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Isolation of Soil Bacteria Secreting Raw-Starch-Digesting Enzyme and the Enzyme Production

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Two strains (No. 26 and 143) of bacteria which secrete both pectinase and raw-starch-digesting amylase simultaneously, were isolated from various domestic soil samples. The two bacteria were identified as *Pasteurella ureae* judging by their morphological and physiological characteristics. The optimal culture conditions for the production of raw-starch-digesting enzyme by the *Pasteurella ureae* 26 were using NH_4NO_3 as the nitrogen source at 37°C with the pH of 7.5, and 15 of C/N ratio. Since the enzyme was produced only when raw or soluble starch was used as a carbon source, but not when glucose or other sugars was used, the enzyme was considered to be an inducible enzyme by starch. Thin layer chromatography of the hydrolyzed product of starch by the raw-starch-digesting enzyme of the strain No. 26 showed that glucose, maltose and other oligosaccharides were present in the hydrolyzates, and therefore the enzyme seemed to be α-amylase. The enzyme had adsorbability onto raw corn starch in the pH range of 3 to 9.

The process of hydrolyzing starch to glucose includes the sequential addition of α-amylase and glucoamylase. But, the conventional process of hydrolysis has several faults, for example, the cost of energy consumption for heating and cooling is expensive, ca. 30~40% of energy is spent for alcohol production (15), and the concentration of produced glucose is low because it is difficult to liquefy high concentration of starch which resulted due to the swelling of starch during cooking. Therefore, it takes a long time to obtain a high concentration of glucose from saccharification process, and a removal of impurities formed during cooking is necessary.

To solve these problems, a direct hydrolysis of raw starch employing the raw-starch-digesting enzyme has been investigated. Abe *et al.* (1) reported that procedures for the biochemical and biological conversion of raw starch into glucose and alcohol have attracted much interest from the viewpoint of energy saving. Raw-starch-digesting enzyme has been mainly found in fungi such as *Aspergillus* sp. (1, 17), *Aspergillus usamii* (5), *Chalara paradoxa* (15), *Corticium rolfsii* (20), and *Rhizopus* sp. (14, 18, 21). The enzyme has been also found in bacteria such as *Bacillus subtilis* (8) and *Streptomyces* sp. (4).

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In this investigation, new bacterial strains which secrete raw-starch-digesting enzyme were screened, and a basic study was performed on the enzyme.

MATERIALS AND METHODS

Preparation of Soil Samples

One hundred soil samples were collected from various areas of Yumyoung mountain in Kangwon-Do, Jebu island in Kyunggi-Do, and Dukyu mountain in Jeonrabuk-Do, etc. Soils 5~10 cm deep from the surface were collected and labelled with the sampling site, date, and name of collector, and stored at room temperature until use.

Isolation of The Microorganisms

Soil sample (1 g) was well suspended in a test tube containing 10 m*l* of sterile saline water. After the soil sample settled down, the clear solution was diluted to 10^{-2}, and 0.1 m*l* of the diluted solution was spread onto the modified Benett's agar medium (Table 1) containing raw corn starch, for the isolation of microorganism which produce raw-starch-digesting enzyme. The raw corn starch powder was purchased from a local super-market.

Colonies which formed halo on the agar medium were picked up after 3~7 days of incubation at 30°C, transferred to agar slant, and incubated at 30°C for 4~6

days. The slants were stored at 4℃.

The halo formation was confirmed again by using a basal agar medium (Table 1) containing raw corn starch as unique carbon source.

Preparation of Raw-Starch-Digesting Enzyme

Raw corn starch powder previously sterilized in a hot drying oven at 180℃ for 2 hours was mixed with 30 m*l* of sterilized basal broth medium in a 100 m*l* Erlenmeyer flask.

Bacterial strain was cultured at 30℃ for 24 hours in a rotary shaking incubator operating at 160 rpm. The culture broth was centrifuged (3,000×*g*, 10 min) and the supernatant was used as the crude enzyme solution.

Assay of Raw-Starch-Digesting Enzyme Activity

100 μ*l* of crude enzyme solution was mixed with 900 μ*l* of 1.11% (w/v) raw corn starch dissolved in 50 mM sodium acetate buffer (pH 5.5) containing 2 mM $CaCl_2$, and the mixture was incubated at 37℃ for 3 hours (2). The reducing sugar formed was assayed by DNS method (3). The enzyme activity was expressed as the concentration (mM) of the reducing sugar formed or relative activity. The relative activity was based on the enzyme activity which is the amount of reducing sugar formed divided by the optical density (as the amount of cell mass) of the cell culture broth from which the crude enzyme solution was prepared.

