Cellulase-free Xylanase: Production by *Thermomyces lanuginosus* TISTR3465 in solid state fermentation and efficiency on bioleaching

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Abstract

The mixed substrate (12g) consisting of NaOH-treated corncob, wheat bran and rice husk at ratios between 5:5:2 to 4:6:2 were optimal ratios for xylanase production 250 ml Erlemeyer flask by *Thermomyces lanuginosus* TISTR3465 in static solid state cultivation at 70% initial moisture content at 50 °C. Addition of 0.12-0.75 g wood xylan to the substrates enhanced the enzyme production 1.30-1.58 folds. Addition of nitrogen sources to the mixed substrates did not increase the enzyme production indicating that wheat bran served as a sufficient nitrogen source. A temperature of 45-50 °C with 70% initial moisture content of the mixed substrates was the optimal conditions for the enzyme production by this strain. Aeration in a static tray solid-state culture was remarkably required for the enzyme production by this strain at higher thickness of substrate beds. Maximum xylanase activity, 1764 U/g dry solid, was obtained at a substrate thickness of 1 cm with an aeration rate of 0.24 l/g/h after cultivation for 5 days. The xylanase from *T. lanuginosus* TISTR3465 was found to be effective in release of reducing sugars from eucalyptus pulp. Treatment of pulp with β-xylanase (220 U/g dry weight pulp) followed with 2% hydrogen peroxide significantly decreased in kappa number (5.7 U) and improved brightness up to 45.2%

Keywords: *Thermomyces lanuginosus*, solid state fermentation, β-xylanases, corncob, biobleaching

บทคัดย่อ

วัสดุหมักผสม (12 กรัม) ซึ่งประกอบด้วยซังข้าวโพดที่ผ่านการแช่ด่าง รำข้าวสาลี และแกลบ ในอัตราส่วนระหว่าง 5:5:2-4:6:2 เป็นอัตราส่วนที่เหมาะสมสำหรับผลิตเอนไซม์ไซลาเนสโดยเชื้อ *Thermomyces lanuginosus* TISTR3465 ในการเพาะเลี้ยงแบบแห้งที่นิ่งในฟลาสก์ขนาด 250 มิลลิลิตร ที่มีปริมาณความชื้นเริ่มต้นที่ 70% ที่อุณหภูมิ 50 องศาเซลเซียส การเติมไซแลนจากไม้ปริมาณ 0.12 - 0.75 กรัม ลงในวัสดุหมักมีผลทำให้การผลิตเอนไซม์เพิ่มขึ้น 1.30 - 1.58 เท่า การเติมแหล่งไนโตรเจนในวัสดุหมักผสมไม่ได้ทำให้การผลิตเอนไซม์เพิ่มขึ้น ซึ่งแสดงให้เห็นว่ารำข้าวสาลีมีแหล่งไนโตรเจนที่เพียงพอแล้ว อุณหภูมิที่ 45-50 องศาเซลเซียส และความชื้นเริ่มต้นที่เท่ากับ 70% เป็นสภาวะที่เหมาะสมต่อการผลิตเอนไซม์ การให้อากาศสำหรับการเพาะเลี้ยงเชื้อในถังหมักระดับห้องปฏิบัติการแบบถาดเป็นสิ่งจำเป็นสำหรับการผลิตเอนไซม์ที่มีชั้นวัสดุหมักที่หนาขึ้น วัสดุหมักที่มีความหนา 1 เซนติเมตร ให้กิจกรรมไซลาเนสสูงสุดที่ 1764 หน่วยต่อกรัมวัสดุแห้งเมื่อให้อากาศ 0.24 ลิตรอากาศต่อชั่วโมงต่อกรัมวัสดุหมักแห้ง หลังการเพาะเลี้ยงเป็นเวลา 5 วัน เอนไซม์จาก *T. lanuginosus* สามารถปลดปล่อยน้ำตาลริดิวซ์จากเยื่อยูคาลิปตัสได้ การใช้เอนไซม์ไซลาเนส (220 หน่วยต่อกรัมเยื่อแห้ง) และตามด้วยไฮโดรเจนเปอร์อ๊อกไซด์ 2% ช่วยลดค่า kappa number ได้อย่างมีนัยสำคัญ (5.7 หน่วย) และเพิ่มความสว่างให้เพิ่มขึ้น 45.2%

