Cellulase Activity in Solid State Fermentation of Palm Kernel Cake with *Trichoderma* sp.

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ABSTRACT

Aims: The effect of different types of fungal inocula to the cellulase activity measured on palm kernel cake (PKC) was studied.
Methodology and Results: Isolate Pro-A1 which was identified as *Trichoderma* sp. was selected as a potential producer of cellulase via solid state fermentation technique (SSF). Two types of PKCs were used; raw PKC (containing residual oil) and defatted PKC. The PKCs were inoculated with different concentrations of conidia and varying amounts (g) of solid mycelia plugs (SMP) for SSF. The effect of ultrafiltered crude fungal filtrate (CFF) as inocula was also being tested. The highest cellulase activity of 2.454 FPU/ml was detected with 60 % (wt/wt) SMP applied to the raw PKC. Conversely, 2.059 FPU/mL of cellulase activity was measured when 80 % (wt/wt) of SMP was applied to the defatted PKC which is 62.3 % higher than the untreated defatted PKC; and more than 100 % increase in enzymatic activity compared to raw PKC. The cellulase activity in the SSF inoculated with 8×10^6 conidia /mL and 12×10^6 conidia /mL were 1.704 FPU/mL for raw PKC and 1.856 FPU/mL for defatted PKC, an enhancement of about 46 % from uninoculated batch. Inoculation with CFF bears corresponding maximum improvement of the cellulase activity on both PKCs of 13.58 % (raw) and 2.86 % (defatted).
Conclusion, significance and impact of study: The current study proves that *Trichoderma* sp. in the form of SMP can enhance the cellulase activity on PKCs effectively with more than 100% increment. Fungal conidia are also a better choice in enhancing cellulase activity of *Trichoderma* sp. permitted that the PKC used is devoid of oil. From this study, *Trichoderma* sp. holds the potential of converting lignocellulosic materials into products of commercial and industrial values such as glucose and other biofuels.

Keywords: Defatted Palm Kernel Cake, Raw Palm Kernel Cake; Cellulase activity, fungal conidia; fungal solid mycelial plug

INTRODUCTION

Every year, food, agriculture and forest industries are producing large volumes of waste worldwide; causing serious disposal problems especially in countries where the economy relies heavily on agriculture. The agricultural industry is under tremendous pressure to find an alternative use for its residual matter. These biological wastes are organic in nature and easily assimilated by the micro-organisms mainly fungi which make such wastes very appropriate for enzyme production under solid-state fermentation (SSF) conditions (Weiland, 1988). The use of these wastes considerably reduces the production costs. Therefore, solid state fermentation (SSF) is finding increasing application in the production of value added products from wastes mostly from lignocelluloses, agro-industrial and etc (Raghavao *et al.,* 2003; Panagiotou *et al*, 2003; Pang *et al*, 2006a; Lee *et al.*, 2011). The applications of SSF are not only at research levels on a laboratory scale (Gupte and Madamwar, 1997; Gutierrez-

Correa and Tangerdy, 1998; Hang and Woodams, 1998; Kotwal *et al*., 1998; Sekar and Balaraman, 1998), but also at pilot and industrial scales (Durand and Chereau, 1988; Xue *et al.,* 1992; Durand *et al*., 1996; Fernandez *et al*., 1996). One of the biggest industries in Malaysia is the oil palm sector. Palm kernel cake (PKC) is one of the abundant by-products that are produced from the palm kernel extraction process. It was being produced at about 1.9 million tonnes yearly in 2003/2004 (Peter, 2005). The PKC constituents are mainly cellulose, hemi-cellulose and lignin used as bio-resource of raw materials for industries considering that it has been produced in large quantities (Iluyemi *et al.,* 2006).

