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SYNERGISTIC ACTIVITY OF ENZYMES PRODUCED BY *EUPENICILLIUM JAVANICUM* AND *ASPERGILLUS NIGER* NRRL 337 ON PALM OIL FACTORY WASTES

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ABSTRACT

The use of palm kernel cake (PKC) and palm oil mill effluent (POME), substances from palm oil factory wastes, for monogastric is limited by their high cellulose and mannan contents. Hydrolytic enzymes have been supplemented to increase the nutrient digestibility. The maximal digestibility was obtained in the synergistic action of all enzyme components including B-D-endoglucanase (CMCase), B-D-glucosidase, B-D-mannanase, p-D-mannosidase, and oc-D-galactosidase. Two kinds of enzymes produced by *Eupenicillium javanicum* and *Aspergillus niger* NRRL 337 on the submerged culture containing 3% coconut meal were selected to hydrolyze PKC or dry POME. Enzyme from *E. javanicum* contained higher CMCase, B-D-mannanase, and a-D-galactosidase activities, while that from *A. niger* NRRL 337 contained more p-D-glucosidase and p-D-mannosidase activities. Saccharification (hydrolytic) activities of enzyme mixtures on PKC and POME were determined at pH 5.0, the optimal pH for p-D-mannanase from *E. javanicum,* and at 5.4 the optimal pH for a-D-galactosidase from *E. javanicum* and P-D-glucosidase from *A. niger* NRRL 337. The enzyme proportions *of E. javanicum* and *A. niger* NRRL 337 were 100 : 0, 80 : 20, 60 : 40, 40 : 60, and 0 : 100%. The highest Saccharification activity on both substrates was observed on the mixture of 80% *A. niger* NRRL 337. The pH levels did not significantly affect Saccharification activity. Fiber components in PKC were more digestable than in POME. Further analysis on the reducing sugar components using thin layer chromatography showed that more monomers were produced in the 60 or 80% of *A. niger* NRRL 337. The glycosidases of *A. niger* NRRL 337 played more important role in the Saccharification activity.

Keywords: Synergistic activity/ palm kernel cake/palm oil mill effluent/ *Eupenicillium javanicum/ Aspergillus niger NRRL* 337

INTRODUCTION

Feeding costs account for 65-70% of the total cost of animal production. Therefore, it is important that most part of the feed can be utilized. Feeding of commercial chickens in Indonesia usually follows the formula applied in their original country (United States). Normally, the feed contains corn, soybean meal, and fishmeal. These feedstuffs are not abundant in Indonesia and have to be imported. On the other hand, rice bran, coconut meal, and palm oil factory wastes: palm kernel cake (PKC) and palm oil mill effluent (POME) are available. PKC is a residue of the oil extraction of palm fruit and constitutes up to 45% of palm oil bunches, while POME is the sludge of crude palm oil process and amounted to 2%

of palm oil bunches. The fiber content of PKC exceeding 20% could occur if shells and fruit fiber are not removed in the extraction process and over its half part is neutral detergent fiber (NDF) containing galactomannan and mannan (Swick and Tan 1995). The crude fiber (cellulose and lignin) of steamed PKC was 21.7% (Supriyati *et al.* 1998). The cellulose, hemicellulose (galactomannan and mannan), and lignin of steamed POME were 17.3, 22.0, and 15.8% (Purwadaria *et al.* 1998).