Selection of Microbial Strains which Produce Pectinase

The isolated strains which secrete raw-starch-digesting enzyme were incubated on a YPP agar medium (Table 2) for 5~7 days at 30℃, and dyed with 5 m*l* of 2% cetyltrimethyl ammonium bromide solution for 15 min. The YPP agar medium was used for the selection of microorganisms which secrete pectinase.

Colonies which formed halo on the YPP agar medium were selected.

Preparation of Pectin Digesting Enzyme

The isolated bacterial strains were inoculated into a 100-m*l* Erlenmeyer flask with 30 m*l* of sucrose-pectin medium (Table 2), and cultured at 30℃ for 5 days in a rotary shaking incubator operating at 160 rpm. The culture broth was centrifuged (3,000×*g*, 10 min) and the supernatant was used as the crude enzyme solution.

Assay of Pectin-Digesting Enzyme Activity

The activity of pectolytic enzyme was determined by measuring the reduction in the viscosity of pectin solution at a given temperature, enzyme concentration, and reaction period. This method gives an appropriate estimation of the activities of a mixture of esterase, polymethylgalacturonases, and polygalacturanases (16).

The solution containing substrate, composed of 5 m*l* of 2% (w/v) pectin in 0.2 M acetate buffer (pH 4.4), was preheated in the Ostwald viscometer at 30℃ for 5 min. and was thoroughly mixed by blowing the preheated 5 m*l* of crude enzyme solution containing 4 m*l* of distilled water. The enzyme reaction was performed at 30℃ for 2 hours. Percent viscosity change was calculated from the equation of Mukherjee *et al.* (16).

$$\text{Percent viscosity change} = \frac{V_o - V_t}{V_o - V_s} \times 100$$

where V_o: flow time in seconds of pectin and heat-inactivated enzyme,

V_t: flow time in seconds of pectin and activated enzyme at reaction time,

Table 1. Composition of media used for the isolation of microorganisms producing raw-starch-digesting enzyme.

Media	Composition (g per liter of distilled water)	
Modified Benett's medium	raw corn starch	10
	peptone	2
	beef extract	1
	yeast extract	1
	nystatin	5×10^{-6}
	pH 7.3 (adjusted with NaOH)	
Basal medium	raw corn starch	5
	NH_4NO_3	1
	KH_2PO_4	1.4
	$CaCl_2$	0.5
	$MgSO_4 \cdot 7H_2O$	0.2
	yeast extract	0.1
	polypeptone	0.5
	pH 6.5 (adjusted with NaOH)	

Table 2. Composition of media used for the isolation of microorganisms producing pectinase.

Media	Composition (g per liter of distilled water)	
YPP agar medium	yeast extract	10
	peptone	20
	pectin	20
	agar	15
Sucrose-pectin medium	sucrose	20
	pectin	20
	NH_4NO_3	2
	$NaNO_3$	2
	Na_2SO_4	0.5
	$MgSO_4 \cdot 7H_2O$	5
	KCl	0.5
	K_2HPO_4	1
	$FeSO_4$	0.1
	pH 5.0 (adjusted with HCl)	

V_s: flow time in seconds of the buffer and heat-inactivated enzyme.

Identification of the Isolated Bacterial Strains

The identification of isolated bacteria was based on the methods described in "Biochemical tests for identification of medical bacteria" (13), "Manual of methods for general bacteriology" (7) and "Bergey's manual of systematic bacteriology" (19). Various morphological and physiological characteristics were examined and the results were used to identify the bacteria.

Optimization of the Culture Conditions for the Production of Raw-Starch-Digesting Enzyme

The optimal conditions for the maximum production of raw-starch-digesting enzyme by the isolated bacterial strain were examined by using the basal medium. The following culture conditions in a rotary shaking incubator were investigated : initial pH of the medium, temperature, incubation time, and inoculum size.

The initial pH of the basal medium containing raw corn starch was adjusted to 4~8 with 0.1 N hydrochloric acid or sodium hydroxide.

The cultivation of strain No. 26 was carried out in 100-m*l* Erlenmeyer flasks containing 30 m*l* of the medium, in a rotary shaker (200 rpm) at 37℃ for 12 hours.