Enzymes can be considered as hot items for industrial applications in Malaysia and has a huge commercial prospect (Pang *et al.,* 2006b). All the enzymes used in Malaysia are mainly imported from Denmark, Netherlands, Belgium and other countries. The import has been gradually increased to around USD 15.0 millions in year

2003, which makes up 5 % of the total market in Malaysia (Department of Statistics Malaysia., 2003). Due to the increase in demand for this enzyme, it has propelled the field of research on cellulases. Several studies are also being made on the use of cellulase in the bioconversion of agro industrial waste (Pricart *et al.,* 2007). Using enzymes from filamentous fungi to recover cellulose from biomass is an example of green method for cellulose production. Cellulolytic enzymes from a filamentous fungus, *Trichoderma* sp. have been a subject of intensive research because this organism secretes large amounts of enzymes needed for complete hydrolysis of crystalline cellulose (Kubicek, 1992; Teeri *et al.,* 1992).

The present study focused on the cellulase activity of raw and defatted palm kernel cake solid state fermentation inoculated with solid mycelia plug and conidia of *Trichoderma* sp. The objective of the research is to determine the cellulase activity on PKC with and without residual oil, as a function of inocula type, vis mycelia plug, conidia and crude extract.

MATERIALS AND METHODS

Isolation and Identification of Isolate Pro-A1, the Potential Producer of Cellulase

The isolation of fungi was done by dipping a sterilized oil palm wood chip into the commercially available agricultural bio-fertilizer from Malaysian company, Pro-Fil Sdn. Bhd. The wood chip was then cultured on nutrient agar for 7 days at 25 °C and 9 h white light under sterilized conditions. The uncontaminated wood chips were taken based on control (sterilized wood chip only). The fungal that grew on the wood chip surface was identified as *Trichoderma* sp. by using the light microscope. Uncontaminated wood chips based on control were removed and transferred to 30 % mixed vegetable agar media, and after a week, the culture grew completely over the media. A piece (2 mm^2) of the culture is then subcultured onto a fresh media and identified. The fungal identification was carried out based on the colony morphologies and structural characteristics as observed under the light and scanning electron microscopy.

Sampling and Preparation of Palm Kernel Cake

Freshly produced palm kernel cake was obtained from a local palm kernel mill namely Fleet Palms Sdn. Bhd. Located at Sungai Jawi, Penang. The fresh samples had undergone the screw press method in extracting the oil and divided into two portions. A portion was immediately stored at 4 °C in a refrigerator until later use and labeled as raw PKC. Another portion was defatted using Soxhlet technique with hexane as solvent and extraction carried out for 8 h. The residual hexane was then removed and the defatted PKC was stored at 4 °C until ready for use. Proximate analysis was carried out for all the substrates based on the methods described by AOAC (1997).

Fungal Cultivation and Inocula Preparation

The mixed vegetable broth for fungal cultivation was prepared by mixing 163 mL of mixed vegetable juice (V8 commercial brand) and 380 mL of sterile distilled. The pH of the medium was adjusted to pH 5.0 and autoclaved at 1.03 x 10^5 Pa, 121 °C for 15min. About 1.0 g of *Trichoderma* sp. mycelia plug was inoculated onto the V8 solid medium, incubated at 30 °C for 5 days. At the end of the fifth day, a piece of the colony with size circa 2 mm^3 was cut from the periphery of the culture where the growth is most active and inoculated into sterile liquid V8 media. The inoculated liquid medium was further incubated for 5 days at 30 °C on a rotary shaker at 150 rev/min. The liquid medium was then filtered and both the biomass and the filtrates collected (containing conidia to be used as inocula). The biomass was rinsed with the sterile distilled water to completely remove any trace of the medium. The conidia concentration was determined using a haemocytometer.

Ultrafiltration of fungal culture to remove the conidia

The filtrates from the fungal culture, was further filtered by using the ultrafiltration 0.45 µm of nylon filter in order to remove the conidia to obtain the crude fungal filtrate and used as an inocula for the SSF.

