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ISOLATION AND LIGNOCELLULOLYTIC ACTIVITIES OF FIBER-DIGESTING BACTERIA FROM DIGESTIVE TRACT OF TERMITE (*Cryptothermes sp.*)

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ABSTRAK

Tujuan penelitian ini adalah untuk memperoleh isolat mikroba pencerna serat dari saluran pencernaan rayap dan mengetahui kondisi optimum pertumbuhan dan produksi enzim selulase, xilanase dan ligninase dari isolat. Penelitian I adalah penelitian deskriptif untuk mengisolasi dan menyeleksi bakteri pencerna serat dari saluran pencernaan rayap berdasarkan aktivitas selulolitik (S), xilanolitik (X) dan lignolitik (L) yang tinggi. Penelitian II adalah optimalisasi kondisi pertumbuhan isolat dan produksi enzim akibat pengaruh aras substrat jerami padi dan nitrogen. Bahan yang digunakan adalah rayap (*Cryptothermes sp.*), jerami padi dan medium kultur. Rancangan penelitian yang digunakan pada penelitian II adalah rancangan acak lengkap pola faktorial. Faktor I adalah aras substrat jerami padi (1, 2 dan 3% W/V), dan faktor II berupa aras nitrogen (0,1; 0,2 dan 0,3 % W/V). Variabel yang diukur adalah aktivitas enzim selulase, xilanase dan ligninase. Hasil penelitian tahap I menunjukkan bahwa isolat yang diperoleh terdiri dari 3 jenis bakteri selulolitik (S_1, S_2, dan S_3), 3 jenis bakteri xilanolitik (X_1, X_2, dan X_3) dan 3 jenis bakteri lignolitik (L_1, L_2, dan L_3). Hasil penelitian tahap II menunjukkan bahwa isolat S_2, X_3, dan L_1 mempunyai aktivitas tertinggi dibandinkan dengan yang lain, yaitu 1,894 U/mL, 1,722 U/mL dan 0,314 U/mL. Kesimpulan penelitian adalah penambahan aras jerami padi 1% dan nitrogen 0,3% menunjukan aktivitas enzim tertinggi baik pada selulase, xilanase maupun ligninase.

Kata kunci: isolasi, bakteri pencerna serat, rayap, aktivitas enzim

ABSTRACT

The objectives of this study were to obtain the fiber-digesting bacteria isolates from termite digestive tract and to determine the optimum conditions of growth and production of cellulase, xylanase and ligninase enzyme of isolate. The first study was conducted to isolate and select the fiber-digesting bacteria from the digestive tract of termites based on the highest activity of cellulolytic (S), xylanolytic (X) and lignolytic (L). The second study was optimation of the growth conditions of bacteria and the enzyme production due to effect of rice straw substrate and nitrogen. The material used were dry wood termites, rice straw, and culture medium. The design used was a completely randomized factorial design, in which the first factor was rice straw substrate (1, 2, and 3% W/V), while the second factor was nitrogen (0.1, 0.2 and 0.3% W/V). Variables measured were cellulase, xylanase and ligninase activities. Results of the first sudy showed that the isolates obtained consisted of 3 types, those were cellulolytic bacteria (S_1, S_2, and S_3), 3 types of bacteria xylanolytic (X_1, X_2, and X_3) and 3 types of bacteria lignolytic (L_1, L_2, and L_3). Meanwhile, results of the second study showed that isolates of S_2, X_3, and L_1 had the highest activity, those were 1.894 U/mL, 1.722 U/mL and 0.314 U/mL, respectively. In conclusion, the

addition of 1% level of rice straw substrate and 0.3% of nitrogen showed the highest enzyme activity on cellulase, xylanase and ligninase.
Keywords : isolation, fiber-digesting bacteria, termite, enzyme activity

INTRODUCTION

Agricultural waste, such as straw is a waste that was the most given to ruminants. Utilization rice straws as animal feed have weaknesses, because the rice straw has high cellulose content and the protein of its is low. In the termite digestive tract contain cellulolytic, lignolytic and xylanolitic microbes. The microbes release some enzymes which has an important role to digest the rice straw.

