Evaluation of partial characterization of cellulase production by *Aspergillus fumigatus* SK1 under untreated Oil Palm empty Fruit Bunch (OPEFB) in solid state fermentation

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**Abstract**

Evaluation of utilization of untreated Oil Palm Empty Fruit Bunch (OPEFB) as a substrate for the reducing sugars by *Aspergillus fumigatus* SK1 in solid state fermentation (SSF). A pH value of 5 and temperature of 60°C were found to be optimal for maximum cellulase enzyme production. *Aspergillus fumigatus* capable to produce CMCase and β-glucosidases enzymes with highest activity indicated as 1.352 and 0.272 U/mL respectively on a sixth day of incubation except FPase which indicated that *Aspergillus fumigatus* not suitable for FPase production. The partial purification of the cellulase enzyme produced by *Aspergillus fumigatus* SK1 under untreated Oil Palm empty Fruit Bunch (OPEFB) in solid state fermentation. The endoglucanase (CMCase) proteins were detected as 72.6 kDa and 42.4 kDa bands and exoglucanase (FPase) as 72.6 kDa band. The molecular mass of β-glucosidase was found to be about 42.4 kDa by SDS-PAGE.

**Keywords:** Oil Palm empty Fruit Bunch (OPEFB); *Aspergillus fumigatus* SK1; SDS-PAGE

1 Introduction

Nowadays, one of the most important concepts in agro-industrial is how to manage the agro-industrial residues. Agro-industrial residue are the most plentiful resources and could use as a main source for valuable products. Accumulation of agro-industrial waste in large quantities are use as combustion fuel in factory, consequently, not only in the deterioration of the environment, but also in the loss of potentially valuable sources which can be used through a valuable products, such as a variety of enzyme (cellulase, xylanase, etc) which conducted to human food, animal feed and cosmetic industry. The agro-waste is significant concept in environmental management. Thus, it can be consumed to produce enzyme which can be very useful to the environment and industrial products and reduce the environmental problem (Okeke and Obi 1995, 1994). However, investigations on enzymatic degradation of raw materials such as oil palm empty fruit bunch (OPEFB), oil palm trunk (OPT), maize straw, corn stover, wheat straw and rice straw have been carried out by several workers (Noratiqah et al. 2013; Ang et al. 2013; Chen et al. 2008; Jeya et al. 2009). In recent developments in biotechnology are yielding new applications for production of enzyme (Gutarra et al. 2005; Pandey et al. 1999). Cellulose present in renewable lignocellulosic material is considered to be the most abundant organic substrate on earth as chemical feed stock (Das and Singh 2004). Cellulase comprises of three different major composites which consisted of CMCase (Endoglucanase), FPase (Exoglucanase), β-glucosidase (Sun and Cheng 2002). Cellulase is the most abundant and renewable biopolymer on earth. Twenty to thirty percent of the primary plant cell wall consists of cellulose (Bhat, 2000; Bastioli, 2005). Cellulose consists of linear chains of several thousand glucose residues, linked by -1,4 glycosidic bonds (Crawford 1981). The nature of the solid substrate employed is the most important factor affecting SSF processes and its selection depends upon several factors mainly related with cost and availability and, thus, may involve the screening of several agro-industrial residues. The breakdown of cellulose into sugar can be achieved by enzymatic hydrolysis as a preferred method. SSF evaluated as a best system producing enzyme with high yield than SmF and also appropriate for thermolabile products (Muller dos Santos et al. 2004). In large-scale SSF processes have been developed successfully in Japan for the manufactures of a variety of products, including fermented foods are and food-products, enzymes, and organic acids. Some SSF system do not require any nutritional supplements, as it induces enzyme-synthesis, provides balanced growth conditions for mycelia-colonisation and biomass formation, as well as prolonging the production of secondary metabolites (Suryanarayan 2003). Cellulases are a group of enzymes which capable to hydrolyze the cellulose polymer (Schülein 1988). Cellulases are produced by a wide variety of bacteria (Krishna 1999), actinomycetes (Tuncer et al. 1999) and fungi (Bollok and Reczey 2000) (Table 1), nevertheless, the most common microorganism for cellulose production is fungi (Shahrierinour et al. 2010) and relatively only few fungi and bacteria produce high levels of extracellular cellulase capable to solubilize crystalline cellulose extensively (Bhat and Bhat 1997) in addition, *Aspergillus fumigatus* SK1 studied in various substrate (Table 1) and although the productivity of cellulase has been reported quite volumetric high (Soleimaninanadegani et al. 2014). However, research investigations on enzymatic degradation of agro-industrial waste indicated that the filamentous fungi which considered as strong
cellulases and xylanase activities strains perform better using solid-state fermentation (SSF). Besides, SSF involves more advantageous, resulting in greater volumetric productivities, higher product stability, low contamination risk and lower instrumental costs. Another advantage is the use of cheap solid agro-lignocellulose wastes which acts as carbon and energy source and further reduce the need of expensive nutrient medium (Hölker et al. 2004). Cellulase major application is to catalyze the hydrolysis of cellulosic material and obtained fermentable sugars as its end product. By using its hydrolyzing reaction, cellulase usage has been applied in various industry fields. Nowadays, cellulases are important enzymes in large quantities for use in different industrial applications, for example, food, brewery and wine, animal feed, textile and laundry, pulp and paper, as well as in agriculture industry. To author's knowledge, no report has been found characterization on enzymatic degradation of OPEFB by using self production cellulase enzyme in in solid state fermentation (SSF) by Aspergillus fumigatus SK1. The enzymes are commonly used in many industrial applications, and the demand for more stable, highly active and specific enzymes is growing rapidly (Ögel et al. 2001). Aspergillus fumigatus SK1 (GenBank Accession No. JQ665711) was isolated from cow dung and our preliminary studies showed that strain SK1 was the strongest xylanases producer with high level of cellulases secretion compared to 16 other screened fungi (Ang et al. 2013). In addition, this paper examines the effects of cellulases production, characterization of crude cellulases as well as capacity of the crude enzyme cocktail for sugars production by saccharification of OPEFB.

