Starch-peptone broth					±	++	
Anaerobic growth in nutrient broth					−	−	
Biochemical properties							
Hydrolysis of							
Starch	Hydrolyzed						
Casein	Hydrolyzed						
Gelatin	Hydrolyzed						
Utilization of							
Citrate					Utilized		
Inorganic nitrogen source					Utilized		
Reduction of nitrate to nitrite					Reduction		
Methyl red (MR) test					Negative		
Voges-Proskauer (VP) test					Positive		
Anaerobic production of gas from nitrate					Negative		
Formation of							
Indole					None		
Hydrogen sulfied					None		
Urease test					Negative		
Oxidase test					Positive		
Catalase test					Positive		
pH for growth					7-11		
Temperature for growth					55℃		
Utilization of sugars:**							
L-Arabinose	+	D-Xylose	+	D-Glucose	+	D-Mannose	+
D-Fructose	−	D-Galactose	+	Maltose	+	Sucrose	+
Lactose	−	Trehalose	+	D-Sorbitol	+	D-Mannitol	+
Inositol	+	Glycerol	+	Ribose	−		

Symbols: *; −, no growth; ±, poor growth; +, normal growth; ++, growth abundant
**; +, positive; −, negative

Table 2. Comparison of protease productivity

Strain	Protease activity (unit/ml)
No. 71 (parent)	160
No. A-76 (lst stage)	800
No. F-22 (2nd stage)	2500
No. H-92 (3rd stage)	4100
No. M-71 (4th stage)	8000

*Cultivation of each strain was carried out at 37℃ for 3 days with shaking in liquid screening medium.

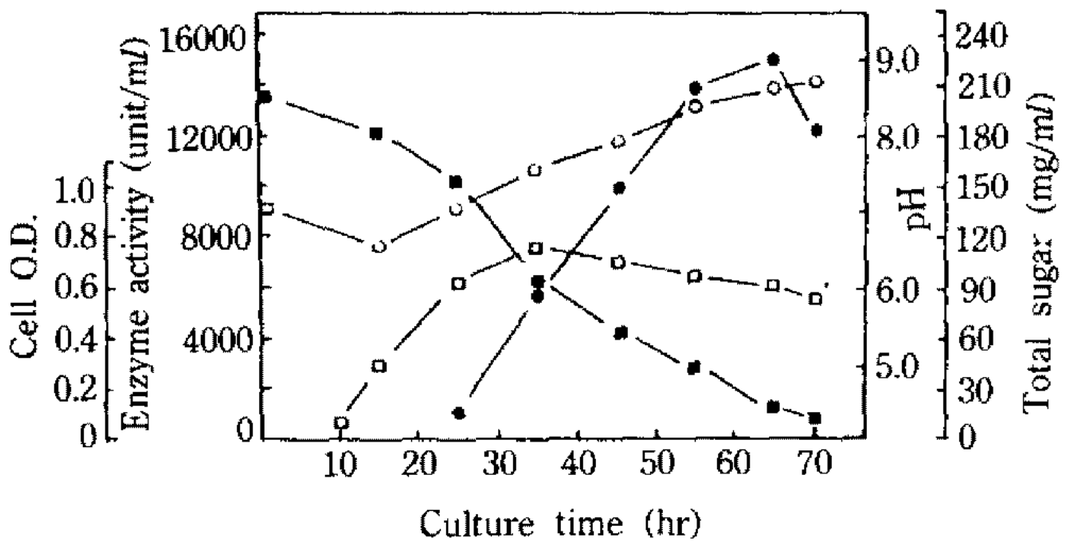

Fig. 3. Time course of alkaline protease production.
*Symbols: ○—○; pH, ●—●; Enzyme Activity, □—□; Cell O.D., ■—■; Total Sugar
**Bacillus* sp. No. M-71 cultivated in the protease production medium at 37℃ using 30 *l* fermentor (Marubishi Co.)

dissolved in 0.05 M Borax-NaOH (pH 10.0), incubated at various temperatures for 10 min, and residual activities were measured at pH 11.0. As shown in Fig. 7, the residual activity was 100% when heated at 20~45℃, and 60% at 50℃.

