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Carbohydrate and Protein Digestions on Palm Kernal Cake by Mannanase BS4 and Papain Cocktail Enzymes

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ABSTRAK.

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Penggunaan enzim mannanase BS4 pada bungkil inti sawit (BIS) dapat meningkatkan energi metabolis. Pencampuran enzim BS4 dengan protease diharapkan dapat meningkatkan kecernaan protein BIS. Penelitian ini bertujuan untuk menentukan proporsi optimal campuran enzim β-mannanase dan protease getah papaya dalam menghidrolisis bungkil inti sawit (BIS). β-Mannanase diproduksi melalui fermentasi substrat padat (FSP) menggunakan bungkil kelapa sebagai substrat. Enzim papain diekstraksi dari getah pepaya (GP) yang diperoleh dari penyadapan getah pada kulit buah pepaya yang belum matang kemudian dikeringkan pada suhu rendah. Evaluasi pencampuran enzim pada BIS dilakukan pada pH 5,8 dan 40°C yang sesuai dengan kondisi lingkungan pencernaan unggas. Perbandingan volume enzim campuran (β-mannanase BS4: papain-v/v) adalah 100 : 0, 75 : 25, 50 : 50, 25 : 75, dan 0 : 100%. Aktivitas *E. javanicum* BS4 β-mannanase adalah 86 U.ml^{-1} dan papain dari getah pepaya (dari KB, DB, DT, dan GP) masing-masing adalah 7, 3, 1, dan 18.000 U.g^{-1}. Getah pepaya dipilih sebagai protease dalam campuran enzim dan dibandingkan dengan enzim komersial produksi Merck (CMP) sebagai kontrol positif. Hasil menunjukkan bahwa β-Mannanase BS4 mempunyai aktivitas degradasi karbohidrat, dan aktivitas kecernaan protein tidak terdeteksi. Papain menunjukkan aktivitas degradasi protein, dan tidak mempunyai aktivitas degradasi terhadap karbohidrat. Rasio campuran enzim 50 : 50 menunjukkan aktivitas sinergis tertinggi dalam degradasi protein BIS, terjadi sedikit peningkatan pada produksi asam amino sebagai produk hidrolisis protein PKC, namun gula reduksi yang terbentuk jauh lebih rendah daripada campuran enzim 100 : 0 dan 75 : 25, yang produksi gula reduksinya hampir sama. Dapat disimpulkan campuran enzim yang paling optimum adalah pada perbandingan volume mannanase BS4 dengan getah pepaya adalah 75 : 25, atau masing-masing setara dengan perbandingan aktivitas enzim 14 U : 10 U.

Kata Kunci: Mananase BS4, Papain, Campuran Enzim, Bungkil Inti Sawit

ABSTRACT

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Supplementation of the mannanase BS4 enzyme on palm kernel cake (PKC) increased its metabolisable energy (ME), and supplementation with protease is expected to increase its protein digestibility. Therefore, the purpose of this research is to determine the optimum proportion of cocktail enzymes between BS4 β-mannanase (produced by *Eupenicillium javanicum*) and protease (papain) and their degradation activities on carbohydrate and protein of PKC. The β-Mannanase was produced by the mold through solid substrate fermentation (SSF) using coconut meal as the substrate. The papain was extracted from papaya latex (PL), collected by longitudinal incisions on unripe papaya fruit and oven dried overnight. The evaluation of enzyme cocktails for PKC hydrolysis was done at pH 5.8 and 40°C which are similar with poultry intestine condition and both enzymes are still active. The β- mannanase BS4 : papain were mixed with some proportions, i.e.: 100 : 0; 75 : 25; 50 : 50; 25 : 75 and 0 : 100% (by volume) in order to study the optimum cocktail composition ratio. The activities of β-mannanase towards gum locust bean was 86 U.ml^{-1} and papains PL activity was 18,000 U.g^{-1}respectively. PL was chosen for synergistic reaction and compared with a commercial Merck papain (CMP, 20,000 U.g^{-1}) as positive control. β-Mannanase BS4 showed carbohydrate digestion activity, and protein digestion activity was not detected. Papain showed protein digestion activity and no carbohydrate digestion activity. Enzyme cocktails of 50 : 50 from PL protease showed slightly increased in synergistic protein digestion activity in PKC. However, its reduction sugar production was much lower than 100 : 0 and 75 : 25. Amino acids production by enzyme cocktails 75 : 25 were higher than that of 100 : 0. As a result, the best volume composition of β-mannanase BS4 and papaya latex was 75 : 25 (v:v) or 14 : 10 (U:U).

