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# Screening and Identification of a Potent Fungus for Producing Raw Corn Meal Saccharifying Enzyme

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# 옥수수 생 전분 당화 효소 생산 곰팡이의 분리 및 동정

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**We have been searching microorganisms which produce highly active raw starch saccharifying enzyme and also have a good cultivation characters in submerged culture. About 170 strains of molds isolated from soil and compost were tested for their amylase productivity on plate contained 2% raw corn meal. Thirty-four strains out of 170 strains produced clearance on the plates, and were tested for their raw starch saccharifying activity. Then, 4 strains which had shown relatively high levels of saccharifying activity were selected. Among them, Strain No. 55 was found to have highest level of raw starch saccharifying activity, and selected for the further studies. In this paper, the morphological, physiological and cultural characteristics of Strain No. 55 were described. Based on the results obtained in these experiments, Strain No. 55 was identified to be a similar species to *Aspergillus niger*.**

In recent years, the importance of enzymatic saccharification of raw starch without heating has become well recognized, mainly from the viewpoints of energy saving and effective utilization of the biomass.

Many microorganisms are capable of producing extracellular amylases. However, only a few fungi have been reported to be producers of active amylases capable of degrading raw starch (1-6). Ueda *et al.*(7) have studied raw starch digesting enzyme of *Aspergillus awamori*. Park and Rivera (8) reported on the glucoamylase from *Aspergillus niger* and *Asp. awamori*. Yamamoto *et al.* (9) also studied on the alcohol fermentation of cassava without cooking using glucoamylase obtained from *Aspergillus* sp..

It is unfortunate for commercial use that *Aspergillus* produces large amounts of glucoamylase in solid culture but not in submerged culture extremely strong hydrolyzing activity toward raw starch compared to other glucoamylases. Therefore, our primary research aim here was to find an active saccharifying enzyme producer for raw corn meal and then to investigate the microbiological characters of isolated microorganism. Also we have studied the production of glucoamylase in submerged culture by a species of *Aspergillus niger* screened for its high glucoamylase activity and the action of the enzyme on starch granules.

Key words: Raw corn meal saccharifying enzyme, *Aspergillus niger*
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## Materials and Methods

### Microorganisms

All the microorganism producing the saccharifying enzyme capable of degrading raw corn meal were isolated from soil and compost from various places.

### Media for screening

The midia used in the screening were as follows. Medium A contained (g/*l*): Raw corn meal, 20.0: $(NH_4)_2SO_4$, 1.4: Urea, 0.3: $KH_2PO_4$, 2.0: $CaCl_2$, 0.3: $MgSO_4 \cdot 7H_2O$, 0.3: Polypepton, 5.0: Yeast extract, 1.0: Agar, 20.0 and Triton X-100, 0.3 m*l*. For Medi-

um B, the agar and Triton X-100 in Medium A were replaced by 2.0g/*l* of Tween 80, and then the medium was used for shaking cultures. Raw corn meal was ground to pass a 60-mesh sieve and then was separately sterilized with ethylene oxide gas at 27°C for 48 hrs.

**Screening method**

Each soil sample was suspended at the approximate ratio of 1g of moist soil to 10 m*l* of sterile water, and then serially diluted. 0.1 m*l* aliquots of the dilutions were aseptically placed onto agar plates of Medium A, and then spread evenly over the plates. The plates were incubated at 30°C for appropriate periods. Fungi and actinomycetes that formed clear zones were pure cultured after streaking on plates. The isolates were incubated at 30°C for 7 days on glucose peptone yeast extract agar slants and then maintained at 4°C.

The initial screening was based on the ratio (*R*) of the diameter of raw corn meal clearance to the diameter of the agar-block. The colonies with large values of *R* were assayed for the activity of saccharifying enzymes in shake flasks.

**Classification and identification of the isolates**

Each of the isolates was inoculated onto Czapek's solution agar and malt extract agar, and classified according to the methods described by Barron (10) and Arx (11).

**Enzyme production**

About 5 m*l* portions of the cultured broths (Medium B) were taken and centrifuged at 3000 rpm for 20 min at 4°C to remove mycelia and residual corn meal powder. The supernatant was used to assay amylase activity.

**Assay of raw starch saccharifying activity**

Raw starch digesting-ability (RDA) was determined by measurement starch saccharifying activity according to Oh *et al.* (6). Raw staroh-digesting activity was assayed using a reaction mixture consisting of 20 mg of corn meal, 0.2 m*l* of 0.1 M acetate buffer solution (pH 4.5) and 1.6 m*l* of distilled water.

