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Screening of a Potent, Raw Naked Barley Saccharifying Enzyme Producer and Its Application on the Uncooked Alcohol Fermentation

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쌀보리 전분 당화효소 생산균의 분리 동정 및 무증자 알코올 발효에의 이용

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Microorganisms capable of degrading the raw naked barley were isolated from soil, and the amylase productivity of each strain was examined on plate contained 2% raw naked barley. Of the fungi and actinomycetes tested, 71 strains were subjected to subsequent testing for amylase production, and 4 strains were selected as potent amylase producers. Among them, Strain No. 281 produced the most potent raw naked barley saccharifying enzyme, and was identified as genus *Rhizopus* from morphological and physiological studies. The ratio of raw starch saccharifying activity (RDA) of the crude enzyme derived from the *Rhizopus* sp. No. 281 was showed 2-3 fold higher than that of commercial enzyme when the raw naked barley was used as the substrate. In the case of uncooked alcohol fermentation using *Rhizopus* sp. No. 281 glucoamylase preparation, the alcohol yield of the broth was 2% higher than that of the commercial enzyme.

There are naked barley,tapioca and sweet potato as the major carbohydrate sources for alcohol production in Korea. Of these sources, the naked barley accounts for about 50% of the total sources for alcohol production. And the government of Korea has directed its activity toward the production of alcohol from naked barley.

Only a few fungi and bacteria have been reported to be producers of active amylases capable of degrading raw starch. Park and Rivera(1) reported on the uncooked alcohol fermentation of cassava. Yamamoto *et al.* (2) worked on the uncooked alcohol fermentation of sweet potato and cassava. Ueda *et al.* (3-5) have studied the uncooked alcohol fermentation from cassava, sweet potato and rice powder. In recent years, *Chalara paradoxa* was also reported as new sources of amylases which are capable of hydrolyzing raw starch(6).

But until now, there is little work on the uncooked alcohol fermentation of naked barley. Futhermore the alcohol yield is low when using the naked barley as the substrate for alcohol fermentation.

Therefore, in the present work, we tried to isolate an active saccharifying enzyme producers for the naked barley and investigated the microbiological characters of isolated microorganism. Also, we have studied the production condition of saccharifying enzyme from the isolated microorganism and the possibility of the application of the uncooked alcohol fermentation using this enzyme.

Materials and Methods

Media for screening

The composition of various media for screening was shown in Table 1. Naked barley grain was ground to pass a 30-mesh sieve and then sterilized with ethylene oxide gas at 27℃ for 48hrs. Agar

Key words: Raw naked barley saccharifying enzyme, *Rhizopus* sp.
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Table 1. Composition of various media for screening

Components (g/liter)		Medium A	Medium B	Medium C
$(NH_4)_2SO_4$	1.4	+	+	+
KH_2PO_4	2.0	+	+	+
$CaCl_2$	0.3	+	+	+
$MgSO_4 \cdot 7H_2O$	0.3	+	+	+
Urea	0.3	+	+	+
Polypepton	1.0	+	-	+
Yeast extract	0.1	+	-	+
Agar	18.0	+	+	-
Raw naked barley	20.0	+	+	+
Mineral solution	1.0 (ml)	+	+	+
Tween 80	2.0	-	-	+

* Mineral solution (g/liter): $FeSO_4 \cdot 7H_2O$ 5.0
$MnSO_4 \cdot H_2O$ 1.6
$ZnSO_4 \cdot 7H_2O$ 1.4
$CoCl_2$ 2.0

* Raw naked barley was separately sterilized with ethylene oxide gas at 27°C for 48 hr

* Medium B: For isolation
Medium A: For first screening
Medium C: For second screening

plates containing raw naked barley powder were prepared by the following procedure:

Autoclaved agar media in flasks were cooled to 45-50°C and then sterilized raw naked barley powder was aseptically added to the media to a concentration of 2%. After thorough mixing by shaking, the media were rapidly poured into sterile Petri dishes and left to harden.

Isolation and culture of microorganisms

The microorganisms producing the saccharifying enzyme capable of degrading raw naked barley were isolated by the method described by Sasaki *et al.* (7). The isolates were incubated at 27°C for 10 days on Potato dextrose agar slants and then maintained at 4°C.

Characterization and identification of the isolates

Microbiological properties of the isolated Strain No. 281 were investigated according to the methods and procedures described by Barron(8) and Arx(9).

The clearance in the media contained raw naked barley (*R* value)

The initial screening was based on the ratio(*R*) of the diameter of raw naked barley clearance to the diameter of the agar block. The colonies with high value of *R* were assayed for the saccharifying acticvity of raw naked barley digesting enzymes.