Key Words: Mannanase BS4, Papain, Cocktail-Enzymes, Palm Kernel Cake

INTRODUCTION

World palm oil production dominated by Indonesia and Malaysia wich covered for 80 to 90% of total global palm oil production. In 2014 Indonesia was at the top as oil palm producer with 33 million metric ton and followed by Malaysia (19.8), Thailand (2), Colombia (1.11) and Nigeria (0.93) million metric tons (Index Mundi in 2016). The total area of Indonesia oil palm plantation in 2014 was 8 million hectares and will be growing to 13 million hectares by 2020.

Palm Kernel Cake (PKC) is a by-product of the Palm (*Elaeis guineensis* Jacq.) kernel oil industry. Indonesian PKC production in 2014 was 4550 million MT. Proximate analysis showed that PKC contains crude protein 14-20%, lignin 8-15%, fat 5-11%, crude fiber 13-20% and gross energy 4408 kkal/kg, (Eziezhi et al. 2007; Alimon 2004). In term of the protein content PKC is considered as a good feed ingredient. However, PKC is also high in non- starch polysaccharides (NSP) comprised between 46.6% and 78% (Omar & Hamdan 1998). The high NSP content which is dominated by mannan (78%), cellulose (12%), arabinoxylan (3%), glucuronoxylan (3%) caused the digestibility of PKC poor (Dusterhoft et al. 1991). High content in NSP leads to stickiness in fecal and NSP is also as a trapping matrix for protein and other nutrients. Therefore, the NSP is considered as an anti-nutritional factor in poultry feeding.

In poultry feeding regime, supplementation with exogenous enzymes such as carbohydrase (cellulose), protease and phytase (Ravindran 2013) is a common practice, lately. Enzyme inclusion will minimize the effect of the anti-nutritional factors. Enzyme such as β-Mannanase (endo-1,4-β-mannan mannanohydrolase, EC 3.2.1.78) randomly hydrolyzes β-1,4 mannosidic linkages in mannan, glucomannan, galactomannan and galactoglucomannan into mannose, glucose and galactose (Gilbert et al. 2008). The β- Mannanase BS4 enzyme will degrade mannan link become monosaccharide and resulted in increasing of fiber digestibility and opening the NSP trapping to release the protein. However, the protein digestibility was still considerred low. Supplementation of proteases such as papain will increase protein digestibility. Enzymes cocktail consist of carbohydrase and protease in good proportion will benefit in increasing of nutrient digestibilities of feed ingredients, especially from agricultural by-product such as PKC.

Some enzyme manufacturers have developed enzymes cocktail,. For example, *Novozymes* developed a versatile enzyme cocktail with increased catalytic activity and thermostability by introduction into a *Trichoderma reesei* to produce cellulases mixture, cellobiohydrolase II and beta-glucosidase. Supplementation of the PKC diet with an enzyme complex resulted in a reduction in jejunal contents viscosity (Sundu 2006), improvement in feed conversion in broilers (Chong et al. 2008), and amelioration of the negative effects of feeding a diet containing PKC on the productive performance of laying hens (Soltan 2009).

Utilization of β- mannanase BS4 to increase digestibility of solid heavy phase for poultry diet had been reported (Pasaribu et al. 2009). Solid heavy phase (SHP) is a by-product material of palm oil factory obtained by ceramic filtration from liquid waste. Another report showed that the supplementation of β-mannanase BS4 enzyme to fermented palm kernel cake increased the metabolizable energy of the fermented PKC. Substitution of soy bean meal between 25% and 50% by enzyme-treated fermented PKC did not show any detrimental effect on the performances of laying hens (Sinurat et al. 2014).

The cocktail enzymes of crude β-mannanase from *Eupenecillium javanicum* BS4 and papain of papaya latex was studied and reported here. The objective of this experiment was to determine the optimum proportion of cocktail enzymes between *Eupenicillium javanicum* BS4 β-mannanase and papain and its degradation activity toward carbohydrates and protein of PKC.