To examine the effect of the inoculum size, strain No. 26 was seed-cultured at 37℃ in the nutrient broth until the absorbance of the culture broth reached 0.4 at 600 nm. Different sizes of the seed-culture were inoculated into 100-m*l* Erlenmeyer flasks containing 30 m*l* of the basal medium, and cultivated in a rotary shaking incubator operating at 200 rpm.

To study the effect of different carbon and nitrogen sources on the production of raw-starch-digesting enzyme, various carbon and nitrogen sources were added to the basal medium. The effects of different carbon sources were tested in the basal medium which contained dextrin, tapioca, glucose, maltose, or soluble starch instead of raw corn starch. The effect of different nitrogen sources, including both inorganic and organic compound, were also examined. 0.1% (w/v) of various nitrogen sources were added to the basal medium containing 0.5% (w/v) raw corn starch as the carbon source.

The effect of C/N ratio was investigated according to the results of the examination for optimal carbon and nitrogen source. The optimum C/N ratio of the medium for the enzyme production was determined by using raw corn starch as the carbon source and NH_4NO_3 as the nitrogen source. In this study, 0.5% (w/v) of raw corn starch was used and the amount of nitrogen source was varied to attain the desired C/N ratios.

Thin Layer Chromatography

Thin layer chromatography of the hydrolysis-product from starch by raw-starch-digesting enzyme was carried out by using pre-coated TLC plates Silica gel 60 F-254 (Merck, Sweden) with the solvent system of n-butanol : acetic acid : water (12 : 3 : 5) and the ascending method, and the spots were detected by spraying aniline-diphenylamine phosphate reagent. The aniline-diplenylamine phosphate reagent was composed of 5 m*l* of 1% (w/v) aniline in acetone, 5 m*l* of 1% (w/v) diphenylamine in acetone and 1 m*l* of 85% (v/v) H_3PO_4.

Adsorbability of Raw-Starch-Digesting Enzyme Activity to Raw Corn Starch

4 m*l* of distilled water, which had been adjusted to pH 1~13 with 1 N hydrochloric acid and sodium hydroxide, was mixed with 1 m*l* of crude raw-starch-digesting enzyme of strain No. 26. Raw corn starch (1 g) was added to the mixture and left to stand at 4℃ for 15 min. After centrifugation, the raw-starch-digesting enzyme activity of the supernatant was assayed and compared with that of original raw-starch-digesting enzyme solution. The adsorption rate (AR) was defined by the following equation (8) :

$$AR\ (\%) = [(B-A)/B]\ 100$$

where A: the raw-starch-digesting enzyme activity of the supernatant
B: the raw-starch-digesting enzyme activity of original enzyme

RESULTS AND DISCUSSION

Isolation and Selection of Microorganisms

About 200 strains of bacteria, actinomycetes and fungi, which produced raw-starch-digesting enzyme (judging by the halo-formation around their colonies) were isolated from the soil samples. The pectinase activities of the isolated strains were tested by the halo formation. As results, 50 strains of the isolated strains were shown to secrete pectinase, and three strains which secrete both pectinase and raw-starch-digesting enzyme simultaneously were selected.

In addition to the raw-starch-digesting enzyme, other enzymes such as pectinase and cellulase are required for the efficient degradation of plant biomass (14). In the past, Gram-negative soil bacterium, *Cellvibrio mixtus* that secretes various enzymes which can digest diverse polysaccharides has been isolated, and this was shown to secrete pectinase, cellulase, amylase, and chitinase (22). But the three strains selected in this investigation did not secrete chitinase nor cellulase.

Enzyme Activities of the Isolated Bacterial Strains

Among the three strains (No. 26, 121, and 143) which

produce both raw-starch-digesting enzyme and pectinase, the enzyme activities of only two strains (No. 26 and 143) were measured since No. 121 only showed a small size halo formation due to its small amount of the raw-starch-digesting enzyme produced.

The raw-starch-digesting enzyme activities of No. 26 and No. 143 strains were examined and the results are shown in Fig. 1 and Table 3. No. 26 produced a larger clear zone on the basal agar medium and more amount of reducing sugar from raw corn starch than No. 143.