Solid State Fermentation

Solid state fermentation of fungus was performed with 5gm ground PKC as the solid substrate. The substrate was autoclaved at 1.03 x 10^{-5} Pa, 121 °C for 15 min and cooled to room temperature before inoculated with different concentrations of fungi conidia: 4x10^6, 8x10^6, 12x10^6, 16x10^6 and 20x10^6 conidia/mL. Another set of substrates were inoculated with different volume of crude extract to 5.0 g PKC % (vol/wt): 10 %, 20 %, 40 %, 60 %, 80 % and 100 %. Different weight ratios of the solid mycelia plug (SMP) to 5.0 g PKC (wt/wt): 10 %, 20 %, 40 %, 60 %, 80 % and 100 % were prepared. The PKC and the SMP are mixed thoroughly and allowed to ferment for five days. Sterile distilled water was added just enough to keep the PKC moist. The initial pH was 5.2 and the entire samples and controls without inoculation were incubated for five days at ambient temperature.

Enzyme Extraction

At the end of the fifth day of fermentation, as much as 20 mL of sterile distilled water was added to the SSF substrate PKC and swirled until it homogeneous. All the flasks were vigorously shaken on the rotary shaker at 200 rev/min for 30 min. The solid biomass was separated from the suspension by filtration through Whatman filter paper no.1. The extract was used as the source of enzyme preparation. The procedure was repeated with different treated raw and defatted PKCs.

Measurement of Enzyme Activity

Measurement of enzyme activity was carried out based on the method of filter paper assay for saccharifying cellulose (FPU Assay) outlined by (Ghose, 1987; Mandels *et al.,* 1976). One mL of 0.05 M citrate buffer (pH 4.8) (pre-warmed to 40.0± 0.1 °C) was added to the test tube containing 1 Whatman 1 filter paper strip (1 cm x 6 cm). After that, 0.5 mL of sample solution (supernatant) also pre-warmed to 40.0 ± 0.1 °C was added to the citrate buffer solution. The resulting solution was mixed thoroughly and then it was transferred to a water-bath maintained at 50.0 ± 0.1 °C. After 60 min (reaction step) the test tubes were removed from the water bath, and 3 mL of 3, 5-dinitrosalicylic acid (DNS) solution was added and mixed thoroughly to stop the enzymatic reaction. Tubes were covered and placed in a boiling water bath for 5 min. All the tubes were cooled to room temperature with a cooling water bath. The absorbance was determined at 540 nm against water blank. Enzyme activity was expressed as FPU/mL (Amount of reducing sugar released per mL of filtrate per h).

Statistical Analysis

The data in this experiment were analysed using Analysis of Variance (ANOVA) from Minitab 15 with 95 % of confidence interval.

RESULTS AND DISCUSSION

Palm kernel cake consists of between 15 % to 18 % protein and 60 % to 70 % polysaccharides. The majority of the polysaccharides are non starch, comprising of beta-mannans type of hemicelluloses (Choi and Gandipon, 2006). The solid state fermentation of both raw PKC which still contains residual oil and defatted PKC, having the oil completely removed were tested with *Trichoderma* sp. in cellulase activity.

## Estimation of Cellulase Activity by *Trichoderma* sp. on Raw and Defatted PKC

Addition of the solid mycelia plug (SMP) 60 % by weight of the SSF exhibits enzyme activity of 2.454 FPU/mL yielding 5.67 mg glucose per mL of extract. **Figure 1** shows the growth of *Trichoderma* sp. fungal mycelia on the surface of raw PKC. This represents the highest cellulase activity of the range tested. *Trichoderma* sp. has been shown to produce cellulase in breaking down the cellulose present in palm kernel fiber (Pang *et al.,* 2006b; Hong *et al.,* 2011). The fungal presence resulted in higher enzyme activity, subsequently yielding more glucose. This is based on the action of cellulase produced by the fungus which can degrade the cellulose in the PKC to glucose. Saenphoom *et al.* (2011) reported a significant increase in the total reducing sugar after treatment of palm kernel expeller (PKE) using exogenous enzyme, suggesting the effective carbohydrates hydrolysis into monosaccharides sugar. Furthermore, Mackul'ak *et al.* (2010) proved that it

is possible to decompose cellulose substrate to glucose by enzymes and figured out that the products of enzymatic degradation of substrates are glucose and lower aldehydes which are highly degraded in anaerobic processes.

