Amylase Production by *Rhizopus oryzae* Using Solid State Fermentation with Cassava Solid Waste as Substrate

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Abstract. Amylase is an enzyme that is used in various industries such as food, paper, and textile industries. Amylase can be produced by microorganisms through fermentation. *Rhizopus oryzae* is one of microorganisms that can produce amylase through fermentation. Tapioca solid waste has a good potential as a carbon source for microorganisms for producing amylase since it contains high carbohydrate. Tapioca solid waste produced in Toba Regency, North Sumatera, reaches 126 tonnes per day, and the use is limited as compost and forage. It is possible to use tapioca solid waste as a substrate for amylase production thus providing added value to it. Fermentation was carried out for 7 days at 35 °C. After extraction, amylase was partially purified by ammonium sulfate at 40% and 60% saturation level followed by dialysis. Amylase activity was determined spectrophotometrically using dinitrosalicylic acid (DNS) method. Partially purified amylase in 60% ammonium sulfate fraction has the highest activity of 47.6278 U/mL and the highest yield of 97.51% with purity level of 0.653 times. Optimum condition of amylase activity was at pH 6.5 and temperature of 60 °C with 30.1797 U/mL activity. The ability of amylase in hydrolyzing starch was analyzed in various time duration (3, 6, 9, and 12 h) and various ratio of enzyme against substrate (1: 2, 1:5, and 1:10). The highest hydrolysis activity of amylase was 44.3778 U/mL, obtained from the ratio 1:10 of enzyme against substrate and 12 h of hydrolysis time.

Keywords: Amylase · Cassava solid waste · Central composite design · *Rhizopus oryzae* · Solid state fermentation

1 Introduction

Amylase is an enzyme that are capable of breaking glycosidic bonds in starch molecules and producing dextrins and oligosaccharides [1]. Amylase has numerous significant uses in various industries; as a desizing agent for starch dissolving in the textile and paper industry, as the main ingredient for detergents manufacturing in the detergent industry, as a starch hydrolyzing agent to produce sugar for ethanol production in the fuel production

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industry, as a starch hydrolyzing agent for dough making in the bakery industry, and as a hydrolyzing agent for chocolate porridge making in the chocolate industry [1–3].

Amylase is an extracellular enzyme produced by microorganisms and intracellular enzyme found in plants. Microorganisms producing amylase are fungi and bacteria [1], and plants producing amylase are bean sprout [4], yam [5], mango [6], cassava [7], Seraya-type local corn [8], glutinous corn [9], sweet potato [10–12], sorghum [13], and jackfruit [14].

Amylase from plants is obtained through extraction, while amylase from microorganisms is obtained through the isolation process from the growth media. The media can either be synthetic or plant substrates. One source of plant-derived microorganism growth media used by microorganisms to produce amylase is tapioca solid waste. The tapioca solid waste has high carbohydrate content as a source of carbon nutrients for microorganisms and does not interfere with other food source.

The tapioca flour factory located in Toba Regency produces 126 tonnes of solid waste per day, and so far, the waste has only been used as compost and forage. The tapioca solid waste contains carbohydrate (68.30–67.93%), protein (1.70–1.45%), fat (0.22–0.30%), fiber (9.42–10.54%), and water (19.70–20.20%) [15]. The high carbohydrate has the potential to increase the added value of tapioca solid waste as a substrate for microorganisms to produce amylase enzyme. Microorganisms from fungi group used in solid state fermentation to produce amylase enzyme are mainly from the *Aspergillus* group and *Rhizopus* group using several agroindustrial waste, such as rice bran, wheat bran, paddy straw [16–20], but to the best of author's knowledge, this research is the first study using *Rhizopus oryzae* for enzyme production using tapioca solid waste.

The goals of this study are to identify the potentials of tapioca solid waste as a growth substrate of *Rhizopus oryzae* to produce amylase enzymes using the solid-state fermentation method, and to determine the optimum pH and temperature conditions for amylase activity.