**Determination of the optimum reaction conditions and enzyme stability**

The optimum pH of the reaction was determined as relative activity after incubation for 30 min at 40°C in a shaker. The reaction mixture consisted of 0.1 m*l* of enzyme solution, 0.4 m*l* of 0.1 M buffer solution of different pH value and 0.5 m*l* of the substrate suspension (10 mg corn meal). To determine the pH-stability, 0.4 m*l* of the enzyme solution was treated with 0.2 m*l* of 0.1 M buffer solution of various pH value for 60 min at 40°C. Then the remaining enzyme activity was determined. The thermostability of the enzyme was determined by treating 0.4 m*l* of the enzyme solution for 15 min with 0.2 m*l* of 0.1 M acetate buffer (pH 4.5) at various temperature. Then the remaining activity was determined at 40°C. The optimum temperature of the reaction was determined by incubating 0.1 m*l* of the enzyme solution with 0.4 m*l* of 0.1 M acetate buffer (pH 4.5) and 0.5 m*l* of the substrate suspension (10 mg) at various temperatures in the shaker for 30 min.

**Action pattern of the enzyme on various raw starches**

Fifty mg of various substrate were treated by the crude enzyme solution at 40°C for 10 hr. The increase of the reducing sugar was followed by the Somogyi-Nelson method (12).

## Results and Discussion

**Screening for raw corn meal hydrolyzing fungi**

At first, we isolated 170 strains of molds from soil and compost. All strains were tested for their ability to produce clear zones in Medium A. Only thirty-four of isolates were selected on the basis of higher and lower ratios of diameter of the halo to that of the colony on the Medium A. Therefore, they were used for subsequent studies. Each of the selected amylase producers was inoculated into test tubes containing 15 m*l* of Medium B. After cultivations with shaking, raw starch saccharifying activity was determined. Four strain showing potent activty, Strain No. 55, 121, 82 and 170 were selected, and used for further experiments. The *R* value of Strain No. 55, 121, 82 and 170 were 3.3, 2.5, 2.2 and 1.8, respectively (Table 1, Fig. 1). The 4 strains that produced the highest levels of amylase in the preliminary tests were inoculated into 500 m*l* Sakaguchi flasks containing 100 m*l* of Medium B and incubated. The time course of the enzyme production by each strain is shown in Fig. 2. Saccharifying activity toward raw corn meal was higher than 80 U/m*l* in the cultural filtrate of Strain No. 55 and 121. The RDA values of the crude enzymes produced by Strain

**Table 1. *R* values and RDA of selected fungi.**

| Strain No. | Diameter of agar block (cm) | Diameter of clearance (including the colony within, cm) | *R* value | RDA |
|---|---|---|---|---|
| 55 | 0.4 | 1.3 | 3.3 | 91 |
| 121 | 0.4 | 1.0 | 2.5 | 65 |
| 82 | 0.5 | 1.1 | 2.2 | 42 |
| 170 | 0.6 | 1.1 | 1.8 | 31 |