MATERIALS AND METHODS

E. javanicum BS4 was IRIAP collection, coconut kernel cake (CKC) was obtained from Indofeed, palm kernel cake (PKC) from Charoen Pokphand. Green and unripe of papaya fruit was obtained from local market. Papain of *Carica papaya* fruit: Papaya peel (PP), flesh (PF), stalks and leaves (PSL) and latex (PL). Mannanase enzyme BS4 production was carried on a solid state fermentation system at tray incubation system with 100 kg capacity.

Mannanase production and papain extraction

β-Mannanase produced on solid substrate fermentation was prepared by addition of of 1.5 dosage Mandels mineral solution (Mandel & Reese 1957) to a sterilized coconut meals, and a 10% of a homogenate culture inoculum. The moisture content of the substrate was adjusted to 60%. Incubation for enzyme production was 7 days at 28°C. Crude enzyme was extracted using 0.05 M acetate buffer pH 5.8 at 1 : 10 ratio and ten times concentrated using $(NH4)_2SO4$ precipitation.

Papains were obtained from papaya peel (PP), flesh (PF), stalks and leaves (PSL) macerated in 0.1M phosphate buffer pH 7 (1 : 1). Juices were filtered with a double cloth to obtain crude enzyme. Saturated ammonium sulfate solution was used to precipitate the crude papain of PP, PF and PSL. The pellets were collected after centrifugation at 4°C, 16980 G, for 10 minutes. The latex of the Carica papaya (PL) was

collected by cutting the skin of the unripe fruit, then dried under 60°C and powdered.

Enzyme activity assay

β-Mannanase activity was determined by using 0.5% Locust Bean Gum (LBG) as the substrate. Reducing sugar was measured by DNS method as described by Miller (1959). Reactions were carried out in the buffer of 0.2 M Na-acetate at pH 5.8 and incubated at 40°C for 30 minutes, otherwise for determination of optimal pH and temperature. pH and temperature were chosed to mimic the gastrointestinal condition of chicken. One unit of β-mannanase activity is defined as the amount of enzyme that hydrolyzed locust bean gum equal into 1 µmol mannose per minute under the assay condition. The activity was calculated after substraction the mannose production from the samples those were from the reactions of substrate and enzymes with controls those were from the same reactions but without incubation.

For synergistic evaluation the saccharification activity was determined on the PKC substrate, its product reducing sugar was determined as glucose with DNS method. Reactions were also prepared at the same pH and temperature as mannanase activity but incubated for 2 hours. One unit of saccharification activity is defined as the amount of enzyme hydrolyzed the carbohydrate of PKC into 1 µmol glucose per minute under the assay condition. Substraction with the control were also calculated.

Protease activity of papain was assayed by the method of Anson (1938) on casein incubated at 0.2 M phosphate buffer at pH 7 and 37°C. The amino acid produced by the protease was stained with Folin Ciocalteau reagent. One protease unit is defined as that quantity of enzyme that liberates the equivalent of 1 µmol of tyrosine per minute under the condition of the assay.

Enzyme evaluation (single and cocktail) was conducted at pH 5.8 and 40°C. Enzyme optimum condition studies were conducted as followed: β-mannanase BS4 activity was determined in a range of pH 4.5-6.2 and temperature of 40-55°C. Papain activity was determined in a range of pH 4.5-7.5 dan temperature of 40-75°C.

Synergistic activity of BS4 and papain

The effect of enzymes in carbohydrate digestibility were tested similar to saccharification activity determination of β-mannanase BS4 and protein digestibility similar to protease activity determination of PL papain activity but on PKC instead of casein. Different ratios of β-mannanase BS4 and papain mixtures were prepared in order to determine the optimum ratios to improve the nutrient digestibilities of PKC as shown in Table 1. pH and temperature condition were determined from the optimal condition where two enzymes would be working and similar with poultry gastrointestinal condition. The data were analysed using descriptive analysis.

RESULTS AND DISCUSSION

Enzymes activities of Papaya Latex (PL) and β-mannanase (BS4) are presented in Table 2. The activity of β-mannanase (BS4) used in this experiment was 86 U/ml and the highest activity of protease was shown in PL papain (18,000 Units/ml). Commercial papain (Merck EC 3.4.22.2) activity was 20,000 Units/g under the same assay condition. Papaya latex protease activity up to 30534 Units had been reported (Nitsawang et al 2006). For further study, PL papain was used to make an enzyme cocktail with BS4 β-mannanase.