Preparation of crude enzymes

About 3m*l* portions of the cultured broths (medium C) were taken and centrifuged at 3000 rpm for 20 min at 4°C to remove mycelia and residual naked barley powder. The supernatants were used for assaying enzyme activity.

Enzyme assays

Raw starch-digesting activity was assayed using a reaction mixture consisting of 20mg of raw naked barley powder, 0.2m*l* of 0.1M acetate buffer solution (pH4.5) and 1.6m*l* of distilled water. After preincubation at 40°C for a few minutes, 0.2m*l* of enzyme solution was added and shaked during the enzyme action. Then, the mixture was centrifuged to remove residual naked barley, and the reducing sugar liberated was determined by the Somogyi-Nelson method (10) using glucose as the standard. Gelatinized starch-digesting activity was assayed with substrate solution consisting of 0.25m*l* of 0.1M acetate buffer solution(pH4.5) and the mixture was incubated at 40°C for 30 min. One unit of enzyme activity was defined as the amount of enzyme which liberated 1μmole of reducing sugar per minute under the respective conditions.

Estimation of raw naked barley digesting-ability (RDA)

Raw naked barley digesting-ability (RDA) was defined as the ratio of raw starch saccharifying activity to gelatinized starch saccharifying activity and a high RDA value exhibited a high saccharifying activity toward raw starch.

RDA was calculated with the following equation:

$$RDA(\%) = \frac{A}{B} \times 100$$

, where A is gelatinized starch-digesting activity and B is raw starch-digesting activity.

The production condition of raw starch saccharifying enzyme from *Rhizopus* sp. No. 281 in wheat bran media

Optimum pH, temperature and cultivation time

for the production of raw starch saccharifying enzyme was determined. Also, in order to investigate the addition effect of different carbon and nitrogen sources on the raw starch saccharifying enzyme production in wheat bran media, different carbon and nitrogen sources were added.

Alcohol fermentation

Naked barley grain (Kochang, Korea, 1986) was ground in Waring Blender and Wiley Mill to pass a 20-mesh sieve. The basal composition of fermentation broth consisted of 100g of naked barley powder, 10m*l* of 0.84% sodium sulfite and 280m*l* of distilled water, were put in a 1 l Meissel's fermentation vessel, and adjusted to pH 3.8 with 95% sulfuric acid. After steeping (55 °C, 2hr), the broth was adjusted to pH 4.8 with 20% sodium hydroxide. And then, 20m*l* of saccharifying enzyme solution (4500 unit) and 40m*l* of yeast culture broth (*Saccharomyces cerevisiae,* 3.0×10^8 cells/m*l*) were added. The Meissel's fermentation vessel was kept at 30 °C in the thermostat. The vessel was weighed every day and the produced ethanol was calculated from the evolution of carbon dioxide. Ethanol contents in distillates were determined with an alcohol meter (11).

Results and Discussion

Isolation of raw naked barley saccharifying microorganisms

First screening; Eighty-four of isolates and 90 fungal cultures from our laboratory were used for this experiment. Seventy-one strains out of 174 strains produced varying degrees of clearance on the plates containing raw naked barley, only four strains with an *R* value of 1.4 or above were selected (Table 2, Fig. 1).

Table 2. *R* values of selected fungi

Strain No.	Diameter of agar-block (cm)	Diameter of clearance (including the colony within) (cm)	*R* value	RDA
281	0.5	1.5	3.0	45
106	0.7	1.0	1.4	20
21	0.6	1.0	1.7	15
177	0.6	1.4	2.3	17

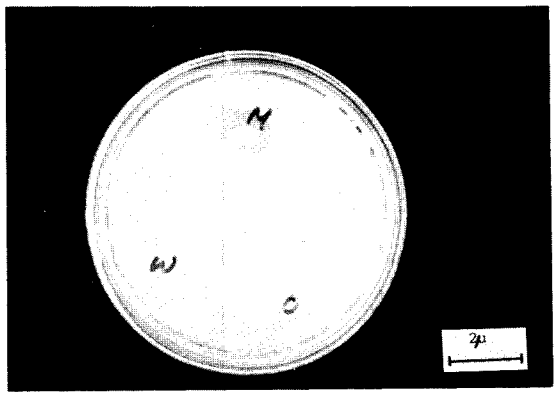

Fig. 1. Formation of clearing zone in the media contained raw naked barley powders

M; The strain which could form clear zone in the media contained 2% raw naked barley powder.
O; The strain which could grow in the media contained 2% raw naked barley powder, but did not form clear zone.
W; The strain which could not grow in the media contained 2% raw naked barley powder.