คำสำคัญ: *Thermomyces lanuginosus*, การหมักแบบแห้ง, เบต้าไซลาเนส, ซังข้าวโพด, การฟอกขาวทางชีวภาพ

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1. Introduction

Xylan-degrading enzymes, particularly endo-β-xylanases, are one type of enzymes with potential for industrial applications. They have been used in facilitating the bleaching of Kraft pulp, improving the digestibility of animal feeds by reducing the viscosity of cereal diet and preparing dough in baking process [1]. In addition enzymatic saccharification of xylan contained in agricultural and industrial wastes may contribute to utilize of lignocellulosic biomass, resulting in useful chemicals and fuels [2, 3, 4]. The use of agricultural wastes as raw substrates for production of enzymes and protein by microorganisms with solid state fermentation is expedient to increase the productivity with cost reduction [5, 6]. Thermophilic fungi are attractive for use in solid state fermentation since they will reduce the problems of heat accumulated in large scale production [7]. Thermophilic fungus, *Thermomyces lanuginosus* formerly named *Humicola lanuginosa* [8], has been reported as a potent strain for production of endo-β-xylanase in solid state fermentation using wheat bran, and corncob as substrates [9, 10, 11]. In Thailand, abundant corncob as well as other agricultural wastes are available but still is not practically utilized. Furthermore corncob also contains large amount of hemicellulose, 30-35% of dry weight [2]. Therefore, this raw material should be employed as a substrate for β-xylanase production. Purkarthfer *et al.* reported that high activity of xylanase by *T. lanuginosus* was obtained from solid state culture using corncob which had a moisture content of 70% and contained 1.75% yeast extract [11]. However, our preliminary experiments were also attempted to utilize corncob as substrate for the enzyme production and for xylooligosaccharides production by enzymatic hydrolysis [12]. Therefore, in this study the production of β-xylanase by this strain in solid state culture using alkaline treated corncob as basal substrate has been investigated. Some factors affected on the production of β-xylanase in static tray culture were reported. Besides, Efficiency of crude cellulase free β-xylanase of *T. lanuginosus* on bleaching of Kraft eucalyptus pulps was also studied.

2. Materials and methods

2.1 Raw materials used for fermentation

Raw materials used for this study were corncob, wheat bran and rice husk. Dry corncob was milled up to 20 meshes using a grinding mill with 1-mm screen. The milled corncob was mixed with 500 ml of 1% NaOH at room temperature for 1 h. The alkaline treated corncob was washed with tap water until neutral and dried up in a hot air oven at 100 °C. Wheat bran used was 40-100 mesh in size, whereas rice husk were used in its original form.

2.2 Fungal strain and inoculum preparation

Thermophilic fungus, *Thermomyces lanuginosus*, formerly named *Humicola lanuginosa* (Griffon and Maublance) Bunce, was used in this study. The fungus was kindly provided by Prof. Dr.Tsutomu Morinaga of Hiroshima Prefecture University. The strain is preserved at MIRCEN culture collection, Bangkok, Thailand. This strain was reported as a potent strain for β-xylanase production [9]. The fungus was cultured at 50 °C for 10 days on 28 ml of 10% wheat bran agar medium in 250 ml Erlenmeyer flask. After that, 15 ml of 0.1% steriled Tween 80 was added to the culture and using sterile spatula to gently scratch the mycelium mat. Then further dilution was made to obtain concentration of 105-106 spores/ml (counted with haemacytometer). This spore suspension was used as inoculum.

2.3 Factors affecting growth and β-xylanase production by *T. lanuginosus* in solid state fermentation

Otherwise stated, solid state fermentation experiment was carried out in 250 ml Erlenmeyer flasks that contained of a total of 10 g of alkaline-treated corncob and wheat bran at various ratios with 2 g of rice husk to increase the porosity of the medium. Other nutrients and wood xylan were supplemented to the medium. The well-mixed substrates were moistened with distilled water to 70% moisture content before sterilization at 121.5 °C for 20 min. After cooling, 2 ml of the spore inoculum was inoculated to media and incubated at 50 °C controlled incubator. The cultured solids were sampled in 24 h intervals for further analysis.