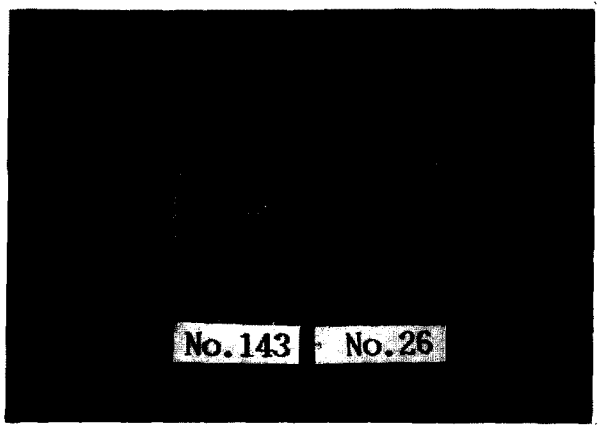

Fig. 1. The microorganisms showing clear zones around the colonies in the basal medium agar plate containing raw starch.

Table 3. The activities of the raw-starch-digesting enzymes secreted from the isolated microorganisms.

Strain No.	Diameter of clear zone[a] (cm)	Reducing sugar[b] (mM)
26	4.0	8.45
143	3.2	7.64

[a]The diameter of clear zone was measured after cultivation at 30℃ for 5 days in the basal medium agar plate containing raw corn starch.
[b]The reducing sugar was determined after the enzyme reaction mixture containing raw corn starch was incubated for 3 hrs.

Table 4. The activities of the pectinase secreted from the isolated microorganisms[a].

Strain No.	Pectinase activity (%)[b]
26	9.69
143	17.10

[a]The isolated strains were incubated for 5 days at 30℃ in sucrose-pectin medium.
[b]The pectinase activity was expressed as the percent viscosity change.

But No. 143 showed a pectinase activity two times higher than that of No. 26 (Table 4).

Identification of the Isolated Bacterial Strains

The morphological and physiological properties of the isolated bacterial strains (No. 26 and 143) which produced raw-starch-digesting enzyme and pectinase simulta-

Table 5. General characteristics of the isolated strains.

Biochemical tests	Isolated strains	
	No. 26	No. 143
Growth at 4℃	Negative	Negative
25℃	Positive	Positive
37℃	Positive	Positive
42℃	Posotive	Positive
Gram staining	Negative	Negative
Cell shape	Rod	Rod
Pigmentation	Negative	Negative
Motility test	Nonmotile	Nonmotile
Aesculine test	Negative	Positive
Indole test	Negative	Negative
Methyl Red test	Negative	Negative
Voges-Proskauer test	Negative	Negative
Gas production	Negative	Negative
Optochin disc test	Positive	Positive
Simmons citrate test	Negative	Negative
Catalase test	Positive	Positive
Oxidase test	Positive	Positive
Urease test	Positive	Positive
Gelatin liquefaction test	Positive	Positive
Starch hydrolysis test	Positive	Positive
Casein hydrolysis test	Positive	Positive
Arginine utilized test	Negative	Negative
Nitrate reduction test	Positive	Positive
Hydrogen sulfide test	Negative	Negative
MacConkey agar test	No Growth	No Growth
Oxidation/Fermentation test	Inert/ Fermentative	Inert/ Fermentative
Carbohydrate fermentatation test		
Adonitol	Negative	Negative
Arabinose	Acid	Negative
Cellobiose	Acid	Acid
Dulcitol	Negative	Negative
Fructose	Acid	Acid
Galactose	Negative	Negative
Glucose	Acid	Acid
Inositol	Acid	Negative
Lactose	Negative	Negative
Mannitol	Acid	Acid
Raffinose	Negative	Negative
Rhamnose	Negative	Negative
Sorbitol	Negative	Negative
Sucrose	Acid	Acid
Salicin	Negative	Negative
Xylose	Negative	Negative

neously, are shown in Table 5.

Bacteria No. 26 and 143 were Gram-negative rods and didn't exhibit any pigmentation. Physiologically they showed negative motility, negative catalase activity and positive urease activity. They didn't grow in the MacConkey agar medium at 25℃, 30℃ nor 37℃. They responded positively to nitrate reduction test, and negatively to Voges-Proskauer test and Methyl red test. They could degrade starch and casein. In the test for the utilization of carbohydrates, the two bacterial strains could not utilize lactose, dulcitol and rhamnose, but could assimilate glucose, fructose, sucrose and mannitol, etc. These two strains were physiologically different from each other in that No. 26 responded negatively to Esculin test and No. 143 responded positively to the Esculin test, and they also showed different results in the use of carbohydrates such as arabinose and inositol, etc. But when their morphological and physiological characteristics were compared with those shown in the "Bergey's manual of systematic bacteriology" (19) and "Biochemical test for identification of medical bacteria" (13), the two strains showed little difference with *Pasteurella ureae*. Therefore both of them were identified as *Pasteurella ureae*.