Effect of surfactants and chelating agents

The crude enzyme was completely inactivated by DFP, BKCL and ABS inhibited the crude enzyme activities by 33% and 30%, respectively. The crude enzyme was characteristically stable in the presence of other surfactants and chelating agents, such as EDTA, Tween 20, Triton X-100, PCMB, STPP, SPB and SLS (Table 3).

The crude enzyme from *Bacillus* sp. No. M-71 which has an optimum pH of 11.0, retains the activity at a relatively low temperature. The No. M-71 protease was completely inactivated by DFP, therefore this enzyme can be classified as a serine enzyme. Therfore, the No. M-71 protease can be used

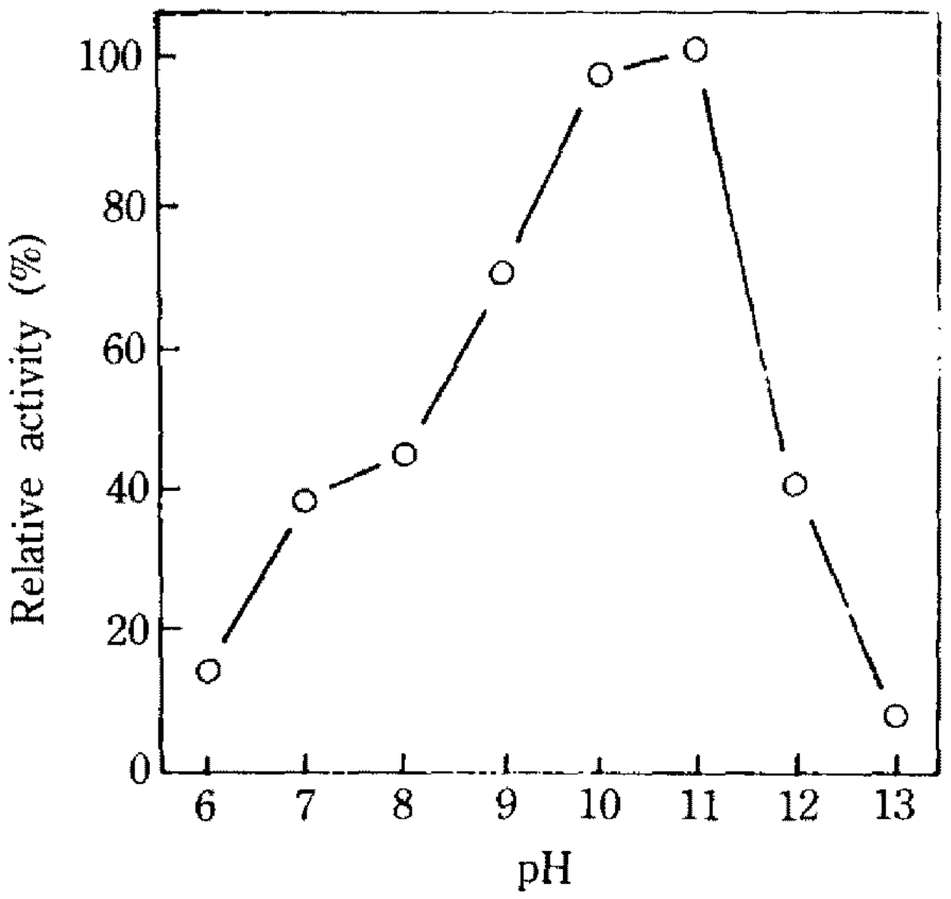

Fig. 4. pH-Activity profile of the alkaline protease.
*The following buffer systems were used: 0.1 M Potassium phosphate-0.05 M sodium borate (pH 6~9); 0.1 M Sodium carbonate-0.1 M Boric acid-Potassium chloride (pH 9~11); 0.1 M Disodium hydrogenphosphate-Sodium hydroxide (pH 11~12); 0.02 M Potassium chloride-Sodium hydroxide (pH 12~13).
*Each buffer solution (0.5 m*l*) containing 2% milk casein was added to 50 μ*l* of the crude enzyme solution to prepare a test solution.

