



Lignocellulosic enzymes characterization and scanning electron microscope analysis on rice bran surface structure changes

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ABSTRACT

Rice bran was one of livestock food ingredients with high crude fibers contents, including cellulose and hemicelluloses. Lignocellulosic enzymes such as xylanase and cellulase functioned in degrading cellulose and hemicelluloses. The objectives of this study were to qualitatively screen *Actinobacillus* sp. (G6) and *Bacillus pumilus* (G7) which produced xylanase and cellulase, determining molecular weight of xylanase and cellulase enzymes using SDS-PAGE and Zymogram, and analyzing structure changes on rice bran surface after Scanning Electron Microscope treatments using xylanase and cellulase enzymes. The findings of this study indicated xylanolytic indexes for G6 and G7 were 2 and 1.7 respectively while cellulosic index for G6 and G7 were 1.6 and 2. Enzymes produced by *Actinobacillus* sp. (G6) had molecular weight 25-35 kDa and above 100 kDa while enzymes produced by *Bacillus pumilus* (G7) had molecular weight 25 kDa up to above 100 kDa. The result of rice bran SEM analysis indicated profile change of the samples before and after enzymes treatment.

Keywords: rice bran, lignocellulosic enzymes, cellulose, hemicelluloses

INTRODUCTION

Rice bran is one of rice milling by-products. It is commonly used as an ingredient of livestock feed. The structure of primary cell wall of rice plant is complex, consisting of: 1) polysaccharide (β -1 polymer and 4-glucose polymer), hemicelluloses (xylose, galactose, and primary mannose), and pectin (consisting of polymethyl galacturonic acid and polygalacturonic acid; 2) Lignin (phenylpropane polymers) and: 3) Glycoproteins^[1].

A problem regarding rice bran utilization as an ingredient of livestock feed is mostly caused by its high crude fiber contents consisting of cellulose, hemicelluloses, and lignin. Cellulose and hemicellulose are crude fibers which are the main constituents of plants cell walls and two of organic materials which are abundant in nature and potentially serve as renewable energy source for livestock. A factor which inhibits the utilization of cellulose as poultry feed is because poultry do not have microbes which produce lignocellulosic enzymes unlike ruminants which own lignocellulosic enzymes microbes inside their rumen which enable them to degrade cellulose and hemicelluloses^[2]. The result of proximate analysis of rice bran nutrient contents (based on dry matter) is: 7.45% crude proteins, 37.39% crude fibers, 21.39% ash, 27.13% nitrogen free extract (NFE), metabolic energy 1813.82 kcal/kg, and cellulose 23.21%. In order to solve rice bran nutrition quality problem, several innovations on enzymatic technology should be done. One of them is through feed manipulation, by adding lignocellulosic enzymes produced by lignocellulosic bacteria (*Bacillus pumilus* and *Actinobacillus* sp) which are able to degrade the bonds of cellulose, hemicellulose, and lignin during agro-industry waste degradation process for livestock feed. Livestock feed production process which applies biological approach is much safer than production process using chemical ingredients that may pollute the environment. The utilization of cellulosic and hemicellulosic enzymes may improve

the nutrition value of rice bran. Cellulosic enzyme consists of three components, namely C1 (β -1,4-glucan cellobiohydrolase or exo- β -1,4-glucanase), Cc (endo- β -1,4-glucanase), and cellobiase (β -glucocidase). Meanwhile, hemicellulosic enzymes consist of endo- β -1,4-xylanase, β -xylosidase, α -L-arabinofuranosidase, α -D-glucuronidase, and acetyl-xylan-esterase^[3]. There was only a little information regarding lignocelluloses as enzymatic process supplementation on agricultural waste (i.e. rice bran) used as poultry feed.

Cellulose biodegradation utilizing cellulosic enzyme produced by microbes was vital in processing agricultural waste (rice bran) onto poultry feed^[4,5]. Supplementing cellulosic enzymes into poultry feed can improve feed efficiency and poultry performance by improving in-vitro^[6,7], in-site^[8,9], and in-vivo^[10] fiber digestibility. This study was intended to determine qualitative screening index of *Actinobacillus sp.* and *Bacillus pumilus* bacteria as the producers of xylanase and cellulase, measuring molecular weight of xylanase and cellulase based on SDS-PAGE and Zymogram, and analyzing rice bran surface structure change after being treated using xylanase and cellulase by utilizing Scanning Electron Microscope (SEM).

