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Indirect Method for Quantification of Cell Biomass During Solid-State Fermentation of Palm Kernel Cake Based on Protein Content

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Abstract: Solid-State Fermentation (SSF) of *Aspergillus niger* FTCC 5003 on Palm Kernel Cake (PKC) is a practical approach to upgrade PKC into value added product. Present study was conducted on *Aspergillus niger* FTCC 5003 growth profile and models that are able to describe the growth in SSF using PKC substrate. Due to the difficulties of separating cell biomass quantitatively from the substrate for SSF systems, indirect method for measurement of cell growth during SSF of PKC by *Aspergillus niger* FTCC 5003 was studied based on the estimation of glucosamine and protein content. Preliminary relationships between glucosamine and protein contents to fungal dry cell weight (D_w) were developed using simulated homogenous SSF data using glass beads as support materials. Both glucosamine and protein contents were well correlated to the fungal dry cell weight in SSF on support materials for protein and glucosamine, respectively. The equations obtained were used for the estimation of cell biomass profile during SSF of PKC from the data of glucosamine and protein as growth indicator study. The estimated fungal dry cell weight based on protein concentration and β-mannanase activity as metabolic activity for microbial growth were well correlated to PKC dry weight which, indicating that both were suitable marker in describing the growth of *A. niger* FTCC 5003 in this system. In contrast, estimated fungal dry cell weight based on glucosamine concentration was not suitable to describe the growth of *A. niger* FTCC 5003.

Key words: Solid-state fermentation, palm kernel cake, *Aspergillus niger*, protein, glucosamine, dry cell weight (D_w)

INTRODUCTION

Solid-State Fermentation (SSF) is a process whereby microbes of interest will grow and utilise the moist substrate materials in the absence of free water. Many bacteria, yeast and fungi are able to grow on solid substrate and find application in SSF processes. However, filamentous fungi are the most important group of microorganism for SSF processes and dominate in research work owing to their physiological capabilities and hyphal mode of growth. During microbial growth, secretion of hydrolytic enzymes and production of other useful metabolites will upgrade the quality of low nutrient value materials such as PKC. Utilization and optimization of *Fungi* sp. in SSF for PKC digestibility

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improvement have been extensively studied by Noraini *et al.* (2000) and Jaafar *et al.* (2001). Previous study showed that *Aspergillus niger* FTCC 5003 was a suitable microbe for further investigation due to its ability in the depolymerisation of PKC fibre (Noraini *et al.*, 2001).

There are different types of techniques available in measuring the growth of microorganism in SSF. Different direct biomass determination such as matrix removal methods by using gelatine matrix, enzyme digestion and membrane filter has been used as an attempt for recovery of fungal biomass in SSF. However, foreign substance and non-digestible residue were found to interfere with the measurement for methods using gelatine matrix and enzyme digestion. The method of direct peeling off the membrane filter for biomass recovery was reported to be able to prevent the penetration of the fungal hyphae, *Rhizopus oligosporus* into the substrate (Mitchell *et al.*, 1989). However, this method obviously cannot be used in actual SSF but could find application in the calibration to indirect method of biomass determination.

Biomass is a fundamental parameter in the characterisation of microbial growth. Its measurement is essential for kinetic studies on solid-state fermentation. Complete recovery of fungal biomass from the substrate is very difficult in solid-state fermentation because the fungal hyphae penetrate into and binds tightly to the solid substrate particles. Many authors have described indirect methods to estimate biomass in solid-state fermentations. These indirect methods are based on metabolic measurement such as respiratory metabolism (Saucedo-Castañeda *et al.*, 1990; Smits *et al.*, 1996), extracellular enzymes (Smits *et al.*, 1996; Mitchell *et al.*, 1991) and organic acids (Soccol, 1992) or specific component measurement like protein content (Saucedo-Castañeda *et al.*, 1990; Favela-Torres *et al.*, 1998), glucosamine (Ooijkaas *et al.*, 1998; Papagianni *et al.*, 2001), ergosterol (Nout *et al.*, 1987) and nucleic acids (Bajracharya and Mudgett, 1980).

The content of the different cell components can be used to estimate the biomass as long as the composition of the biomass is constant and stable. Protein content is the most readily measured biomass component. Raimbault and Alazar (1980) used soluble protein content to measure the growth rate of *A. niger* on cassava meal. Mycelia biomass from solid-state fermentation was determined indirectly from measurements of soluble proteins contents (Favela-Torres *et al.*, 1998). Glucosamine is a useful compound for the estimation of fungal biomass as it is an essential and stable component in chitin of mycelia cell walls. Although the proportion of chitin in the mycelium will vary with age and the enviroumental conditions; Desgranges *et al.* (1991) reported that this parameter was reliable for solid-state culture carried out in media containing the same components, regardless of their concentrations. Glucosamine was used as an indirect method for biomass determination and an efficient parameter for growth in solid-state fermentation (Papagianni *et al.*, 2001; Krishna and Nokes, 2001).