Supplementation of mannanase (galactomannanase) enzyme complex to improve the nutritional value is potential for improving feed quality. The components of the enzymes are p-D-endoglucanase (CMCase), p-D-glucosidase, P-D-mannanase, P-D-mannosidase, and a-D-galactosidase. *Eupenicillium javanicum* isolated from palm seed (Purwadaria *et al.* 1994) produced less P-D-glucosidase and more p-D-mannanase, P-D-mannosidase, and a-D-galactosidase than *Aspergillus niger* NRRL 337 known as mannanolytic fungus (Araujo and Ward 1990; Haryati *et al.* 1997). The different activities of the enzyme component open up the possibilities of using the enzyme mixtures to improve the saccharification (hydrolysis) activity in PKC and POME. The synergistic effect using enzyme mixture of *Trichoderma viride* and *A. ustus* on alkali treated bagasse had been reported (Manonmani and Sreekantiah 1987). The saccharification on the substrate was 63% when using enzyme mixture of 1:1, while when individual enzymes were used 13.5 and 22.9%, respectively. The information on optimal mixture of such enzyme composition will be useful in enzyme application for animal feeding.

The objective of the present study is to determine the synergistic activity of enzymes produced by *E. javanicum* and *A. niger* NRRL 337 to hydrolyze PKC and POME.

MATERIALS AND METHODS

Palm kernel cake (PKC) and palm oil mill effluent (POME)

PKC was obtained from palm oil factory by solvent extraction, while POME was obtained from the centrifugation of palm oil mill effluent. After centrifugation POME was dried under the sun. Both dry materials were ground to a fine powder (0.5 mm) using Wiley mill.

Enzyme production

Enzymes were produced by *E. javanicum* (RIAP collection) or *A. niger* NRRL 337 in the medium containing yeast extract 3g/l, coconut meal 30g/l and minerals in g/1 $(NH_4)_2SO_4$ 1.4, KH_2PO_4 2.0, $MgSO_4$ 0.3, urea 0.3, and $CaCl_2$ 0.3 and in ppm $FeSO_4$ 5, $MnSO_4$ 16, $ZnSO_4$ 14, and $CoCl_2$ 20. Molds were cultivated in 50 ml medium in a 250 ml flask at 29°C using reciprocal shaker (150 rpm), after inoculation with 2 ml of spore suspension (5 x 10^{13} and 30 x 10^{13}/ml for *E. javanicum* and *A. niger,* respectively) from five-day PDA culture slant. The

incubation time for *E. javanicum* was five days, while that for *A. niger* was six days (Haryati *et al.* 1997). Sodium azide was added at 0.2% final concentration. The culture was then centrifuged (12000 rpm, 20 min, 4°C) and supernatant was collected for enzyme assays and saccharification.

Enzyme activities

The activity of carboxymethylcellulase (CMCase) and β-D-mannanase were assayed by determining the reducing sugars produced from CMC and gum locust bean (mannan) as glucose or mannose, respectively (Haggett *et al.* 1979; Araujo and Ward 1990). One unit was defined as enzyme which liberates one p.mol glucose or mannose per minute. The glycosidase activities (β-D-mannosidase, β-D-glucosidase, and α-D-galactosidase) were assayed using nitrophenyl glycosides as substrates and one unit was defined as enzyme which liberates one umol nitrophenol per minute (Ide *et al.* 1983). Specific activity of all enzymes was calculated in unit/mg extracellular protein.

Determination of protein and fiber component concentration

Protein concentration was determined by Bradford method (1976) and Bovine serum Albumin was used as a standard. The concentrations of fiber components (cellulose, hemicellulose, lignin and silica) were calculated as neutral and acid dietary fiber according Van Soest and Robertson (1968).

Determination of optimum pH and temperature

The activities of both enzymes were determined at 50°C at different pH (4.6, 5.0, 5.4, 5.8, and 6.2) to obtain the optimum pH, while for determination of the optimum temperature, the enzyme assays were carried out at maximum pH and different temperatures (35, 40, 45, 50, 55, and 60°C).

Saccharification activity towards PKC and dry POME

Saccharification activities were determined following determination of avicelase (Haggett *et al.* 1979) using PKC and dry POME as substrates. The incubation time of the reaction was two hours and reducing sugars produced was determined with DNS method (Miller 1959). The activity value was expressed in unol glucose/ml liberated in one minute.

