Production of Raw Cassava Starch-Digesting Amylase of *Streptomyces* sp. No.4 by Solid State Fermentation

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ABSTRACT

The effect of culture conditions and medium components on raw cassava starch-digesting enzyme production were investigated with Streptomyces sp. No. 4 under solid state fermentation. The enzyme production on a basal solid medium composed of rice or wheat bran as a main component by the strain reached the maximum after 3 days cultivation. The optimum pH, temperature, moisture content of medium for the enzyme production were around 6.0, 35C and 50-60%, respectively. Addition of 12%(w/w) raw cassava starch, 0.24%(w/w) CSL and 20 mM $MgSO_4$ into those medium improved the enzyme production. The maximum production of the enzyme on improved medium was performed after 4 days cultivation. The main enzymatic products from hydrolysis of raw and gelatinized cassava starch by the enzyme were mainly maltose and maltotriose.

INTRODUCTION

Cassava is one of the most efficient crops in term of carbohydrate production. Approximately 85-90% of its carbohydrate contents in the form of starch (FAO, 1990) which serves as a raw material for the production of various sugars for fermentation .

The enzymatic hydrolysis of starch to fermentable sugar is proceeded by cooking a cooking/gelatinized pretreatment. This process requires the input of energy at a considerable cost. With the purpose to eliminate the pretreatment, several researchers have isolated raw-starch digesting microorganism. However data on the production of enzymes which preferably digest raw cassava starch are still limited (Ueda et al., 1981; Fujio et al., 1984; Tani et al., 1986; Tani et al., 1988; Nishise et al., 1988).

Recently, a *Streptomyces* sp. No.4 was isolated from soil around cassava plantation in Indonesia which produces amylase with the ability to degrade raw cassava starch when cultivated on a solid culture medium containing wheat or rice bran.

Wheat bran has been found as medium component in solid culture for the production of enzymes from microorganisms such as *Rhizomucor pusillus* (Kanlayakrit et al., 1987), *Bacillus megaterium* 16M (Ramesh and Lonsane, 1987) and *Penicillium brunneum* (Haska and Ohta, 1991). Supplementation of wheat bran with other solid and/or water-soluble nutrients was found to enhanced enzyme formation in solid state fermentation processes (Kumar and Lonsane, 1987).

To maximize the yield and utilization of rice bran and cassava starch as alternative component medium to wheat bran, the present study was conducted to examine the optimum conditions for the production of raw-starch digesting amylase from *Streptomyces* sp. No.4 under solid state fermentation.

MATERIALS AND METHODS

Materials

The cassava starch used was a commercial grade (Setia Co.,Bogor, Indonesia). Other starches were obtained commercially from Wako Pure Chemicals Co., (Japan). Wheat bran and rice bran was purchased locally in Hiroshima. All other chemicals were commercial products of first grade.

Microorganism

Streptomyces sp. No. 4 was isolated from soil around cassava plantation in Indonesia and maintained on Gauze inorganic salt starch agar slant (William and Cross, 1971).

Medium composition and cultivation.

The basal medium was composed of 5 g wheat bran (WB) or rice bran (RB) of 0.2-0.4 cm particle size and had a moisture content of 9.5 %. It was moistened with distilled water to give various range initial moisture content,charged in 100 ml Erlenmayer flask and autoclaved at 12°C for 20 min. Cassava starch was sterilized separately by dry heat at 120°C, for 2 h and then added in the medium. The sterilized medium was inoculated with 1 ml of spore suspension and incubated at 35°C for 3-4 days.

Preparation crude enzyme solution.

The enzyme from fermented bran was extracted twice with 50 ml of 10 mM sodium acetate buffer solution (pH 5.5) after keeping for 2 h, at 4°C. The slurry was squeezed through cotton gauze. The extract was centrifuged at 10,000 x g for 15 min (4°C). The supernatant was filtered through filter paper No.2 (Advantec Co., Tokyo), and the resulting clear supernatant was used for the enzyme assay.

Enzyme assay and analytical methods

Raw starch-digestingactivity was assayed using a reaction mixture composed of 1 ml of 2% raw cassava starch suspension, 0.2 ml of 0.1 M sodium acetate buffer (pH 5.5) and 0.6 ml of deionized water. After pre incubation for 5 min at 40°C, 0.2 ml of enzyme solution was added and incubated for 30 min at 40°C with shaking on a Monod-type shaker (Taitec Co.,Tokyo). After removing the undigested starch granules by centrifugation, the reducing sugar liberated into the supernatant was measured by Somogy-Nelson method (Fukui, 1978).