2.4 Static tray solid state fermentation

The solid state fermentation in static tray culture with moisten aeration was studied at 50 °C in tank reactor (PVC-dessicator) equipped with a porous

bamboo tray 22 cm in diameter as shown in Figure 1. The react or and other accessories were then put in at 50 °C controlled incubator. A mixture of substrates consisting of NaOH-treated corncob, wheat bran, rice husk and wood xylan in ratio of 5:5:0.25 at 70% initial moisture content was used. The desired amounts of mixed substrates were sterilized at 121.5 °C for 30 min. The mixed substrates were inoculated with a 2% (v/w) of spore suspension and then transferred into the tray under aseptic conditions to make 1, 2 or 5 cm thick substrate beds. Fermentation was carried out without aeration and with aeration rates of 0.06, 0.24 and 0.45 l air/g/h, respectively. In the case of aeration, was supplied through membrane filtration. The airflow rate was controlled with a regulator valve. The air was bubbled through a water reservoir and passed to the bottom of the tank reactor to maintain constant of moisture content of solid. The fermented solid was periodically sampled to determine moisture content, fungal growth and extracted with distilled water for the enzyme assay.
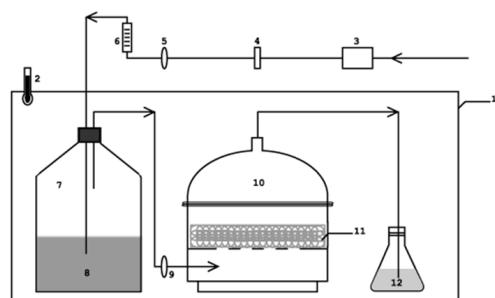
1. Incubator 50 °C	7. Saturation vessel
2. Thermometer	8. Sterilized deionized water
3. Air pump	9. Air filter
4. Air controller	10. Bioreactor
5. Air filter	11. Substrate bed
6. Flow meter	12. 5% $CuSO_4 \cdot 7H_2O$

Figure 1. Diagram of tray culture with aeration accessories.

2.5 Enzyme treatment of pulp

Unbleached pulp, eucalyptus pulp was obtained from Advanced agro Co. Ltd. (Prageenburee, Thailand). The pulps were thoroughly washed with distilled water until a neutral pH of washed waters was attained. The xylanase treatment of the pulp was done in two stages.

Stage I:

Washed pulp equivalent to 40 g of dry weight was palace in flasks and 360 ml of distilled water was added. These flasks were then incubated in a water bath set previously at 50 °C. 40 ml of 1 M phosphate buffer, pH 6.0, was added to bring reaction mixture. A control pulp was run without enzyme. An enzyme equivalent to 220 U (per g dry weight pulp) was added to experiment. The pulp was mixed every 30 min. After 3 h, the pulps were filtered through Whatman filter paper No.4 using a Buchner funnel and the filtrate was retained for reducing sugars analysis. The treated pulp was then washed with distilled water and was used for determination of the kappa number and the brightness.

Stage II:

The consistency of the pulp at this stage was 10%, twenty g of enzymatically treated pulp from stage I was put in Erlenmeyer flask and 200 ml of 2% H_2O_2 was added. The pulp was incubated at 50 °C for 3 hr. After incubation, the pulp was filtered and the filtrate retained for analysis. The pulp was washed thoroughly with distilled water before determination of determination of the kappa number and the brightness.

3. Analysis

The fermented solid was extracted with distilled water at 4 °C for 1 h. The extracted solution was centrifuged and the clear supernatant obtained was used for pH measurement and determination of enzyme activity. The β-xylanase assay was carried out in 0.5% oat spelt xylan (Sigma Chemicals, USA) in 50 mM acetate buffer, pH 6.0 at 50 °C for 10 min as described in a previous paper [9]. One unit of enzyme activity was defined as the amount of enzyme that liberated of 1 μmole reducing sugars per min under assay employed and was expressed as IU per g dry solid. Moisture was determined by weight loss after being kept in 105 °C oven overnight. Growth was estimated by determination of glucosamine (Glc NH$_2$) using a modification of the method of Morgan-Elson [13] after the extraction and acid hydrolysis of chitin from fungal biomass in dry solid by method of Nishio *et al.* [14]. The kappa number and the brightness were analysed described by TAPPI Test Methods T236 cm-85 and TAPPI Test Methods T452 om-92, respectively [15]. The amount of released reducing sugars from pulp was measured according to the Somogyi-Nelson [16].

Table 1. β-Xylanase production and growth of *T. lanuginosus* after cultivation for 5 days at 50 °C on mixed substrates consisting alkaline treated corncob, wheat bran and rice husk in different ratios with 70% initial moisture content.

