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ACTIVITY OF AN α-L-RHAMNOSIDASE PRODUCED BY *Aspergillus niger* DURING SOLID STATE FERMENTATION OF COFFEE PULP WASTES

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Abstract. *An α-L-Rhamnosidase released by Aspergillus niger during solid-state fermentation (SSF) using coffee pulp (CP) wastes media has been investigated. The activity of α-L-Rhamnosidase based on reducing sugar production against 2% CP alkali extract substrate in 50 mM acetate buffer pH 5. The maximum activity of α-L-Rhamnosidase was obtained in sixth-day SSF with reducing sugar production of 13 μg/mL. The enzyme is actively hydrolyzed 0.1% p-nitrophenyl-α-L-rhamnopyranoside (PNP-Rha) to 95% from initial concentration. Purification using DEAE-Toyopearl 650M increased hydrolysis activity ten times against the substrate, reaching 134 μg/mL of reducing sugar. Optimum enzyme activity at pH 4.5 and 50°C, while stable at pH and temperature in a pH range of 3.5-7 and below 50°C.*

Keywords: *α-L-Rhamnosidase, Aspergillus niger, coffee pulp wastes, solid-state fermentation*

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INTRODUCTION

An α-L-Rhamnosidase is an enzyme that can specifically release terminal α-L-Rhamnose from several flavonoids glycosides, glycolipids and some natural products (Caldini et al., 1994; Vila-Real, et al., 2011; Yadav, et al., 2011; Yadav, 2018). This enzyme is widely distributed in nature and has been successfully purified from animal tissue, plants and several species microorganisms such as yeast, fungi and bacteria (Gastón et al., 2007; Yadav et al., 2010; Ding et al., 2017). This enzyme has been widely used in several industrial applications as a catalyst (Habelt & Pittner, 1983; Bokkenheuser et al., 1987; Caldini et al., 1994; Wang et al., 2005; Birgisson et al., 2007). The application of enzymes in the bioconversion process is the veracious strategy because it does not harm either have no negative impact to the environment and further its stability of the biodiversity in ecosystem function will always be maintained (Prakasham et al., 2007; Muzakhar et al., 2015; Muzakhar et al., 2017). However, to produce enzyme still requires a quite costly process including the materials either purification process. Utilization of coffee pulp (CP) waste material to produce this enzyme is a breakthrough that can be considered because of its huge availability in the environment. Preliminary investigation also revealed that this *Aspergillus niger* easily grow in coffee pulp as carbon and nitrogen without any nutrient added and much black spores appear after 3-4 days incubated at 30°C. Based on these reasons, the investigation of microbial utilization of CP to produce α-L-Rhamnosidase was reported.

MATERIALS AND METHODS

The coffee pulp was collected from a coffee plantation at mountain of Ijen mountain area of Bondowoso district. Anion exchanger chromatography DEAE Toyopearl 650 M was purchased from TOSOH Corp. and all chemical substances were obtained from Sigma.

Cultivation of *Aspergillus niger* and Optimization of Crude α-L-Rhamnosidase Production

The stock culture of *Aspergillus niger* was preserved in PDA medium every two weeks and used for producing crude α-L-Rhamnosidase. To optimize α-L-Rhamnosidase production under solid state fermentation (SSF), 50 g of sterilized CP in a one liter Erlenmeyer flask was inoculated with three loops full of *A. niger* and incubated at 30°C for 5 days. The crude enzyme was daily harvested and measured for the activity. To harvest the enzyme was conducted by adding 500 mL of distilled water containing 1% NaCl and 0.1% toluene (v/v) and shaking at 120 rpm and 30°C for 9 hours. The suspension was filtered using glass filter, centrifuged to recover the supernatant, dialyzed through a column (5 x 25 cm) DEAE-cellulose against 20 mM acetate buffer pH 5 and used as a source enzyme for next analysis. Under the same procedure, the large quantity of crude α-L-Rhamnosidase production, SSF of 500 g CP in 5 L erlenmeyer flask was done.

Preparation of Substrate Coffe Pulp (CP) Alkali Extract and Coffee Bean Arabinogalactan

Five hundred grams of CP powder was suspended in two liters of 10% NaOH, stirred for 12 hours and filtered. The filtrate was centrifuged at 8000 rpm for 20 minutes, recovered the supernatant and adjusted to pH 5 with acetic acid. The supernatant was made to 50% ethanol and centrifuged 8000 rpm for 20 minutes to obtain the pellets and dried under reduced pressure at 50°C. The dry matter as CP alkali substrate was about 4.3% of the starting material.