EXPERIMENTAL SECTION

This research applied lignocellulosic enzymes obtained from *Actinobacillus sp.* (G6) and *Bacillus pumilus* (G7).

Research Procedures

Qualitative Screening of Xylanase and Cellulase Producing Bacteria

Qualitative examination on cellulase and xylanase enzymes activity was conducted by growing *Actinobacillus sp.* (G6) and *Bacillus pumilus* (G7) on solid medium Luria Bertani (LB) containing 1% Carboxyl Methyl Cellulose (CMC) and Oat-spelt Xylan (OX) substrates at 40° C temperatures for 16-18 hours. Enzymes activities was identified by halo zones (clearer areas around the bacteria growing locations. The medium was colored using Congo Red 0.1% and being washed in NaCl 1M to highlight halo zones in red background^[11]. Xylanolytic and cellulolytic indexes were measured by:

$$\text{Xylanolytic or Cellulolytic Index} = \frac{\text{halo zone diameter}}{\text{bacteria colony diameter}}$$

Cellulase and Xylanase Production

Singular colonies of *Actinobacillus sp.* (G6) and *Bacillus pumilus* (G7) rumen bacteria isolates were cultured on 5 mL LB liquid medium containing 1% CMC substrate and 1% OX substrate at 40° C and being shaken using shaker incubator at 150 rpm for 16-18 hours. About 1% of liquid culture were inoculated in 100 mL LB liquid medium at 40° C and being shaken on shaker incubator at 150 rpm for 16-18 hours. The suspensions were centrifuged at 3500 rpm and 4° C for 15 minutes. The resulted supernatants were raw extracts of cellulase and xylanase enzymes.

Enzymes Assay

Cellulase and xylanase enzymes activities were examined based on 3,5-dinitrosalicylic acid (DNS) method namely by mixing 100 μ L enzymes and 100 μ L substrate (i.e. CMC 1% and OX 1% substrates) in 100 mM pH 7 Phosphate-Citrate (PC) buffer solution incubated in water bath at 50° C for 60 minutes. The results of incubation were added into 600 μ L DNS reagent and stored into boiled water bath for 15 minutes and being cooled down inside ice water bath for 20 minutes. Reduction sugar produced in these processes was measured using spectro-photometer UV-Vis at 550 nm. One unit of enzyme activity is defined by amount of enzyme required to produce 1 μ mol product per time unit and per mL enzyme^[12].

SDS-PAGE and Zymogram Analysis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed using 12% (w/v) polyacrylamide gel with a 5% stacking gel and the Mini-Protean II system (BioRad) according to the method described by Laemmli^[13]. After electrophoresis, the gel was stained for 30 min with Coomassie Blue R250 dye in a methanol-acetic acid-water solution (4:1:5, by volume) and then destained in the same solution without the dye.

Cellulase and Xylanase analysis using Zymogram was initiated by creating separating gel 12% containing 1 mL CMC 0.1% or OX 0.1% substrate and stacking gel 5%. The sample (i.e. enzyme) was mixed with buffer sample in composition 4:1. 25 μ L sample was put into gel wells. The gels were soaked into 1x electrophoresis buffer solution. Electrophoresis was conducted at 100 Volt voltages for about 1.5 hours.

Zymogram was conducted by soaking the gels into 2.5% Triton X-100 solution for an hour at room temperature while constantly shaken on shaker machine. Cellulase and xylanase enzymes in gel measurement was conducted by soaking the gels inside 100 mM PC buffer pH 7 and being incubated at 50° C temperatures for 4-5 hours. Active cellulase and xylanase ribbons was identified by soaking the gels inside 0.1% *Congo Red* solution^[14] for 30 minutes at room temperature followed by washing the gels several times in NaCl 1M solution. Colorless bands in red background indicated active protein ribbons.

Rice Bran Preparation

Rice bran as sample on this research was obtained from Surabaya, East Java-Indonesia. The sample was washed, powdered, and dried inside an oven at 40° C until reaching its constant weight.

Rice Bran Hydrolysis

40 mL enzymes were added into 4 grams rice bran. The mixture was incubated at 50° C for 24 hours. This process applied three treatment variations, namely 4 grams rice bran was hydrolyzed using 40 mL xylanase; 4 grams rice bran was hydrolyzed using 40 mL cellulase; and 4 grams rice bran was hydrolyzed using 20 mL xylanase and 20 mL cellulase.