Ratios of mixed substrates	Residual moisture content (%)	β-Xylanase (U/g dry solid)	Glucosamine content (mg/g dry solid)
10 : 0 : 2	61.4	3.5	1.5
8 : 2 : 2	65.0	222.0	7.1
6 : 4 : 2	68.0	713.0	10.2
5 : 5 : 2	68.7	722.0	12.8
4 : 6 : 2	69.6	728.8	17.9
2 : 8 : 2	70.8	326.3	23.7
0 : 10: 2	70.1	105.3	23.8
0 : 5 : 7	67.8	100.1	14.8

4. Results

Effect of ratio of alkaline treated corncob and wheat bran on enzyme production.

Our preliminary study found that only alkaline treated corncob alone was not sufficient for growth and the enzyme production by *T. lanuginosus* in solid state culture. Therefore, the effect of wheat bran supplement as nitrogen source and other nutrients to alkaline treated corncob on β-xylanase production was investigated to determine the optimum ratio of these substrates. The fungus was cultivated in solid state culture using 10 g of the mixed substrates: alkaline treated corncob and wheat bran, in different ratios of 10:0, 8:2, 6:4, 5:5, 4:6, 2:8, 0:10 and with 2 g of rice husk. As results shown in Table 1, the fungus produced very low β-xylanase activity; 2.6 U/g dry solid with slight growth on a medium containing only alkaline treated corncob as a substrate. However, xylanase activities, 222, 713, 722, 729 and 326 U/g dry solid were obtained when *T. lanuginosus* grown for 5 days on the mixed substrates of alkaline treated corncob and wheat bran in ratios of 8:2, 6:4, 5:5, 4:6 and 2:8, respectively. Furthermore, growth of *T. lanuginosus* as determined by glucosamine content was 8.2, 12.8, 12.1, 18.0 and 23.7 mg/g dry solid, respectively. Higher amounts of wheat bran in the mixed substrates increased growth, but these adversely decreased the enzyme production. Lowest enzyme activity, 105 U/g dry solid, but with good growth, 23.8 mg glucosamine/g dry solid, was obtained when the fungus was grown on wheat bran as sole substrate. The production of enzyme significantly decreased in the medium without alkaline corncob. *T. lanuginosus* was cultured in a higher porosity solid medium, consisting of 5 g of wheat bran with 7 g of rice husk, which yielded only 100 U/g dry solid with 14.8 mg glucosamine/g dry solid (Table 1).

This indicated that alkaline treated corncob played important role as substrate for the induction of xylanase.

The optimal ratio of alkaline treated corncob and wheat bran for the enzyme production was in the range of 6:4-4:6. In addition though property of water adsorption of each raw material is different, moisture content of the solid media, consisting of different ratio of alkaline treated corncob and wheat bran, then were slightly different as shown in Table 1.

Therefore, we concluded that the major effect on the enzyme product was due to nutritional values of materials rather than property of water adsorption of raw materials.

4.1 Effect of wood xylan addition

The effect of addition of exogenous wood xylan on β-xylanase production was investigated. Addition of wood xylan in the range of 0.12 to 0.75 g to the 12 g of mixed substrates, which consisted of alkaline treated corncob, wheat bran and rice husk in ratio of 5:5:2 enhanced the production rate and the extent of enzyme production. β-Xylanase activities of 944, 974, 1,052 and 1,149 U/g dry solid were obtained when *T. lanuginosus* was grown for 5 days in 70% moisture content of the mixed substrates consisting of 0.12, 0.25, 0.50 and 0.75 g of wood xylan, respectively. However, addition of wood xylan did not show any significant effect on growth of the fungus. Though addition of exogenous wood xylan increased

the production of β-xylanase, the increase was not in linear proportion to the wood xylan added. Excess xylan addition did not significantly increase the enzyme production.

4.2 Effect of addition of nitrogen source

Each nitrogen source, peptone, urea, $NH_4H_2PO_4$, $(NH_4)_2 SO_4$ and NH_4NO_3, was added at a concentration equivalent 0.1 g nitrogen to 70% moisture content mixed substrates that consisted of alkaline treated corncob, wheat bran, rice husk and wood xylan in a ratio of 5:5:2:0.25. It was found that addition of peptone and urea did not promote the enzyme formation; yields were in the range of 1,250-1,320 U/mg dry solid after 5 days incubation (data not shown). Though the increment of glucosamine content from 11.7 to 15.7 mg/dry solid was found in the medium to which peptone was added. Addition of $NH_4H_2PO_4$, $(NH_4)_2SO_4$ and NH_4NO_3, however, showed significant adverse effects, yielding 185, 210 and 236 U/g dry solid, respectively.