2 Materials and Methods

2.1 Substrate and Fermentation Media Preparation

Tapioca solid waste was obtained from tapioca flour processing plant at Toba Regency, North Sumatera. The solid waste was dried using oven at 60 °C and grounded using IKA MF 10 basic grinder with 0,5 mm filter size. The composition of fermentation media used is 1% $(NH_4)_2SO_4$ (g/g), 1% NH_4NO_3 (g/g), and 1% $MgSO_4.7H_2O$ (g/g). Nitrogen sources like $(NH_4)_2SO_4$ and NH_4NO_3 are important in the formation of the main components of cells. Mineral sources like $MgSO_4.7H_2O$ are also important in the formulation of media and the activity of *Rhizopus oryzae* [21].

2.2 Cultivation of *Rhizopus oryzae* and Inoculum Preparation

Rhizopus oryzae culture was obtained from Laboratory of Microbiology, SITH, ITB. *R. oryzae* culture was reproduced in PDA (Potatoes Dextrose Agar) media for 72 h at 35 °C. In the making of a standard curve and a growth curve, *R. oryzae* was cultured in

liquid YPG (Yeast Pepton Glicerol) medium, and the absorbance measurements were carried out at wavelengths of 600 nm. In the preparation of fermentation inoculum, 5 ose *R. oryzae* culture was added to every 100 mL YPG medium, then the inoculum was incubated at 35 °C until reaching the absorbance of 1.339 at wavelengths of 600 nm and cell concentration of 0.091 g/mL. The preparation was conducted aseptically.

2.3 Solid State Fermentation

The fermentation was carried out on 100 g of tapioca solid waste substrate in 500 mL Erlenmeyer flask for 3 times. 1% $(NH_4)_2SO_4$, 1% $MgSO_4.7H_2O$, and 1% NH_4NO_3 g/g was added to the fermentation substrate. The tapioca solid waste was sterilized at 121 °C for an hour. Once cool, the inoculum was added with the ratio 70 mL for 100 g substrate. The fermentation was conducted for seven days at 35 °C. As a control, 70 mL distilled water was added to 100 g substrate.

2.4 Isolation and Partial Purification of Amylase

After seven days of fermentation, 200 mL distilled water was added to each flask and stirred at 35 °C for 60 min. The mixture was centrifuged at 8.500 rpm for one hour and the supernatant containing the amylase enzyme was separated. The volume of enzyme crude extract was recorded and the protein level as well as the activity were determined.

The partial amylase enzyme purification used the sedimentation method by ammonium sulfate at 40%–60% saturation level and continued with the dialysis process. Ammonium sulfate was added to the enzyme crude extract until it reached 40% saturation level. The mixture was then homogenized using magnetic stirrer for 12 h at 4 °C. The solution was centrifuged at 8.500 rpm for one hour at 4 °C. Forage containing amylase enzyme was separated from the supernatant and dissolved in buffer Tris HCl 0.1 M pH 7.4.

The following step of purification was the sedimentation process by ammonium sulfate with 60% saturation level to the supernatant of ammonium sulfate with 40% saturation level. The mixture was then homogenized using magnetic stirrer for 12 h at 4 °C. The solution was centrifuged at 8.500 rpm for one hour at 4 °C to obtain the forage containing amylase enzyme. The forage from the sedimentation process was dissolved in buffer Tris HCl 0.1 M pH 7.4.

Dialysis process for each fraction of partial purification result used the cellophane tube with 10 kDa cut-off. Forage re-suspended was put into dialysis tube, then the tube was put into buffer Tris-HCl 1 mM pH 7.4 for 12 h at 4 °C and stirred slowly using magnetic stirrer. Buffer Tris-HCl was replaced regularly every 4 h, to maintain the pH level inside the dialysis tube. After the dialysis process, the protein level and the activity were determined for each fraction of the partial purification.

2.5 Protein Content Determination of Amylase

The protein level of amylase enzyme based on the sample was determined using the Bradford method with Bovine Serum Albumin (BSA) as the standard. BSA standard was

prepared by making solution stock of 0.5 g/mL, and making the variation of standard solution concentration of 2.5, 5, 7.5, and 10 μg/μL. As much as 1 mL of Bradford solution was added to each concentration variation and left at room temperature for two minutes. The absorbance level of each concentration variation was measured at wavelength of 595 nm. The BSA standard curve was determined by flowing the absorbance against the BSA standard curve. After that, the linier regression equation was determined to get R2 $\geq$ 0.95. To determine the protein concentration in the sample, the BSA standard solution was replaced with the sample solution.