Second screening; Each of the selected saccharifying enzyme producers was inoculated into test tubes containing 15m*l* of medium C. After cultivation for 7 days at 30 °C with shaking, raw starch-digesting ability was determined (Table 2). The RDA values of the crude enzymes produced by Strain No. 281, 106, 177 and 21 were 45, 20, 17 and 15, respectively.

The crude enzyme of Strain No. 281 exhibited an extremely high RDA value, the value being about 2-3 fold toward raw naked barley in comparison with the other selected strains. Therefore, we used this strain for further studies. A plot of RDA vs. *R*. values from Table 2 did not find a good correlation. This could be due to the growth properties of the strain on the agar block, diffusional characteristics of the enzymes from the agar block into the underlying agar, the rate of migration of enzymes along the agar and so on.

Microbiological characters of fungi, Strain No. 281

The isolated fungi which had a strong raw starch-digesting activity was identified by morphological observation. The fungi grew on a malt agar medium. Colonies on malt agar medium grew rapidly

Table 3. Morphological characteristics of *Rhizopus* sp. No. 281

Sporangiospore	globose or elliptical, smooth yellowish, brown in mass	size: 0.65-0.8×0.6-0.75μm
Sporangia	globose, smooth, black	size: 2.0-2.5×2.3-3.0μm
Columellae	globose or ovoid, usually with truncate base, smooth, colourless	size: 1.5×2.1μm (globose) 2.6×3.7μm (ovoid)
Sporangiophore	branched sympodially and monopodially, pale brown	size: length 29.6-284.0μm diameter 0.5-1.1μm
Rhizoid	detected	

* Medium: Potato-glucose agar
Incubation temp: 35°C, for 7 days

Table 4. Physiological characteristics of *Rhizopus* sp. No. 281

Growth temperature	8-40°C	optimum 30°C
Growth pH	3-9	optimum 4-5
Hydrolysis of starch	positive	
Utilization of carbon sources:		
Arabinose +	Rhamnose −	Maltose + + +
Xylose + + +	Lactose −	Raffinose +
Glucose + + +	Mannose + + +	Dextrin +
Galactose + + +	Sucrose +	Soluble + starch
Fructose + + + +	Cellobiose + + +	

+ good utilization − poor or not utilization
* 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 (mg dry cell/100 ml medium)

Table 5. Cultural characteristics of *Rhizopus* sp. No. 281 on various media

1. Potato dextrose agar	Good growth, Turf 10 mm in height, Young turf white
2. Czapek Dox agar	Poor growth
3. Malt extract agar	Abundant growth, Turf over 15 mm in height, Young turf white, later yellow

at 30°C with white turf. Sporangia were globose shape with the size of 41.6-115μm, and black, smooth walled. Sporangiophore was growing vertically from hyphae with a long cylindrical form and formed rhizoid. According to the morphological observation, the strain should be belong to the genus *Rhizopus*. Other characters of *Rhizopus* sp. No. 281 are shown in Table 3, 4, 5 and an photomicrograph in Fig. 2.

The production conditions of raw starch saccharifying enzyme from *Rhizopus* sp. No. 281 in wheat bran media

1) Effect of pH, temperature and cultivation time

The temperature and pH for the production of raw starch saccharifying enzyme are illustrated in Fig. 2 and 3, respectively, As shown in Fig. 3, the maximal enzyme activity was attained at 30°C. Therefore, this enzyme is suitable for uncooking ethanol production in the view point of low temperature saccharification. Fig. 4 shows the effect of initial pH of the medium on production of the enzyme, and pH 4.5-5.0 was revealed most favorable

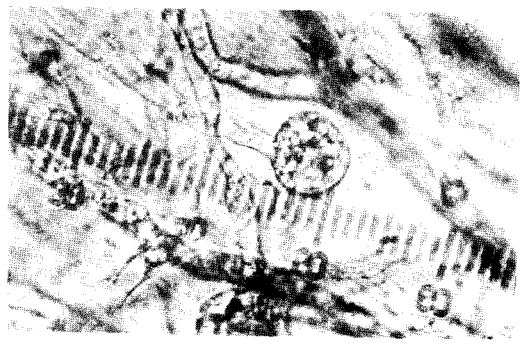

Fig. 2. Photomicrograph of morphology of *Rhizopus* sp. No. 281
The strain was grown on potato-glucose agar at 30°C for 7 days