Gelatinized starch-digesting activity was done with the same method as that for raw starch-digesting activity, after raw starch (2%) has been gelatinized in a boiling water for 5 min.

The reaction mixture and procedure for the determination xylanase and carboxymethylcellulase activities were the same as in the case of raw starch-digesting enzyme, except that 1% xylan or carboxymethylcellulase was used as substrate.

One unit of enzyme activity for each of the substrate was defined as the amount of enzyme that liberated 1μmol of reducing sugar per min.

The type of sugars released during the hydrolysis of the starches was monitored by HPLC (Hitachi L-3300, Hitachi Co., Japan) equipped with RI detector and gel pack column (GL-C 620, Hitachi Co., Japan). Elution was carried at a flow rate of 0.6 ml/min at 60°C using water as a carrier.

RESULTS AND DISCUSSION

Effect of moisture content

Optimum initial moisture content for the enzyme production on wheat bran medium was found at 60%. While about 50% of initial moisture content was optimum for the enzyme production on rice bran medium (Fig.1). The enzyme production was decreased when the initial moisture content of rice or wheat bran higher or below those value. The amount of moistening agent used was shown to influence the physical properties of the moist solid (Feniksova, et al., 1960).

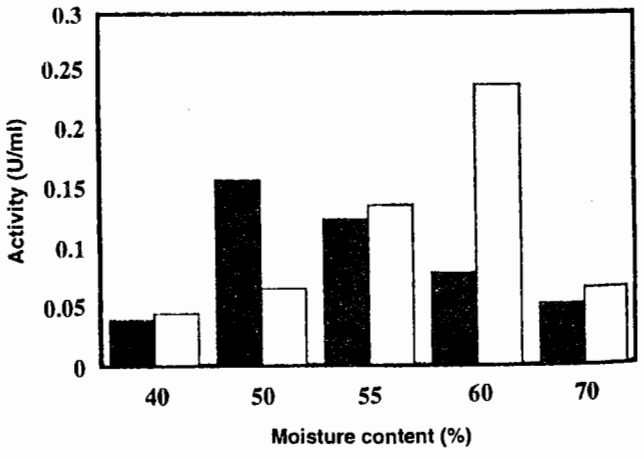

Fig. 1. **Effect of moisture content on enzyme production**
Symbols: ■, **rice bran medium;** □ , **wheat bran medium**

In fungal and bacterial SSF systems such effect of higher moisture content were reported to involve decrease porosity, loss of substrate particle structure, development of stickiness, reduction in gas volume, decreased gas exchange, lowered oxygen transfer due to decreased diffusion and enhanced formation of aerial mycellium (Ramesh and Lonsane, 1990). On the other hand, the solubility of the nutrients present in substrate were reduced by insufficient moisture (Feniksova et al., 1960).

The temperature optimum for production of raw starch-digesting enzyme was found to be between 30-35°C. The production decreased rapidly when incubated above 37°C, around 40% of its maximum activity found in the culture filtrate. Almost no activity was detected in the culture filtrate when cultivated at 20°C. The initial optimum pH of both medium was around 6.0 respectively.

Based the results obtained above (50-60% initial moisture content, pH 6.0 and incubated at 35°C for 3-4 days) , we examined the effect of addition of nutrients on raw cassava starch enzyme production .

Effect of starch

Addition of cassava starch to the medium had positive effect on enzyme production. The optimum concentration of the starch was found to be between 8-12% (w/w) for the wheat bran medium and between 12-16% when using rice bran medium (Fig.2). In the subsequent experiment , 12% raw cassava starch was added to the wheat or rice bran medium.