The objective of the present study was to develop correlations for cell growth determination during SSF of PKC by *A. niger* FTCC 5003 based on estimation of glucosamine and protein content.

MATERIALS AND METHODS

Microorganism

Aspergillus niger FTCC 5003 provided by Food Technology Centre, MARDI, Malaysia was used throughout this study. The preserved stock culture of *A. niger* FTCC 5003 was grown on the sterilised potato dextrose agar slants and incubated at 30±2°C for three days. The spores were collected with sterilised distilled water containing 0.01% (v/v) Tween 80. The spores numbers were obtained via total cell count method using haemocytometer and the spore size were fixed at 10^7 spores mL^{-1}.

Solid-State Fermentation on Support Material

In order to clarify that the glucosamine and protein determined solely came from the fungus, simulated homogenous SSF on support material was carried out in 250 mL Erlenmeyer flasks,

containing 40 g of glass beads, 2% of mannose solution and 1% nitrogen source from urea. The flasks with glass beads were sterilised at 121°C, 15 psi for 15 min prior to inoculation. After cooling, 9.9 mL of mannose solution, 0.1 mL urea solution and 1 mL of spore suspension were added aseptically. The glass beads, inoculum, mannose and urea solution were then shaken to thoroughly mix the system. The flasks were incubated at 30°C for 9 days fermentation time. Samplings were done in triplicates.

Solid-State Fermentation on Palm Kernel Cake

Solid-state fermentation was carried out in 250 mL Erleumeyer flasks, containing 30 g of PKC and 1% nitrogen source from urea. The flasks with substrate were steam sterilised at 121°C, 15 psi for 15 min prior to inoculation. The media was then allowed to cool down; appropriate amount of sterilised distilled water and 6 mL of 3 days old spore suspension were added. The substrate, inoculum and water were manually mixed aseptically with sterilised spatula. The flasks were incubated at 30°C for 10 days fermentation time.

Fungal Biomass Harvesting from Solid-State Fermentation on Support Material

The fungal biomass from the SSF on support material was harvested by adding 10 mL sterilised distilled water. Three milliliter of sample suspension were withdrawn and kept at 4°C for protein and β-mannanase analysis.

Sampling

Samples were collected triplicate every day for 10 days fermentation. The fermented solid material was manually mixed under aseptic condition. The collected samples were used for PKC dry weight determination, protein concentration and glucosamine concentration analysis.

Analytical Procedures

Fungal Dry Cell Weight (D_w) Determination for Solid-State Fermentation on Support Material

For fungal dry cell weight analysis, the suspension after removal of 3 mL for protein analysis was then filtered through pre-weight Whatman filter paper No. 1. The fungal biomass was washed with sterilised distilled water and it was repeated until the filtrate was clear. Then, the residue was vacuum filtered through a pre-weight 0.2 μm membrane filter. For dry cell weight measurement, the filters were dried in an oven until constant weight and re-weighed after cooling the filters in a desiccator as described by Ooijkaas *et al*. (1998).

PKC Dry Weight (D_w) Analysis for Solid-State Fermentation on Palm Kernel Cake

Empty flask was weighed prior to incubation. Every day, the flask containing fermented material was weighed to obtain total wet weight (ww) by calculating the difference between weight of flask with fermented material and empty flask weight. Approximately 40 g of fermented material from each sample (in triplicate) were dried in oven until constant dry weight was achieved. The PKC dry weight (D_W) is defined using Eq. 1:

$$\text{PKC } D_W = \text{Total wet weight} \times \frac{\text{Constant dry weight (g)}}{\text{40 g (ww)}} \quad (1)$$

Protein Assay

Thirty milliliter of sterilised distilled water was added into the flask containing approximately 3 g (ww) of wet sample. Cool extraction was carried out by orbital shaking at 140 rpm, 4°C for 24 h. Ten milliliter of slurry was centrifuged at 5000 rpm for 10 min. The filtered supernatant was then

digested with 1 N NaOH at 1:1 ratio, where the digestion was carried out for cell breakage and protein solubilisation at 100°C for 15 min. After cooling, the soluble protein concentration was determined by Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard.

