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ISOLATION AND CHARACTERIZATION OF RAW STARCH DEGRADING AMYLASE FOR IMPROVING ENERGY EFFICIENCY OF STARCH-BASED INDUSTRY

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***Abstract*— Raw starch degrading amylase (RSDA) refers to amylase that can act directly on raw starch granules without gelatinization of starch. RSDA is commercially important enzyme useful in industry, since it saves much energy in gelatinizing starchy material. This study was aimed to isolating raw starch degrading microorganism and characterization of RSDA enzyme produced by selected microorganism. Two different samples, farm soil and cassava waste, were used as microorganism sources. The isolation was performed by serial dilution and plating method. Among 4 isolates obtained (TK6f, TK6b, TK6c, and O5c), TK6b showed highest RSDA activity and was selected for RSDA production. A 58, 77 kDa enzyme was obtained after precipitation with ammonium sulfate at 70% saturation. The enzyme had highest activity at temperature 40 ^{0}C and pH 7, and relative stable at temperature 30-50 ^{0}C and pH 7-8. Rice starch had the highest digestibility by enzyme followed by corn, potato, and cassava starch. Enzyme activity was enhanced with the presence of calcium ion, while ferrous, barium, copper, sodium, and magnesium ions inhibited enzyme. The properties of the enzyme proved its potential commercial value to be used for improving energy efficiency in starch-based industry.**

***Keywords*— Amylase, isolation of microorganism, raw starch**

I. INTRODUCTION

Enzyme have attracted attention from researchers all over the world because of its wide range of physiological, analytical, and industrial application. More than 3000 different enzymes have been identified and many of them found their way into industrial application [1]. Among the most important industrial enzymes, amylase constitutes 25% of the sales in enzyme market [2]. This enzyme plays a vital role in many industrial processes such as food, fermentation, textile, paper, detergent, and pharmaceutical industry [3]. In spite of its tremendous application in industrial sectors, the most widespread applications of amylase are in starch-based industry [4]. Amylase are mostly used for starch hydrolysis that converts starch into low molecular weight products such as dextrin, maltose, and glucose [5].

Conventionally, hydrolysis of starch by amylase requires three-step processes namely gelatinization, liquefaction, and saccharification. This process is energy-intensive thus increasing the production cost of starch-based products [6]. With the view of reducing energy consumption, there is considerable research on raw starch degrading amylases (RSDA) currently. RSDA refers to amylase that can act directly on raw starch granules without gelatinization of starch [7]. Those RSDA that active towards raw starch have one or more starch-binding domain that aid binding of the enzyme to the relatively inert substrate [8].

RSDA is commercially important enzyme useful in industry, since it can increase energy efficiency by saving much energy in gelatinizing starchy material [9]. The removal of gelatinization step also has the potential to minimize the formation of undesirable Maillard reaction by-product and increase the value of the co-products since valuable components would undergo less thermal stress [9]. In addition, thermally gelatinized and liquefied starch can have a viscosity higher than that of the starch slurry by a factor of 20-fold and is difficult to pulp and/ or stir. Low temperature liquefaction provides an additional energy benefit stemming from the lower viscosity. Reduced viscosity may also increase the capacity of equipment applied in industry[10].

RSDA are produced by plants, animals, and microorganism [7]. However, RSDA from microbial sources are the most preferred one for industrial application because of their broad biochemical diversity, feasibility of mass culture, ease of genetic manipulation, consistency, and ease of process modification [1, 7]. Only limited number of microbial amylases seem to be able to degrade raw starch. Several reports on RSDA production from microorganism belonging to *Aspergillus niger* [11], *Bacillus amyloliquifaciens* ABBD [12], *Streptomyces* sp. [13], *Bacillus aquamaris* MKSC 6.2 [8], and *Bacillus subtilis* 65 [14]. In the present study, we report the isolation of novel RSDA producing microorganism from cheap and readily available sources, farm soil and cassava waste. The best isolate were selected for further production and characterization of RSDA.