SEM Analysis

Changes on rice bran surface structure which had been hydrolyzed using enzymes at optimum condition were analyzed using SEM (phenon). Samples were layered by gold before being analyzed In this process, samples which had not treated using enzymes and samples which had been incubated using aquadest were also examined (used as control groups) to examine changes on rice bran surface structure.

RESULTS AND DISCUSSION

Qualitative Screening of Xylanase and Cellulase Producing Bacteria

The activity of cellulase and xylanase enzymes produced by *Actinobacillus sp.(G6)* and *Bacillus pumilus (G7)* bacteria could be identified from the existing halo zones (clear/colorless area) around the location where bacteria grown (See Figure 1 below). The activity of cellulase enzyme could be seen on LB medium containing CMC 1% substrate while the activity of xylanase enzyme could be seen on LB medium containing OX 1% substrate. Xylanolytic index of G6 and G7 and cellulolytic index of G6 and G7 were presented on Figure 2 :



Figure 1: G6 and G7 Isolates Qualitative Screening Result

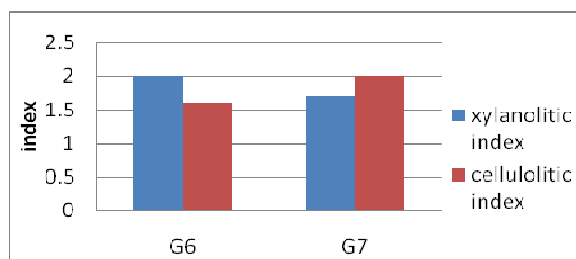


Figure 2: Cellulolytic and Xylanolytic Indexes of G6 and G7

Research finding data on G6 bacteria indicated that the activity of xylanase enzyme was higher than cellulase activity. On the other hand, research finding data on G7 bacteria indicated cellulase activity which was higher than xylanase activity. Halo examination result indicated qualitatively cellulase and xylanase activities. This method

utilized *Congo Red* dye for its strong interaction with polysaccharide containing D-glucopyranocyl and β -(1,4), β -(1,3)-D-glucan bonds and several hemicellulosic bonds, including galactoglucomannan. Based on this characteristics, *Congo Red* dye could be used to detect the existence of cellulose and hemicelluloses on agar plate [14]. Halo zones formed through *Congo Red* coloration indicated substrates had been hydrolyzed into simple sugar products [15]. NaCl flushing was intended to preserve color stability and inhibit enzyme activities [16]. Qualitative data could be obtained by examining enzymes activities on CMC and OX substrates.

Enzymes Assay

The result of xylanase and cellulase activity was presented on Figure 3:

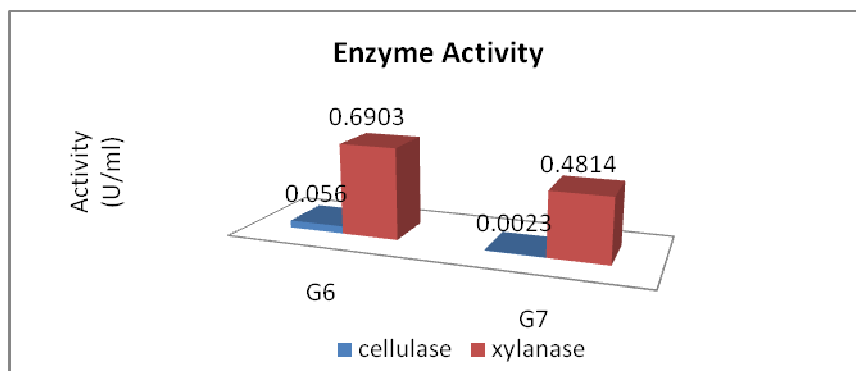


Figure 3: Graphic describing the activity of Xylanase and Cellulase produced by G6 and G7 bacteria

The activity of xylanase on G6 and G7 were higher compared to the activity of cellulase. Cellulase was examined by mixing CMC substrate with DNS reactor while xylanase activity was examined by mixing OX substrate with DNS reactor. The amount of reducing sugar was measured by applying spectrophotometric DNS method at wave-length (λ) 550 nm [12]. One activity unit of cellulase was defined as the amount of glucose (in μ mol) produced by one mL enzyme per minute. Meanwhile, one activity unit of xylanase was defined as the amount of xylose (in μ mol) produced by 1 mL enzyme per minute. Different results of cellulase and xylanase activity on G6 and G7 were related to enzymes different capability in hydrolyzing substrates.

