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OPTIMIZATION OF CELLULASE PRODUCTION WITH *PENICILLIUM NALGIOVENSE* Sll GROWN ON PRETREATED WHEAT POLLARD

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ABSTRACT

The cellulase production with *Penicillium nalgiovense* S11 on wheat pollard was enhanced using substrate pretreatments, i.e.: (i) mechanic process by Wiley milling, (ii) reducing sugars removal by water soaking, and (iii) chemical pretrcatment by 0.5% NaOH soaking at 100°C. The enzyme production stated as enzyme activities of all prctrcated substrates were higher than the untreated substrate. Although soaking with water showed significant increase in enzyme activities, the highest CMCase (EC 3.2.1.4), FPase (filter papcrase) and p-glucosidase (EC 3.2.1.21) were observed on NaOH pretreated pollard. The NaOH prctreatment also enhanced the enzyme production by increasing substrate concentration from 2 to 4%. The optimal incubation time in the cellulase production on 4% NaOH-pretrcated pollard was observed on the fifth day. Addition of 250 ppm glucose also increased the enzyme activities. The optimal treatments increased the specific activities of CMCase, FPase, and |3-glucosidase into 60, 4, and 198 times, respectively, as compared to the specific activities on 2% unpretreated pollard.

Keywords *:* Cellulase production/ pollard pretreatments/ water soaking/ NaOH soaking

INTRODUCTION

Most poultry feedstuff such as corn and soybean meals, rye, barley, rice bran, wheat bran, coconut meal, and palm kernel cake from plants contain fibers including hemicellulose, cellulose, and lignin. In contrast with ruminants, poultry digestive track does not digest hemicellulose and cellulose. Recently, it was reported that fermentation occurred in the caecum of poultry due to microbial activity (Josefiak *et al.* 2004), the fermentation could break down cellulose in a small quantity. The addition of cellulases and hemicellulases in the poultry feed had been reported to increase body weight, efficiency of feed utilization, the apparent metabolizable energy, and dry matter digestibility (Campbell and Bedford 1992; Friesen *et al.* 1992; Marquardt *et al.* 1996). The effectiveness of the enzymes was related to the adequate activity of enzyme added.

Cellulase production has been studied since *Thrichoderma reesei* was isolated by Reese in 1950. However, the enzyme activity was limited by its low specific activity. *Penicillium nalgiovense* Sll was isolated from the nest of termites *Glyptotermes montanus* (Nurbayti 2002). The mold had the ability to produce cellulase that digested high crystalline cellulose (filter paper), the most common components in natural cellulose. The enzyme could be produced in the submerged culture containing 2% wheat pollard. Increasing the substrate into 3% concentration

reduced the enzyme activity due to limited oxygen availability and increasing the repression effect caused by soluble carbohydrate, such as starch and reducing sugars (Gong and Tsao 1979). The repression effect from the pollard could be omitted by reducing the soluble sugars using water soaking. In addition, the production of enzyme could be enhanced by mechanic treatment on the substrate using milling and extrussion, and chemical treatment using phosphoric acid, hydrochloric acid, sodium hydroxide (NaOH), and ammonia (Klyosov 1986; Marsden and Gray 1986). Among the chemical pretreatments, NaOH was the most commonly used. The NaOH treatment swells the crystalline regions of cellulose and extends the amorphous regions. The amorphous regions are more digested than the crystalline regions. Recently, Shin *et al.* (2000) found that the NaOH pretreatment at room temperature extracted the short molecules in the amorphous region of tenzel, a cellulose fiber produced from wood pulp into the open-up structure. This condition enhanced the cellulase hydrolysis activity.

In this experiment the cellulase production of *P. nalgiovense* S11 was optimized by substrate pretreatments i.e.: (i) mechanic process by Wiley milling; (ii) removal of reducing sugars by water soaking; and (iii) chemical treatment by NaOH soaking at 100°C. The activities of carboxymethylcellulase (CMCase) and filter paperase (FPase) in hydrolyzing the backbone of amorphous and crystalline cellulose, as well as p-glucosidase that cleaves the cellooligomers into glucose were determined as parameters for enzyme production.