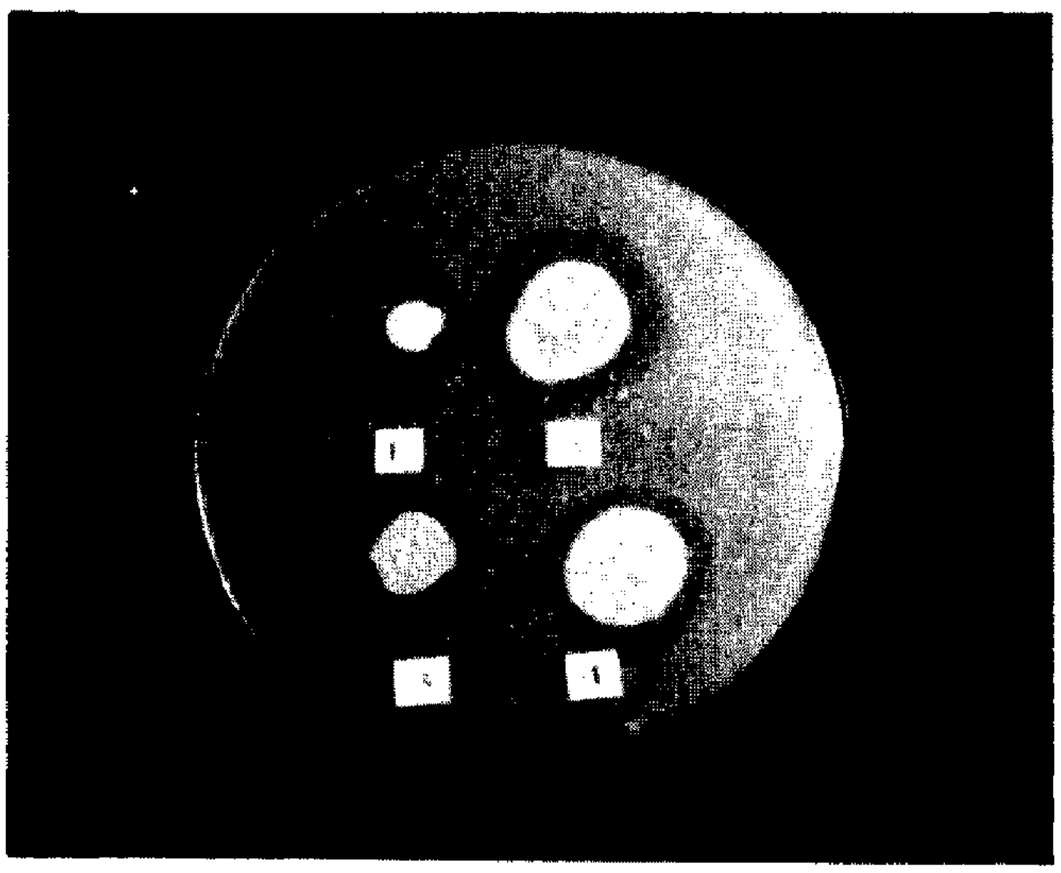

**Fig. 1. Formation of clearing zone in the media contained raw corn meal powders.**
Symbols: 1, Strain No. 55: 2, Strain No. 82: 3, Strain No. 121: 4, Strain No. 170

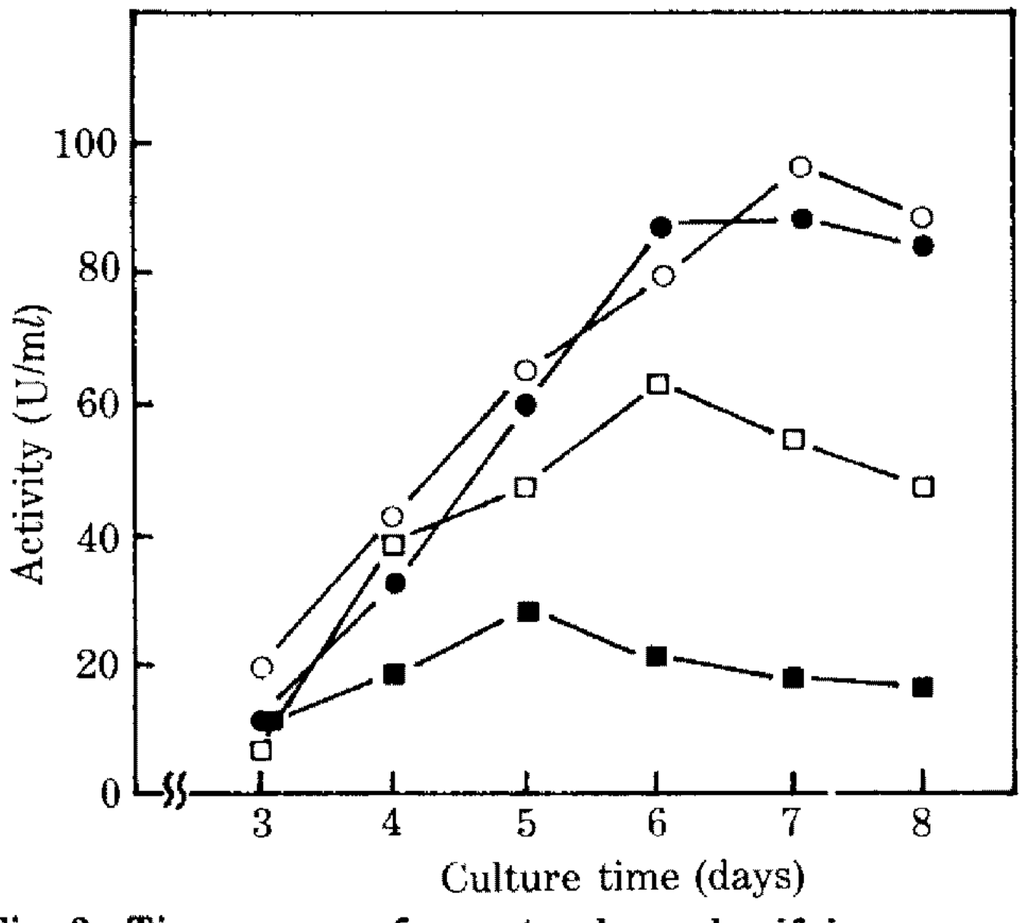


**Fig. 2. Time course of raw starch saccharifying enzyme production by selected fungi.**
Symbols: ○, Strain No. 55: ●, Strain No. 121: □, Strain No. 82: ■, Strain No. 170