This reaction was also used to determine the synergistic activity of the enzyme mixtures. Several proportions of enzymes from *E. javanicum* and *A. niger* NRRL 337 (0:100, 20:80, 40:60, 80:20, and 100:0%) were used. Aside from the concentration of reducing sugar produced in the reaction, the sugar components of the product were also determined using thin layer chromatography (Lestari *et al.* 2001). The samples and standards (glucose, cellobiose, mannose, mannobiose, and

galactose) were spotted on plates of silica gel 60 (20 x 20 cm). The reducing sugar concentrations of spots from samples were 3-8 чg, while the concentration of each standard was 8 *чg.* Elution was carried out using the mixture of ethanol, n-propanol and water (30:150:20). The elution was stopped when the solvent reached 2 cm from the top. The gel was then dried, and the elution was repeated to get better resolution. Spots were detected by spraying the plates by a mixture of aniline (1 ml), diphenylamine (1 g), 80% H_3PO_4 (7.5 ml) in 50 ml acetone and heated at 100°C for 1 hour.

RESULTS AND DISCUSSION

The activities of enzyme components (CMCase, (β-D-mannanase, (β-D-manno-sidase, β-D-glucosidase, and α-D-galactosidase) involved in the fiber hydrolysis were determined (Table 1). The enzymes produced from *E. javanicwn* contained higher activities of CMCase, β-D-mannanase, and a-D-galactosidase, while those produced by *A. niger* contained higher β-D-mannosidase and P-D-glucosidase activities. It is already known that *Aspergillus* spp. produce more glycosidases (Ghose *et al.* 1985; Manonmani and Sreekantiah 1987; Haryati *et al.* 1997). The different major enzyme components of both enzymes suggested the possibility of combination between enzymes.

Table 1. Specific activities of CMCase, P-D-mannanase, P-D-mannosidase, p-D-glucosidase, and a-D-galactosidase of enzymes pr oduced by *E. javanicum* and *A. niger* NRRL 337 grown on coconut meal.

Molds	Specific activities (U/mg)					
	[Protein] (ug/ml)	CMCase	p-D-man nanase	P-D-Gluco sidase	a-D-galacto sidase	P-D-manno sidase
E. javanicum	435	18.6	1172.2	1.5	16.8	1.1
A. niger NRRL 33'	498	9.9	14.1	3.1	2.3	3.3

The optimal synergistic activity was obtained when the optimal pH and temperature conditions were applied. The optimal pH and temperature *of E. javanicum* β-D-mannanase and α-D-galactosidase were at 5.4 and 50°C, and 5.0 and 55°C respectively, while those of *A. niger* β-D-glucosidase were at 5.0 and 55°C (Figures 1 and 2). The temperature difference gave less effect than the pH difference. A temperature increase from 50 to 55°C reduced β-D-mannanase activity at 22.7%, while the temperature reduction from 55 to 50°C reduced activities of α-D-

galactosidase and (β-D-glucosidase at 11.3 and 10.9%, respectively. An increase of pH from 5.0 to 5.4 increased 39.5% of the β-D-mannanase activity, and reduced 24.9 and 13.5% α-D-galactosidase and β-D-glucosidase, respectively. Further analyses at these conditions were used to determine saccharification activity towards PKC and POME (Table 2).

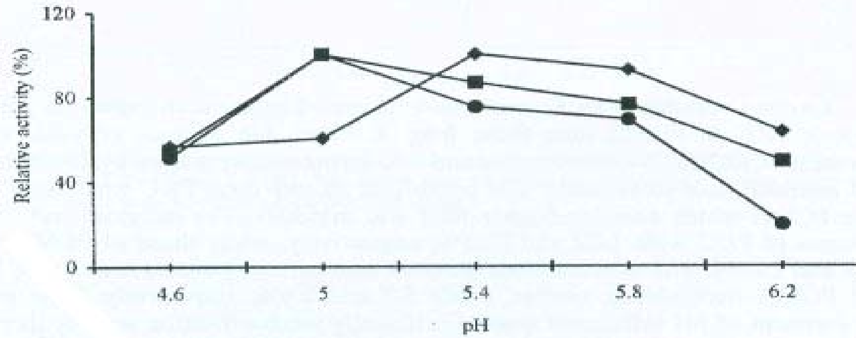


Figure 1. Optimum pH for *E. javanicum* α-D-galactosidase (•) and β-D-mannanase (♦), and *A. niger* β-D-glucosidase (■).