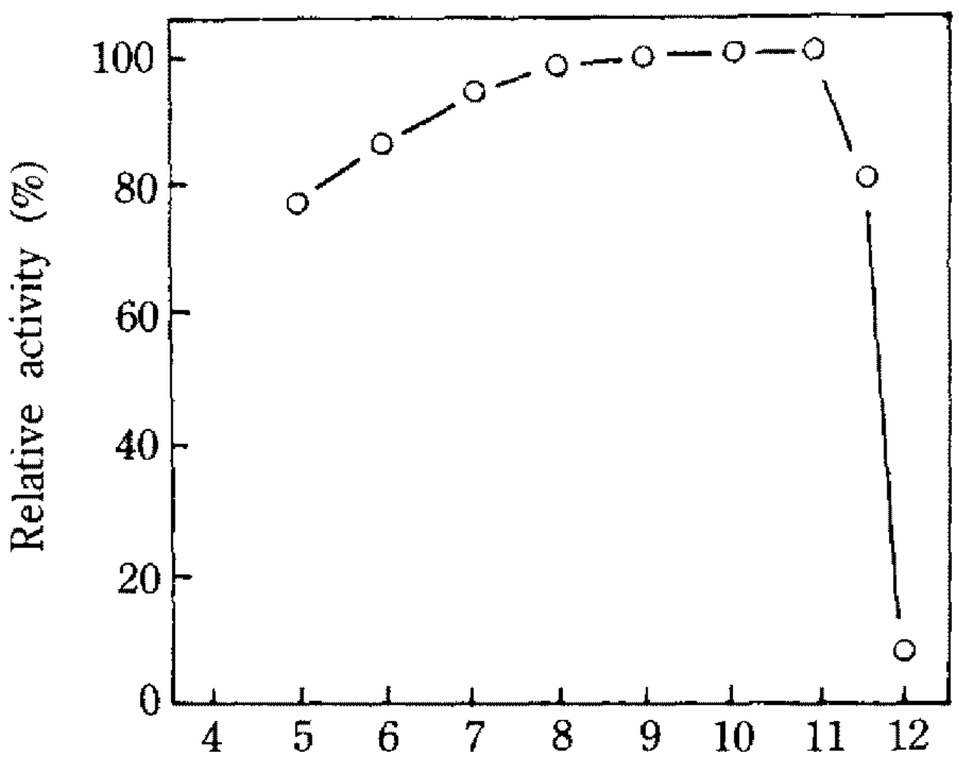

Fig. 5. Effect of pH on stability of the enzyme.
*The following buffer systems were used: 0.1 M Citric acid-0.2 M Disodium hydrogenphosphate (pH 4~8); 0.1 M Sodium carbonate-0.1 M Boric acid-Potassium chloride (pH 8~11); 0.15 M Disodium hydrogenphosphate-Sodium hydroxide (pH 11~12).
*Each buffer solution (0.5 m*l*) was added to 50 μ*l* of the crude enzyme solution and the mixture was incubated at 60℃ for 10 min. After incubating 9.5 m*l* of a 0.1 M carbonate buffer solution containing 2% casein and having a pH of 11.0 was added to the mixture. The enzymatic activity of the mixture was determined.