**Table 2. Morphological characteristics of *A. niger* No. 55.**

| | | |
|---|---|---|
| Conidia | globose or elliptical smooth or slightly rough | diameter: 3.0-5.0 $\mu$m |
| Conidiophore | brown, smooth brittle | length: 1.0-1.5 mm diameter: 5-20 $\mu$m |
| Conidial heads | globose | diameter: 200-300 $\mu$m |
| Sterigmata | two series | 5.5-10.0*3.0-4.0 $\mu$m |
| Vesicles | globose, light brown | diameter: 25-50 $\mu$m |

*Medium: Potato dextrose agar. Incubation temp: 30°C, for 7 days

**Table 3. Physiological characteristics of *A. niger* No. 55.**

| | | |
|---|---|---|
| Growth temperature | 10-40°C | Optimum 30°C |
| Growth pH | 3-8 | Optimum 4-5 |
| Utilization of carbon sources: | | |
| Sucrose +++ | Cellobiose ++ | Maltose ++ |
| Rhamnose + | Glucose +++ | Fructose +++ |
| Sorbose − | Mannose ++ | Fucose − |
| Arabinose +++ | Ribose + | Xylose +++ |

+++: good, ++: moderate, +: poor, −: no growth
*Medium was composed of carbohydrate 1.0%, polypepton 0.5%, yeast extract 0.2%, $KH_2PO_4$ 0.1% and $MgSO_4 \cdot 7H_2O$ 0.05%.
*Incubation was carried out at 30°C for 4 days.

No. 55, 121, 82 and 170 were 91, 65, 42 and 31, respectively. The crude enzyme of Strain No. 55 exhibited an extremely high RDA value (Table 1).

**Identification of fungi, Strain No. 55 by morphological observation**

Morphological, physiological and cultural characterization of the fungi, Strain No. 55 was performed. Colonies on malt agar medium grew rapidly at room temperature. Conidia were globose shape with the size of 3.0-5.0 $\mu$m, and uncolored, smooth walled. Conidiophores growing vertically from hyphae with a long cylindrical from consisting of 2 to 3 septa. According to the morphological observation, the strain was identified as *Aspergillus niger* (Table 2). Other characters of *Aspergillus niger* No. 55 are shown in Table 3,4 and an photomicrograph in Fig. 3.

**Table 4. Cultural characteristics of *A. niger* No. 55 on various media .**

| | | | | |
|---|---|---|---|---|
| Czapek's solution agar | good growth, dark chocolate brown | 24-26°C, | 5-7 cm, | 10 days |
| Malt extract agar | abundant growth, deep chocolate brown | 24-26°C, | 7-8 cm, | 10 days |
| Potato dextrose agar | good growth, deep brown | 24-26°C, | 6-7 cm, | 10 days |

**Fig. 3. Photomicrograph of morphology of *A. niger* No. 55 (×800).**
The strain was grown on potato dextrose agar at 30°C for 7 days