SDS-PAGE and Zymogram Analysis

The results of cellulase and xylanase enzymes analysis based on SDS-PAGE and zymogram were presented on Figure 4:

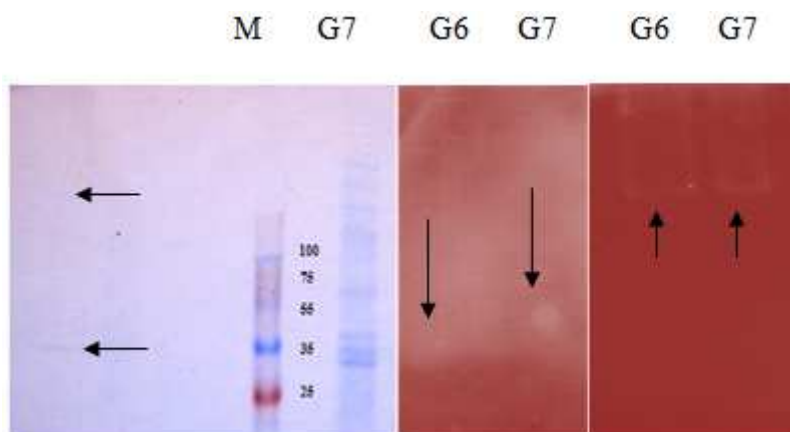


Figure 4: 1 (SDS-PAGE): M: Protein Marker (5 bands 100, 70, 55, 35, 25 kDa); G6 (1 band between 25 kDa and 35 kDa, 1 band above 100 kDa), G7 (2 bands between 25 kDa and 35 kDa, 1 band between 35 kDa and 55 kDa, 1 band between 55 and 70 kDa, 4 bands above 100 kDa). 2 (Xylanase zymogram): G6 (halo zone between 25 kDa band and 35 kDa), G7 (halo zone between 35 kDa and 55 kDa). 3 (Cellulase zymogram): G6 and G7 halo zone above 100 kDa

Result of SDS-PAGE indicated the molecular weight of the enzyme produced by G6 between 25 kDa and 35 kDa and above 100 kDa. The enzyme performed xylanase activity on protein bands 25-35 kDa and cellulase activity on protein band above 100 kDa. Meanwhile, the molecular weight of the enzyme produced by G7 ranged from 25 kDa to above 100 kDa. The enzyme performed xylanase activity on protein bands weighed 35-55 kDa and performed cellulase activity on protein band above 100 kDa. The documentation on SDS-PAGE and zymogram results was not

very clear. It may be caused by enzymes low protein contents. This issue can be solved by purifying the enzymes and increasing its concentration which made protein bands clearer during SDS-PAGE and zymogram process.

In this research, [17] reported that relative molecular weights of cellulase enzymes were varied, for example endoglucanase weighed 12.5 - 90 kDa, exoglucanase 41.8 – 65 kDa, and cellobiase 47 kDa. Cepeljnik *et al* (2001) [18] also reported that relative molecular weight of β -1,4-endoxylanase was 58 kDa.

SEM Analysis

The results of SEM analysis indicated changes of rice bran surface structure after enzyme treatment. These changes were presented on Figure 5, 6, 7, and 8 below:

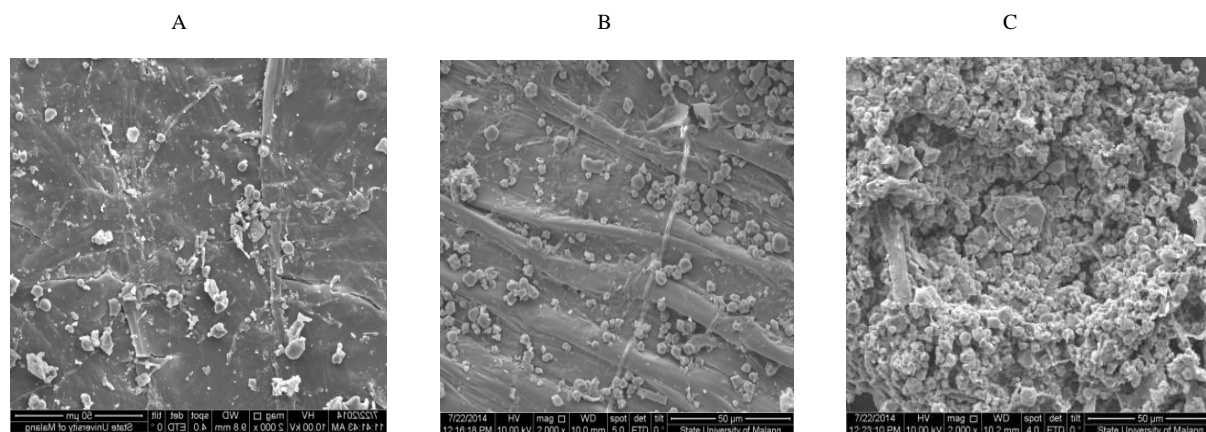


Figure 5: Rice bran surface structures (2000x magnification). A: before treatment; B: treated using water; C: treated using xylanase produced by G6