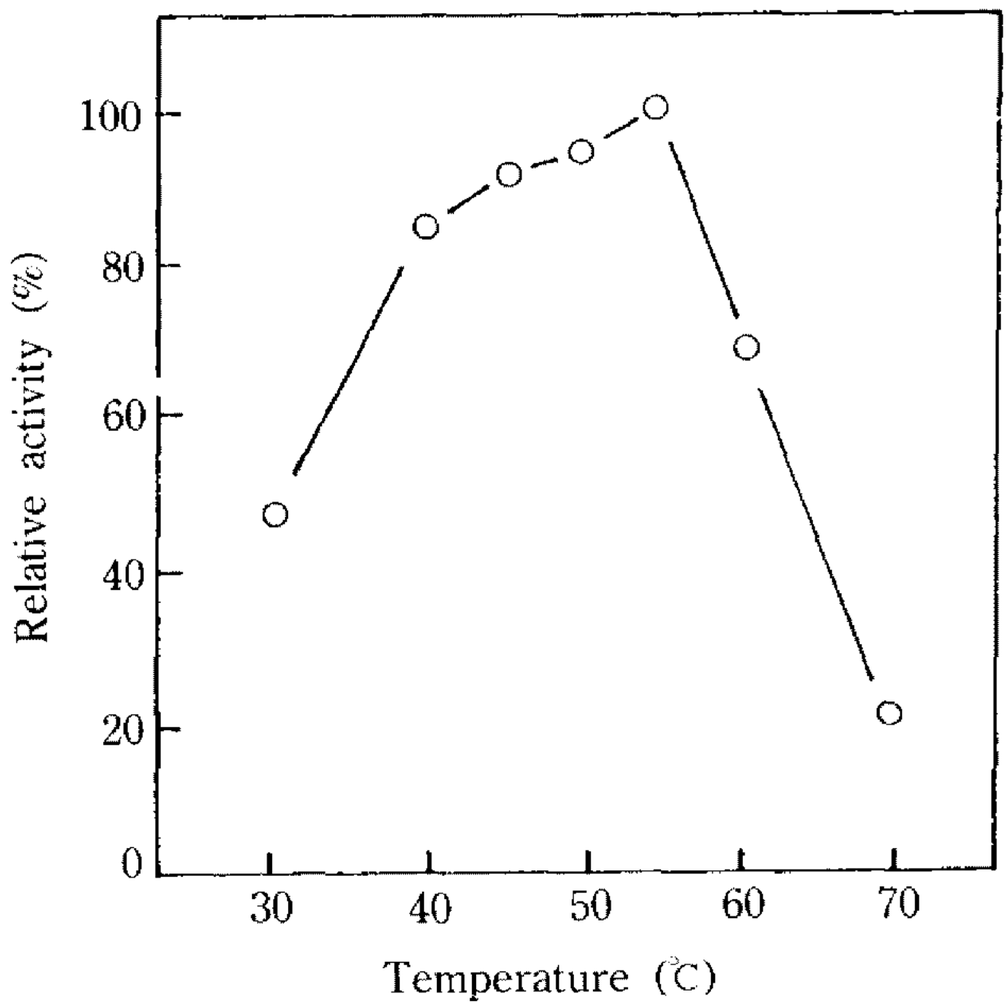

Fig. 6. Optimum temperature for enzyme action.
*The reaction was carried out at the temperature indicated for 30 min at pH 11.0.