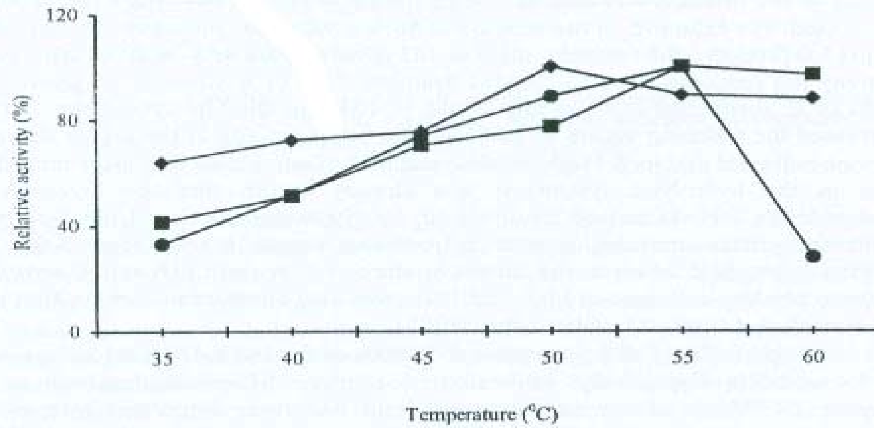


Figure 2. Optimum temperatures for *E. javanicum* α-D-galactosidase (•) and β-D-mannanase (♦), and *A. niger* β-D-glucosidase (■).

Table 2. Saccharification activity of enzymes produced by *E. javanicum* and *A. niger* NRRL 337 towards PKC and dry POME at various pH and temperatures.

Molds	Substrates	Saccharification activity (μmol/ml)		
		pH 5.0 ; 50°C	pH 5.4 ; 50°C	pH 5.0 ; 55°C
E. javanicum	PKC	11.11	13.70	12.50
	POME	0.44	0.56	0.55
A. niger	PKC	1.90	0.55	1.48
NRRL 337	POME	0.29	0.33	0.43

Enzymes produced by *E. javanicum* showed higher Saccharification activity towards PKC or POME than those from *A. niger* due to high activities of *E. javanicum* CMCase, β-D-mannanase and α-D-galactosidase in hydrolyzing cellulose and hemicellulose compounds. The hydrolysis activity upon PKC was also higher than POME which contains higher fiber and minerals. The cellulose and lignin contents of PKC were 14.2 and 20.5%, respectively, while those of POME were 20.8 and 25.6%. The hemicellulose (mannan and galactomannan) contents of PKC and POME were almost similar, being 5.3 and 5.6%, respectively. The micro-environment of pH influenced more significantly Saccharification activity than the temperature, therefore the synergism activities towards PKC and POME were carried out at pH 5.0 and 5.4 which were the optimum pH for glycosidases and β-D-mannanase, respectively. Both pH conditions were applied at 50°C.

