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Effect of Oil Palm Empty Fruit Bunch Particle Size on Cellulase Production by *Botryosphaeria sp.* Under Solid State Fermentation

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Abstract: Locally isolated *Botryosphaeria sp.* showed the ablity to produce cellulases (FPase, CMCase and β-glucosidase) from oil palm empty fruit bunch (OPEFB) as substrate. Different particle sizes (0.25-0.3 mm, 0.42-0.6 mm, 0.84-1.0 mm and 5.0-10 mm) of OPEFB were investigated under solid state fermentation on the cellulase production. The highest production of FPase and β-glucosidase were obtained from OPEFB particle size of 0.42 – 0.60 mm with 3.261 ± 0.011 U/g and 0.115 ± 0.008 U/g, respectively. It was found that among the four different OPEFB particle sizes studied, particle size of 0.84 – 1.0 mm gave the highest activity of CMCase (8.134 ± 0.071 U/g). Highest concentration of reducing sugars produced in this experiment was 4.303 ± 0.095 mg/ml.

Key words: substrate particle size, cellulase, solid state fermentation, *Botryosphaeria sp.*

INTRODUCTION

Oil palm mills produce a large amount of biomass waste from its daily operation. Generally, 17.08 million tones per annum of oil palm empty fruit bunches (OPEFB) have been produced continuously in 2005 (MPOB 2006). Fully utilization of OPEFB can be achieved by generating value added products such as activated carbon, enzymes, citric acid and others. OPEFB is categorized as lignocellulosic feedstock since it is rich in cellulose contents .Moreover the usage of OPEFB as substrate in cellulases production can reduce the operation cost since substrate cost became one of the major operational costs, representing 30-40% of total production cost (Tanaka *et al.*, 2006; Zhang *et al.*, 2007).

Insolubility of OPEFB is one of the limitations in submerged fermentation. Solid state fermentation (SSF) is more capable in producing certain enzymes and metabolites that usually produced with low yield in submerged fermentation. The bioconversion of OPEFB into polyoses by using SSF resembles the natural condition of growth for the majority of fungi. Bacteria, yeast and fungi are able to grow on solid substrate but filamentous fungi are the best adapted for SSF (Krishna, 2005). The hypha of the fungi has the power to penetrate into the solid substrate.

There are several factors involved in the selection of a suitable substrate for SSF such as macromolecular structure, particle size and shape, porosity and particle consistency (Krishna, 2005; Tao *et al.*, 1997). The substrate must be in a limited size range for an optimal production of cellulase. This process can be facilitated by chipping, milling and grinding the biomass into a fine powder to increase the surface area/volume ratio of the cellulose particle. An optimal sized particle lead to better nutrient absorption, gas exchange and heat transfer thus high enzyme production.

The species of *Botryosphaeria* fungus attacks woody host and it is described as an endophyte (Crous, 2006) and pathogen on plants. The ascomycete fungus *Botryosphaeria sp.* produces a broad range of lignocellulolytic enzymes such as laccases (Barbosa *et al.*, 1996), pectinases (Da Cunha *et al.*, 2003) , cellulase and xylanases (Dekker *et al.*, 2001). These enzymes play an important role in the degradation process of lignocellulosic materials through a synergistic action (Lynd *et al.*, 2002; Zhang and Lynd, 2004).

Less report are available on cellulases production by *Botryosphaeria sp.* using lignocellulose material. Dekker *et al.* (2001) only reported low activity of filter paper cellulase in the media containing veratryl alcohol. The main objective of this study is to evaluate the potential of locally isolated fungus, *Botryosphaeria sp.* to produce cellulases under solid state fermentation by investigating the effect of different substrate particle size.

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MATERIALS AND METHODS

Microorganism:

Botryosphaeria sp. was obtained from Biomass Technology Centre, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. The fungus was grown on potato dextrose agar (PDA) at 30°C to a 7 days culture for development.

Substrate:

OPEFB fiber was obtained from Seri Ulu Langat Palm Oil Mill in Dengkil, Selangor, Malaysia. The dry-mixed substrates were subjected to a sieving procedure employing sieves mesh-size of: 18, 20, 30, 40, 50 and 60 (Mc Cabe *et al*., 2001). These fibers later were classified into four different diameter sizes according to which of the sieves mesh-size that the fiber retained. The smallest fiber size 0.25-0.30 mm were collected from fractions between meshes 50 and 60, followed by the fibers size of 0.420-0.60 mm collected from fractions between meshes 30 and 40, and fibers size of 0.84-1.0 mm which were collected from fractions between meshes 18 and 20. Another fiber size that will use as substrate in this experiment is unsieved shredded OPEFB fiber (5-10 mm).

Substrate Pretreatment

Pretreatment of OPEFB fibers were done by soaking in 2.0 % (w/v) NaOH per 5.0 g of OPEFB followed by autoclaving at 121 °C for 90 minutes. The pretreated OPEFB was filtered, washed with distilled water until no traces of alkaline were detected and then dried in oven for 24 hours at 60 °C.