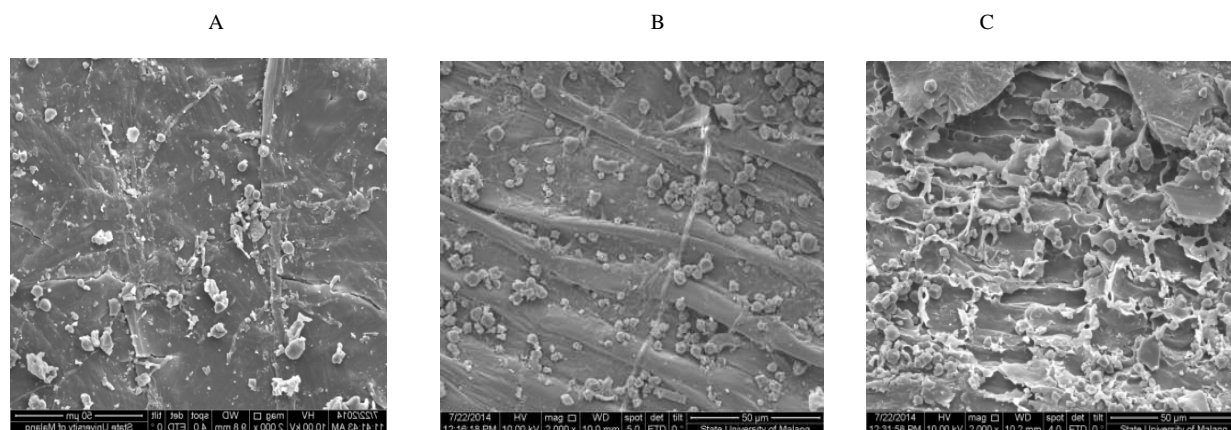


Figure 6: Rice bran surface structures. A: Before treatment; B: after being treated using water; after being treated using xylanase produced by G7

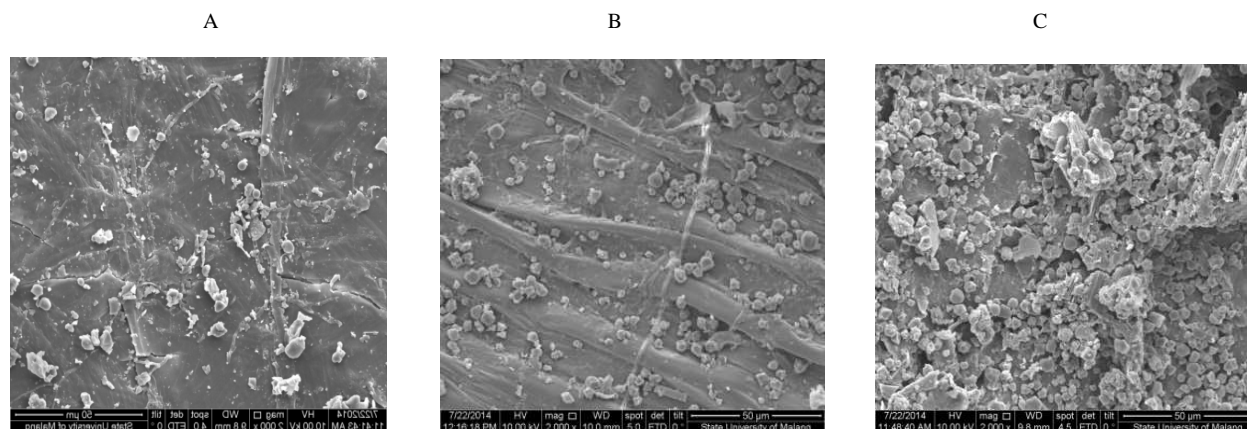


Figure 7: Rice bran surface structures. A: before treatment; B: after being treated using water; C: after being treated using xylanase and cellulase produced by G7