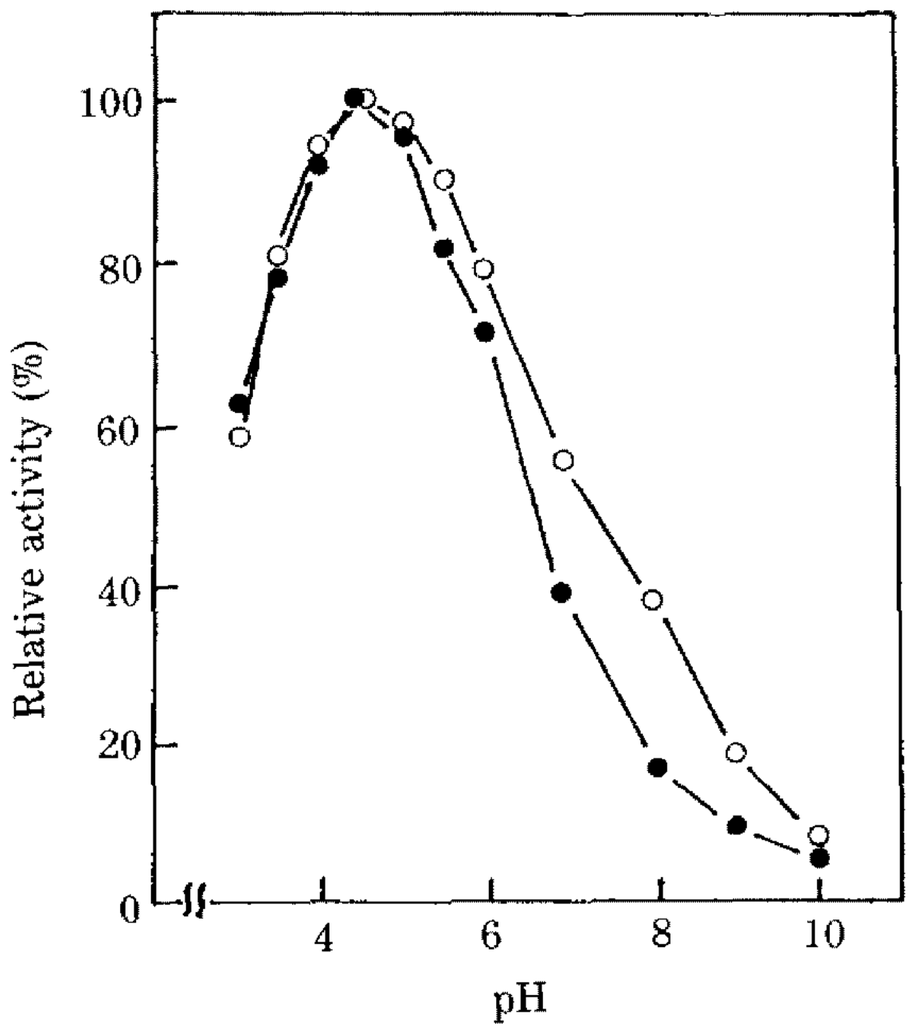


**Fig. 4. pH-Activity curves of the crude *A. niger* No. 55 amylase.**
Symbols: ○, gelatinized starch-saccharifying activity, ●, raw starch-digesting activity. The relative activity was expressed as % activities at various pH values compared to the enzyme activity at pH 4.5. The buffers (0.1 M) used were as follows: pH 3.0-4.0, citric acid: pH 4.5-6.0 Na-acetate: pH 7.0-8.0, Na-phosphate: pH 9.0-10.0, boric acid