Glucosamine Assay

Five milligram of dry sample was hydrolysed with 5 mL of 6 M HCl in glass vial with screw cap at 100°C for 4 h. After the acid hydrolysis, the sample was dried under vacuum at 70°C in a rotary evaporator. The dried residue was dissolved in 5 mL deionised water and the solution was then analysed using HPLC by sugar sp0810 column (Shodex, 8 mm ID 300 mm L). Deionised water was used as mobile phase with a flow rate of 1.0 mL min^{-1} and column temperature was maintained at 70°C. Peak was detected with a reflective index detector (Jasco, RI-1530) and commercial glucosamine (Sigma, G4875) was used as standard.

Endo-1,4-β-D-Mannanase Activity Assays

Beta-mannanase activity was measured by method described by Michael (2000) using azo-carob galactomannan (2%) diluted in 2 M sodium acetate buffer, pH 4.5 as substrate.

Statistical Analysis

The correlation coefficient was used to determine the relationship between two properties in present study. The equation for the correlation coefficient is shown in Eq. 2:

$$\rho_{x,y} = \frac{\text{Cov}(x,y)}{\sigma_x \ \sigma_y} \tag{2}$$

Where:

$$-1 \le \rho \le 1 \quad \text{and} \quad \text{Cov}(x,y) = \frac{1}{n\sum_{i-1}^{n}(x_i - \mu_x)(y_i - \mu_y)}$$

RESULTS AND DISCUSSION

An initial study of simulated homogenous SSF using glass bead as support materials has been carried out as to obtain information of microbial growth and complete recovery of fungal biomass during SSF on PKC. The complete recovery of fungal biomass was also aimed to find application for biomass estimation from indirect method of biomass determination during SSF on PKC.

Solid-state fermentation using support material had several potential applications in scientific studies and industrial process. Due to its less complicated product and biomass recovery, SSF on support material offers an advantage for easier recovery of biomass from the support material with fewer impurities compared with the natural substrate (Ooijkaas *et al*., 1998). Solid-state fermentation on support material impregnated with defined media has been carried out. The fungal dry weight, protein and glucosamine content in SSF on support material were determined in order to obtain a suitable relationship for biomass estimation of *Aspergillus niger* FTCC 5003.

Figure 1 shows the relationship between fungal dry cell weight and protein concentration, while the relationship for glucosamine content is shown in Fig. 2. For both cases, the evidence of a straight line relationship were observed and the statistical analysis for the relationship is shown in Table 1. Based on statistical analysis, strong correlation values have been observed between fungal dry cell weight and protein concentration (0.997) and between fungal dry cell weight and glucosamine concentration (0.962), respectively.