Termite is one of the animals which in its digestive tract contains aerobe and anaerobe microbes (Wenzel *et al*., 2002). Cellulase enzyme from termites microbes showed a high ability to degrade fiber or cellulose (Watanabe *et al*., 1998). Uhi *et al*. (2001) reported that supplementation of 1.5% termite *Glyptotermes Montanus* in the chicken feed performed the lowest feed conversion ratio (FCR) and the gain of chicken was the highest . Researches on the ability of fiber-digesting enzymes of termites have been conducted, but up to now, in Indonesia the researches on the isolation of fiber-digesting microbes in the digestive tract of termites are lacking.

Fermentation is one of the efforts in improving the quality of ruminant feed ingredients. Fermentation is defined as a process to convert the complex compounds into simple compound which has a high economic value through the service of microbes (Stanbury *et al*., 2003). The fermentation process has many advantages such as no negative effects, easy to do, relatively not require specialized equipment and relatively low cost. The fermentation process is done by adding microbes or fungi as a starter. A starter is chosen which has an ability to be cultured and easy to be obtained.

The purposes of the study were to obtain fiber-digesting microbes isolated from the digestive tract of termites, to test enzyme activities having cellulase, xylanase and ligninase activities and to determine the effect of substrate and nitrogen levels on enzymes activity. The invent of the study was the preparation techniques to study microbial isolation accomplished by washing termites first with 70% alcohol to sterilize microbes outside the body of termites. The optimum conditions of growth and production of cellulase enzymes included xylanase and ligninase of selected microbes also studied to determine the effect of different levels of nitrogen substrate and medium, and long incubation time. The results of the selection of superior fiber-digesting microbes were expected to be used to perform the processing of fibrous feedstuffs in order to increase quality.

MATERIALS AND METHODS

The experiment was conducted approximately 6 months in Feed Technology Laboratory, Faculty of Animal Science and Agriculture, Diponegoro University and in Microbiogenetic Laboratory, Faculty of Mathematics and Natural Sciences, Diponegoro University. The material used was a dry wood termite (*Cryptotermes sp.*), and rice straw. Chemicals used in the isolation and selection process included alcohol 90%, 0.85% physiological saline, culture medium according to Eutick *et al*. (1978) in 1 liter reagent consisted of 1 g K_2HPO_4, 1 g $MgSO_4$ .7 H_2O, 2 g of $(NH_4)2SO_4$, 0.5 g KCl, 0.02 g CaC_2 (anhydrous), 0.01 g of $FeSO_4$ .7H_2O, 0.001 g $MnSO_4$. 4H_2O) and carbon source 0.5 to 1% W/V in the form of powder, agar, 1% HCl, 0.25 M NaOH, urea, and distilled water. The activity test of cellulase and xylanase enzymes was done according to the 3,5-dinitrosalicylate (DNS) method (Sinegani and Emtizi, 2006) and ligninase according to modified method of Kawakami and Samingan cited by Martani *et al*. (2003). Some of the equipments used in the implementation of the first phase of the study included erlenmeyer, scale cups, petri dish, ose, micro pipette (1 mL), 5 mL pipette, 10 ml pipette, beaker, test tubes, bunsen, incubators, autoclaves and microscopes

Experiment I
Isolation of Microbes

Isolation and selection of isolate and select the fiber-digesting bacteria were conducted in the first experiment descriptively. Isolation of microbes was begun by washing the 10-15 termites (*Cryptotermes sp*.) with alcohol 70% to clean. Furthermore, the head of termite was

removed. The stomach of termites was crushed, then the stomach was suspended at 10 mL of physiological NaCl 0.85%. The suspension of stomach was taken and incubated on 10 days in sterile selective liquid medium containing 1 % of rice straw (Eutick *et al*., 1978) at neutral pH(7). Inoculum was then inoculated into selective solid culture medium (agar) substrates containing cellulose, xylan and lignin, then was incubated aerobically at room temperature for 5 x 24 hours in a petri dish at neutral pH (Wenzel *et al*., 2002). Bacterial colonies growing on each substrate (cellulose, xylan and lignin) were purified, and then the enzyme activities (cellulase, xylanase and ligninase) were tested. The kind of fiber-digesting bacteria was obtained from this test, those were S, X and L for cellulolytic, xylanolytic and lignolytic, respectively.