A sharply decline in pH in the medium supplemented with ammonium salts, from pH 6 to pH 3.6-3.8 after 24-48 h incubation, was the significant reason for the lower enzyme formation by this strain of *T. lanuginosus*.

4.3 Effect of initial moisture content and temperature

Production of β-xylanase by *T. lanuginosus* was investigated at 45 °C by adjusting different initial moisture content between 60-76% of the mixed substrates. As shown in Table 2, moisture content of 70% resulted in the maximum production; 1,294 U/g dry solid after 120 h cultivation. Moisture contents higher or lower 70% decreased the enzyme production by the strain. Evaluations of the production of β-xylanase by *T. lanuginosus* in 70% moisture content mixed substrates at 35, 40, 45, 50, 55 and 58 °C were carried out. Results, shown in Table 3, indicated that maximum enzyme production was obtained when the culture was grown at 45-55 °C for 4-5 days incubation; yielded approximately 1,200 U/g dry solid. However, at 55 °C the fungus prolonged to synthesis the enzyme. It was found that the production of enzyme severely decreased to 207 U/g dry solid when the cultures were grown at 58 °C. Less enzyme production was obtained at 40 °C, the yield being 386 U/g dry solid. No growth

and enzyme production was found at 35 °C, indicating that the fungus is thermophile. It was also found that the moisture content of solid materials slightly decreased after 3 days culture at temperature employed.

4.4 Effect of aeration rate on β-xylanase production in static tray culture

Effect of various moisten air aeration rates, 0.06-0.45 l/h/g dry solid, on growth and β-xylanase-xylanase production were investigated at 50 °C using static tray culture in a tank reactor equipped with a 22 cm diameter bamboo tray. The mixed substrates consisting of alkaline treated corncob wheat bran and wood xylan in the ratio of 5:5:2 at thickness of 1, 2 and 5 cm were used. As shown in Figure 2, it was found that aeration was required for the maximum production of enzyme by *T. lanuginosus*, particularly at higher substrate thickness.

The culture without aeration had a longer lag phase; 36 h, with lesser growth and enzyme production. The production of β-xylanase by *T. lanuginosus*; was 1,308, 272 and 108.2 U/g dry solid with contents of glucosamine, 13.4, 10.0 and 8.2 mg /g dry solid, at substrate thickness of 1, 2 and 5 cm respectively, after 5 days cultivation.

However under the aeration the fungal had a shorter lag phase, 24 h., with higher biomass and enzyme production. The xylanase production and growth were decreased when the substrate bed reached up to 5 cm as shown in Table 4. The maximum production of xylanase by *T. lanuginosus* was 1,764 U/g dry solid with content of glucosamine; 16.1 mg /g dry solid at aeration rates of 0.24 l/h/g dry solid after 5 days cultivation on 1 cm bed thickness.

4.5 Effect of xylanase on the bleaching

Eucalyptus pulps (20 g) were brought to bleach at 50 °C for 3 h with *T. lanuginosus* β-xylanase produced by solid state culture of 220 U/g dry weight pulp. The enzyme treatment resulted in 1.4 unit reductions of Kappa number and increase in brightness of 33.1% compared to the values of the pulps treated with buffer as control (Table 4). Whereas, the brightness value of Eucalyptus pulps that subjected to the peroxide treatment was 40.6%. The reduction of Kappa numbers by 5.7 unit and brightness value of 45.2% pulps were obtained after treated with enzyme and peroxide as shown in Table 4.

Table 2. Effect of initial moisture content on β-Xylanase production of *T. lanuginosus* after cultivation at 5 days on mixed substrates consisting alkaline treated corncob, wheat bran rice husk and wood xylan in ratios 5:5:2:0.25.

Initial mositure content	Residual moisture content (%)	β-Xylanase (U/g dry solid)
62.3	60.6	745
66.5	65.2	926
70.1	68.9	1,294
73.4	73.4	1,150
76.3	76.4	951

Table 3. Effect of temperature on β-Xylanase production of *T. lanuginosus* after cultivation on mixed substrates consisting alkaline treated corncob, wheat bran, rice husk and wood xylan in ratios 5:5:2: 0.25 with initial moisture content of 70%.