MATERIALS AND METHODS

Pretreatment of wheat pollard

Wheat pollard was obtained from PT Bogasari wheatmill and used as substrate for *P. nalgiovense* S11 (RIAP collection) in producing cellulase. The wheat pollard was pretreated before being used as substrate i.e.: (i) wheat pollard without pretreatment as a control referred as pollard; (ii) milling; (iii) water soaking; and (iv) NaOH soaking, referred as water and NaOH solution pretreated, respectively.

In the Wiley mill, the pollard was ground to a fine powder of 0.5 mm. For the water treatment, 25 g pollard was soaked for one hour in 500 ml of water at room temperature, then the water was squeezed out using cotton cloth, the solid material was dried in the 40°C oven, and ground with Wiley mill (0.5mm). The method for NaOH pretreatment was carried out following Purwadaria (1988). The same amount of pollard (25g) was soaked in 500 ml of 0.5% NaOH solution (W/V) for 1 hour at 100°C. Then the pollard was filtered through cotton cloth and washed under running tap water until the wash water was neutral to pH paper. Excess water was squeezed out, dried in the 40°C oven, and ground with Wiley mill.

Enzyme production

Enzymes were produced by *P. nalgiovense* S11 in the Mandels medium containing yeast extract 3 g/1, 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 and pollard (Purwadaria *et al.* 2003a). The concentration of pollard and pretreated pollard was 2, 3, or 4%. Molds were cultivated in 50 ml medium in a 250 ml erlenmeyer flask at 29°C using reciprocal shaker (150 rpm), after inoculation with 2 ml of spore suspension from five day PDA culture slant (15x10*'spores/ml). The incubation time was carried out for three, four, or five days. Sodium azide was added at 0.2% final concentration. The culture was then centrifuged (12 000 rpm, 20 min, 4°C) and supernatant was collected for enzyme assays.

Enzyme activities

The activity of carboxymethylcellulase (CMCase) was assayed by determining the reducing sugars produced from CMC as glucose or mannose, respectively (Haggett *et al.* 1979). One unit was defined as amount of enzyme which liberates one umol glucose per minute. The p-D-glucosidase and (3-D-cellobiosidase were assayed using p-p-D-nitrophenylglucoside and p-p-D-nitrophenylcellobioside (from Sigma Company) as substrates and one unit was defined as enzyme which liberates one umol nitrophenol per minute (Ide *et al.* 1983; Deshpande *et al.* 1984). Filterpaperase (FPase) was determined by using filter paper Whatman no 1 (1 cm x 6 cm) as substrate following Mandels and Sternberg (1976). The production of 2 mg glucose in the reaction was stated as 0.185 unit of FPase. All assays considered the controls prepared as samples, but without incubation time. Specific activity of all enzymes was calculated in Units/mg protein (U/mg).

Determination of protein, reducing sugar, and fiber component concentrations

Protein concentration was determined by Bradford method (1976) and Bovine Serum Albumin was used as standard. Reducing sugar concentration in pollards was determined after water extraction using DNS method (Miller 1959). The concentrations of fiber components (cellulose, hemicellulose, lignin and silica) of pollard before and after pretreatment were calculated as neutral and acid dietary fiber according to Van Soest and Robertson (1968).

RESULTS AND DISCUSSIONS

Every pretreatment of the pollard enhanced the enzyme production (activities) of *P. nalgiovense* S11 (Table 1). Milling increased the ratio of surface area to volume resulting to better mold growth and enzyme production. The results are in agreement with cellulase production of *Bacillus subtilis* CBTK 106 on banana

wastes (Krishna 1999). It was reported that the increase of the bacterial enzyme activities (CMCase, FPase, and p-D-glucosidase) was parallel with particle reduction. However, the optimum reduction was observed at particle size of 0.4 mm. In the case of *P. nalgiovense* S11, compared with untreated pollard, milling at particle size of 0.5 mm increased FPase and P-D-glucosidase, but slightly decreased CMCase activities. In this condition, oligosaccharides and the size reduction of crystalline and amorphous celluloses might induce more FPase and p-D-glucosidase, but repressed the CMCase production.