II. MATERIALS AND METHODS

A. Sample collection

Farm soil and cassava waste were collected from cassava farm and tapioca factory in Malang, East Java, Indonesia. 50 g of sample was collected in sterile bottles and taken to the Central Science Laboratory of Brawijaya University for analysis [15].

B. Preparation of media

Raw starch broth media consisted of 1% starch (Merck), 0,5% bacteriological peptone (Pronadisa) , 0,5% yeast extract (Pronadisa), 0,1% K_2HPO_4 (Merck), and 0,02% $MgSO_4.7H_2O$ (Merck) in distilled water was prepared according to standard methods described in [13]. All the above ingredients, except the starch, were sterilized at 121 ^{0}C for 15 min with autoclave (TOMY). Starch was sterilized separately by dry heating at 121 ^{0}C for 2 h and added aseptically in the sterilized medium. While raw starch agar had exactly the same composition as

raw starch broth with addition of 1,5% bacteriological agar as solidifying agent.

C. Isolation and identification of RSDA producing microbes

25 g of sample was weighed into 50 mL of sterile raw starch broth media and then incubated at 37 ^{0}C for 48 h. After incubation, serial dilutions were prepared up to 10^{-7} and then 1 mL of the dilution was added, using pour plate method, to raw starch agar. The agar plates were incubated at 37 ^{0}C for 48 h. After incubation, a clear zone around the growth indicates positive result of RSDA activity. The potential isolates were purified by subculturing on fresh raw starch agar to obtain pure culture. The isolates were characterized based on Gram staining, spore staining, colony surface, colony elevation, and colony color [15].

D. Microbial growth and RSDA activity curve

0,5 mL of isolate was inoculated in 10 mL starch broth medium and incubated in a shaker incubator (New Brunswick) at 120 rpm and 37 ^{0}C for 24 h. After the incubation period, 9 mL of isolate was transferred into 180 mL starch broth medium. These medium were incubated in a continuous shaker incubator at 120 rpm and 37 ^{0}C. Microbial growth curve was established by measuring optical density at 430 nm. Samples for growth curve and RSDA activity were collected until next 24 h [16].

E. Enzyme production and partial purification

0,5 mL of isolate was inoculated in 10 mL starch broth medium and incubated in a shaker incubator at 120 rpm and 37 ^{0}C for 24 h. After the incubation period, 9 mL of selected isolate was inoculated in 180 mL of raw starch broth medium in a 500 mL flask. The flask was incubated in a shaker incubator, operated at 120 rpm and 37 ^{0}C until RSDA activity reach its maximum (based on RSDA activity curve). After the incubation period, the resultant broth was centrifuged (Thermo Scientific) at 10,000 rpm for 15 min and the supernatant was collected as the source of crude enzyme [15]. Partial purification of enzyme was achieved by ammonium sulfate (Merck) precipitation followed by dialysis. Ammonium sulfate was slowly added to supernatant up to 70% saturation and kept at 4 ^{0}C for overnight. The solution was centrifuged at 10,000 rpm for 15 min. The pellet was suspended in 50 mM phosphate buffer pH 7 and dialyzed for 24 h against the same buffer at 4 ^{0}C [17].

F. Enzyme and protein assay

Enzyme activity is determined by DNS method. 0,5 mL of enzyme is incubated for 1 h at 37 ^{0}C with 0,5 ml of substrate, 1% of soluble starch in 50 mM phosphate buffer pH 7. The reaction was stopped by the addition of 1 ml of DNS reagent (1 g of DNS (Sigma-Aldrich) dissolved in 20 ml of 2 M of NaOH (Merck), to which 30 g of potassium sodium tartarate (Merck) were added and filled with water up to 100 ml). The reaction mixture was heated at 100 ^{0}C for 15 min and then diluted with 8 ml of distilled water. The absorbance of the mixture was determined spectrophotometrically at 540 nm (Jenway). Enzyme activity was determined based on the glucose standard curve of amylase activity. One unit of amylase is defined as amount of micromole of reducing sugar that was liberated per ml enzyme per minute under assay conditions [18, 19]. The protein contents of the enzyme were measured at 280 nm using a Nanodrop spectrophotometer (Thermo Scientific).