Table 1. Ratio of β-mannanase BS4 and papain in enzyme cocktail

Enzyme Ratio (% by volume)	
Mannanase BS4	PL protease
100	0
75	25
50	50
25	75
0	100

Table 2. Protease activity of papaya fruit parts and mannanase activity of BS4*

	Source of Enzyme	
	Activity ($U.ml^{-1}$)	Activity ($U.g^{-1}$)
PP	7	ND
PF	3	ND
PSL	1	ND
PL	ND	18,000
CMP	ND	20,000
BS4	86	ND

*Assay condition: pH 5.8 and 40°C;** Casein as substrate; PL was papaya latex; CMP was commercial papain

The effect of pH and temperature on β-mannanase BS4 activity and for PL-papain were presented in Figure 1 and in Figure 2 respectively. The optimum activity for β-Mannanase was at pH 5.8 and 50°C, and active in the range of pH 4.5-6.2 and temperature of 40-

55°C. Papaya latex papain had an optimum protease activity at pH 6.5 and 70°C and shows activity in the range of pH 4.5-7.5 and 40-75°C. These informations showed that both enzymes will work well in the poultry gastrointestinal condition, even though not reach the optimal activities. The pH of the poultry gastrointestinal condition is laid between pH level of 4.90 Venda (local) chicken and 6.73 of the broiler chickens (Mabelebele et al. 2014). These enzymes were also active in the range of temperature of 40 and 55°C that was closed to the chicken digestive tract temperatures, i.e., 42°C (Dawson & Whittow 2000 *in* Husmaini et al. 2011). The protease optimum temperature and pH activity of PL papain was similar with of commercial papain obtained from Merck (Figure 3), except for the activity in which the commercial papain (20000 $U.g^{-1}$) was higher than the PL papain (18000 $U.g^{-1}$; Table 2). Previous studied reported that papain was active in the range of temperature 60-70°C and pH level of 3 and 9 (Amri & Mamboya 2012). For evaluation the synergistic activity condition reactions were carried out at pH 5.8 and 40°C.

As a common knowledge, enzyme is protein. In designing of enzyme cocktail especially when mixed with protease, the possibility that protease will hydrolyze the companion enzyme should be noted. In this study, both enzymes were subjected for hydrolysis of PKC carbohydrates especially mannan and PKC protein (Figures 3 and 4). It was shown that papaya latex did not digest carbohydrates of the PKC (Figure 3).