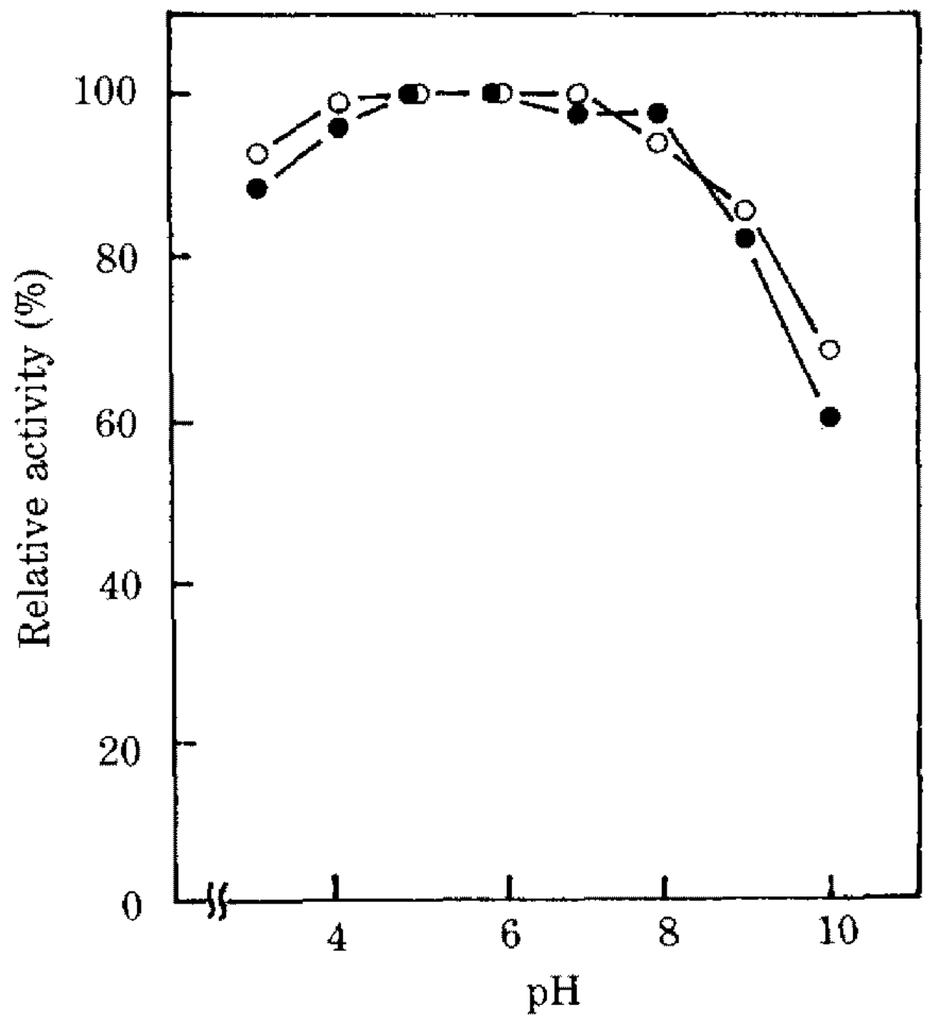


**Fig. 5. pH Stability curves of the crude *A. niger* No. 55 amylase.**
Legends same as in Fig. 4

**Properties of amylases produced by *Aspergillus niger* No. 55**

The optimum pH of the enzyme was found around pH 4.5 for both raw and gelatinized starches (Fig. 4). After being kept at various pH values for 60 min at 40°C in the absence of substrate, the enzyme retained about 90% of its activity between 3 and 8 (Fig. 5). Fig. 6 and 7 show the optimum temperature of the activity and the thermostability of the enzyme at various temperatures. The optimum temperature of the enzyme was found around 50°C for both raw and gelatinized starches. The enzyme was rather unstable against heat treatment. It retained all activity after incubation for 15 min at 40°C, and lost 70% activity for raw starch-digesting activity and lost 83% activity for gelatinized starch-digesting activity at 50°C.