2 Materials and methods

The fungus, Aspergillus fumigatus SK1, used in this study for cellulase production was obtained from Biorefinery laboratory, Biotechnology and Medical Department, Universiti Teknologi Malaysia UTM. Inoculum was prepared by maintaining the fungus on PDA plates at room temperature for 7 days then the fungus grown well. Spores were harvested using 1% (v/v) Tween-80 and collected by centrifuged at 4000 rpm for 20 min. The spores were diluted in order to obtain spore inoculums of 10⁶ spores/g of OPEFB.

2.1 Microorganism and cultivation conditions

OPEFB used in this study was obtained from Department of Biotechnology, Universiti Putra Malaysia (UPM), Serdang. Then OPEFB was grinded into fine particle (125-250 μm) by Toko Tenaga Keluarga Sdn. Bhd., in addition, cellulases (CMCase, FPase and β-glucosidase) productions were considerably affected by OPEFB particle size. Generally, cellulases activities increased dramatically as the particle size of substrates decreased (Ang et al. 2013). The final moisture level was determined by using commercial moisture analyser (MX50, AD Weighing Co., Ltd., Japan) to determine the OPEFB water contain and adjustment of standard curve for moisture which is a one of the selected factors in this study. And OPEFB in this study used based on untreated substrate. Solid state fermentation was carried out in separate sets of 250 ml Erlenmeyer flasks with appropriate volume of production medium and spores suspension (10⁶ spores/g of OPEFB) to generate final moisture level of 80%. The medium used for the crude cellulase production from Aspergillus fumigatus SK1 was modified by Mandels and Weber (1969), Mendel medium with 1.4 g/L (NH₄)₂SO₄, 2 g/L KH₂PO₄, 0.3 g/L urea, 0.3 g/L CaCl₂, 0.3 g/L MgSO₄, 0.005 g/L FeSO₄, 0.0016 g/L MnSO₄·H₂O, 0.0014 g/L ZnSO₄·7H₂O, 0.002 g/L CoCl₂, 0.75 g/L peptone and 2 mL/L Tween.

2.2 Effect of initial moisture and water activity

Different moisture levels were tested (65-85 %, on dry weight basis). In all cases OPEFB was used as the solid substrate. The mineral medium containing 0.2 % (w/v) KH₂PO₄, 0.1 % (w/v) MgSO₄, and 0.1 % (w/v) NaCl was used as the moistening agent. The final moisture level was examined using commercial moisture analyser (MX50, AD Weighing Co., Ltd., Japan).