G. SDS-PAGE and zymogram.

SDS-PAGE was performed using 10% polyacrylamide gel supplemented with 0,2% soluble starch (Merck). After electrophoresis, half of the gel was stained with 0,25% Coomassie brilliant blue dye (Sigma-Aldrich) in methanol-acetic acid-water solution (4:1:5 by volume) and destained in the same solution without dye [20]. For zymogram of amylase activity, SDS was removed by washing the gel in solutions containing 50 mM Na_2HPO_4 (Merck), 50 mM NaH_2PO_4 (pH 7.2) (Merck), isopropanol 40% (Merck) for 1 h and 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2) for 1 h, respectively. Renaturation of protein was carried out by keeping gel overnight in a solution containing 50 mM Na_2HPO_4, 50 mM NaH_2PO_4 (pH 7.2), 5 mM β-mercaptoethanol (Merck) and 1 mM EDTA (Merck) at 4 ^{0}C. Gel was stained in Iodine solution (Iodine 5g/l, KI 50 g/l) for 30 min, clear band indicate the presence of amylase activity [20].

H. Effect of pH on enzyme activity and stability

The optimum pH was determined by incubating the assay reaction mixture in buffer pH 4, 5, 6, 7, 8, and 9. To determine the stability of enzyme, the enzyme was pre-incubated in the same buffer systems utilized for enzyme activity (pH 4-9) for 60 min at 4 ^{0}C. The enzyme activity in standard reaction mixture was taken as control [21].

I. Effect of temperature on enzyme activity and stability

The activity of enzyme at different temperature was determined by incubating reaction mixture at temperature ranging from 30-70 ^{0}C in 50 mM phosphate buffer pH 7.

TABLE I. RSDA ACTIVITY OF ISOLATES

Name of Isolates	Source	RSDA Activity (U/ ml)
TK6f	Farm soil	0.986
TK6b	Farm soil	1.130
TK6c	Farm soil	0.906
O5c	Cassava waste	0.710

Thermal stability of enzyme was examined by incubating the enzyme at 30-70 ^{0}C in 50 mM phosphate buffer pH 7 for 60 min and followed by determining residual activity under standar assay conditions. The non-heated enzyme was considered as control [21].

J. Effect of metal ions on enzyme activity

To assess the effect of metal ions on enzyme activity, the enzyme was pre-incubated in the presence of 2 mM metal ionsuch as calcium, copper, barium, magnesium, and sodium, for 60 min at 4 ^{0}C and then the enzyme assay was carried out. The enzyme activity in standard reaction mixture was taken as control [21].

K. Raw starch degradation

Each of raw starch isolated from potato, corn, rice, and cassava were suspended in 50 mM phosphate buffer pH 7 to obtained 1% raw starch solution. The solutions then were used for determining enzyme activity under standard reaction assay [22].

III. Result And Discussions

A. Microorganism

Among isolates that obtained in this study, four isolates with largest clear zone were selected for further examination. Clear zone formation in starch agar medium showed extracellular RSDA activity of isolates in hydrolyzing raw starch [16]. Three isolates were obtained from farm soil named TK6f, TK6b, and TK6c. While one isolates was obtained from cassava waste named O5c. These isolates were tested further for their RSDA activity by measuring reducing sugar formation in standard reaction mixture (Table 1). Based on those data, all of isolates had amylase activity in starch broth medium (Table 1). The highest enzyme activity after 24 h incubation was shown by isolate TK6b. isolate TK6b was then chosen for further studies because of its largest clear zone formation in starch agar medium and highest RSDA activity in starch broth medium.

It was important to know characteristics of the isolate by performing several tests. Morphological test was carried out on isolate TK6b. Isolate TK6b produced oval, somewhat raised, yellowish colony on starch agar plates. It was coccus-shaped, Gram positive bacteria. Based on endospore staining, it was known that isolate TK6b did not perform endospore. The identification using morphological techniques is difficult, but the molecular approach of 16S rRNA sequencing has now been established as a much accurate and reliable procedure for recognizing and distinguishing between microorganism species [21]. Further verification of microorganism species must be done with 16S rRNA sequencing.