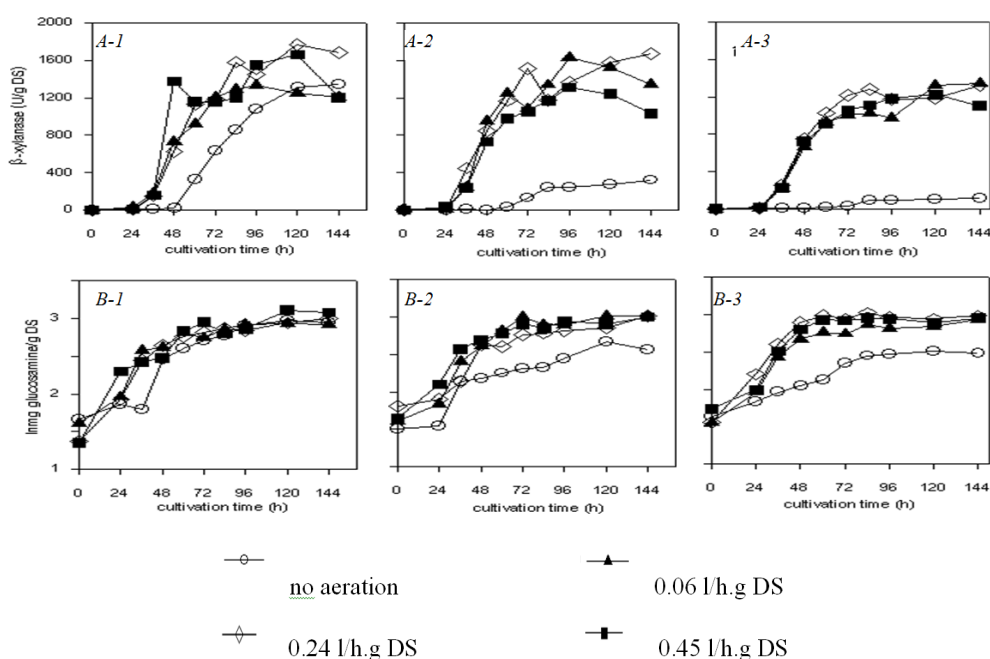| Temp (°C)	Moisture content (%) cultivation time(days)			β-Xylanase (U/g dry solid) cultivation time (days)		
	3	4	5	3	4	5
35	69.2	68.6	71.2	1.7	3.4	8.0
40	70.5	69.7	68.5	263	386	393
45	69.1	70.2	68.8	946	1,200	1,294
50	68.9	67.8	65.8	934	1,210	1,150
55	67.0	66.8	62.2	296	1,220	1,160
58	65.3	62.8	59.0	36.9	207	296

Figure 2. Effect of aeration on β-xylanase production (A) and growth (B) of *T. lanuginosus* when cultivated in tray solid state culture at 50 °C on the mixed substrates at different substrate bed thinkness: 1 cm. (1) 2 cm. (2) and 5 cm. (3).

Table 4. Reducing sugar, brightness and kappa number of eucalyptus pulp bleached xylanase enzyme and 2% hydrogen peroxide.

Condition	Reducing sugars liberated (mg/ml)	Kappa no. (unit)	Brightness (%)
Buffer	0.227	12.26	27.8
Xylanase	0.372	10.95	33.1
Buffer +H_2O_2	0.097	10.16	40.6
Xylanase +H_2O_2	0.012	6.64	45.2