Microbe Selection Based on Enzyme Activity

Selection was done by growing of each colony of bacteria having cellulolytic, xilanolitic and lignolitic properties in a liquid medium with 1% of rice straw substrate in aerobic and neutral pH for 5, 10 and 15 days. Testing activity of the enzyme (cellulase, xylanase and ligninase) was performed at each period of incubation (5, 10 and 15 days). Extracellular enzyme fractions were separated by centrifugation of 5,000 rpm for 20 min. Cellulase enzyme activity assay was performed by measuring product reducing sugar (glucose), whereas xylanase enzyme activity assay was performed by measuring the xylose product. For ligninase enzyme activity assay was performed by measuring the vanillin products resulting from the degradation of lignin.

Reducing sugar measurements were performed by the DNS method (Sinegani and Emtiazi, 2006). Reducing sugar was measured by mixing the 0.5 mL of filtrate and 0.5 mL of enzyme substrate suspension (carboxymethyl-cellulose or xylan at 1% levels). The mixture was then incubated for 30 min at 50^{o}C. After 30 minutes, the mixture was added with 1 mL of DNS reagent and heated for 5 minutes at a temperature of 100°C, then cooled at room temperature. Furthermore, this was added with 8 mL of 0.5 M citrate buffer pH 4.8 in the mix and read on a spectrophotometer at a wavelength of 540 nm to 560 nm for the xylose and glucose, then converted to a standard curve.

Ligninase activity was measured based on vanillin products formed in the process of lignin degradation (Martani *et al*., 2003). Crude enzyme extract was obtained by inoculating bacterial culture as much as 10% in the medium (V/ V), in which at a temperature of approximately 30°C was incubated for 3 days (3 x 24 hours). At the end of incubation cultures, the medium consisting bacteria were centrifuged at a speed of 5,000 rpm for 15 minutes. The supernatant containing crude enzyme extract (enzyme ligninase) was used as inoculum for lignin degradation in acetate buffer solution (pH 7) using alkali lignin.

Vanillin concentration measurements was performed by mixing 0.5 mL of the filtrate with 0.1 g substrate enzyme lignin in 0.5 mL of 50 mM acetate buffer at pH 5.5. The mixture was then incubated for 60 min at 30 °C, then was centrifuged at 5,000 rpm. The filtrate was mixed with 4 mL of methanol. The absorbance was read on a spectrophotometer at a wavelength of 335 nm. The results were calculated with a standard curve (Samingan, 1998; Hayani and Fatima, 2002).

The enzyme activity was internationally considered as activity units (IU = international units). One unit of enzyme activity (1 IU) was defined as the amount of enzyme which can convert 1 micro mol (10-6 mol) substrate for 1 min at the optimal state (Nelson and Cox, 2000). Calculation of enzyme activity was in units/mL substrate/min following formula:

Enzyme activity = [(mg (reducing sugar x 1000 x 20]/[(moleculr weight) (reducing sugar) x 30)] = Unit/ ml substrat/ minute = U/ mL

After the enzyme activities were tested, one of each type of bacteria (cellulolytic, xilanolytic and lignolytic) having the highest enzyme activity was selected and taken.

Experiment II

Experiemnt II was conducted to isolate and select the fiber-digesting bed on the highest cellulolytic, xylanolytic and lignolytic activity. The material used was a fiber-digesting microbe (cellulolytic) resulted from experiment I. The activities were carried out by treatment incubation differing levels of substrate (1, 2 and 3% W/V) and nitrogen levels (0.1, 0.2 and 0.3% W/V) of the medium, and long incubation for 5, 10 or 15 days (depending on the incubation time at experiment II).

Microbia was harvested in each treatment

(difference of substrate level and nitrogen levels), then the cellular extra enzyme fractions were separated by centrifugation 5,000 rpm for 20 min. Enzyme activity of cellulase, xylanase and ligninnase and were measured, then enzyme showing highest activity was selected.

Experimental Design and Statistical Analysis

The experimental design used was 3 x 3 of completely randomized factorial design with 3 replication. The first factor was rice straw substrates (1, 2, and 3% W / V), second factor was nitrogen levels (0.1, 0.2 and 0.3% W / V) . Measured variables was the enzyme activities of cellulase, xylanase and ligninase. The data were analyzed by using analysis of variance, then was followed by Duncan's multiple range test when obtained the difference among treatments (Steel and Torrie, 1991).