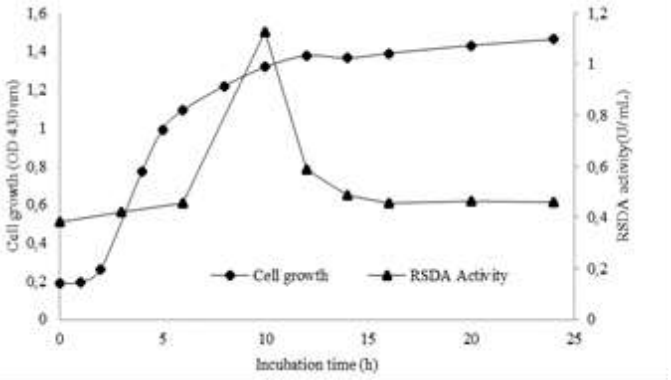


Fig 1. Growth curve and RSDA activity of isolate TK6b in starch broth medium

RSDA production

The result on the time profiles for both amylase activity and growth of isolate TK6b are shown in Fig. 1. Lag phase was reached in 2 h incubation time. This short period of lag phase was due to pre-fermentation that carried out before enzyme production. Previous research has explained that rapid growth in lag phase was related to rapid bacterial adaptation from seed-culture medium to fermentation medium with same composition [16]. When the strain was cultivated in a flask, RSDA activity peaked (1.124 U/ mL) at 10 h and was found to decrease during next 2 h. It was observed that maximum amylase production occurred at the end of exponential phase. Amylase production by this strain was found to be growth dependent as maximum enzyme production was observed during the end of exponential phase. Previous research [22] explained that amylase activity was affected by other enzyme activity. Decreasing amylase activity was also caused by repression by glucose and other readily metabolizable substrates which had been produced previously by degradation of starch in medium [7].

After cultivation supernatant of isolate TK6b was harvested by centrifugation. The raw starch degrading activity of the supernatant was determined to be 0,108 U/ mg. The enzyme was purified from the supernatant as described in "Materials and Methods". The isolation and purification of the enzyme was monitored by ability of enzyme to hydrolyze substrate. The results of the purification are summarized in Table 2. The purification procedure yielded amylase with specific activity of 0.563 U/ mg.

RSDA characteristics

The apparent molecular weight of the enzyme was estimated to be 58.77 kDa according to SDS-PAGE and zymogram analysis (Fig 2). Different molecular masses of RSDA from various microorganism ranging from 32-68 kDa have been reported previously [13, 14, 22].

TABLE II. Summary of The Partial Purification Steps of RSDA from Isolate TK6b

Step	Total Activity (U)	Total Protein (mg)	Specific Activity (U/ mg)	Purification (fold)	Recovery (%)
Supernatant	934,48	8670	0,108	100	1
Ammonium sulfate precipitation	67,71	251,98	0,269	7,25	2,49
Dialysis	71,62	127,26	0,563	7,66	5,22

The effect of the temperature on enzyme activity was shown in Fig 3. Although the enzyme was active at a range temperature from 30-70 ^{0}C, the optimum temperature for enzyme activity was found to be at 40 ^{0}C. At least 70% of the activity was detectable at temperature between 30 and 70 ^{0}C. Other investigator were reported for optimum temperature of RSDA ranging from 30 to 85 ^{0}C [10-14]. Thermal stability of enzyme determined at temperature range 30-70 ^{0}C. There were still up to 60 % activity remaining after pre-incubation for 1 h in all samples (Fig. 4}. Factor influenced thermal stability of enzyme was the denaturation of enzyme protein that caused loss activity [8]. Generally the optimum temperature for cell growth and RSDA characteristic is identical [7].