The arabinogalactan from coffee bean substrate was prepared with the following steps. Fifty grams of coffee bean was extracted with 2 parts of ethanol and benzene solution (1:2), 10 parts water and 20 parts 1.0% sodium hypochlorite. The residue produced, by extraction, was boiled with 20 parts of water for eight hours, concentrated to an adequate volume (about 200 times from initial concentration) under reduced pressure and mixed with three volumes of cold ethanol about 4°C. The precipitate obtained was dried by washing with ethanol and ether. Final yield was 2.2% of the starting material.

Enzyme Assays

Enzyme activity was determined by measuring the release of reducing sugars by Somogy-Nelson method. One unit of enzyme activity defined as reducing sugar produced at a rate of 1 µmol per minute in 1 mL total volume mixture of enzyme and substrate. The α-L-Rhamnosidase activity was also determined by measuring the amount of p-nitrophenol released from the appropriate p-nitrophenyl-α-L-rhamnopyranoside.

The Degree of Hydrolysis and Total Sugar Content Analysis

The degree of hydrolysis was examined in a series of time during incubation of reaction mixture of crude enzyme and 2% CP alkali extract substrate at 37°C. The release of reducing sugars was measured and the degree of hydrolysis was calculated as follows:

$$Degree\ of\ hydrolysis(\%) = \frac{Total\ reducing\ sugar\ of\ hydrolyzate\,(w/v)}{Total\ substrate\,(w/v)} * 100\%$$

The total sugar content of the substrate was measured by the phenol-sulfuric acid method (34).

Purification

Purification steps were carried out at 25°C, using 20 mM acetate buffer, pH 5. The initial stages in the purification process, namely the dialysis process using hollow fiber dialysis column 5Kda against acetate buffer 20mM pH 5 to remove the remaining sugar and polysaccharides dissolved. The enzyme was then concentrated using 60% of saturated ammonium sulfate followed by centrifugation at a speed of 12000 rpm to obtain pellets. The pellet was dissolved in small volume and re-dialysis with the same buffer. Subsequently, the dialyzed of crude enzyme was loaded into an on DEAE Toyopearl 650M column which was pre-equilibrated with the same acetic buffer used. Purification was done by elution 5mL per second using a 500mL buffer with a gradient concentration of 0 to 0.5M NaCl, and for each fraction volume was 5 mL. Each fraction was assayed for α-L-Rhamnosidase activity against 2% CP substrate.

Enzyme Properties

The effect of pH and temperature on activity and stability of the enzyme was determined at the pH range of 2 to 10 and a temperature range of 20 to 70°C. The effect of pH on the α-L-Rhamnosidase activity was measured after 10 minutes incubation at 37°C in 1 mL 1% CP substrate. Molecular weights of enzymes were estimated by comparison of their migration rates with those of protein standards in gel filtration Superdex 75 with eluent 1 mL/min using 20 mM acetate buffer pH 5. Proteins standard (in Dalton) used, were phosphorylase (97,400), albumin (66,300), aldolase (42,400), carbonic anhydrase (30,000), trypsin inhibitor (20,100) and lysozyme (14.400).

RESULTS AND DISCUSSION

Optimization of α-L- Rhamnosidase

Extracellular enzyme activity was released by *A. niger* during the SSF process using the CP substrate has been identified as α-L-Rhamnosidase. It has been previously known that *A. niger* can produce various types of enzymes to convert their living substrate or medium into biomolecules as material for their metabolism during the growth process. It has been reported that *A. niger* in the SSF process released hydrolase enzymes such as cellulase, xylanase, pectinase and from several groups of lipase enzymes (Hatzinikolaou et al., 1996; Ellaiah et al., 2004; Lúcia et al., 2011; Narasimha et al., 2015; Reddy et al., 2015).

In this study, during the SSF process, showed that *A. niger* was able to grow as indicated by the huge of black spores in the CP medium. As shown in Figure 1, optimum production of α-L-Rhamnosidase occurred on day sixth in room temperature of 30°C without additional nutrients into the growth medium. The enzyme hydrolyzed CP and produced reducing sugar 13 μg/mL. The activity of α-L-Rhamnosidase based on reducing sugar production against 2% CP alkali extract substrate in 50 mM acetate buffer pH 5. This study also calculated that the enzyme maximum hydrolyzed CP medium at 78% degree of hydrolysis. It can be explained that *A. niger* is able to utilize carbon, nitrogen and mineral sources from the CP medium.

Purification and Molecular Weight α-L-Rhamnosidase

Elucidate the existence of α-L-Rhamnosidase, a purification process was done. As shown in Figure 2, fractions 57 to 63 were the active hydrolyzed CP substrate. The active fractions were pooled and re-dialysis against the same buffer to remove remaining NaCl.