**Hydrolysis of various raw starches**

As seen in Fig. 8 rice, wheat and corn belong to

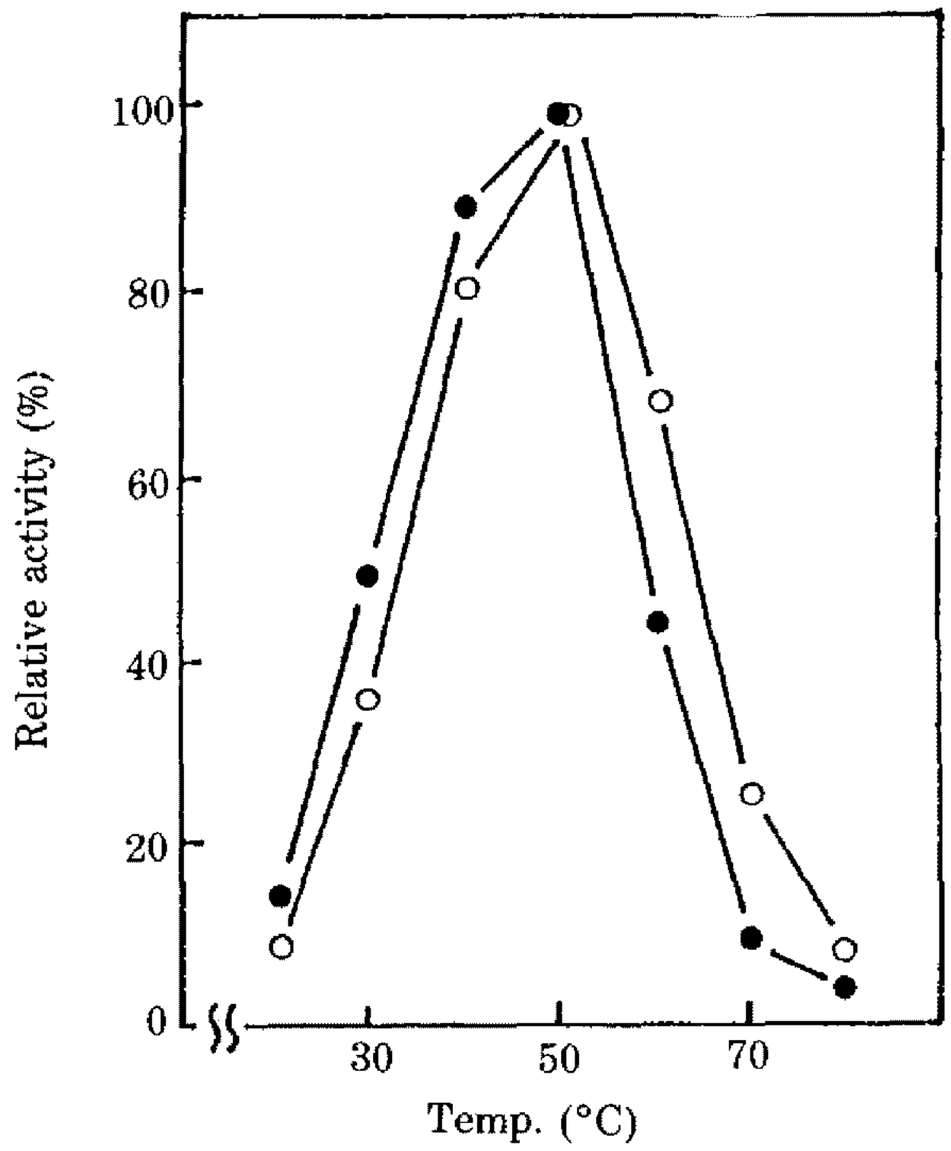


**Fig. 6. Optimum reaction temperature of the crude *A. niger* No. 55 amylase.**

Legends same as in Fig. 4. The relative activity was expressed as % activities at various temperatures compared to the enzyme activity at 50°C (pH 4.5).

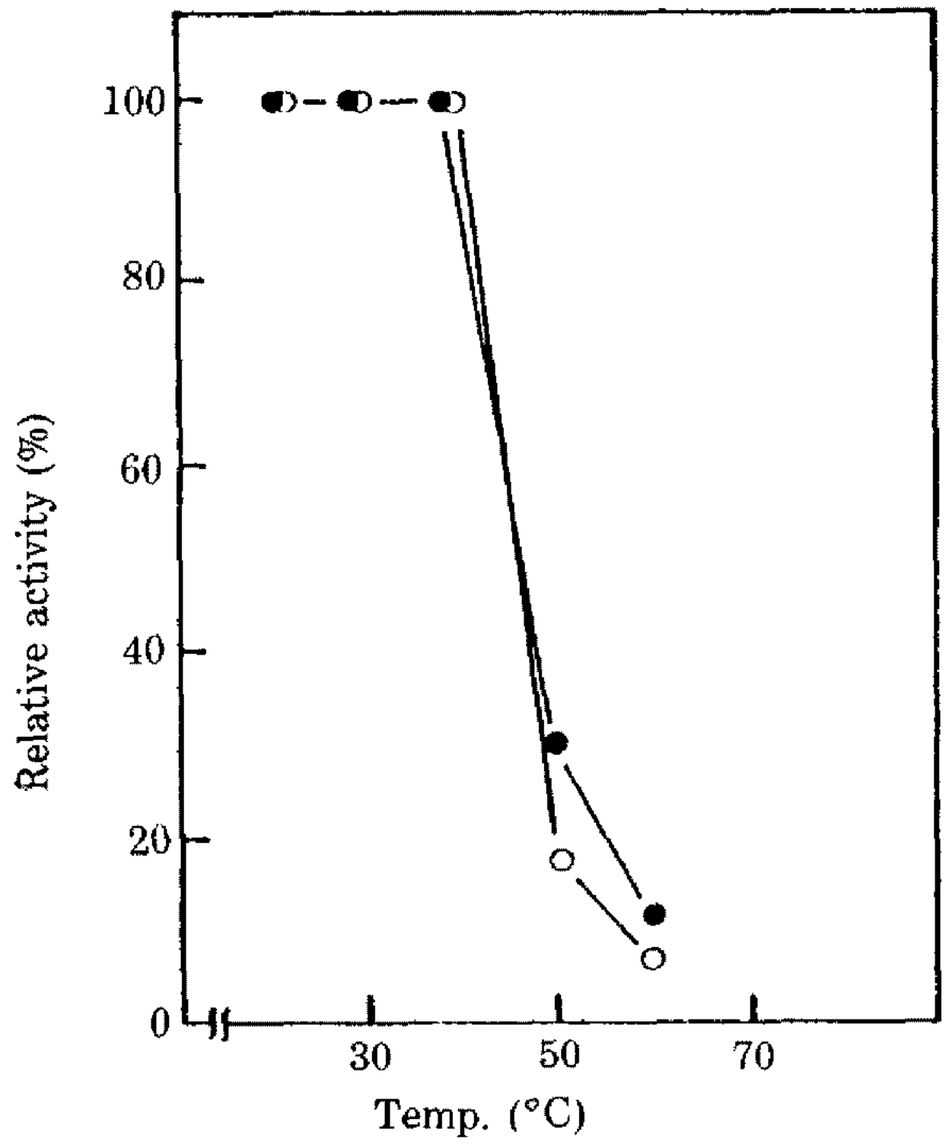


**Fig. 7. Thermostability of the crude *A. niger* No. 55 amylase.**

Legends same as in Fig. 4. Enzyme solutions were heated for 15 min at various temperatures (pH 4.5). The remaining activities were determined under the standard assay conditions immediately after heating for 15 min.