Application of *Trichoderma* sp. SMP to the defatted PKC enhanced the cellulase activity by 62.3 % compared to uninoculated substrate. With 60 % (wt/wt) SMP, more than 100 % increase of cellulase activity can be attained on raw PKC substrate. On the other hand, the highest cellulase activity measured on substrate inoculated with conidia, was about 1.856 FPU/mL which occurred when 12×10^6 conidia/mL were used on defatted PKC, and 1.704 FPU/mL cellulase activity on raw PKC inoculated with 8×10^6 conidia/mL. Approximately 46.4 % and 46.5 % higher cellulase activity were achieved with raw PKC and defatted PKC respectively. The growth of *Trichoderma* sp. conidia on PKC can be seen in **Figure 2**.
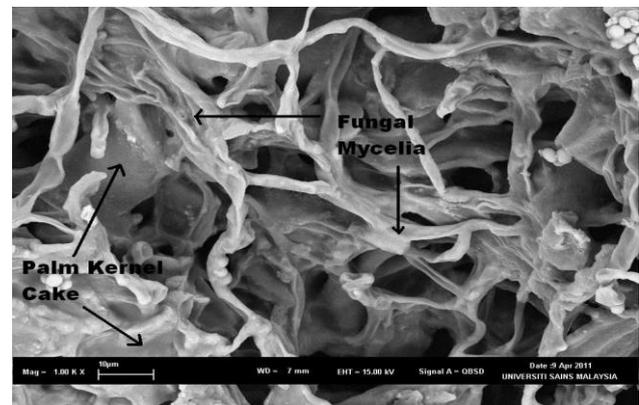

Figure 1: Five day old solid state fermentation showing *Trichoderma* fungal mycelia on the surface of raw palm kernel cake (1.0 K magnification)
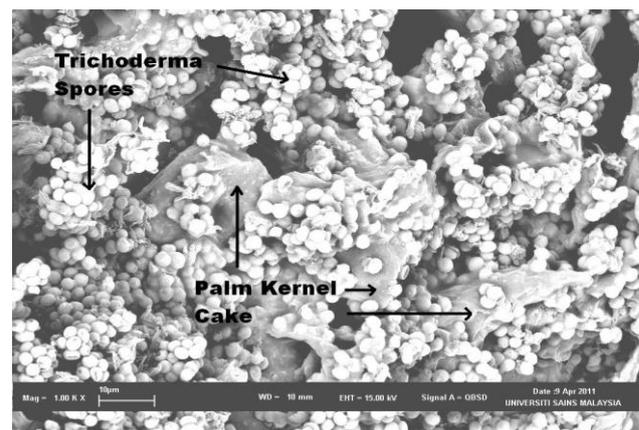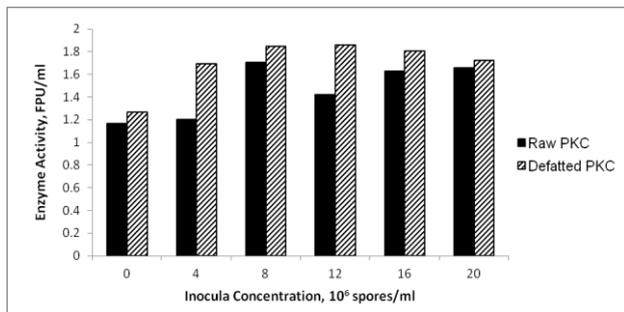

Figure 2: Five day old solid state fermentation showing *Trichoderma* spores on raw palm kernel cake (1.0K magnification)

Difference in Enzyme Activities between Solid Mycelial Plug (SMP) and Conidia as Inocula