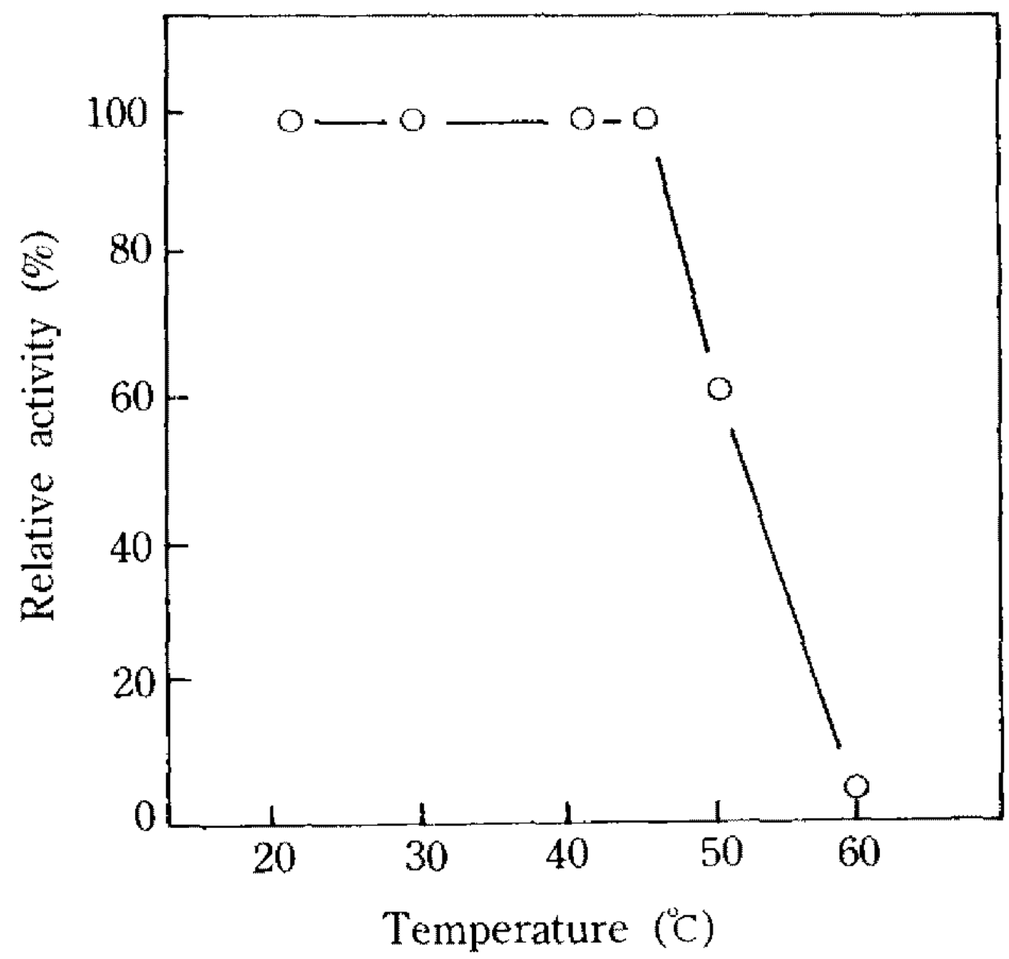

Fig. 7. Effect of temperature on stability of the enzyme.
*The enzyme was dissolved in 0.05 M Borax-NaOH buffer (pH 11.0), incubated at various temperatures for 10 min, and the residual activities were measured.

with a detergent builder and thus it can be useful as a detergent additive, since it is stable at a pH of 7 to 11.5 and the maximum enzymatic activity is attained at a pH of 11.0. Especially, the enzyme retains its high activity even at a relatively low temperature, as compared with conventional enzymes for a detergent (20). Consequently, the enzyme is a most desirable type of alkaline protease as an

Table 3. Effect of various inhibitor on the action of alkaline protease from the isolated *Bacillus* sp. No. M-71

Inhibitor	Concentration	Relative activity
None	–	100
PCMB	10^{-1} M	100
EDTA	5×10^{-1} M	98
DFP	2.5×10^{-1} M	2
Tween 20	0.5%	100
Triton X-100	0.05%	98
SLS	0.2%	93
BKCL	0.2%	67
ABS	5×10^{-1} M	70
STPP	0.2%	96
SPB	0.1%	99

*The mixture of 1.0 m*l* of enzyme, 2.0 m*l* of Tris-HCl buffer, pH 7.5, 0.02 M and 1.0 m*l* of the chemical reagent indicated were incubated at 30℃ for 1 hr, and the residual activity was measured.
*PCMB = ρ-Chloromercuribenzoate; EDTA = Ethylenediamine tetraacetic acid; DFP = Diisopropylfluorophosphate; Tween 20 = Polyoxyethylene sorbitan monolaurate; Triton X-100 = Isooctylphenyl polyethoxy alcohol; SLS = Sodium lauryl sulfate; BKCL = Benzalkonium chloride; ABS = Alkylbezenesulfonate; STPP = Sodium tripolyphosphate; SPB = Sodium perborate

additive for a household detergent, in such a country where clothes are washed at a room temperature as Korea.

요 약

Alkaline protease 生産力이 높은 細菌 No. 71을 土壤에서 分離하였다. 分離된 細菌을 同定한 結果 *Bacillus* sp.인 것으로 判明되었으며, 이 No. 71 細菌에 變異劑를 반복 處理한 結果 alkaline protease 生産性이 母菌보다 약 50배 向上된 高 生産性 變異株 No. M-71를 얻었다. 부분 精製된 alkaline protease는 casein에 대해 pH 11.0에서 가장 높은 活性을 보였고, pH 5.0~11.0 사이에서 安定性을 나타내었다. 또한 酵素의 最適 溫度는 55℃이었다.

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