All mixtures of enzymes at pH 5.0 or 5.4 showed the synergistic Saccharification either towards PKC or POME (Figures 3 and 4). The highest synergistic action of the mixture was obtained when higher volumes of *A. niger* (60 or 80%) were used. For example, in the mixture of 80% *A. niger* the hydrolysis upon POME at pH 5.0 produced the reducing sugar at 162 uj/ml (Figure 4A). Without synergistic activity the reducing sugars produced from the 80% of *A. niger* (86.4 uj/ml) and 20% of *E. javanicum* (17.8 uj/ml) would be 104.2 uj/ml. The synergistic activity increased the reducing sugars up to 55%. The better activity in the higher *A. niger* rations indicated that its β-D-glucosidase and β-D-mannosidase play more important role in the hydrolysis system. It was already known that the digestion of disaccharides (cellobiose and mannobiose) by glycosidases reduced the feed back inhibition effects on endoglucanase and endomannanase. It was reported that the highest synergistic action in the culture mixture of *T. reesei* D-16 and *A. went//* Pt 2804 to produce cellulase and hemicellulase was also observed at composition of 1 *(T. reesei): 4 (A. wentii) (Ghoseetal.* 1985).

The optimum pH of *E. javanicum* β-D-mannanase and α-D-galactosidase were at 5.4 and 5.0, respectively, while that *of A. niger* β-D-glucosidase was at 5.0 (Figure 1). Which of the enzyme component had more important role in the synergistic activity was not clearly indicated by varying pH conditions. All pH conditions and substrates produced similar pattern and showed the highest synergistic activity at 60 or 80% *A. niger* application. Moreover, considering the reducing

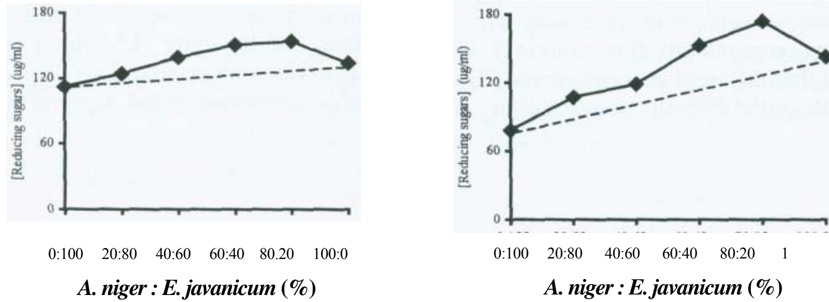
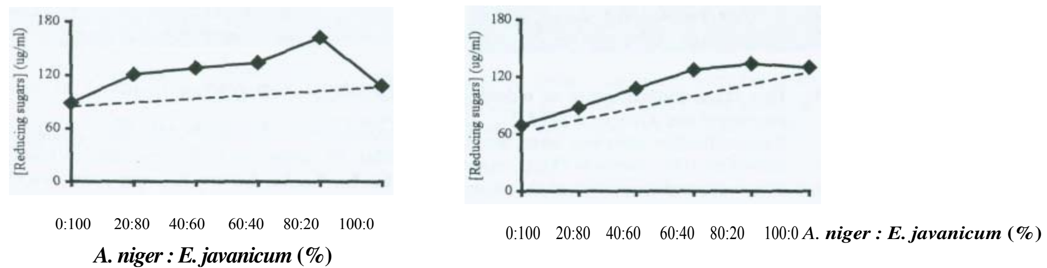


Figure3. Synergistic saccharification towards PKC by enzyme mixtures *of A. niger and E. javanicum* at pH 5.0 (A) and 5.4 (B). The dotted lines represent the theoretical reducing sugar values expected for a non-synergistic saccharification.



Fiaure4. Syncrmstic saccharification towards POME by enzyme mixtures of *A. niger* and *E. javanicum* at pH~5.0 (A) and 5.4 (B). The dotted lines represent the theoretical reducing sugar values expected for a non-synergistic saccharification.

sugar produced by PKC and POME at pH 5.4 by 100% *A. niger* (143 jg/ml and 130 uj/ml, respectively) was higher than that of 100% *E. javanicum* (78 uj/ml and 69 fg/ml, respectively), then the proportion of *A. niger* should be higher than 80% on PKC and 60% on POME (Figures 3 and 4). Although the optimum pH of *A. niger* B-D-glucosidase was at 5.0, only saccharification upon POME showed a better synergy at pH 5.0, while that upon PKC was better at pH 5.4. The obtained result might be due to the high mineral concentration in POME that influenced the activity of each enzyme component differently. The ash and silica contents of PKC were 8.5 and 1.7%, respectively, while those of POME were 17.6 and 9.5%. The high mineral or cation contents in POME might have influenced the buffering capacity of the substrate and affected the pH in the reaction.