Solid mycelia plug (SMP) as inocula generated higher enzyme activity than conidia culture when tested on both raw and defatted PKC (**Figures 3 and 4**). The cellulase activity from the raw PKC inoculated with SMP was higher than from defatted PKC based on SMP/PKC ratio. The cellulase activity enhancement was about 49.6 % for both raw and defatted PKC. The SMP/PKC ratio is crucial as long as the PKC as a substrate for *Trichoderma* sp. is not limiting to stimulate the production of cellulase. This means increasing cellulase activity will be observed until all the substrates, vis the PKCs are being broken down by the mounting mass of *Trichoderma* sp. present in the substrate. As the substrate is being reduced to simple sugars, the cellulase enzymatic process will be slowed down and eventually inhibited.
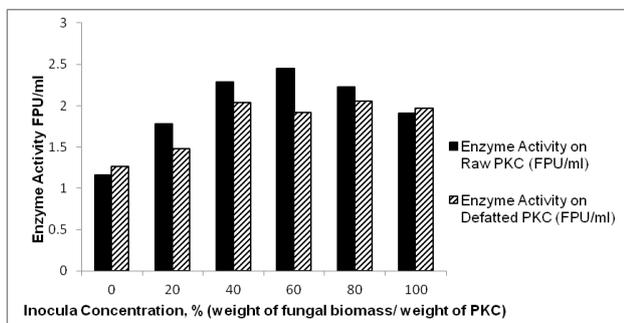
Figure 3: Cellulase activity of raw and defatted palm kernel cake solid state fermentation (PKC SSF) with different ratios of solid mycelia biomass to PKC
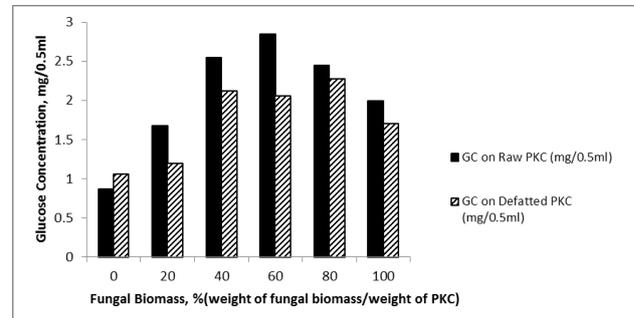

Figure 4: Cellulase activity of raw and defatted palm kernel cake solid state fermentation (PKC SSF) with different conidia concentrations

Relationship between the production of glucose and cellulase activity by *Trichoderma* sp. on PKC

Different enzymes have developed different ways of breaking down cellulose. Cellulolytic enzymes can be characterized as C_1 enzyme, β-1, 4 glucanases (Exo-β-1, 4 glucanases and endo- β-1, 4 glucanases) and cellobiases (King and Vessal, 1969). According to **Figures 3 and 5**, enzyme activity and glucose concentrations produced a linear relationship which

means any increment in the enzyme activity will also increase the glucose concentration. The same results are observed in **Figures 4 and 6**.

Figure 5: Glucose concentrations from raw and defatted palm kernel cake solid state fermentation (PKC SSF) with different ratios of solid mycelia biomass to PKC
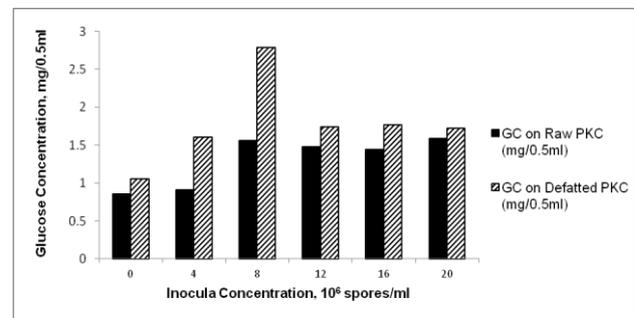
Figure 6: Glucose concentrations from raw and defatted palm kernel cake solid state fermentation (PKC SSF) with different conidia concentrations