Studies of the effect of the pH on the enzyme activity and stability were also performed. The effect of the pH on the amylase activity was analyzed by carrying out assays at different pH conditions. The result was bell-shaped curved showing an optimal activity at pH 7 (Fig. 5). These dinding were similar to earlier report of RSDA properties from *Bacillus amyloliquifaciens* ABBD [12]. The enzyme showed 60% of maximal activity between pH 5 and 8. Such characteristics may be important for industrial processes in which a broad-pH range

is needed. It was noteworthy to state that significant loss of activity occured at pH 9. The enzyme was stable in a pH range of 6-7 after 1 h pre-incubation at 4 ^{0}C (Fig. 6).

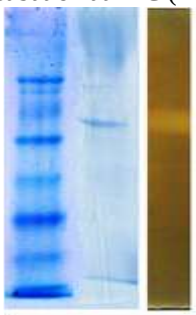


Fig 2. SDS-PAGE and zymogram analysis by using 10% homogenized polyacrilamide gel supplemented with 1.2% soluble starch (From left to right: Marker – RSDA SDS-PAGE – RSDA Zymogram)

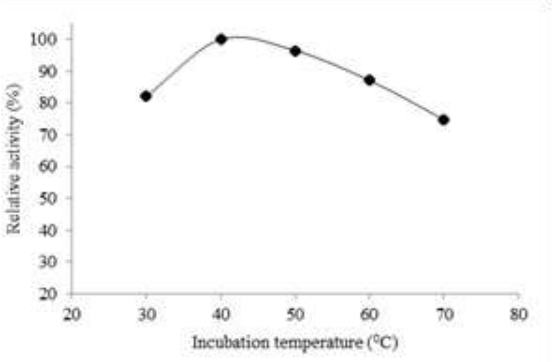


Fig 3. Effect of temperature on the activity of isolate TK6b RSDA

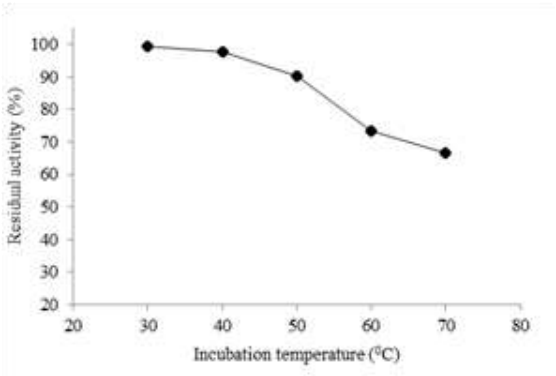
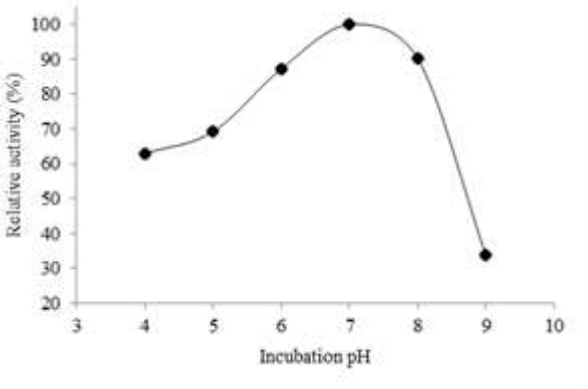
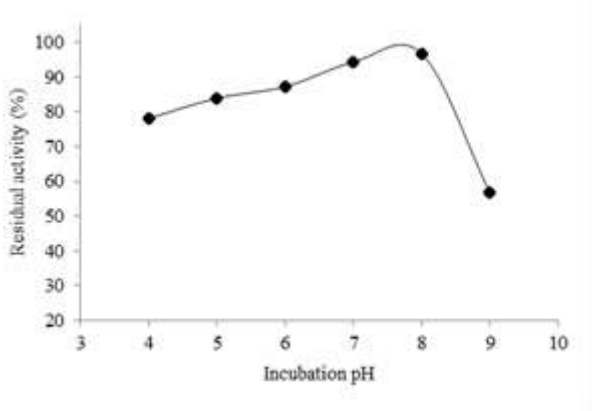