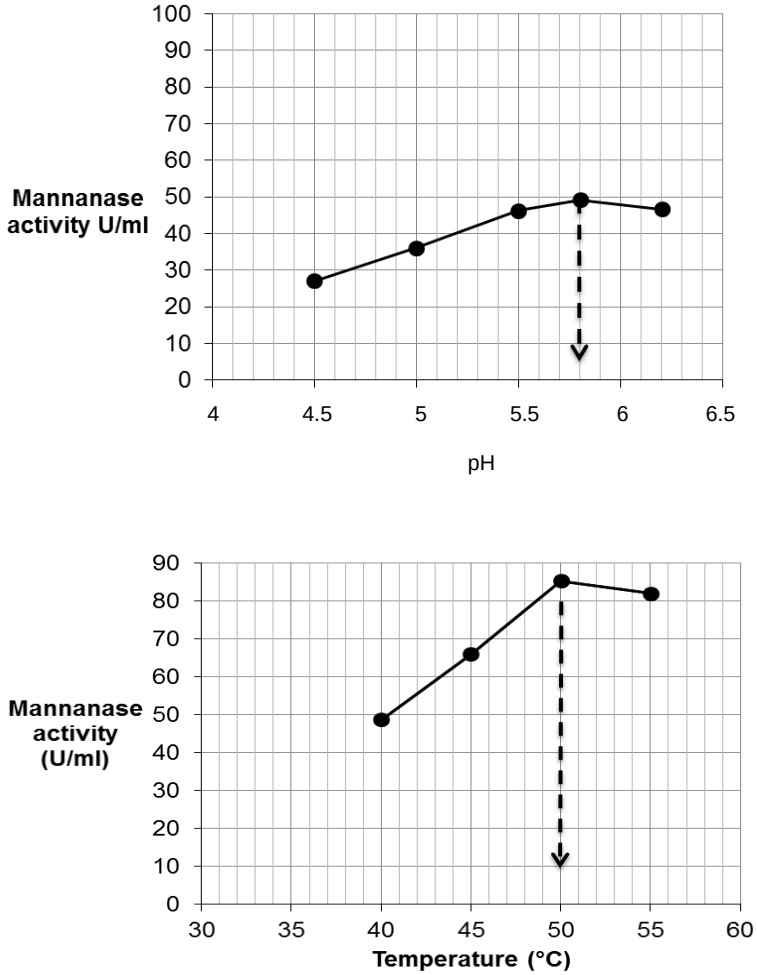

Figure 1. The effect of pH and temperature on β-mannanase BS4 activity

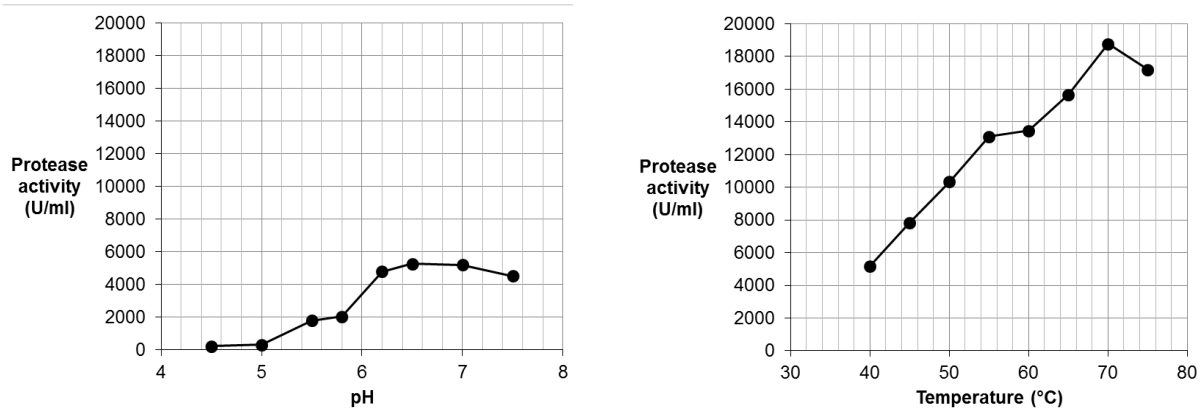

Figure 2. The effect of pH and temperature on protease activity of Papaya latex

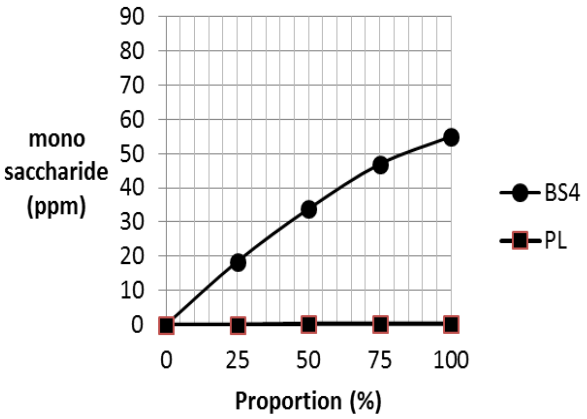

Figure 3. Reducing sugars production from hydrolysis of palm kernel cake by proportion mixtures between β-Mannanase BS4 and papaya latex

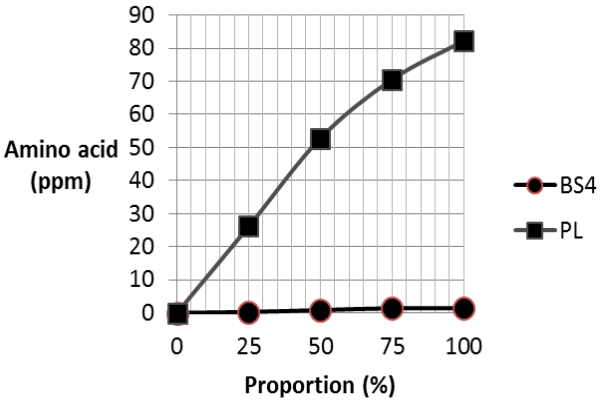

Figure 4. Amino acids production from hydrolysis of palm kernel cake by different proportion mixtures between β-mannanase BS4 and papaya latex