Table 1. Ccllulaso production *of P. nalgiovense* SI 1 on pretrcatcd substrate

Pollard pretreatments	[protein] (ug/ml)	Activity(U/ml)			Specific activity (U/mg protein)		
		CMCase	FPase	P-gluc	CMCase	FPase	P-gluc
None (Control)	322±34	0.10+0.05	0.90+0.02	0.004+0.001	0.31+0.16	2.82+0.33	0.014+0.006
Milling	334±55	0.06+0.02	1.89+0.18	0.024±0.012	0.19+0.02	5.8H1.35	0.070+0.021
Water soaking	181+ 4	1.02+0.02	1.77+0.07	0.091+0.004	5.61+0.03	9.78+0.50	0.504+0.017
NaOH soaking	229+30	2.96+0.11	1.93+0.07	0.199+0.031	13.08+1.38	8.56+1.41	0.870+0.080

FPase was filter paperase, p-gluc was p-glucosidase

Milling also slightly increased extracellular (soluble) protein. Therefore, the ratio of specific activities (U/mg protein) of each enzyme between milling and non-treatment was similar with the activities (U/ml). FPase and P-D-glucosidase of specific and enzyme activities were higher in milling, while CMCase was lower than those of untreated pollard.

In the case of water pretreatment, soaking decreased the reducing sugar content (Table 2) that reduced the repression effect. Some starch and pectin were solubilised and rinsed with water resulting in the decrease of the viscosity of the medium (Pawlik *et al.* 1990). Then, the mold had better growth and enzyme production than in untreated pollard due to better oxygen transportation and substrate homogen-ization. Although, all enzyme activities increased in water pretreatment, extracellular protein was decreased than in untreated pollard. These indicated that the protein in the water pretreatment process might be only related to enzyme production as the soluble protein from pollard was entirely rinsed. The increase of enzyme activities and the decrease of protein concentration resulted in the increase of the specific activities of CMCase, FPase, and P-D-glucosidase to 18, 3, and 35 times, respectively, than those in untreated substrate, while the activities were only 10, 2, and 23 times higher than those in untreated substrate, respectively.

Table 2. The fiber and reducing sugar contents of pollards before and after treatment

pretreatments	[Reducing sugars] (%)	[Fiber] (%)		
		Hemicllulosc	Cellulose	Lignin
None (Control)	0.16	23.0	7.4	1.5
Milling	0.17	23.0	7.4	1.5
Water soaking	0.13	37.8	12.0	2.0
NaOH soaking	0.01	49.5	26.9	7.1

The highest enzyme activity was observed in NaOH pretreatment. In this process milling, water soaking and NaOH action in the fiber, all together changed the pollard composition (Table 2) and altered the substrate into more digestible fibers (Khrisna 1999, Shin *et al.* 2000; and deVrije *et al.* 2002). Among pretreated pollard, NaOH pollard had the highest hemicellulose, cellulose, and lignin contents (Table 2) due to the solubilisation of some dry materials including amino acids, starch, pectin, and short oligosaccharides. Although its fiber content was the highest, more digestible fiber in NaOH treatment resulted better growth and enzyme production.

Since the reducing sugars of NaOH pretreated pollard was very low, it was possible that S11 used only glucose or energy to grow from cellulolytic hydrolysis. The less repression effect of small amount of reducing sugars and the need for energy highly induced the enzyme production and increased the extracellular protein content (Table 1). The NaOH pretreatment also altered the fiber structure into more swelling materials as a result it can be more submerged in the culture. Before treatment, the pollard was more floating. These conditions affected better substrate homogenization and oxygen transferred in the culture, which is important for the mold growth.

The high protein content in the NaOH pretreated pollard resulted in lower specific activity of FPase than of the water pretreatment. However, other enzyme activities and protein content were much higher than in the water pretreated pollard. Therefore, NaOH pretreatment was applied for further experiment.