RESULTS AND DISCUSSION

Isolation and Selection of Fiber Digest Bacteria

The results of fiber-digesting bacteria isolation from the digestive tract of termites are presented in Table 1. The growingof fiber-digesting bacteria growth on selective media (the substrate of cellulose, xylan and lignin) showed a good growth.

Most of the bacteria isolates (Tabel 1.) had white, shiny, and convex circular. Most of them had nature of gram-positive, only one showed a negative gram (isolate S_3). According to Matteoti *et al*. (2012), most of the fiber-digesting bacteria isolated from the gut of termites showed gram-positive, particularly the nature xilanolitic. (Anthony and Hill, 1988) stated that gram-positive bacteria have the property to absorb (maintain) the color violet in gram staining process, have a peptidoglycan cell wall thicker and more sensitive to iodine than gram-negative bacteria. The selection of bacterial isolates are presented in Figure 1, Figure 2 and Figure 3.

To determine the pattern of growth, primarily logarithmic phase, the isolates were grown in liquid medium and observed every 4 hours. Every 4 hours, each of culture isolates was taken and the growth was measured with a spectrophotometer at a wavelength of 620 nm. The growth of cellulolytic, xylanolytic and lignolytic bacteria from termite digestive tract are presented in Table 2. As shown in Table 2, the most of cells number occurred in the growth phase for 16 hours, meaning that at the time of the case or the logarithmic phase of growth accelerated resulting in the most amount of cells compared to the other time periods. Tampoebolon (1997) found that the fiber-digesting microbes logarithmic phase (cellulolytic) from buffalo rumen would be occurs in 12-15 hours after incubation using the substrate glucose 1%. This study was in agreement to Kurniawati (1999) in

Table 1. The Results of Isolation and Morphology Observed and Gram Color Microbes in Termite Digestive Tract

Isolate	Colony Characteristic	Form	Gram	Substrate
S_1	White milk, shinny, circular, entire, convex	oval	(+)	Cellulose
S_2	Light blue, shinny, circular, entire, convex	oval	(+)	Cellulose
S_3	White milk, shinny, large colony, entire	oval	(-)	Cellulose
X_1	White, shinny, irregular, convex, entire	oval	(+)	Xylan
X_2	Yellow with circular white milk, circular, entire	rod	(+)	Xylan
X_3	Dark white, low convex, entire	oval	(+)	Xylan
L_1	White milk, shinny, convex, circular, entire	rod	(+)	Lignin
L_2	Dark white, convex, entire	rod	(+)	Lignin
L_3	Dark white, shinny, large colony, entire, circular	rod	(+)	Lignin

S = cellulolytic bacteria isolate; X = xylanolytic bacteria isolate, and L = lignolytic bacteria isolate

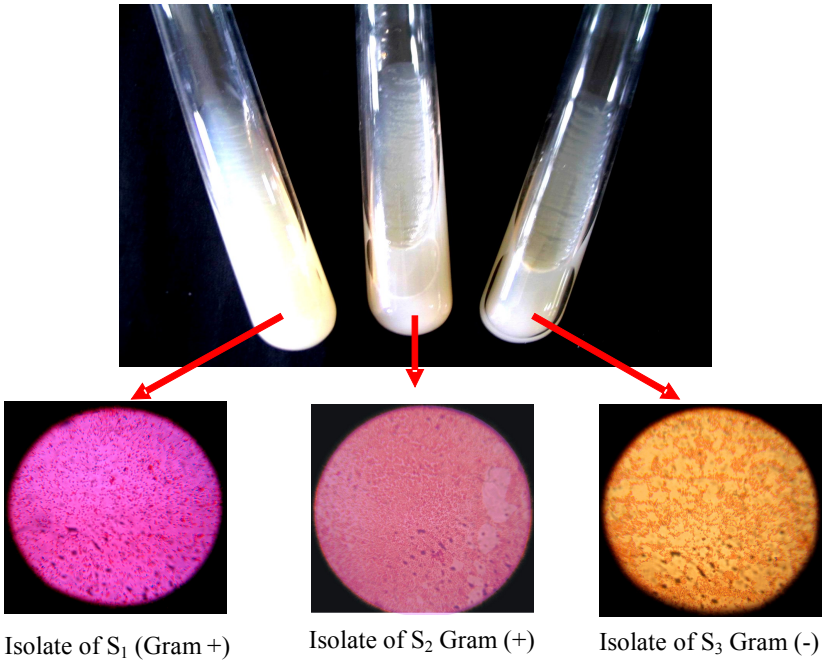

Figure 1. Cellulose Bacteria Isolate of S_1, S_2 and S_3 (Enlarge 400x)