2.6 Standard Curve of Glucose

The activity test of amylase enzyme needs a glucose standard curve as a comparison in determining the glucose concentration resulting from the hydrolysis of starch. To make the glucose standard curve, the stock solution with 10 mg/mL concentration was diluted to obtain variations in the standard solution concentration of 0, 1, 2, 4, 6, and 8 mg/mL. Solution incubated at 50 °C was added to 3 mL of dinitrosalicylic acid (DNS) solution. The mixture was then incubated at 100 °C for 5 min and let cool under running water for 5 min or using ice bath and the absorbance was measured at wavelength of 540 nm. The glucose standard curve was determined by flowing the absorbance to the glucose standard curve. After that, the linier regression equation was determined to get R2 $\geq$ 0, 95.

2.7 Determination of Amylase Activity

The activity of amylase enzyme was determined using spectrophotometry method. The enzyme activity was tested by adding 0, 4 mL of crude enzyme extract to 3,2 mL starch solution with concentration of 5 g/L in the buffer Tris-HCl 0.1 M pH 7.4. The mixture was then incubated at 37 °C for 30 min. 1 mL of DNS solution was added to every 3 mL solution and incubated in water bath with 100 °C for 5 min. The mixture was cooled in running water for 5 min or in ice bath and the absorbance was measured at wavelength of 540 nm. Based on the result of amylase enzyme activity test, %-yield and the amylase enzyme purity level were determined.

$$\%\text{-yield} = \frac{\text{Final total units}}{\text{Initial total units}} \times 100 \tag{1}$$

$$\text{Purity level} = \frac{\text{Final specific activity}}{\text{Initial specific activity}} \tag{2}$$

2.8 Characterization of Amylase

The characterization of amylase enzyme was performed against the enzyme resulted from the partial purification with the highest enzyme activity. The combination of pH (3–10) and (30 °C–90 °C) was determined using the Minitab application with CCD-RSM method. The results obtained were 13 trial variations as shown in Table 1. To get pH 3 and 6, the citrate phosphate buffer of 0.2 M (pH 3–7) was used, and in order to get pH 10, the glycine buffer of NaOH 0.2 M (pH 9–10) was used.

Table 1. Experiment variations of pH and temperature

Run	pH	Temperature (°C)
1	3	30
2	3	90
3	10	30
4	10	90
5	6.5	30
6	6.5	90
7	3	60
8	10	60
9	6.5	60
10	6.5	60
11	6.5	60
12	6.5	60
13	6.5	60

2.9 Hydrolysis Ability of Amylase Determination

The hydrolysis ability test of amylase enzyme used starch as the substrate. The ratio of enzyme against substrate was 1:2, 1:5, and 1:10. Buffer with pH optimum was used to dissolve the starch, and the amylase enzyme was added and homogenized using magnetic stirrer for 15 min at optimum temperature. The mixture was incubated for 1, 3, 6, 9, and 12 h. The hydrolysis was stopped by adding 1 mL DNS to every 3 mL mixture and incubated in water bath at 100 °C for 5 min. The mixture was cooled for 5 min and the absorbance was measured at wavelength of 540 nm.

3 Results and Discussion

The crude extract of amylase enzyme isolated from the tapioca solid waste substrate (control) and from the fermentation result was partially purified using ammonium sulfate at 40%–60% saturation level. Based on the data on Table 2, it can be seen that, in line with the increase in the saturation level of ammonium sulfate, the highest total protein concentration produced was found in the extract fraction of 60%, at 15.561 mg/mL with the protein molecular weight of 4045.86 mg.

The amylase was characterized using starch as the substrate and the hydrolysis activity was obtained from the glucose concentration produced. The higher the glucose concentration, the higher the amylase enzyme activity. The highest amylase enzyme activity was obtained at the extract fraction of 60%, at 47.6278 U/mL with the total activity of 12383.2280 U (Table 3), with amylase yield of 97.51% and the purity level 0.653 times (Table 4).