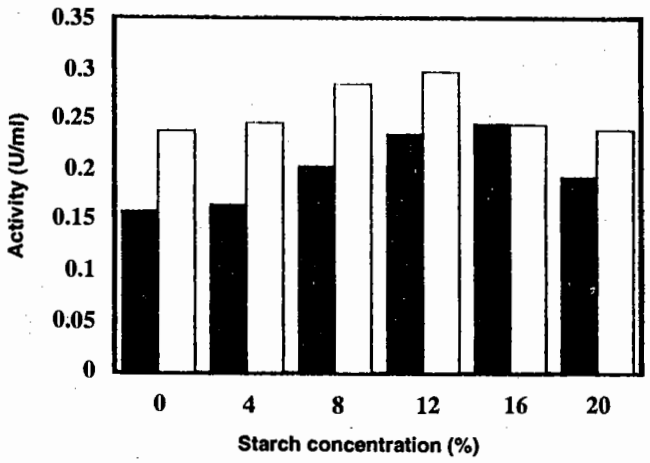

Fig. 2. **Effect of starch concentration on enzyme production**
Symbols: ■ , **rice bran medium** □ , **wheat bran medium**

Additional nitrogen source

It was found that corn steep liquor (CSL) slightly improved the raw starch-digesting activity. In the presence 12% raw cassava starch, the maximum production of the enzyme on both media was observed at 0.24% (w/w) of CSL (Fig.3).

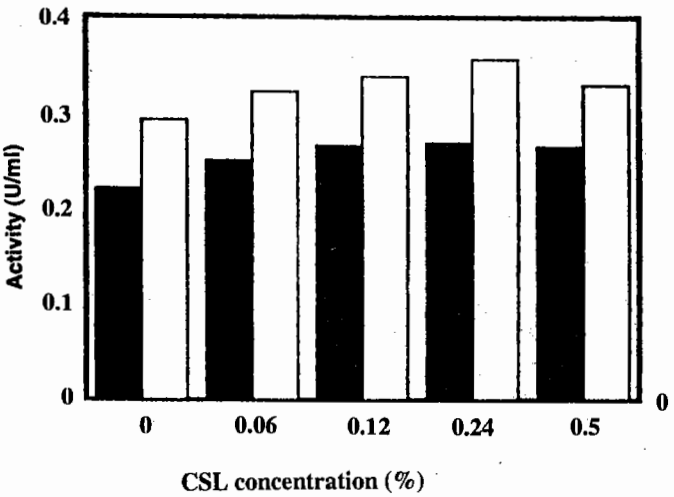

Fig. 3. **Effect of CSL, concentration on enzyme production**
Symbols: ■ , **rice bran medium;** □ , **wheat bran medium**

Effect of mineral

To determined the effect of mineral on enzyme production 7 kinds of mineral were tested and each was added into wheat or rice bran medium containing 12% raw cassava starch and 0.24% CSL (Fig.4). Among all

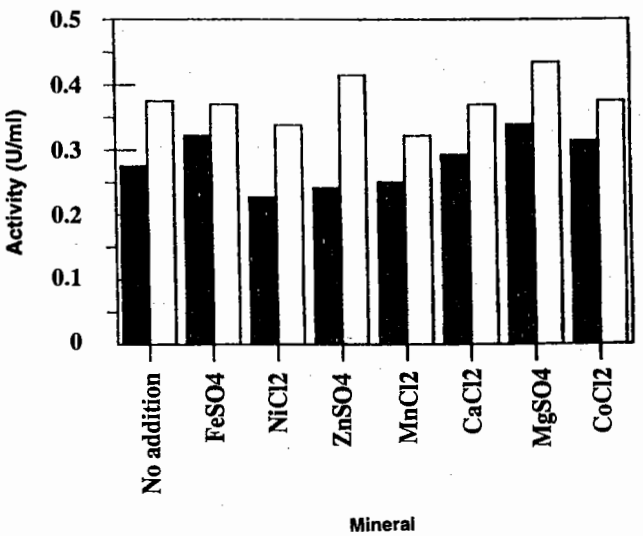

Fig. 4. **Effect of minerals on enzyme production**
Symbols: ■ , **rice bran medium** □ , **wheat bran medium**

the minerals, $MgSO_4$ was the most effective for stimulating the enzyme production. Magnesium, customarily provided as sulfate, probably has it its principal essential function the activation of enzymes necessary to normal metabolism and growth (Cochrane, 1959).