Substrate specificity of α-L-Rhamnosidase activity also tested using various substrates such as carboxymethyl-cellulose, arabinogalactan, xylan, pectin and p-nitrophenyl-α-L-rhamnopyranoside. As shown in Table 1, when CP and p-nitrophenyl-α-L-rhamnopyranoside substrates were used, 134 µg/mL of reducing sugar and 56 µg/mL of p-nitrophenol was released, with the degree of hydrolysis 78 and 94%. In contrast, no reducing sugars were detected when carboxymethyl-cellulose, arabinogalactan, xylan and pectin substrates were used. This evidence proved that all active fractions only contain α-L-Rhamnosidase. Further analysis of enzyme molecular weight as described in the method, the α-L-Rhamnosidase has 32,000 Da, approximately.

Optimum and Stability of α-L- Rhamnosidase on Activity

The α-L-Rhamnosidase exhibited maximum activity at pH 4.5 (Figure 3A) and retained nearly 100% activity in a pH range of 3-5.5 after 30 minutes exposure to corresponding pH values (Figure 3B). α-L-Rhamnosidase showed optimum activity at 55°C respectively (Figure 3C) and are nearly 100% stable below 60°C after 30 minutes exposure to respective temperatures (Figure 3D).

The successful microbial utilization of CP to produce α-L-Rhamnosidase and other extracellular enzymes, is a new strategy in increasing the added value of CP waste. However, research efforts are needed to improve the efficiency of the process.

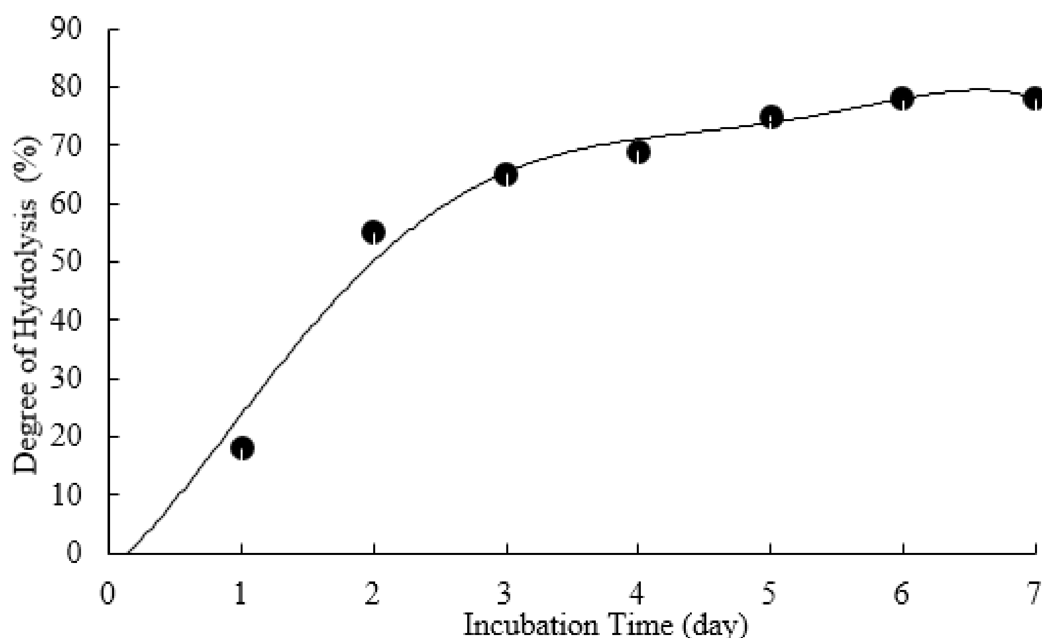


Figure 1. Optimum of α-L-Rhamnosidase produced during SSF on CP Substrate after Six days Incubation at 37°C.

Table 1. Purified α-L-Rhamnosidase activity on various 1% substrates

Substrate	The degree of Hydrolysis (%)
Coffee Pulp	78
carboxymethyl-cellulose	ND
arabinogalactan	ND
xylan	ND
pectin	ND
p-nitrophenyl-α-L-rhamnopyranoside*	94