Solid State Fermentation of OPEFB:

Three gram of pretreated OPEFB was placed in 250 ml flasks and then was autoclaved at 121 °C for 15 minutes. Mandel medium (Mandels *et al.*, 1974) was added as nutrient to initial moisture content of 70%. The composition of the Mandel medium is as followed (g/l): 1.4, $(NH_4)2SO_4$; 2.0, KH_2PO_4; 0.3, $CaCl_2{\cdot}2H_2O$; 0.3, $MgSO_4{\cdot}7H_2O$; 0.005, $FeSO_4{\cdot}7H_2O$; 0.0016, $MnSO_4{\cdot}H_2O$; 0.0014, $ZnSO_4{\cdot}7H_2O$; 0.002, $CoCl_2{\cdot}6H_2O$. Each flask was inoculated with 3 mycelium plugs (0.5 cm diameter each). The flasks were incubated at 30 °C for 10 days. Sampling was done everyday to measure the cellulase activity and the reducing sugar concentration.

Enzyme Extraction:

Thirty ml of 0.2 M acetate buffer at pH 4.8 was added in each flask and shake at 150 rpm, room temperature for 30 minutes. For sample analysis, the resulting homogenate was centrifuged at 4000 rpm, 10 minutes and 4°C to remove fungi cells. The cell free supernatant was then used for sample analysis.

Analytical Analysis:

The activity of FPase, CMCase and β-glucosidase was assayed according to the method explained by Wood and Bhat (1988) with some modifications. One unit of FPase or CMCase activity was expressed as 1 µmole of glucose liberated per ml enzyme per minute. While, one unit of β-glucosidase activity was determined as 1 µmole of p-nitrophenol liberated per ml enzyme per minute. The cellulases were expressed as U/g of dry OPEFB. Reducing sugars concentration was assayed with DNS method (Miller, 1959).

RESULTS AND DISCUSSION

Bioconversion of lignocellulosic biomass into useful products is a complex process involving synergistic actions of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Zhang and Lynd 2004). The results illustrated that the locally isolated *Botryosphaeria sp*. which is described as an ascomycete fungus produced cellulases (endoglucanase, exoglucanase and β-glucosidase) from OPEFB.

Cellulase activities were measured quantitatively based on the individual cellulase production (exoglucanase, endoglucanase and β-glucosidase). FPase activity is one of the assays to determine the amount of exoglucanase enzyme present in the sample. Among all of the different sizes investigated, particle size of 0.42 - 0.60 mm produced the highest FPase activity of 3.261 ± 0.011 U/g which almost more than 2 fold higher compared with particle size between 5.0 to 10.0 mm (Fig 1).

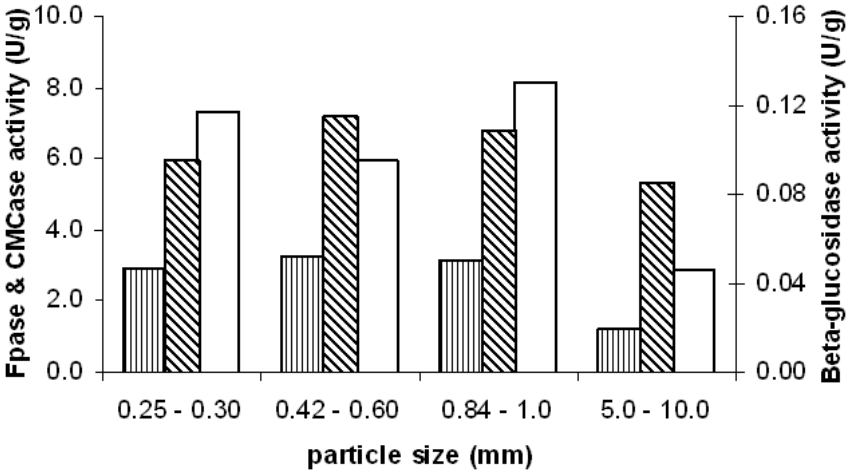

Fig. 1: Cellulases activity (Fpase ◫ , CMCase □ and B-glucosidase ▧ activities) on four different particle sizes of OPEFB

Higher CMCase activity of *Botryosphaeria sp.* was observed using 0.84 – 1.0 mm OPEFB particle size as compared to FPase and β-glucosidase which showed maximal activities with substrate particle size of 0.42 – 0.6 mm (Fig 1). Membrillo *et al.* (2008) showed that *Pleurotus ostreatus* CP-50 produced maximum FPase and CMCase on different particle size of sugarcane baggase. FPase activity was formed on 0.92 mm particles while CMCase activity was on 1.68 mm substrate particle size. Study by Blandino *et al.* (2002), shown that two factors affect the rate of *Aspergillus awamori* growth are particle size and chemical composition of milled wheat and wheat grains mixture. The growth of the fungus on different particle size had a significant effect on cellulase production.