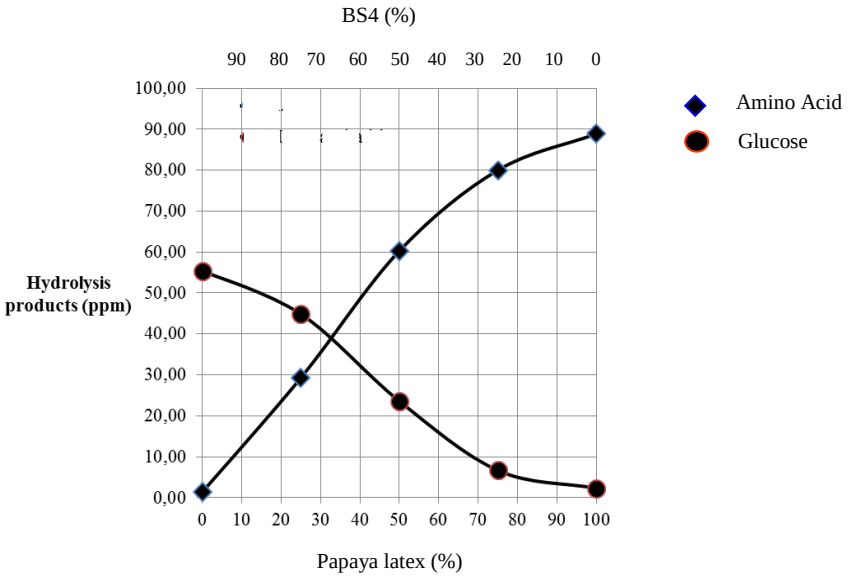

Figure 5. Protease and carbohydrase activities of cocktail enzymes with different proportion of Papaya Latex (PL) and mannanase (BS4) in hydrolyzing palm kernel cake

However, crude β-mannanase BS4 enzyme showed slightly protease activity (Figure 4) and liberated the PKC amino acids in a low concentration, i.e., up to 1.5 ppm.

The β-Mannanase BS4 enzyme showed to degrade the carbohydrates and slightly degraded the protein of the PKC. The Papaya latex degraded the protein but did not degrade the carbohydrates of the PKC. The addition of protease increased the value of protein digestibilities. Supplementation of papain alone increased the digestibility of PKC protein. The protein which is trapped in the PKC fiber fraction would be hard to be attacked by the PL protease since it showed a low saccharification activity. Carbohydrase enzyme disrupted the encapsulating effect of the cell wall and released the structure protein (glycoprotein) in soybean meal had been reported (Meng & Slominski 2005). It was assumed that exogenous carbohydrases and protease increase nutrient digestibility as the effect of disruption in cell wall integrity, production of fermentable disaccharides, low-molecular weight polysaccharides and oligosaccharides will then improving protein solubility and digestion (Cowieson & Ravindran 2008). An improvement of 16% protein digestibility in the corn-soybean based diet due to the effect of multi-carbohydrase enzymes addition was also reported (Cowieson 2010).

The volume ratios of cocktail enzymes of mannase BS4 : papaya latex (50 : 50) (Figure 5), showed the increase of amino acids up to 15% when compared to 50% volume papaya latex alone (Figure 4). The PKC protein was released when mannan fiber was degraded by mannanase BS4. The protein and protein-bound carbohydrates or acid detergent insoluble crude protein (ADICP) are digested. In contrast to the production of amino acids, the reducing sugar production as the result of PKC carbohydrates degradation by enzyme mixture of 50 : 50 ratio decreased up to 42% of β-mannanase BS4 alone (Figure 3). The synergistic effect of β-mannanase BS4 and papaya latex in the proportion of 75 : 25 showed increased production of amino acids up to 8% and reduction sugar up to 25%. The optimum cocktail composition was 14 U of β-mannanase BS4 enzyme and 10 U of papaya latex papain. This ratio showed a good synergistic activity between protease and BS4 carbohydrase in producing reducing sugars and amin acids of palm kernel cake.

CONCLUSION

Optimum activity for β-mannanase at pH 5.8 and 50°C, and active in the range of pH 4.5-6.2 and 40-55°C. Papaya Latex had an optimum protease activity at pH 6.5 and 70°C and of pH 4.5-7.5 and 40-75°C. Both enzymes work well in the poultry gastrointestinal environment, i.e., at pH 5.8 and 40°C. The optimum cocktail composition to digest palm kernel cake was 14 U of β-mannanase BS4 enzyme and 10 U of papaya latex papain protease since it increased both carbohydrates/fiber and the protein digestibilities.

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