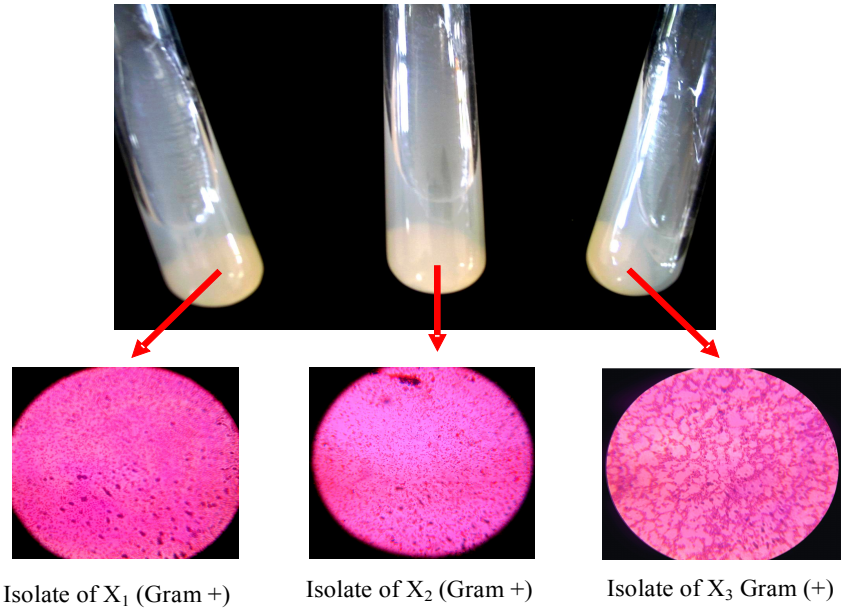

Figure 2. Xylanolitic Bacteria Isolate of X_1, X_2 and X_3 (Enlarge 400x

which the buffalo rumen cellulolytic microbes also showed logarithmic phase at relatively the same time (14-16 hours).

Isolat Selection Based on Enzyme Activity Test

Average of enzyme activity of fiber digesting bacteria isolated from termites is presented in Table 3. The ellulolytic bacteria isolates (Code S) at the time of incubation of 5, 10 and 15 days indicated that S_2 isolates had the highest activity compared to other cellulolytic bacteria isolates. The best incubation time occured at 10 days

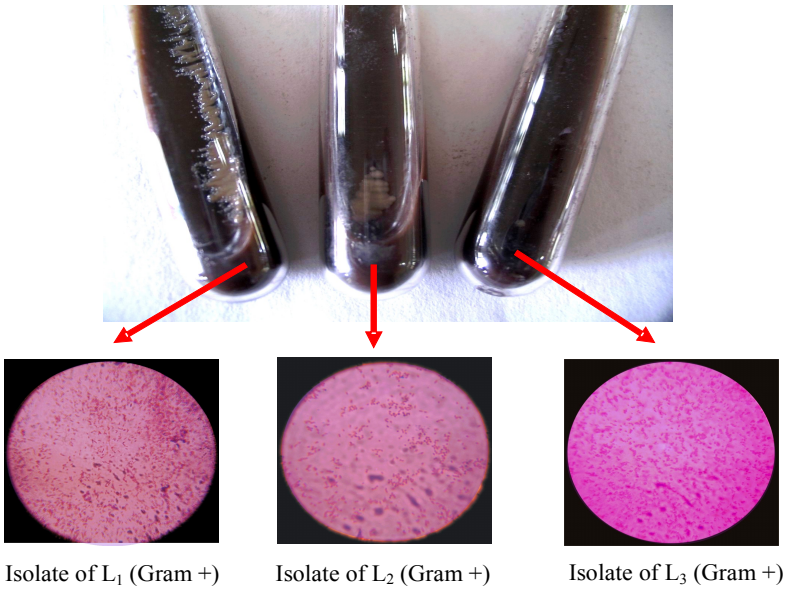

Figure 3. Lignolitic Bacteria Isolate of L_1, L_2 and L_3 (Enlarge 400x)