The highest β-glucosidase activity was obtained using particle size of 0.42-0.6 mm, 0.115 ± 0.008 0.148 U/g while the lowest enzyme activity production (0.085 ±0.024U/g) was obtained from OPEFB particle size of 0.25-0.30 mm (Fig 1). Overall, β-glucosidase activity produced during the fermentation was considered low (< 0.2 U/g). This may due to the low productivity of β-glucosidase enzyme by *Botryosphaeria sp.* The highest reducing sugars concentration was 4.303 ± 0.095 mg/ml using 0.42-0.60 mm OPEFB particle size.

Generally low OPEFB particle sizes (400μm) produced high cellulases due to larger specific surface area in fine particles but low porosity property (Tao, 1997). Due to the inverse correlation of porosity and surface area factors, most researchers claimed that 400 μm substrate sized particles contribute to the optimum fungal growth and cellulases production (Tao *et al.*, 1997; Krishna and Chandrasekaran 1996). The low porosity caused less penetration of fungus hypha into the pores of the substrate and fungal growth only can be observed on the surface of the substrate. When larger substrate particle size (> 400 μm) was applied in the fermentation, a network of aerial hypha grows into the inter-particle space low fungal growth on surface of the substrate particle and decreased the resulted enzymes.

Highest FPase and CMCase activity was obtained on day 3 (Fig 2). FPase activity was increased exponentially before reach the optimum day and thereafter the enzyme activity decreased. Fifty percent particle degradation was reported for the period of 12 to 36 hours of fermentation period. In figure 2, the FPase activity increased significantly on 2nd day until it reach the maximal value on 3rd day for 0.42-0.60 and 0.84-1.0 mm particle size. After 3rd day, the FPase production started to decline thereafter followed by a slight decreasing pattern until the end of the fermentation.

The study by Nandakumar *et al.* (1993) and Giese *et al.* (2008) are in agreements with our observation. Study by Nandakumar *et al.* (1993) showed that cellulases, xylanases and reducing sugar production by *Aspergillus niger* occurred after 36 hours of fermentation and escalated up to 72 hours. Giese *et al.* (2008) reported that *Botryosphaeria rhodina* grown on orange bagasse (peel,seed and pulp) in solid state fermentation produced high titers of pectinase was on 72 hours while laccase on 96 hours.

Figure 2 shows that β-glucosidase activity increased rapidly within the first 2 days of fermentation, after which a more or less stationary phase was observed. The relative distribution of β-glucosidase was quite different with FPase and CMCase activities. β-glucosidase activity always lagged behind FPase and CMCase. This is an interesting pattern which seems to be due to the increased levels of disaccharides (the hydrolysis

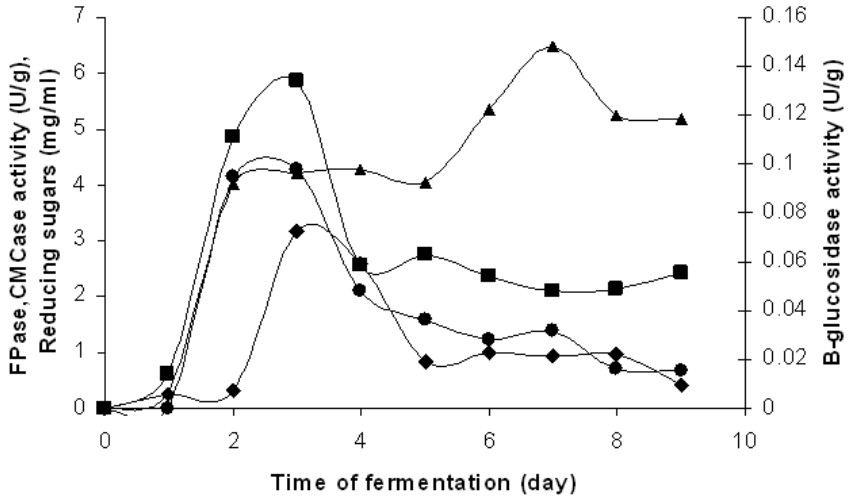

Fig. 2: FPase (♦), CMCase (■), β-glucosidase (▲) activities and reducing sugars (●) production with 0.42-0.6 mm particle size of OPEFB

products of glucanases) at this stage. The optimum day to produce β-glucosidase was around day 5 to day 7. This may due to the substrate (cellobiose) for β-glucosidase is only obtained in large quantity after cellulose being hydrolysed by exoglucanase and endoglucanase where the optimum day for those enzymes are at day 3.

The level of reducing sugars during this solid state fermentation showed a major increase until it reached the highest peak on day 3 (Fig 2). Fungi cannot directly absorb polysaccharides, so they are induced by low molecular weight compounds to synthesize and secrete the enzymes to hydrolyse the macromolecules into smaller metabolizable compounds (Zhang *et al.*, 2004). However, the reducing sugars content decreased gradually towards the end of the fermentation.

In a nutshell, *Botryosphaeria sp.* had shown its ability to convert the OPEFB into cellulases and simple sugars and the optimal particle size to obtain high yield of cellulases is 0.42-0.6 mm.

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