Growth Curve of the Isolated Microorganisms and Effect of the Temperature on the Production of Raw-Starch-Digesting Enzyme

The growth curve of No. 26 was examined and the results are shown in Fig. 2. When it was cultured in the basal medium containing raw starch as a carbon source, it showed a more rapid growth rate at 37℃ than at 30℃.

Fig. 3 shows the production of raw-starch-digesting enzyme when the strain was cultured at 30℃ and 37℃. At 37℃ the culture exhibited a more rapid enzyme production compared with that at 30℃. The maximum activity of enzyme was shown after 11 hours at 37℃ and after 18 hours at 30℃, respectively. After that time, the enzyme activity rapidly decreased.

Abe *et al.* (1) reported that the production of the raw-starch-digesting enzyme by *Aspergillus* sp. stopped after the growth period.

Fig. 2 and 3 show that maximum amount of raw-starch-digesting enzyme was produced during exponential phase and that when the stationary phase was reached, the production of the enzyme rapidly decreased at 30℃ and also at 37℃. When the enzyme production at 30℃ was compared with that at 37℃, some differences in the pattern of the enzyme production were observed. At 37℃, the production was maximum during the early exponential phase and rapidly decreased during stationary phase, but at 30℃ the enzyme-production started during the early exponential phase and gradually increased and became maximum during the final stage of exponential phase and decreased dramatically during stationary phase. This trend was the same with No. 143 (Fig. 4). The reason is supposed that since the cells grew faster at 37℃ than at 30℃, the cells produced the raw-starch-digesting enzyme earlier at 37℃ than at 30℃. In conclusion, the optimal temperature was 37℃ since the production of the enzyme and the growth rate became maximum during early exponential phase. No. 26 was chosen to examine the optimum conditions for the production of the enzyme because No. 26 exhibited higher enzyme activity than No. 143 at 30℃.

Effect of the Initial pH of the Medium

The effect of initial pH of the medium on the production of the raw-starch-digesting enzyme was examined and the results are shown in Fig. 5. No. 26 hardly produced any raw-starch-digesting enzyme at pH 4.0 but produced the maximum at pH 7.5.

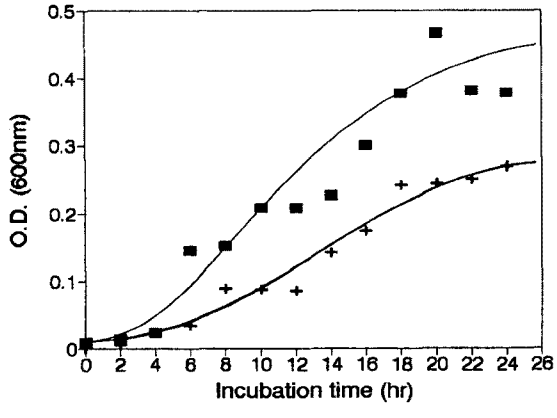

Fig. 2. Growth curve of the isolated strain No. 26 in the basal medium containing raw starch.
Temperature: 30℃ (+), 37℃ (■).

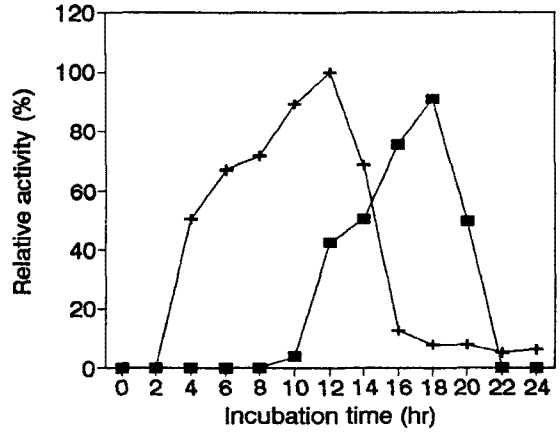

Fig. 3. The time course of the production of raw-starch-digesting enzyme by strain No. 26.
Temperature: 30℃ (■), 37℃ (+).