2.3 Partial characterization of crude cellulase

Information on the optimum conditions for the crude cellulase reactions was necessary to achieve efficient yield and productivity. Thus, the crude cellulase from Aspergillus fumigatus were characterized with respect to temperature and pH optima (Christov et al. 1999). The optimum reaction temperatures for CMCase, FPase, β-glucosidase enzymes were determined using standard assay methods at various temperatures ranging from 30 to 90 °C for 30 min in 0.05 M sodium acetate buffer (pH 5.0). The optimum temperature was observed to be around 60 °C (Liu et al. 2011).

2.4 Enzyme assay

The measurement was performed as described by Wood et al. (1988) (Wood and Bhat 1988). Adsorption was calculated as the measured endoglucanase, exoglucanase and β-glucosidase activity subtracted from the initial endoglucanase, exoglucanase and β-glucosidase added. The reducing sugar was determined using the 3,5-dinitrosalicylic acid (DNS) method. Carboxymethylcellulase (CMCase) activity was indicated in unit defined as 1 mole of glucose liberated per mL enzyme per minute. β-glucosidase activity was measured as releasing of p-nitrophenol from p-nitrophenyl β-D-glucoside. β-glucosidase activity was indicated in unit defined as 1 mole of p-nitrophenol liberated per mL enzyme per minute. The DNS method was based on the characteristic of glucose as the reducing sugar which reduce the oxidized form of DNS reagent (3,5-dinitrosalicylic acid) to reduce form (3-amino,5-nitrosalicylic acid). The reduced form of DNS reagent absorbs light at 535 nm wavelength and the calorimetric change used for the prediction of the amount of glucose in the sample’s supernatants (Miller 1959).
soluble protein was determined by Lowry method using bovine serum albumin as a standard protein (Lowry et al. 1951). Measuring of the protein concentration in the samples was conducted using the protein standard curve.

### 2.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE analysis was conducted using 12% (w/v) polyacrylamide gel. Crude cellulates transferred through the ultracentrifuge process with using Vivaspin 20 polyethersulfone membrane from Sartorius with molecular cut off 10 kDa. The sample and 5 x sample buffer (20 L + 5 L) were mixed in an eppendorf tubes. The mixture was heated at 100°C for 5 minutes and then transferred into ice. The protein solution was spin down for 1 minute at 4000 rpm in microcentrifuge. 15 L of the samples was loaded into the wells. As a reference, 5 L of LMW-SDS Marker Kit Broad Range Protein Molecular Weight Markers was loaded into a separate well. The electrophoresis system was conducted at a constant voltage of 200 V for 45 minutes. After the run has been completed the ladder was cut and stained separately using coomasis blue dye for 20 minutes for visualization of protein bands. The gel was then destained by using 30% (v/v) methanol solution overnight. The destained gel was dried in a vacuum gel dryer for 2 hours (Laemmli 1970).

### 3 Results and discussion

Figure 1 cellulase enzymes solution prepared from *Aspergillus fumigatus* SK1. The maximum reducing sugars production was observed on day 6. Next step was to investigate the effect of initial moisture of OPEFB and the effect of incubation time on CMCase and β-glucosidase production in SSF. Substrate moisture is a crucial factor in SSF and its importance for enzyme production has been well established. Discrepancy in initial moisture content of substrate showed that the enzyme synthesis was associated with the availability of moisture. Substrate with initial moisture of 80% had the reducing sugars (0.1076 U/ml) and was used for the further investigation.