Table 2. Growth of Cellulolytic, Xylanolytic and Lignolytic Bacteria from Termite Digestive Tract on Liquid Medium (Absorbance Value on λ = 620 nm)

Isolate	Incubation Length at Room Temperature (hour)						
	0	4	8	12	16	20	24
S_1	0.0073	0.2367	0.5757	0.9076	0.9256	0.7862	0.8724
S_2	0.0029	0.4173	0.6852	0.9092	0.8995	0.7753	0.7092
S_3	0.0040	0.2608	0.3738	0.6919	0.8885	0.8453	0.8011
Average	0.0052	0.3043	0.5929	0.8362	0.9045	0.8023	0.7942
X_1	0.0058	0.1419	0.3738	0.4861	0.7816	0.5040	0.5137
X_2	0.0088	0.1011	0.2842	0.4206	0.4607	0.4561	0.4209
X_3	0.0132	0.1174	0.2520	0.4174	0.6319	0.4279	0.4319
Average	0.0093	0.1201	0.3033	0.4414	0.6247	0.4627	0.4555
L_1	0.0193	0.1270	0.3013	0.4862	0.4861	0.4953	0.3904
L_2	0.0117	0.1468	0.3165	0.3135	0.5137	0.3135	0.2189
L_3	0.0117	0.1570	0.3949	0.4281	0.5230	0.3184	0.3235
Average	0.0142	0.1436	0.3376	0.4093	0.5076	0.3757	0.3109

S = cellulolytic bacteria isolate, X = xylanolytic bacteria isolate, and L = Lignolytic bacteria isolate

incubation time. According to Inoue *et al*. (1997) and König *et al*. (2013), the optimum degradation time of cellulose and xylan on cellulolitic and xylanolitic microbes occurred on day 11-16 after incubation.

Xylanase enzyme activity of bacterial

isolates xilanolitic X_1, X_2 and X_3 showed the same trend in terms of the best of incubation time, i.e. 10 days. The X_3 isolates showed the highest activity than other isolate. Best ligninase enzyme activity occurred on incubation time of 10 days. In this incubation time, L_1 isolates showed the highest lignilase activity. It was stated by Chen *et al*. (2008), microbial cellulase activity of the digestive tract of termites was higher than the average of rumen microbial activity of cellulase (0.4 U/mL), whereas xylanase activity was significantly lower than rumen microbial xylanase activity (17.23 U/mL). Ligninase activity in rumen microbes was not detected because of very low number. The selection of fiber-digesting bacteria was quantitatively done, those were for isolate cellulolytic bacteria (S_2), for bacterial isolates xilanolitic (X_3) and whereas for bacterial isolates lignolitic (L_1) as presented in Table 3.

Production and Cellulase Enzyme Activity of S_2 Isolate

The results of the calculation of enzyme cellulase activity in the combined treatment of nitrogen levels and different substrates are presented in Table 4. Activity of cellulase enzymes had value ranged from 0.041 to 2.852 U/mL, with an average total was 1.463 U/mL.

There was no interaction effect of combination in differing levels between substrate and nitrogen on the specific activity of the cellulase nzyme. It means that the combined treatment of the addition of substrate levels and nitrogen together did not affect specific activity of cellulase. However, each treatment (difference in substrate levels and nitrogen) gave significant effect ($P<0.05$) to activity of cellulase.

The higher level of nitrogen up to 0.3%, significantly ($P<0.05$) increase the activity of the cellulase enzyme. The highest activity was occurred on the N_3 treatment. This happens because the more sources of nitrogen in the fermentation medium; it would increase the production of cellulase enzymes. Nitrogen was needed to synthesize protein, so that the presence of N would support the enzyme synthesis. Wijanarka (1998) noted tht nitrogen added up to 0.5% was more effectively increased the production and activity of the enzyme, while the addition of the source of N to 0.75% increase the cell biomass from the production of enzymes.