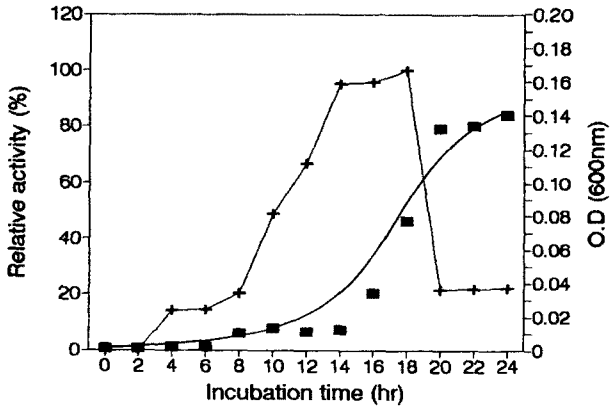

Fig. 4. The time course of the production (+) of the raw-starch-digrsting enzyme by strain No. 143 and its growth curve (■).
Temperature: 30℃

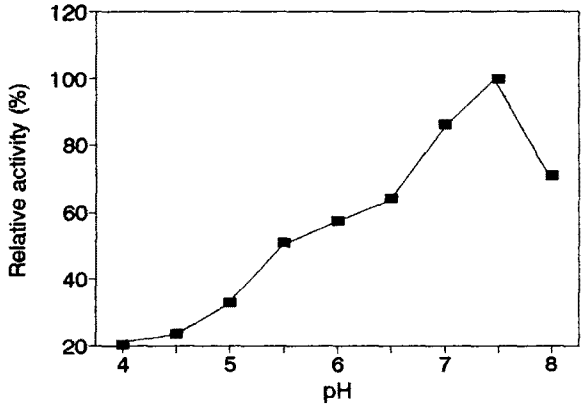

Fig. 5. The effect of initial pH of the medium on the production of raw-starch-digesting enzyme by strain No. 26.

Takao *et al.* (20) and Abe *et al.* (1) reported that the production of raw-starch-digesting enzyme from fungi *Aspergillus* and *Corticium reached* maximum at the initial pH 5.0 and 6.0, respectively. This proves that the No. 26 bacterial cells prefer more alkaline initial pH of medium in the production of the enzyme than the fungal cells do.

Effect of Different Carbon Sources

From the study of raw-starch-digesting enzyme produced by fungus *Corticium rolfsii* isolated from a tomato stem, Takao *et al.* (20) reported that a high level of raw-starch-saccharifying activity was observed not only in the culture grown on raw starch but also in the cultures grown on monosaccharides, such as glucose and fructose, and disaccharides.

No. 26 produced the enzyme in a basal medium which contained dextrin, tapioca, glucose, maltose, or soluble

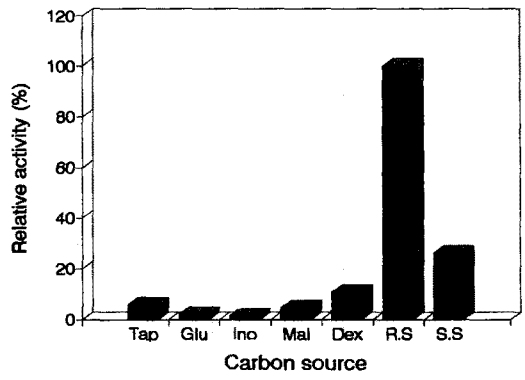

Fig. 6. The effect of various carbon sources on the production of raw-starch-digesting enzyme by strain No. 26.
Strain No. 26 was inoculated into the basal medium containing each 0.5% (w/v) carbon source and incubated for 12 hrs at 37℃.
Tap: Tapioca, Glu: Glucose, Ino: Inositol, Mal: Maltose, Dex: Dextrin, R.S.: Raw starch, S.S.: Soluble starch.

starch instead of raw starch as a carbon source. And the results of the enzyme production from each source are compared (Fig. 6). Raw starch was the most effective carbon source in the production of the enzyme among the carbon sources. This is the same result with that from the study of Kim (10) where a bacterium, *Bacillus circulans*, produced the raw potato starch-digesting amylase only when it was cultivated in a medium containing raw starch as a carbon source, and produced only a small amount of the enzyme when it was grown in a medium containing either soluble starch or maltose.

By putting all the experimental results together, the conclusion was drawn that crude raw-starch-digesting enzyme of No. 26 was induced by starch, particularly by raw starch.