*) 0.5% substrate

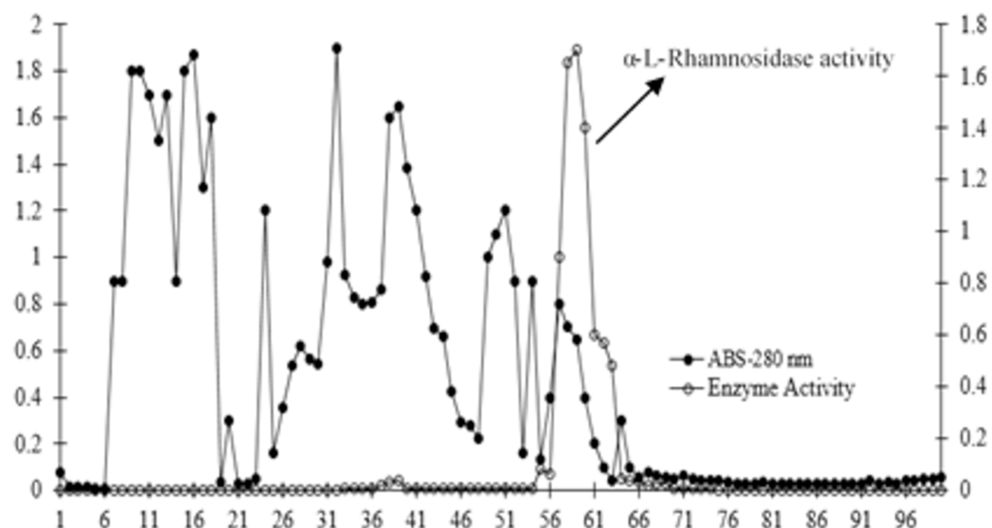
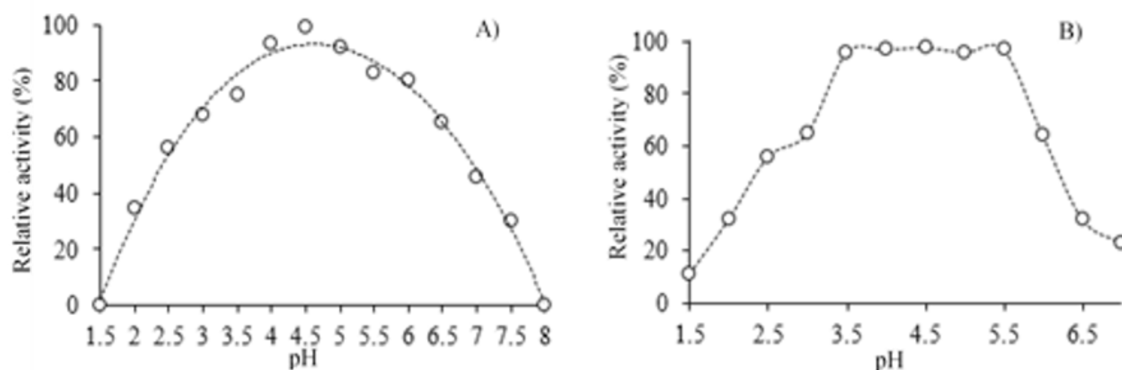


Figure 2. The buffered crude enzyme was loaded an on DEAE Toyopearl 650M column, eluted by using acetate buffer 20 mM pH 5 and gradient 0 - 0.5 M NaCl. The protein concentration (●) was monitored at 280 nm, and the α-L-Rhamnosidase activity (○) was the checked after 10 minutes incubation of the mixture (100 µl crude enzyme in 1ml of 2% CP substrate in the same buffer).



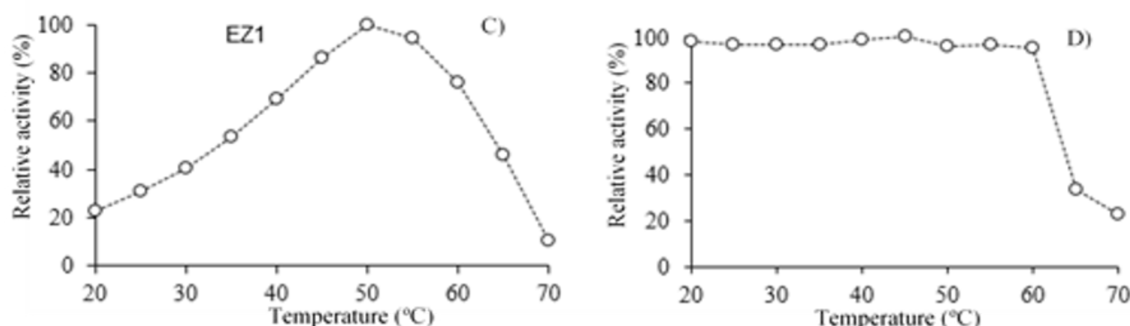


Figure 3. The optimum (A, C) and Stability (B, D) of purified α-L-Rhamnosidase activity on temperature and pH.

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