In the following experiment, the concentration of NaOH pretreated pollard increased from 2 to 3 and 4%. Increasing the substrate content changed the enzyme production in the course of incubation time (Figure 1 and 2). The repression effect in the enzyme production by increasing the untreated substrate from 2 to 3% (Nurbayti 2002) was not observed in this experiment. The highest production of CMCase for digesting amorphous cellulose was observed at 4% pollard. The optimum CMCase activity in 2% pollard was observed at three days incubation time and thereafter was decreasing, while in 3% pollard reached the optimum activity at four days, and in 4% pollard was still increasing even at five days incubation time (Figure 1A). Compared with CMCase activity, FPase showed different patterns (Figure IB). For

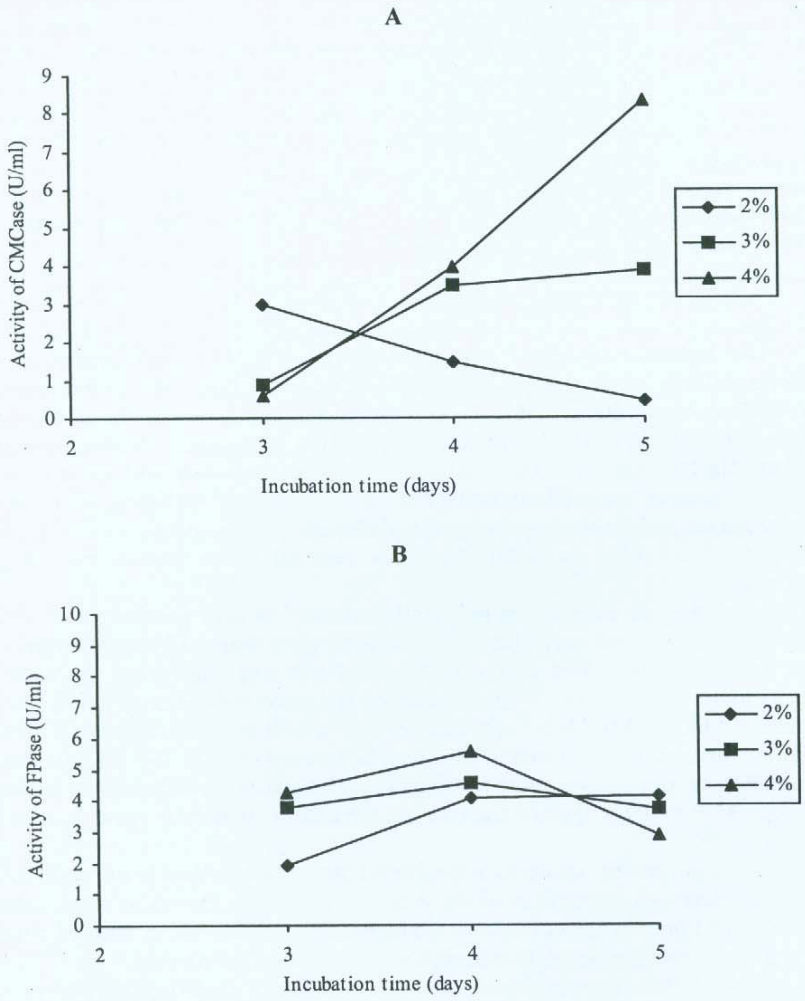

Figure 1. The activity of CMCase (A) and FPase (B) of the enzyme produced by *P. nalgiovense* S11 on various concentrations of NaOH pretreated pollard.