Effect of inocula

Highest cellulase activity was detected from fermented defatted PKC with 12×10^6 conidia/mL as in **Figure 4**. Similar maximum increase of 46 % in the cellulase activity was detected on both raw PKC and defatted PKC. The outcome from the use of crude fungal filtrate illustrated slower increase in cellulase activity. According to **Figure 7**, the cellulase activity on the SSF using crude fungal filtrate show only 13.58 % and 2.86 % increase of the cellulase activity on raw and defatted PKC respectively. **Figure 8** shows glucose production from inoculated defatted PKC is generally higher than raw PKC, except when 100% (v/w) crude filtrate was used. At this concentration, the highest yield with 1.6 mg glucose per 0.5 ml media was determined on raw PKC, counter to the results obtained from lower inocula percentage. Based from the statistical analysis using analysis of variance (ANOVA) provided by Minitab 15, all treatments were significant with $p<0.005$ when tested with 95 % confidence level. The two major factors that affect microbial yield of enzymes in a SSF system in this study are the substrate type and amount of inocula.

DISCUSSION

Estimation of Cellulase Activity by *Trichoderma* sp. on Raw and Defatted PKC

The SSF of defatted PKC requires more than 60 % (wt/wt) solid mycelial plug to produce enough enzymatic activity at 2.059 FPU/mL to breakdown cellulose, to yield as much as 4.544 mg glucose/mL. The cellulase activity (~1.2 to 2.5 IU/mL) per unit volume of enzyme broth and the yield of cellulases were attributed to the growth of the *Trichoderma* sp. on the hemicellulose fraction of the PKC during its first phase and then on a cellulose fraction of the PKC during its later phase for cellulase production, with the PKC substrate in solid-state fermentation (Chahal, 1985). A research conducted by Chahal (1985) on solid-state fermentation of wheat straw with *T. reesei* showed high cellulase activity (16 to 17 IU/mL) per unit volume of enzyme broth and high yields of cellulases were attributed to the growth of *T. reesei* as well as to the close contact of hyphae with the substrate in solid-state fermentation.
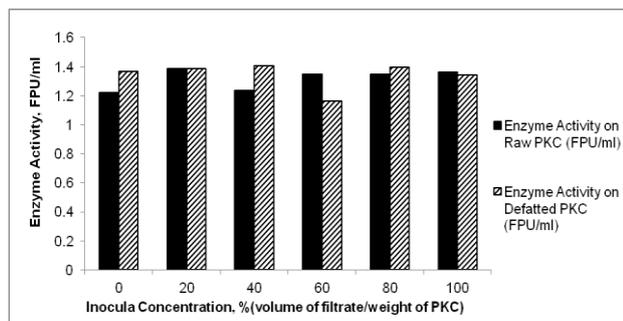
Figure 7: Cellulase activity of raw and defatted palm kernel cake solid state fermentation (PKC SSF) with different volumes of crude fungal filtrate
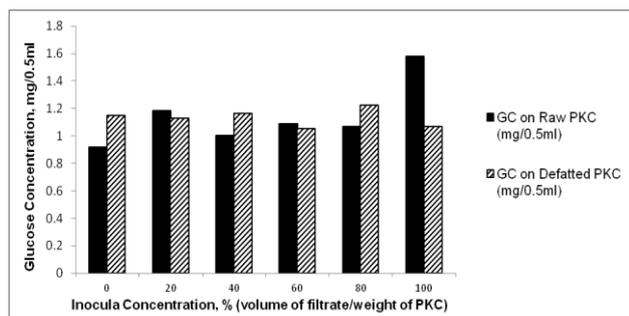
Figure 8: Glucose concentrations from raw and defatted palm kernel cake solid state fermentation (PKC SSF) with different volumes of crude fungal filtrate