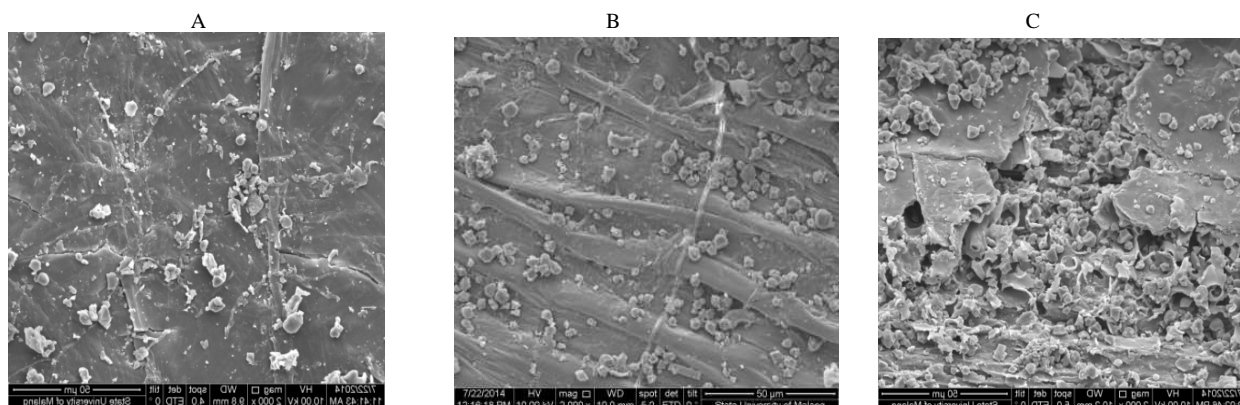


Figure 8: Rice bran surfade structures. A: before treatment; B: after being treated using water; C: after being treated using xylanase and cellulase produced by G6

Scanning Electron Microscope (SEM)

SEM was equipment used to describe surface profile of an object by shooting electrons onto its surface. In order to obtain good description from an object, the object should be metallic. *Eichhornia crassipes*, rice stalks, and rice bran were organic object without metal contents. Therefore, in order to be able to analyze these objects under SEM, they should be metalized by layering them with metals, such as palladium gold. Samples which were analyzed consisted of rice bran samples which had been incubated for 24 hours. Rice bran sample which had not been treated using enzyme and rice bran sample which had been treated using aquadest were also analyzed as control groups.

The results of SEM analysis showed changes on surface profiles among the samples before and after being treated using enzymes (as described on Figure 5, 6, 7, and 8 above). There were changes on rice bran surface structure. Before being treated using enzymes, the surface structure of rice bran was compact and well-organized (See Figure 5A, 6A, 7A, 8A). After enzyme treatment, rice bran surface structures became more hollow and cracked (as seen on Figure 5C, 6C, 7C, and 8C above). There was no significant difference between rice bran which had not been treated (Figure 5A, 6A, 7A, and 8A) and rice bran which had been treated using aquadest (Figure 5B, 6B, 7B, 8B). this finding implied that temperature used during incubation process did not affect the cracks (damages) on rice bran surface structure. SEM analysis results on rice bran which had been treated using xylanase and cellulase (Figure 7C) indicated more cracks than rice bran which had been treated using xylanase (Figure 6C).

Cracks on rice bran surface structure was the effect of enzymes (cellulase and xylanase) activity in damaging rice bran cell wall which mainly composed by cellulose and hemicelluloses (particularly xylan). Xylan was main component of hemicelluloses which was located between lignin and lower cellulose fibers compounds^[19]. Xylanase functioned to hydrolyze xylan into its monomers, such as xylose, xylooligosaccharide, and arabinose. Meanwhile, cellulase functioned to hydrolyze cellulose into glucose^[20]. Previous studies found that enzymes (xylanase, cellulase, and combination of xylanase and cellulase) hydrolysis on rice stalks caused cracks on rice bran surface structure^[21].

CONCLUSION

From the results of this research, it can be concluded that G6 (*Actinobacillus sp.*) and G7 (*Bacillus pumilus*) bacteria were able to produce xylanase and cellulase. The molecular weight of enzymes (xylanase and cellulase) produced by G6 (*Actinobacillus sp.*) bacteria was 25-35 kDa and above 100 kDa while the molecular weight of enzymes (xylanase and cellulase) was 25 kDa and above 100 kDa. The results of SEM analysis indicated changes on samples (rice bran) surface profile before and after enzymes treatment.

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