During growth on basal wheat or rice bran medium, the peak of enzyme production by *Streptomyces* sp. No.4 occurred after 3 days. On improved wheat or rice bran medium the production for enzyme peaked after 4 days of cultivation. However the values of enzyme titers were higher than basal wheat or rice bran medium. Table 1 shows the comparison of maximum enzyme activities of the crude enzyme produced from the basal and improve rice or wheat bran medium.

Table 1. **Properties of the crude enzyme produced under various culture conditions.**

Medium composition	Amylase activity (U/ml) on		CMCase[1] activity (U/ml)	Xylanase activity (U/ml)	Final pH
	Raw cassava starch	Gelatinized cassava starch			
Rice bran	0.16	0.74	0.07	5.21	8.0
Wheat bran	0.24	1.30	0.06	6.61	8.0
Rice bran + *mixture	0.35	2.09	0.08	3.68	7.9
Wheat bran + *mixture	0.44	2.18	0.06	4.50	7.9

*The mixture composed of 12%(w/w) raw cassava starch, 0.24%(w/w) Corn Steep Liquor and 20 mM MgSO4.
[1]Carboxymethylcellulose

The raw cassava starch- digesting activity of crude enzyme produced from improved wheat bran medium was 1.83 times higher than the basal wheat bran medium and 1.26 times higher than improved rice bran medium. While xylanase activity in filtrate from improved rice or wheat bran medium was lower than from basal rice or wheat bran medium.

It is seemed that addition of cassava starch to the wheat or rice bran medium induced starch hydrolyzing enzyme only, but gave negative effect on xylanase production of *Streptomyces* sp. No. 4.

To determine the product of starch hydrolysis by the crude enzyme, the reaction mixture of the starch hydrolysis were analyzed by HPLC. As shown in Table 2, hydrolysis of 1% gelatinized cassava, wheat or potato starch for 24 h by crude enzyme from improved wheat bran medium produced mainly maltose (between 62-66%), about 25% maltotriose and no glucose was detected. The end product profiles of this enzyme differ from *Penicillium expansum* α-amylase or commercial maltose producing enzyme Fungamil (Novo Industry) which has the ability to produce glucose from starch. It was also found that *Streptomyces* sp. No. 4 enzyme produced lower levels of maltose than *Penicillium expansum* α-amylase but slightly higher than Fungamyl which produced 60.4% maltose and 20%maltotriose (Doyle et al., 1989). Hydrolysates of the raw cassava starch were mainly maltose (48%), 18.4% maltotriose and 20.2% dextrin. Similar result was observed on the hydrolysates of the raw wheat starch. The enzyme hardly degraded raw potato starch. It produced only 4% maltose and 13% others oligosaccharide from this starch.

Table 2. **Composition of reaction products from hydrolysis of starches**

Starch	Sugar composition of reaction products (%)				
	G1	G2	G3	G4	D
Raw cassava	ND	48.0	18.4	1.4	20.2
Gelatinized cassava	ND	67.0	28.0	0.5	ND
Raw wheat	ND	45.0	18.4	1.6	9.1
Gelatinized wheat	ND	66.0	27.4	ND	ND
Raw potato	ND	4.0	1.5	0.7	11.4
Gelatinized potato	ND	62.0	25.9	2.3	ND

G1: glucose; G2: maltose; G3: maltotriose; G4: maltotetraose; D: dextrin; ND: Not detected

It has been reported that native potato starch granules was less susceptible toward amylase hydrolysis (Kimura et al., 1995). The resistance of native potato starch granules to amylase could be due to a high degree of double-helical chains in potato granules that involve both the amylose and the amylopectin components, but a high percentage of these double-helical chains are isolated from each other so that they are not highly associated with each other in large crystalline net works. When potato starch granules are gelatinized the non associated , double-helical chains unravel and become susceptible to amylase hydrolysis (Kimura et al., 1996).

The enzyme from *Streptomyces* sp. No. 4 could hydrolyzed a water insoluble, cross linked blue starch (starch azure) which is normally considered to be hydrolyzed only by α-amylases, as reported by Schmidt

and John (1979). Based on this observation and results obtained from the composition of the reaction products from the starch hydrolysis, the crude enzyme could be belong to the α-amylase type.

CONCLUSION

From this study we have realized an improvement in the culture condition for optimizing raw starch-digesting enzyme production by *Streptomyces* sp. No.4. This strain produced preferably in the wheat bran medium, and the enzyme could be classified as α-amylase.

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