Kim *et al.* (11) reported that α-amylase synthesis may be constitutive, but catabolite repressible, or inducible but catabolite repressible according to the individual strain of bacteria even in the same species. In general, synthesis of amylase is repressed by low molecular weight substrates such as glucose. The raw-starch-digesting enzyme of strain No. 26 was produced in the basal medium containing 0.5% (w/v) raw corn starch alone as a carbon source, but was seldom produced in the basal medium containing both 0.5% (w/v) glucose and 0.5% (w/v) raw corn starch. Furthermore, the enzyme was not measured in the medium containing glucose alone as a carbon source (Table 6). These results show that the enzyme synthesis of strain No. 26 can be induced by starch and repressed by a catabolite such as glucose.

Effect of Different Nitrogen Sources

Table 6. Synthesis of raw-starch-digesting enzyme by the cells of strain No. 26[a].

Carbon source (%, w/v)		Synthesis of raw-starch-digesting enzyme
Raw starch	Glucose	
0.00	0.25	−[b]
	0.50	−
0.25	0.00	+
	0.25	−
	0.50	−
	1.00	−
0.50	0.00	+
	0.25	−
	0.50	−
	1.00	−

[a]The cells were cultured in the basal medium containing glucose and raw corn starch as carbon source and incubated for 12 hours at 37℃.

[b]+ represents synthesis of raw-starch-digesting enzyme.
− no synthesis.

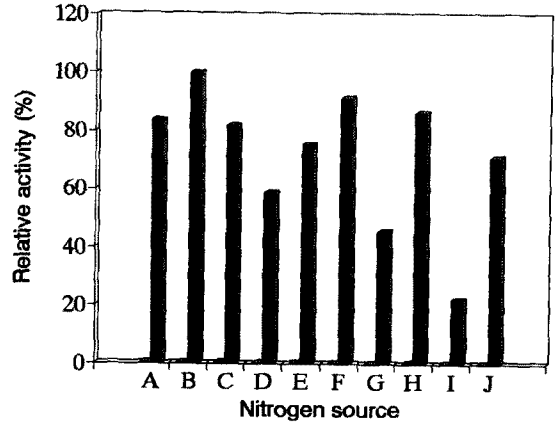

Fig. 7. The effect of various nitrogen source on the production of raw-starch-digesting enzyme by strain No. 26.
Strain No. 26 was inoculated into the basal medium containing each 0.1% (w/v) nitrogen source and incubated for 12 hrs at 37℃.
A: $(NH_4)_2SO_4$, B: NH_4NO_3, C: $NaNO_3$, D: KNO_3, E: $Mg(NO_3)_2$, F: Bacto Peptone, G: Malt extract, H: Yeast extract, I: NH_4Cl, J: CH_3COONH_4.

Effect of different nitrogen sources, including both inorganic and organic compounds, were examined. Fig. 7 shows the influence of different nitrogen sources on the production of raw-starch-digesting enzyme. Of the inorganic nitrogen sources tested, NH_4NO_3 gave the maximal yield of the enzyme. Among the organic nitrogen sources, Bacto peptone was found superior to the others tested. Generally, most of them were good nitrogen sources for the production of the enzyme, but NH_4Cl, among the inorganic nitrogen sources, and malt extract, among the organic nitrogen sources, produced less amount of the enzyme.

Effect of the C/N Ratio

The effect of carbon to nitrogen (C/N) ratio of the medium on the production of raw-starch-digesting enzyme was investigated and the results are shown in Fig. 8. The optimum C/N ratio for the enzyme production was 15.

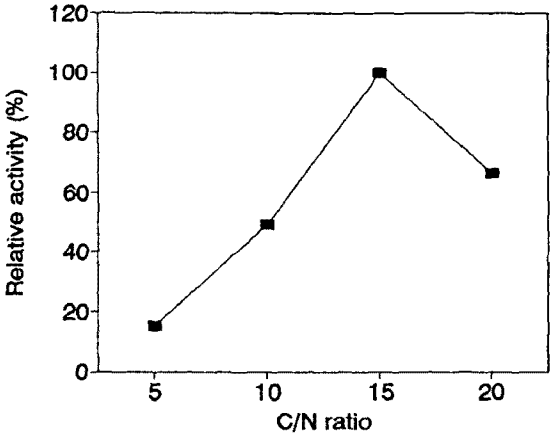

Fig. 8. The effect of various C/N ratio on the production of raw-starch-digesting enzyme by strain No. 26.

Effect of the Inoculum Size

The effect of the inoculum size on the production of the enzyme was examined and the results are shown in Fig. 9. One percent (w/v) inoculum showed the maximum enzyme production. Inoculum sizes larger than 1% (w/v) caused to produce less amount of enzyme.