The role of every enzyme component in the synergistic action ws difficult to be detected since all enzyme components took part in the reaction. Detailed successive reaction in the synergistic action had been clearly observed in the pure enzyme components (Purwadaria 1995). However, results from TLC plates indicated that different concentrations of sugar components were produced on PKC and POME by the hydrolysis action of the mixtures of *E. javanictan* and *A, niger* (Figure 5).

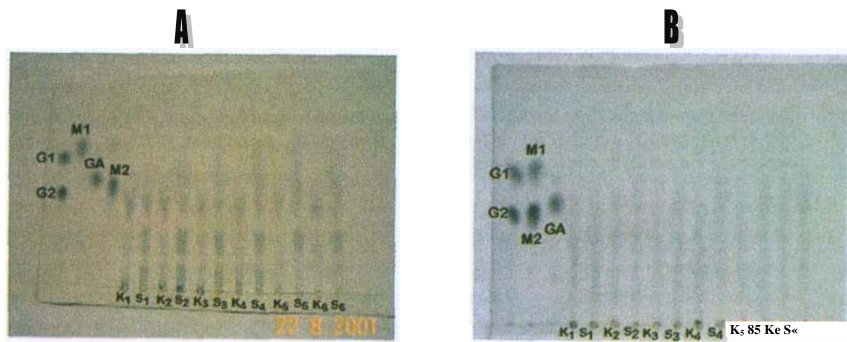


Figure 5. Thin layer chromatogram of reducing sugars produced from synergistic sachanfication of *E. javanicum* and *A. niger* at pH 5.0.
Saccharification activities were determined towards PKC (A) and POME (B). Glucose (Gi), cellobiose *(Gi),* mannose (Mi), mannobiose (M_2), and galactose (GA) were used as reference. The concentration of each standard was 8 \%. KI, Kj, K_3, Kj, K_5, and K,, were controls without incubation, while Si, S_2, S_3, S_4, S_5, and S_6 were samples from the composition of *A. niger : E. javanicum* at 0:100, 20:80,40:60, 60:40, 80:20, and 100:0%.

The chromatogram of PKC digestion was clearer than that of POME due to its higher mineral content which had disturbed the separation (Figure 5). The reducing sugars of controls produced from the mixture of enzymes and substrate without incubation were compared with samples from the mixture of enzymes and substrate with incubation. Smaller amounts of reducing sugars were detected from the controls (substrates) compared to the samples. Oligosaccharides (trimers and more) resulted from endoglucanase or endomannanase activities especially by higher composition of *E. javanicum* in the incubated samples (Figure 5A-S], 82, and 83) were much more than those of controls. The oligosaccharides produced were further digested by glycosidases of *E. javanicum* and the addition of glycosidases from *A. niger* (Figure 5A and B). Addition of 60 or 80% *A. niger* to the enzyme mixtures produced more monomers and dimers including mannose, glucose, galactose, and cellobiose.

The possibility of higher hydrolysis activity resulted from synergistic action will contribute a beneficial effect in the enzyme application on monogastric. The

incorporation of carbohydratases known as inducer enzymes is inhibited by the feed soluble sugar content prepared for energy source. The addition of glycosidases produced by *A. niger* (β-D-glucosidase and β-D-mannosidase) on top of α-D-galactosidase from *E.javanicum* might have increased the digestion of the dimmers such as cellobiose and mannobiose that reduced the inhibition effect of the dimmers (short olygosaccharides) on endoglucanase and endomannanase. Therefore, the application of enzyme cocktails in animal di»ts is considered to be more appropriate than single enzyme.

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