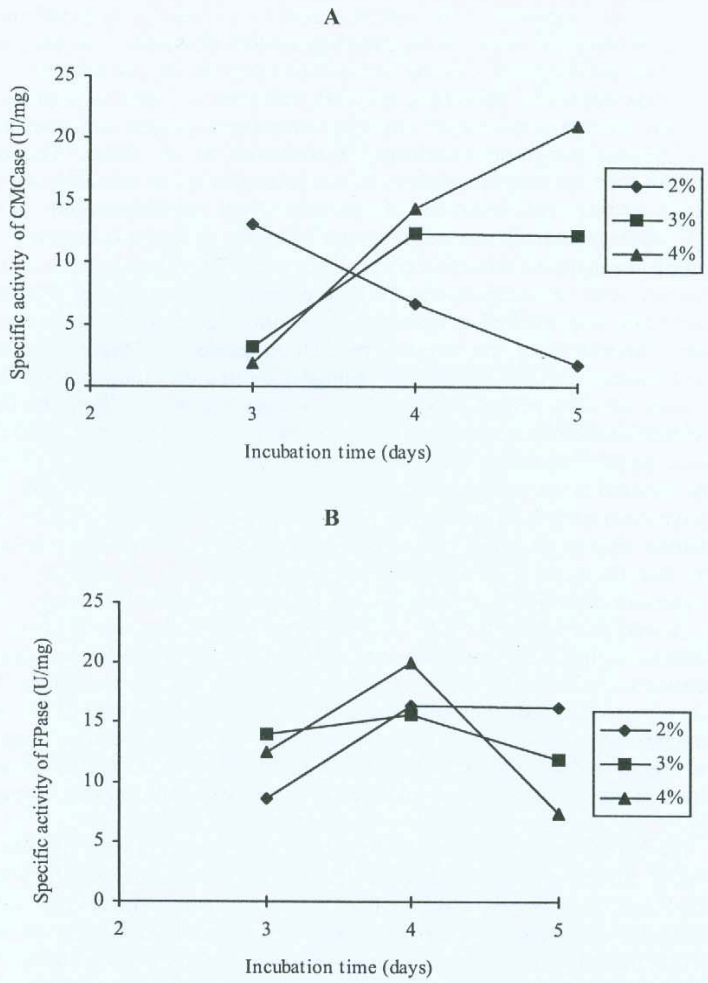

Figure 2. The specific activity of CMCase (A) and FPase (B) of the enzyme produced by *P. nalgiovense* S11 on various concentrations of NaOH pretreated pollard.

every substrate concentration, the optimum activity was reached at 4 days incubation time and thereafter it was declining. The highest FPase activity was observed in 4% pollard that also had the highest reduction at five days incubation time.

The production of hydrolytic enzymes was generally parallel with the growth curve of microorganisms, the activity was increasing until optimum incubation time and then was stable or decreasing (Sachslehner *et al.* 1998). The optimum incubation time for enzyme production was related to substrate concentration. The higher substrate concentration of coconut meal in submerged culture of *EupeniciUium javanicum* resulted in longer lag phase or longer optimum incubation time for p-mannanase production (Purwadaria *et al.* 2003b). In the case of CMCase production with *P. nalgiovense* Sll, the optimum activity in 2% substrate concentration was reached at three days incubation time, after that, due to limited nutrient concentration, the enzyme production ceased. In the higher substrate concentrations, CMCase reached the optimal activities after longer incubation time, four days for 3% pollard, and at least 5 days for 4% pollard. In the more concentrated substrate, the culture needs longer incubation time due to less oxygen transfer. Longer optimum incubation time in the higher substrate concentration rarely occurred in the soluble substrate. Increasing xylobiose from 0.05 to 1.00% for the production of P-mannanase with *Sclerotium rolfsii* resulted to similar optimum incubation time at 15 hours (Sachslehner *et al.* 1998). In this case, concentration higher than 1% might repress the enzyme production.

The reduction of FPase on 4% NaOH pretreated pollard at five days incubation time showed that unlike the CMCase, the FPase was not produced anymore. It is difficult to explain why the CMCase was still produced, while FPase stopped. The reasonable explanation for the reduction of FPase was enzyme instability. Nurbayti (2002) reported the reduction of FPase activity from culture filtrate of Sll stored at room temperature. The highest reduction of FPase in the 4% substrate might be due to the highest reducing sugar concentration produced from the hydrolytic action of cellulase in the substrate, which inhibited the activities of the enzyme especially the FPase.

The curves of specific activities for both CMCase and FPase (Figure 2) were similar to curves of enzyme activities (Figure 1) reflecting those which were not affected by the protein concentration. This result confirmed the data from pretreatment comparison (Table 1), where the extracellular protein produced in NaOH pretreated pollard was more for cellulase, but not for other proteins.

The optimum substrate concentration to produce CMCase and FPase was 4%, however, the optimum incubation time for CMCase and FPase were five and four days; respectively. The high FPase is important for feed supplement application, since most natural celluloses contain crystalline structure. However, in the fifth day the increase of CMCase was much higher than the decrease of FPase. The CMCase was also needed for the synergistic hydrolytic activity on Sigmacell 20, the microcrystalline cellulose (Purwadaria 1995). Therefore, the fifth day incubation in 4% substrate was selected for further experiment in exploring the effect of glucose addition.