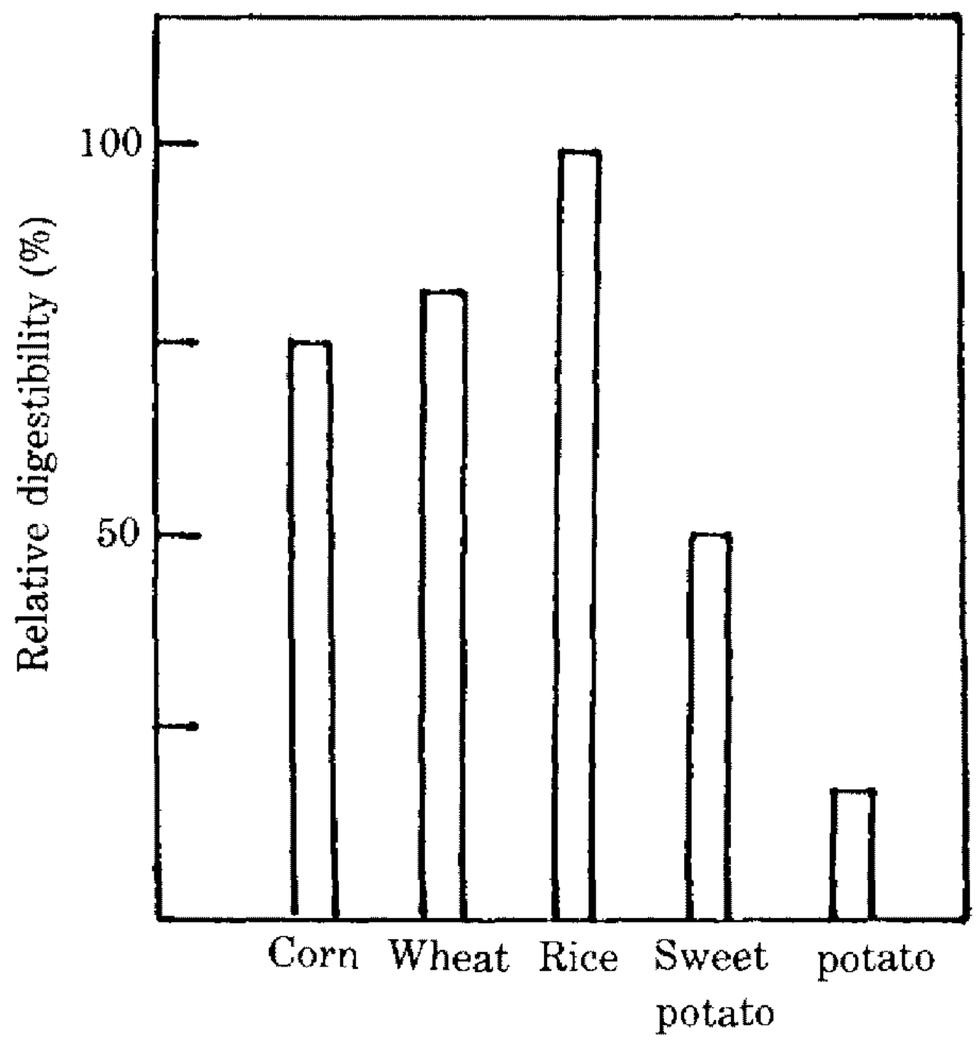


**Fig. 8. Comparison of the digestibilities of various raw starches.**

The saccharifying activities of the amylases produced by *A. niger* No. 55 toward each raw starch were analyzed as described under Materials and Methods.

the highly susceptible starch to the enzyme. Sweet potato and potato starches are more resistant to the enzyme action. These results suggest that *Asp. niger* No. 55 produced a strong raw corn meal saccharifying enzyme, and the enzyme can be used for the saccharification of starch without cooking. The enzyme is useful for the fermentation of alcohol with energy saving process, as well as for the production of starch sugars such as glucose, isomerized glucose syrup etc. from starch granules.

## 요 약

산업적인 응용을 위해, 生 전분 분해력이 높고 액체 배양에 적용 가능한 균을 토양으로부터 분리한 결과, 옥수수 生 전분 당화 효소 생산력이 높은 곰팡이 No.55 균주를 분리하고 이 균주의 형태적, 생리적, 배양특성을 조사, 균 동정을 행한 결과 No.55 분리균은 *Aspergillus niger* 또는 그 유연균으로 동정되었다.

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