Raw PKC originated from a palm oil factory. The oil residues in the PKC have been removed through expeller pressing; however about 6-8 % of residual oil remain in the PKC. The expeller pressing alters the palm kernel physical structure, flattening the cells and this provides the fungi greater ease to breakdown the cellulosic components and potentially enhancing the celulase

production (Roslan *et al.*, 2009). The defatted PKC had undergone oil removal via hexane digestion. The residual hexane in the palm kernel cake could have adverse effect on the microorganism such as fungi or deactivates enzymes produced by the fungi. Until recently, it was assumed that enzymes added to organic solvents would lose activity and likely to be denatured and this issues of enzyme inactivation in organic solvents has been reported and studied by Fagain *et al.* (2003) who have reviewed the bioreactor stability, shelf life and operational stability of a variety of enzymes suspended in a neat organic solvent and aqueous-organic solvent mix. This will explains why the defatted PKC gave lower cellulase activity compared to raw PKC.

Difference in Enzyme Activities between Mycelial Plug and Conidia as Inocula

The conidia inocula produced higher cellulase activity in defatted PKC than raw PKC. The enhancement of cellulase activity between raw PKC and defatted PKC was less than 1 % with conidia as inocula. This may be due to the chemical content within the defatted PKC sample. The usage of hexane in the solvent extraction process of removing the remaining oil in the PKC may change the bond, active sites or functional groups for the enzyme reaction. As reported by Mozhaev *et al.*, (1989) and Khmelnitsky *et al.*, (1991), the addition of increasing concentrations of organic solvents into aqueous solutions of proteins will always results in protein denaturation which show itself in a decrease of enzymatic activity and a sudden change of protein spectra. For example, enzymes lose their activity because the substrates can no longer bind to the active site and because amino acid residues involved in stabilizing substrates transition states are no longer positioned to be able to do so. Autoclaving happened to be a good alternative in improving the accessibility of the PKC for enzymes and to avoid too high sugar loss. In addition, autoclaving can act as sterilization thus minimized the risk of microbial contamination (Jose *et al.*, 2010).

Relationship between the production of glucose and cellulase activity by *Trichoderma* sp. on PKC

The enzyme production by fungi is more favored compared to bacteria when both are used on the oil cakes under solid state fermentation. This is attributed to the morphology and physiology of these molds which enable them to penetrate and colonize solid substrates (Sivaramakrishnan *et al.*, 2007). *Trichoderma* sp. exhibits higher cellulase activity to breakdown the PKC giving higher glucose. For example, with the usage of Trichoderma's 60 % (wt/wt) SMP to the PKC, it can improve the cellulase activity by more than 100 % and doubled the glucose concentrations if compare to the original PKC without inoculations.

Many fungi are able to produce extracellular enzymes that can act on polysaccharides. Cellulose is able to be

decomposed to cellobiose via long1-4 anhydroglucose chains by the extracellular enzymes of many Ascomycetes, Fungi Imperfecti and Basidiomycetes, especially the Homobasidiomycetes. The fungal stages of cellulose decomposition occur within the cell where cellobiase breaks down cellobiose to utilizable glucose units. So, more glucose can be detected. Lignin is thought to be decomposed mainly by Basidiomycete fungi, and the breakdown may be by the release of aromatic units from the lignin molecule. Further breakdown is probably carried out by a wider range of fungi (Smith and Berry, 1975).

CONCLUSION

Overall, raw PKC fermented with 60 % (wt/wt) solid mycelia plug to PKC gave the highest cellulase activity circa 2.454 FPU/mL an increase of more than 100 % in cellulase activity. Fermentation of defatted PKC with 12 x 10^6 conidia/mL yields the maximum cellulase activity of 1.856 FPU/mL with an enhancement of 46.5 % of cellulase activity. Crude fungal filtrate yield maximum increase in cellulase activity on raw and defatted PKC are 13.58 % and 2.86 % respectively using culture filtrate.

The current study clearly indicates that palm kernel cake can be used as one of the carbon sources for the production of cellulase activity by fungus *Trichoderma* sp. in the form of solid fungal mycelia plug, where more than 100 % increased cellulase activity can be observed. Fungal conidia are also a better choice in enhancing cellulase activity of *Trichoderma* sp. permitted that the PKC used is devoid of oil.

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