The addition of glucose at 250 ppm in 4% NaOH pretreated pollard (containing 50 ppm reducing sugars) increased CMCase and FPase, while higher glucose concentration reduced the activities (Figure 3). However, the reduction of CMCase and FPase in the addition of glucose at 1000 rpm were only 6 and 9%, respectively. *P. nalgiovense* S11 is quite resistant to catabolite repression. In another experiment the isolate still produced cellulases in 3% NaOH pretreated pollard added with 10,000 ppm (1%) glucose, but the activity was much lower than that without glucose addition (Sanjaya 2003).

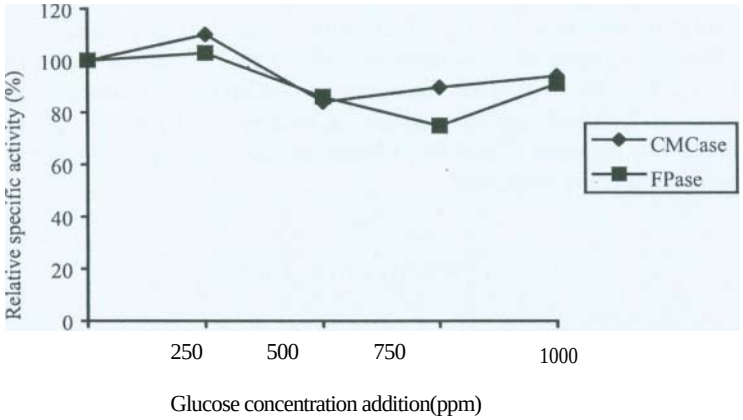

Figure 3. The relative specific cellulase activities from cultures added with glucose

P-glucosidase and cellobiohydrolase activities were determined in the optimum FPase and CMCase production with the addition of glucose at 250 ppm. Compared to the p-glucosidase and cellobiohydrolase produced in 4% pollard without glucose addition, those were 0.87 and 0.14 U/ml, respectively, P-glucosidase was increased to 1.10 U/ml, while cellobiohydrolase was reduced into 0.07 U/ml. Similar to CMCase and FPase, p-glucosidase was enhanced by the addition of glucose at 250 ppm, but the condition repressed the cellobiohydrolase. The cellobiohydrolase might be the least resistant enzyme to catabolite repression.

The cellulase production was induced by short oligosaccharides including disaccharides, such as cellobiose, xylobiose and sophorose, while pure or natural polysaccharides induced more CMCase, xylanase, and mannanase (Sachslehner *et al.* 1998). In the low concentration of monosaccharides such as glucose, galactose, and mannose, the low constitutive cellulase could be produced (Gong and Tsao 1979; Sachslehner *et al.* 1998). Therefore, in the low glucose addition (250 ppm),

the cellulase production of P. *nalgiovense* SI 1 increased, since glucose was used by the mold to start growing. The low addition of glucose (500 ppm) in 1% gum locust bean was also reported to increase the production of mannanase by *E. javanicum* (Haryati *et al.* 1995). Generally in the presence of high reducing sugars, the cellulase is produced after the reducing sugars are used (Sachslehner *et al.* 1998). The same authors also reported that although one dose of cellobiose 3.0 mM (0.5%) could induce the cellulase production of *S. rolfsii*, the ten times incorporation at 0.3 mM at every 2.5 hours induced 2.5 times higher enzyme. In the case of our experiment, the addition of glucose at 250 ppm increased CMCase, FPase, and p-glucosidase, but reduced cellobiohydrolase. Detailed experiment to observe the regulation mechanism of S11 has not yet been carried out.

It could be concluded from this experiment that NaOH pretreatment in wheat pollard, increasing substrate concentration from 2 to 4% and addition of glucose at 250 ppm at 5 days incubation are the optimum conditions to produce cellulase of P. *nalgiovense* S1l based on its specific activities of CMCase, FPase and P-glucosidase. The expense of NaOH pretreatment and the gain of enzyme activities has to be economically evaluated.

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