Table 2. Protein concentration of partially purified amylase

Extract	Tapioca solid waste	Volume (mL)	Total protein concentration (mg/mL)	Protein weight (mg)
Crude	UF	28	0.155	4.34
	F	263	10.296	2707.85
40% fraction	UF	24	7.598	182.35
	F	260	15.270	3970.20
60% fraction	UF	24	4.979	119.50
	F	260	15.561	4045.86

Note: UF = unfermented; F = fermented

Table 3. Amylase activity

Extract	Tapioca solid waste	Volume (mL)	Enzyme activity (U/mL)	Total activity (U)	Protein weight (mg)	Specific activity (U/mg)
Crude	UF	28	5.7061	159.7708	4.34	36.8135
	F	263	48.2889	12699.9807	2707.85	4.6900
40% fraction	UF	24	13.6389	327.3336	182.35	1.7951
	F	260	43.7556	11376.4560	3970.20	2.8655
60% fraction	UF	24	18.9722	455.3328	119.50	3.8103
	F	260	47.6278	12383.2280	4045.86	3.0607

Note: UF = unfermented; F = fermented

pH and temperature were optimized to obtain the optimum condition of pH and the temperature of amylase activity. Based on the analysis using Minitab, the highest amylase activity was 30.1797 U/mL reached at the optimum condition of pH 6.5 at the temperature of 60 °C. Optimized pH determined the ionic charge of amino acid residue side chain at a specific ionization condition affecting the level of enzyme activity, reaction rate, stability, and kinetic constant [22]. pH condition too high or too low could break the hydrogen bond in the enzyme and change the enzyme structure, causing the substrate unable to bond with the active side of the enzyme. A denatured enzyme could lead to an activity decrease and interfere with specific enzyme reactions [23, 24].

In addition, the temperature also influenced the enzyme activity. An increase in temperature would increase the rate of molecules movement causing an increase in the rate of reaction. However, too high temperatures could also cause denaturation of enzyme protein. The enzyme activity increased as the temperature increased due to energy increasing between the enzyme and the substrate. The optimum temperature would produce the highest available energy, enabling it to reach the maximum enzyme

Table 4. The purity of partially purified amylase

Extract	Tapioca solid waste	%-yield	Purity (times)
40% fraction	UF	204.88	0.049
	F	89.58	0.611
60% fraction	UF	284.99	0.104
	F	97.51	0.653

Note: UF = unfermented; F = fermented

activity result. However, when the temperature exceeded the optimum point, the enzyme activity would decrease significantly due to the denaturation of the enzyme protein affecting the stability of the reaction [25].

Central Composite Design-Response Surface Methodology (CCD-RSM), which was used in this research, is a statistical approach that can be employed to optimise operational factors, such as pH and temperature, to maximise enzyme activity. Therefore, there is no further statistical analysis needed for the data obtained. The optimum condition of pH and temperature to different amylase activities depended on the microorganism source and the microorganism growth of substrate source. The optimum condition of amylase activity produced by *Bacillus cereus* grown on synthetic fermentation media [26] resulted in the optimum pH of 6.5 and the optimum temperature of 35 °C. Amylase produced by *Bacillus licheniformis* isolated from hot spring [27] resulted in the high activity at the optimum pH of 8.0 and at the temperature of 55 °C. Meanwhile, the amylase produced by *Aspergillus oryzae* grown on a substrate mixture of soybean and wheat seed [28] produced the highest activity at pH 5 and at the optimum temperature of 60 °C.

Optimum conditions of pH 6.5 and the temperature of 60 °C in this research were used to observe to the ability of amylase in hydrolyzing the starch substrate at different ratios of enzyme against substrate and different incubation times. Based on the observation in this research, it is found that the increase of amylase activity was obtained as the substrate ratio and incubation time increased. The amylase activity to hydrolyze the starch reaching 44.3778 U/mL occurred at the ratio 1:10 of enzyme against substrate with 12 h incubation time.

Based on this research, it can be concluded that tapioca solid waste can be used as a fermentation substrate by *Rhizopus oryzae* to produce the amylase enzyme. The optimum temperature of 60 °C and the optimum pH of 6.5 can produce the amylase enzyme as much as 30.1797 U/mL, with the ratio 1:10 of enzyme against substrate and 12-h incubation time, producing the activity as much as 44.3778 U/mL. In order to obtain a more complete amylase characteristic, it is necessary to further study from the enzymatic kinetics perspective.

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Authors' Contributions. The research was designed, the data was analysed and the manuscript was written by MMM; the research was designed and the manuscript was reviewed by RFK; the research was supervised and the kinetic research was conducted by HNH; the fermentation, purification, and enzyme activity determination were conducted by IS and EES. All authors read and approved the final manuscript.

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