5. Discussion

T. lanuginosus isolate was considered a potent strain for cellulase free β-xylanase production. The isolate produced a large quantity of β-xylanase under solid state fermentation using wheat bran [9]. However, this work indicated that maximum β-xylanase production by *T. lanuginosus* could be obtained when a mixed substrate consisting of alkaline treated corncob and wheat bran in a ratio of 5:5 was employed. Alkaline treated corncob did not only function to increase porosity, but also contained accessible xylan that served as an excellent substrate or inducer for the β-xylanase production. Addition of various exogenous inorganic nitrogen sources did not enhance the enzyme production but showed an adverse effect on enzyme formation due to the severe dropped in the pH of the media. Addition of peptone or urea also did not increase the enzyme production, indicating that wheat bran could serve as a sufficient nitrogen source as well as a good source of nutrients and substrate for growth and the enzyme production by this strain. Wheat bran was reported to be good raw material for the production of β-xylanase by *T. lanuginosus* [9, 16]. It was also reported that a medium consisting of corncob and yeast extract was an excellent medium, which supported growth and enzyme production, by *T. lanuginosus* [11]. However, our results suggested those mixture substrates of wheat bran and alkaline treated corncob can be considered excellent medium that enhances the enzyme production. Although, alkaline treated corncob could provide xylan to as a serve as inducer for xylanase production, addition of wood xylan may readily induce the enzyme synthesis in the beginning of growth. Then, the enzymes further hydrolyzed xylan, which is contained in alkaline treated corncob and may yield successive inducers that enhanced the enzyme formation. This result supported the finding of Alam *et al.* that addition of 0.7 g of wood xylan to wheat bran could increase the β-xylanase production by *Thermoascus aurantiacus* by 28% [17]. In general β-xylanase was induced by xylan and derivatives of xylose [18]. Moisture content is important factor affecting the enzyme production in solid state cultivation. Maximum enzyme was also obtained when the initial moisture of substrates was adjusted to 70%. Higher moisture content reduced the production of enzyme. This may be due to excess water occupying spaces between solid particles causing in poor oxygen accessibility [19]. However, *T. lanuginosus* that was grown on wheat bran medium at 80% moisture content gave the maximum enzyme production [20]. Moisture content of solid materials slightly decreased during 3-4 days culture at each temperature employed as shown in Table 3. This indicated that less efficient on the enzyme production was due to high temperature (> 55 °C) that is a inheritance behavior of the fungal strain, not effect on decreasing of substrate moisture content. In addition, at higher temperature incubation (> 55 °C) reduced the enzyme production caused of less thermostable of the enzyme. The enzyme production by the fungus cultured in static tray culture increased with increasing of aeration rate. Under non-aerated condition,

the growth and enzyme production decreased considerably with the increase in thickness of the substrate. However, in aeration condition, the enzyme slightly decreased when increasing substrate thickness. These results indicated that the culture grown at substrate thickness of 1-2 cm with the aeration rate of 0.24 l/g dry solid/h yielded the maximum xylanase activity, in the range of 1764-1577 U/g dry solid. This was in agreement with the report of Abdullah *et al.* also found that the aeration of 0.12 l/g/dry solid with a 1-2 cm delignified wheat bran bed was the optimized condition for single cell protein of *Chaetomium cellulyticum* using stationary layer solid state fermentation [21]. The solid state fermentation at higher thickness of substrate bed may result in insufficient oxygen supply and accumulation of heat and carbon dioxide derived from the metabolism that caused the lower growth and enzyme production [22, 23].

Xylanase from various fungi and bacteria have been reported to facilitate the bleaching of Kraft pulp, thereby reducing chlorine demand be decreasing the kappa number. The xylanase from *T. lanuginosus* TISTR3465 was found to be effective in release of reducing sugars from eucalyptus pulp as shown in Table 4. The above mentioned data was also consistent with that of Christov *et al.* [24] when xylanases from *Aspergillus oryzae* and *Gliocladim viridae* were applied for pulp bleaching. It was shown that enzymes from both microorganisms could release reducing sugar and increase the brightness of papers. However, the amount of released reducing sugar was significantly different (0.28 and 0.02 mg/ml for *A. oryzae* and *G. viridae*, respectively). There was significant drop in kappa number (5.7 U), brightness was improved up to 45.2% when the bio-treated samples were subjected to peroxide treatment (Table 4). It has been suggested that xylanase treatment improved the accessibility of the pulp for the bleaching chemical, it was acted on re-precipitated xylan on the surface of the micro fibrils allowing for better chemical penetration and thus improve lignin extraction [25]. It was obviously confirmed that decrease in kappa number and increase in pulp brightness by xylanase were resulted from that xylanase would destroy lignin-carbohydrate bond therefore resulting in easily removal for lignin [26, 27]. Moreover, xylanase could also degrade xylan on the surface of pulp and improve the reaction with chemical reagents and there was no effect on kappa number [27].

This is the reason for the decreased kappa number and higher brightness of the enzyme treated pulp at the same bleaching reagents consumption. In contrast to this, hemicelluloses degrading enzymes selectively hydrolyze polysaccharide chain attached to lignin, thereby decreasing the amount of chemicals required for pulp bleaching. Our studies indicated that xylanase activated the pulp towards bleaching with hydrogen peroxide suggesting that the future applications will not be focused solely on chlorine and chlorine dioxide bleaching.

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