Thin Layer Chromatography of Starch-Hydrolyzate Formed by the Raw-Starch-Digesting Enzyme

The raw corn starch was hydrolyzed with the crude enzyme of strain No. 26 for 24 hours at 37℃. The products were analyzed by the thin layer chromatography at various intervals of the reaction times and the results are shown in Fig. 10. The raw-starch-digesting enzyme hydrolyzed starch, and produced glucose, maltose and other oligosaccharides. In the early stage of the hydrolysis, maltose and other oligosaccharides were formed, and in the later stage, glucose, maltose and oligosaccharides were formed. With the lapse of time, the amount of sugars with low molecular weight such as glucose and maltose increased. These type of hydrolysis indicated that the raw-starch-digesting enzyme may be α-amylase but not glucoamylase. The α-amylase randomly cut the internal glycosidic bonds of the starch molecule and produces various intermediates of starch-hydrolyzed produ-

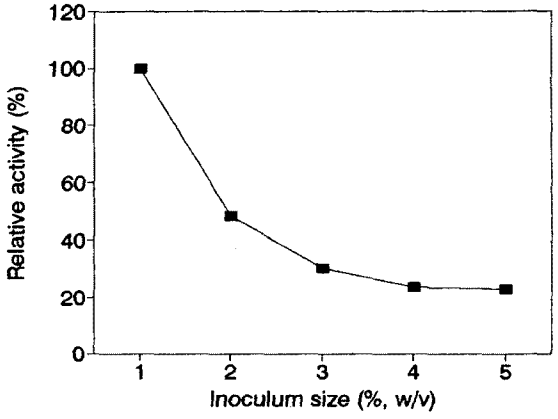

Fig. 9. The effect of the inoculum size on the production of raw-starch-digesting enzyme by strain No. 26.

Fig. 10. Thin layer chromatogram of the starch-hydrolyzates formed by the raw-starch-digesting enzyme of strain No. 26.

An aliquot (0.5 ml) of the enzyme reaction mixture was taken out after 3, 12 and 24 hr of incubation.

Symbols 1: indicates standard sucrose solution, 2: standard mannose solution, 3: standard glucose solution, 4: products at zero time, 5: after 3 hr., 6: after 12 hr., 7: after 24 hr., 8: raw corn starch (substrate) 9~12: crude enzyme solution after 0, 3, 12 and 24 hr., respectively.

cts such as glucose, maltose, oligosaccharides and dextrin (12). Bergman *et al.* (2) isolated three microbial strains which produce strong raw-starch-digesting enzyme, and reported that one fungal and one yeast strain produced α-amylase and glucoamylase and that the final product from starch was only glucose, whereas a bacterial strain produced α-amylase which could produce glucose and malto-, oligosaccharide from starch. The results of this study also shows that the bacterial strain No. 26 produced α-amylase only.

Adsorbability of Raw-Starch-Digesting Enzyme Activity to Raw Corn Starch

Hayashida *et al.* (9) reported that fungal α-amylase is divided into two types: raw-starch-digesting α-amylase I and raw-starch-nondigesting α-amylase II, where the former is raw-starch-adsorbable whereas the latter is raw-starch-unadsorbable. In fungi, the occurrence of the raw starch digestibility was parallel with the raw starch adsorbability and the adsorbability occurred at the raw starch affinity site which is different from the active site on the enzyme molecule (6). In bacteria, the raw-starch-digesting α-amylase of *B. subtilis* 65 digested potato starch granules, but showed no adsorbability onto raw corn starch at any pH (8). The raw-starch-digesting enzyme of strain No. 26 showed adsorbability onto raw corn starch, especially in the pH range of 3 to 9 (Table 7). Thus, bacterial raw-starch-digesting enzyme could be divided into raw-starch-adsorbable and raw-starch-unadsorbable, but the occurrence of the raw starch digestibility was not always parallel with the raw starch adsorbability.

Table 7. Adsorbability of the raw-starch-digesting enzyme of strain No. 26.

pH	Adsorption rate (AR, %)[a]
1	−19.5
3	52.5
5	49
7	48.8
9	51.9
11	23
13	25.5

[a]The adsorption rate (AR) was defined by the following equation AR (%)=[(B−A)/B] 100

where A: the raw-starch-digesting enzyme activity of the supernatant to test adsorbability.

B: the raw-starch-digesting enzyme activity of original enzyme.

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