The addition of up to 1% of substrate level could increase the activity of cellulase (1.769 U/mL). In increasing levels of substrate up to 3%, the activity of celluase could decrease. Each enzyme has a Km (Michaelis-Menten coefficient) specific value and Km showed a certain substrate concentration causing the enzyme activity to a maximum number. Therefore, if the substrate concentration close to Km value, the enzyme

Table 3. Average of Fiber Digest Bacteria Activity Isolated from Termite Digestive Tract (U/mL)

Isolate	Incubation trial (days)		
	5	10	15
S_1	0.751	0.881	0.735
S_2	1.911	2.209	1.562
S_3	0.378	0.447	0.367
Average	1.013	1.179	0.888
X_1	0.308	1.977	1.920
X_2	0.248	1.803	1.790
X_3	0.706	2.469	1.990
Average	0.421	2.083	1.900
L_1	0.172	0.390	0.380
L_2	0.147	0.248	0.196
L_3	0.159	0.308	0.287
Average	0.159	0.315	0.288

Table 4. Effect of Substrate Concentration and Nitrogen Level on Cellulase Production of S_2 Isolate (Enzyme Activity on U/mL)

Concentration of Substrate (%)	Level of Nitrogen (%)			Average
	0.100	0.200	0.300	
0	0.041	0.044	0.060	0.048^{c}
1	1.831	2.286	2.852	2.323^{a}
2	1.551	1.700	1.880	1.710^{b}
3	1.615	1.787	1.905	1.769^{b}
Average	1.260^{c}	1.454^{b}	1.674^{a}	

^{a,b,c} Difference superscript on same column and raw indicate significant differences ($P<0.05$)

activity will be maximum. Substrate level of 1% was used in the study close to the Km value of cellulase enzyme, compared to levels of other substrates. Tampoebolon (1997) and Kurniawati (1999) noted that the calculation results of Km values cellulase enzyme activity and ß-glucosidase were 1.0309% and 0.8%, respectively. The substrate of 1% level was the best level to the average activity of the enzyme. According to Nelson and Cox (2000), Km was defined as the substrate concentration because half the speed of enzyme activity reached its maximum speed.

Production and Xylanase Enzyme Activity of X3 Isolate

The results of the calculation of xylanase enzyme activity levels in the combined treatment of different nitrogen substrates is presented in Table 5. In general, the higher level of nitrogen up to 0.3% could increase the enzyme activity, but the higher level of substrate up to 3% would decrease the enzyme activity.

Xylanases activity were ranged from 0.046 to 2.825 U/mL, with an 1.817 U/mL on average. This activity value was lower than the research of Shimizu *et al*. (1998), who isolated *Bacillus sp*. from the digestive tract of termites with a value of 3.6 IU enzyme activity. There was no interaction effect of combined treatment in differing levels between substrate and nitrogen on the specific activity of the enzyme xylanase. This means that the combined treatment of the addition of substrate levels and nitrogen together did not affect each specific xylanase activity. However, each treatment (difference in substrate levels and nitrogen) performed significant effect ($P<0.05$) of the xylanase enzyme activity.

The higher level of nitrogen up to 0.3% significanly ($P<0.05$) increased the activity of xylanase enzyme. The highest xylanase activity occurred in the N_3 treatment (nitrogen level of 0.3%), the xylanase activity was 2.040 U/mL on average, followed by N_2 (1.828 U/mL) and N_1 (1.583 U/mL). This could happen because the more sources of nitrogen in the fermentation medium that it would increase the production of xylanase enzyme.

The increased levels of nitrogen up to 0.3%, would support a smooth synthesis of the enzyme protein. Wijanarka (1998) stated the addition of up to 0.5% of nitrogen source more effectively increase the production and activity of the enzyme, while the addition of the source of N to 0.75% increased the cell biomass from the production of the enzyme.

The addition of substrate level up to 1% also increased the xylanase activity (2.482 U/mL), but increasing levels of substrate to 3% would decrease the xylanase activity. This could happen that because each enzyme has a specific Km (Michaelis-Menten coefficient). Km showed a certain substrate concentration causing the enzyme activity to a maximum. Therefore, at a certain substrate concentration, the bacteria would have the best growth and also have the most optimum enzyme activity. The Km value of this enzyme closed with standard compared to the value to other substrate levels. Tampoebolon (1997) reported that the level of substrate 1% in the culture medium of buffalo rumen microbial cellulolytic resulted in the highest of enzyme

Table 5. Effect of Substrate Concentration and Nitrogen Level on Xylanase Production of X_3 Isolate (Enzyme Activity on U/mL)

Concentration of Substrate (%)	Level of nitrogen (%)			Average
	0.10	0.20	0.30	
0	0.046	0.053	0.065	0.055^{b}
1	2.163	2.458	2.825	2.482^{a}
2	2.017	2.454	2.618	2.363^{a}
3	2.105	2.345	2.653	2.368^{a}
Average	1.583^{c}	1.828^{b}	2.040^{a}	

^{a,b,c} Difference superscript on same column and or raw indicate significant differences ($P<0.05$)

cellulase activity (Km = 1.0309%). According to Nelson and Cox (2000), Km was defined as a certain substrate concentration causing the speed of enzyme activity reached half of its maximum speed.