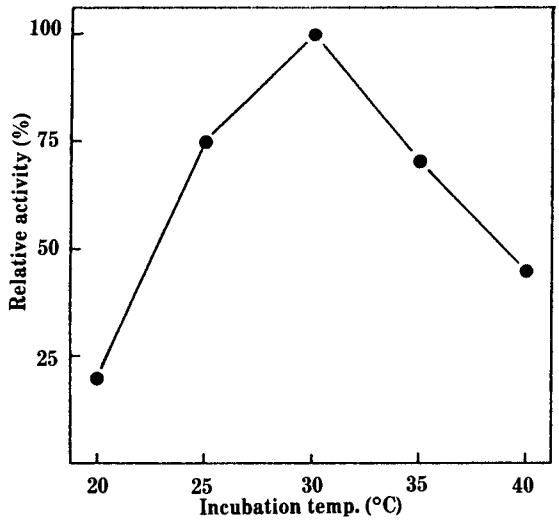

Fig. 3. Effect of temperature for the production of raw starch saccharifying enzyme

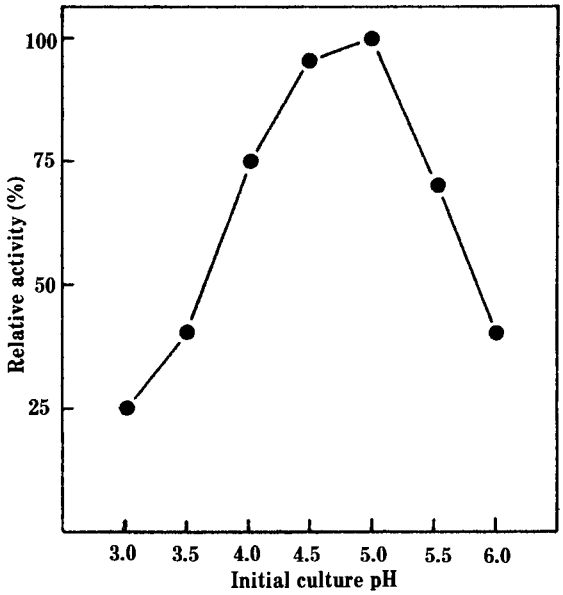

Fig. 4. Effect of pH for the production of raw starch saccharifying enzyme

5g of wheat bran was mixed with 4 m*l* of 0.05M citrate buffer at each pH value and incubated at 30°C for 96 hrs.

for the enzyme production. As shown in Fig. 5, the enzyme was produced maximally in 5 days of cultivation.

2) Effect of carbon source

Rhizopus sp. No. 281 was grown at 30°C in

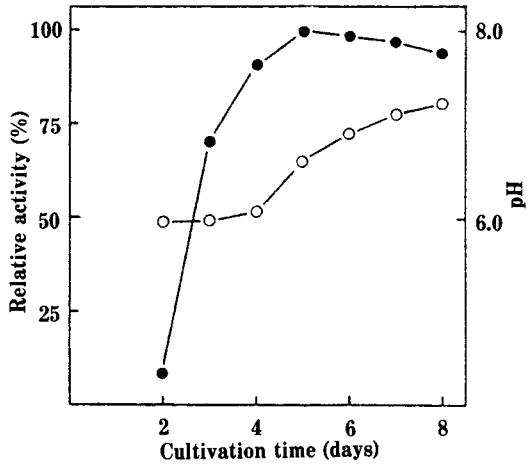

Fig. 5. Time course of raw starch saccharifying enzyme production

Table 6. Effect of carbon source on the raw saccharifying enzyme production

Carbon source	Final pH	Relative activity (%)
D-Glucose	5.8	108
D-Fructose	5.6	110
D-Galactose	6.3	108
D-Mannose	6.0	102
D-Arabinose	5.6	98
D-Ribose	5.3	101
D-Rhamnose	6.0	83
D-Xylose	6.5	101
Lactose	6.0	83
Maltose	5.9	96
Raffinose	6.6	98
Dextrin	6.2	97
Sucrose	5.6	98
Corn starch	6.1	101
None	6.3	100

0.5% of each carbon source was added in wheat bran media and cultivated at 30°C for 4 days

wheat bran media where carbon source indicated in Table 6 was added. The relative activity and pH of cultured filtrate are summarized in Table 6. Among the carbon sources examined glucose, fructose and galactose were effective while rhamnose and lactose showed a little bit inhibitory effect.

3) Effect of nitrogen source

The effect of nitrogen sources was tested by ad-

Table 7. Effect of nitrogen source on the raw saccharifying enzyme production

Nitrogen source	Final pH	Relative activity (%)
$(NH_4)_2SO_4$	6.4	95
NH_4Cl	6.4	89
NH_4NO_3	6.5	95
KNO_3	6.5	102
$NaNO_3$	6.5	87
Urea	7.0	75
None	6.5	100

0.1% of each nitrogen source was added in wheat bran media and cultivated at 30°C for 4 days

ding several kinds of inorganic nitrogen sources. As shown in Table 7, when organic nitrogen sources were used the production of enzyme was less than that of inorganic nitrogen sources.