The highest β-glucosidase and CMCase activity was obtained on day 6 (Figure 1). β-glucosidase activity was increased exponentially before reach the optimum day and thereafter the enzyme activity decreased. FPase activities increased significantly on 2nd day until it reach the maximal value on 12rd day for 0.027. After 6th day, the β-glucosidase and CMCase production started to decline thereafter followed by a slight decreasing pattern. The study by Ang et al. (2013) and Giese et al. (2008) showed that cellulases and reducing sugar production by *Aspergillus fumigatus* SK1 and *Aspergillus niger* occurred after 96 and 36 hours respectively of fermentation and escalated up to 72 hours. The optimum day to produce CMCase and β-glucosidase was around day 5 to day 6. Figure 1 shows the level of reducing sugars during this solid state fermentation which indicated major increase until it obtained the highest peak on day 6. However, *A. fumigatus* SK1 was able to produce cellulates with higher activity compared to other research (Ang et al. 2013). In addition, FPase activity remained low accordingly. This consequence could be due to catabolite repression from excess cellobiose which produced during degradation of cellulose (Lynd et al. 2002). Cellulase enzyme productions also depend on the chemical composition of the substrate on performance activities, incorporating influences of temperature and moisture upon chemical-specific respiration and physiochemical association between its components (Gao et al. 2008). Characterization of crude cellulates enzyme The characterization study was done by means of determination of molecular weight, optimal pH, temperature and enzyme activity (Song and Wei 2010). The effects of pH and temperature on CMCase, FPase and β-glucosidase were shown in Figures 2 and 3. Optimum temperature for CMCase was 60°C while 60°C and almost 70°C for FPase and β-glucosidase (Fig-
The maximum activities of cellulases were indicated at different pH in different buffers (Figure 3). The optimum pH of β-glucosidase was pH 4 while the CMCase and FPase were most active at pH 5 when incubated in 60°C for 30 min. This optimal value was in accord according to the optimal culture pH for Aspergillus fumigatus which is between pH 3.0 and pH 6.0 (Liu et al. 2011).

The presence of protein band of exoglucanase, endoglucanase and β-glucosidase were clustered together and showed series of thick bands due to their close related subunit molecular weight. The endoglucanase (CMCase) proteins were detected as 72.6 kDa and 42.4 kDa bands and exoglucanase (FPase) as 72.6 kDa band (Kim and Kim 2012). The appearance of for bands at 26.4, 42.4, 72.6 and 99 kDa (Figure 4) may be caused by isoenzymes or different subunits of the same enzyme protein on electrophoresis gel (Coral et al. 2002). This result is similar to what has been reported by ang et al. (2013), which reported that a cellulolytic enzyme from A. fumigatus SK1 on OPT substrate separated in SDS-PAGE gel showed molecular weight ranging from 18.2 kDa to 90 kDa protein bands in gel electrophoresis. Detected value of 42.4 kDa, is close to the major protein band suggested the appearance of β-glucosidase enzyme in A. niger EFB1 cellulase system (Yan and Lin 1997). The presence of β-glucosidase enzyme as major protein band showed in Figure 4 may explain the domination of β-glucosidase enzyme activity than exoglucanase and endoglucanase activities (Figure 4). The various molecular mass of endoglucanase enzyme from A. niger and A.fumigatus has been reported by several literatures. These include 26 kDa (Hurst et al. 1977), 43 kDa and 25 kDa (Vidmar et al. 1984), 40 kDa (Akiba et al. 1995), 45 kDa (Ximenes et al. 1996) and 31 kDa (Okada 1985). In addition, Singh et al. (1990) reported that corresponding to molecular mass of 52.5 kDa by A. niger (exoglucanase enzyme).

4 Conclusion

In summary, Aspergillus fumigatus able to produce CMCase, β-glucosidase and FPase enzymes with highest activity indicated as 1.352 and 0.272 U/mL respectively on a sixth day of incubation except FPase which indicated that Aspergillus fumigatus not suitable for FPase production (0.018 U/ml). The three cellulase enzyme presented an optimum temperature at 60°C and optimum pH at pH 5.0. CMCase, FPase and β-glucosidase were more stable in acidic pH condition. The endoglucanase (CMCase) proteins were detected as 72.6 kDa and 42.4 kDa bands and exoglucanase (FPase) as 72.6 kDa band. The molecular mass of β-glucosidase was found to be about 42.4 kDa by SDS-PAGE.

References


endoglucanase from pretreated oil palm empty fruit bunch by bacillus pumilus EB3. Journal of bioscience and bioengineering 106 (3):231-236
Okada G (1985) Purification and properties of a cellulase from Aspergillus niger. Agricultural and biological chemistry 49 (5)
Okeke B, Obi S (1994) Lignocellulose and sugar