Production and Ligninase Enzyme Activity of Isolate L_1

Enzyme activity of ligninase ranged from 0.019 to 0.462 U/mL. The average of ligninase activity was 0.274 U/mL (Table 6).

Martani *et al*. (2003) showed the lignolitic bacteria isolate selected from various natural substrates had average enzyme activity of 0.5 U/mL. The ligninase activity of bacteria isolates from gastro intestinal tract of termite was lower. This could happen due to differences in species or strains of lignolityc bacteria. In general, the higher level of nitrogen up to 0.3% and substrate up to 2% could increase the enzyme activity, but the higher the level of substrate up to 3% would decrease the enzyme activity. The results of the calculation of variance showed that there was no interaction effect of combined treatment of substrates differing levels and levels of nitrogen on the ligninase enzyme activity. This means that the combined treatment of the addition of substrate levels and nitrogen levels together did not affect of ligninase enzyme activity. However, each treatment (difference in substrate levels and nitrogen) performed significant effect ($P<0.05$) on ligninase activity.

Average enzyme activity in treatment N_3 (nitrogen level of 0.3%) was significantly ($P<0.05$) higher than N_2 and N_1 treatments (0.335 *vs* 0.275 and 0.248 U/mL, respectively), but between the N_1 and N_2 treatments there was no different. This could happen because the more sources of nitrogen in the fermentation medium, it would increase the production of ligninase. Nitrogen was a source of N to synthesize a protein (enzyme), so that the presence of N would support the smooth running of the enzyme protein synthesis. Wijanarka (1998) showed the addition up to 0.5% nitrogen source effectively would increase the production and activity of the enzyme, while the addition of source of N up to 0.75% would increase the cell biomass compared to the production of the enzyme.

The addition of substrate level up to 2% increased the activity of ligninase enzyme, but in increasing levels of substrate up to 3%, the ligninase activity enzyme would decrease. Treatment of S_3 (substrate of 2%) was significantly ($P<0.05$) higher than that in S_4, S_2 and S_1 (0.394 *vs* 0.349; 0.373 and 0.0272 U/mL). The enzyme activity of S_4 treatment was significantly ($P<0.05$) higher than the S_1 (0.0272 versus 0.349 U/mL), but the S_2 treatment (0.373 U/mL) did not differ from S_4.

It was stated by Schumm (19930, coefficient of Michaelis-Menten (Km) was the specific substrate concentration showing the activity of certain enzymes into the maximum. Substrate level at 2% resulted in value close to the value of Km of the enzyme ligninase compared to levels of other substrates. Therefore, at the substrate of 2% the ligninase activity had optimum enzyme activity speed.

CONCLUSION

There were 3 kinds of cellulolytic bacterial isolates (S_1, S_2, and S_3), 3 kinds of xilanolitic

Table 6. Effect of Substrate Concentration and Nitrogen Level on Ligninase Production of L_1 Isolate (Enzyme Activity on U/mL)

Concentration of Substrate (%)	Level of Nitrogen (%)			Average
	0.10	0.20	0.30	
0	0.019	0.027	0.035	0.027^{c}
1	0.305	0.363	0.423	0.373^{ab}
2	0.342	0.378	0.462	0.394^{a}
3	0.314	0.331	0.446	0.349^{b}
Average	0.248^{b}	0.275^{b}	0.335^{a}	

^{a,b,c} Difference superscript on same column and or raw indicate significant differences ($P<0.05$)

bacteria (X_1, X_2, and X_3) and 3 kinds of lignolitic bacteria (L_1, L_2, dan L_3) obtained. The addition of 1% level of rice straw substrate and 0.3% nitrogen showed the highest enzyme activity on cellulase, xylanase and ligninase. Isolates of S_2, X_3 and L_1 showed the highest activity.

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