Alcohol fermentation of raw naked barley powders

Fig. 6 shows fermentation curves developed by using raw naked barley powders without cooking and with saccharifying enzyme from *Rhizopus* sp. No. 281. After six days fermentation, the weight decrease by the CO_2 evolved reached 29. The rate of fermentation of *Rhizopus* sp. No. 281 enzyme seemed to be slightly faster than the rates of commerical enzyme. The yield, based on the amount of theoretical alcohol, was 85% when using *Rhizopus* sp. No. 281 enzyme and was 2% higher than that of the commercial enzyme.

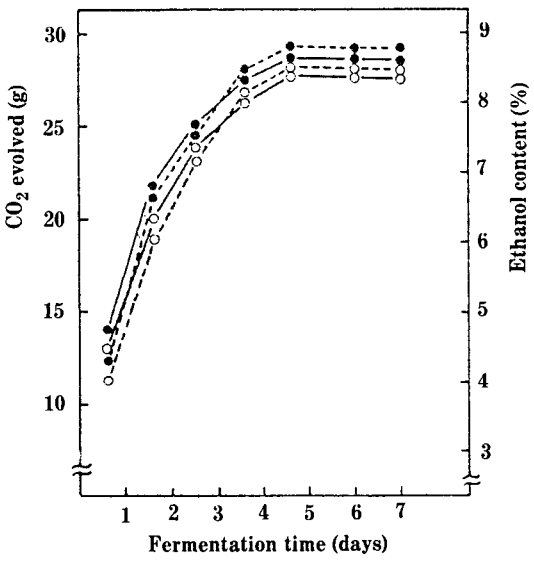

Fig. 6. The time course of uncooked alcohol fermentation on raw naked barley powders.
● enzyme from *Rhizopus* sp. No. 281
○ enzyme from *Aspergillus* sp.
Each saccharifying enzyme (40 units/g of raw naked barley) was used in uncooked alcohol fermentation process. Other conditions of alcohol fermentation were the same as predescribed in Methods and Materials.

요 약

토양으로 부터 生 쌀보리 분해 효소 생산능이 있는 170주의 곰팡이와 4주의 방선균을 분리하여 이중 生 쌀보리 분해 효소 생산능이 가장 높은 No.281 균주를 선정하여 동정한 결과 *Rhizopus* sp.로 밝혀졌으며, 밀기울을 기본 배지로 한 효소 생산 조건을 검토한 결과 효소 생산 최적 온도는 30℃, pH는 4.5-5.0이고 배양 시간 5일 후에 최고의 역가를 보였다. 生 쌀보리를 이용한 무증자 알코올 발효 실험에서는 분리한 *Rhizopus* sp. No.281의 효소를 이용 했을때가 시판 효소 보다도 에탄올 생성이 2% 증가된 결과를 나타내었다.

References

1. Park, Y.K. and B.C. Rivera: *Biotechnol. Bioeng.*, **24**, 495(1982).
2. Svendsby, O., K. Kakutani, Y. Matsumura, M. Itzuka, and T. Yamamoto: *J. Ferment. Technol.*, **59**, 485 (1985).
3. Ueda, S.: *J. Jpn. Soc. Starch Sci.*, **25**, 124(1978).
4. Ueda, S., and Y. Koba: *J. Ferment. Technol.*, **58**, 237(1980).
5. Ueda, S., B.C. Saha, and Y. Koba: *Microbiol. Sci.*, **1**, 21(1984).
6. Kainuma, K., H. Ishigami, and S. Kobayashi: *J. Jpn. Soc. Starch Sci.*, **32**(2), 136(1985).
7. Sasaki, H., K. Kurosawa, and S. Takao: *Agric. Biol. Chem.*, **50**(6), 1661(1986).
8. Barron, G.L.: *The Genera of Hyphomycetes from Soil*, Williams and Wilkins Co., Baltimore (1968).
9. Arx, J.A.: *The Genera of Fungi Sporulating in Pure Culture*, Verlag von J. Cramer, Lehre (1970).
10. Somogyi, M.: *J. Biol. Chem.*, **195**, 19(1952).
11. Honda, N.: *Alcohol Handbook* (in Japanese), 5th. Ed., 128, Japanese Association of Industrial Fermentation, Japan (1978).

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