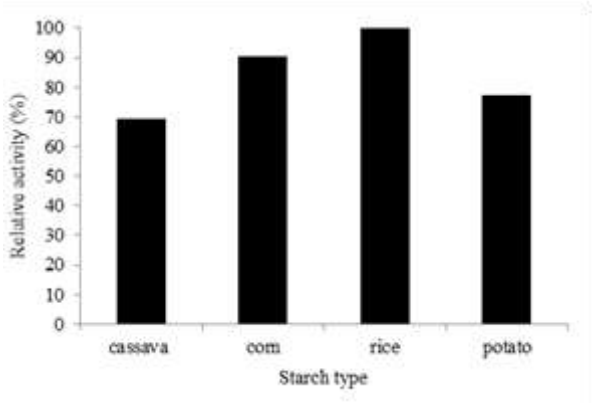
Fig 4. Effect of temperature on the stability of isolate TK6b RSDA



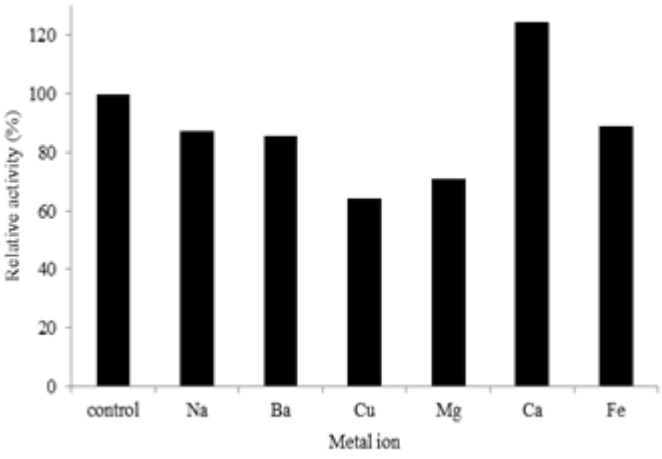
Effect of pH on the activity of isolate TK6b RSDA



Effect of pH on the stability of isolate TK6b RSDA



Effect of stach type on the digestibility of isolate TK6b RSDA



Effect of metal ion on the digestibility of isolate TK6b RSDA

External factor such as cations have been known to affect the activity of enzyme. The presence of the cations could play a vital role in the protein folding or in the catalysis [23]. Figure 7 shows the effects of metal ions on enzyme activity. Calcium ions showed stimulation of the enzyme activity. These result is in agreement with the work of [22] This stimulation by calcium ion has been correlated with the formation of calcium-sodium-calcium metal triad in the main Ca-binding site of the enzyme [22]. Fe, Ba, Na, Cu, and Mg ions inhibited the enzyme activity by 11.21%, 14.24%, 12.56%, 35.91%, and 29.09%.

Enzymes capable of digesting various raw starch granules are economically attractive as the can increase the range of starch sources for direct hydrolysis. the potential raw starch

digestion ability of isolate TK6b enzyme was investigated by monitoring the extent of hydrolysis various raw starch granules. It showed that the enzyme degraded all raw starches tested. The highest hydrolysis was found on rice starch, followed by corn, potato, and cassava starches (Fig. 8). The varying hydrolysis efficiency is very likely correlated to the cristallinity of the starch. Most tuber starches such as potato and cassava have a B-type crystalline structure, while most cereal starches such as rice and corn have an A-type of cristallinity [24]. B-type granules contain much more water, are thicker, have large growth ring, and have longer average amilopectin branch chain length than A- and C-type starches making them more resistant to a enzymatic degradation [25]. Another factor determining the extent to amylase can degrade starch granules is morphology of the granular surface. Potato starch have smooth and even surface while the surface of corn starch granule has cracks and pores going deep in the interior of the granule, enabling a more easy penetration of amylase into the granules [8].

IV. Conclusion

The present study has yielded a novel RSDA by submerged fermentation from isolate TK6b. The advantages of this amylase compared to previously reported ones are wide range of temperature conditions and digestibility of raw starch granules. Enzyme appears to be a good candidate for the direct hydrolysis of diverse raw starch and omitting energy intensive and expensive gelatinization step. Due to the importance of these findings, further studies will be carried on in order to commercialize the production process after necessary optimization in enzyme production and purification.

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