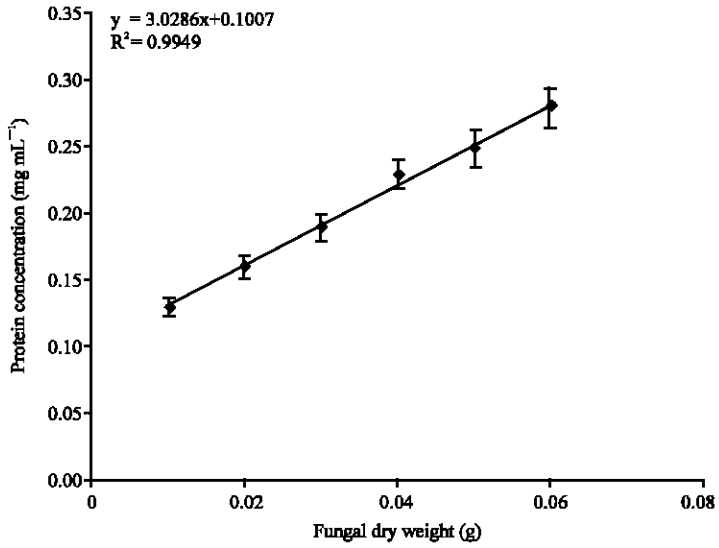

Fig. 1: Relationship between fungal dry weight and protein concentration

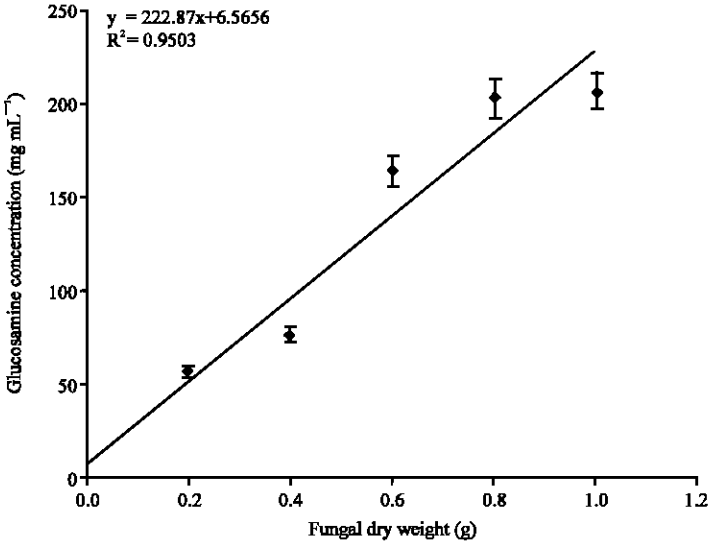

Fig. 2: Relationship between fungal dry weight and glucosamine concentration

Both protein and glucosamine content were well correlated to the fungal dry cell weight in SSF on support material, indicating that protein and glucosamine were suitable calibration methods for indirect biomass estimation in systems that have similar conditions such as SSF on PKC. Therefore, Eq. 3 and 4 (Table 1) were used in the indirect biomass estimation for growth of *Aspergillus niger* FTCC 5003 in SSF using PKC as substrate.

The typical growth pattern of *Aspergillus niger* FTCC 5003 in SSF on PKC which estimated fungal dry cell weight was based on protein and glucosamine are shown in Fig. 3 and 4, respectively. It were observed that the protein and glucosamine concentration increasing as the fermentation time

Table 1: Statistical analysis for fungal dry cell weight in relation to protein concentration and glucosamine concentration

	Fungal dry weight and protein concentration	Fungal dry weight and glucosamine concentration
Equations	$\text{Fungal } D_W = \frac{(\text{Protein concentration} - 0.10)}{3.03}$ (Eq. 3)	$\text{Fungal } D_W = \frac{(\text{Glucosamine concentration} - 6.57)}{222.89}$ (Eq. 4)
Correlation coefficient	0.997	0.962
R^2	0.995	0.926

Experimental unit, n = 54

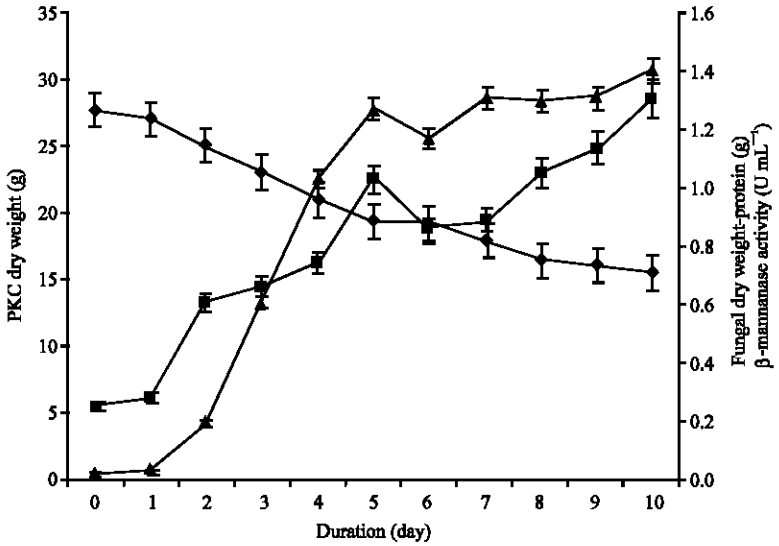

Fig. 3: *Aspergillus niger* FTCC 5003 growth profile in SSF on PKC which estimated fungal dry weight was based on protein concentration (-■-) fungal dry weight, (-◆-) PKC dry weight and (-▲-) β-mannanase activity

increased for both fungal dry weights. Estimated maximum fungal dry weight at day 10 was 1.297 and 0.632 g based on protein and glucosamine concentration, respectively. It shown that β-mannanase as part of the metabolite in the system increased gradually while the PKC dry weight as substrate in the system decreased proportionally with fermentation time.

Table 2 shows the statistical analysis for PKC dry weight in relation to β-mannanase activity and estimated fungal dry cell weight based on the protein and glucosamine concentration. Based on statistical analysis, correlation value of 0.970, was observed between palm kernel cake and β-mannanase activity; on the other hand, estimated PKC and fungal dry cell weight based on protein concentration showed a correlation value of -0.967. This suggested that the growth of *Aspergillus niger* FTCC 5003 in this system was well described by both estimated fungal dry cell weight based on protein concentration and β-mannanase activity.

The statistical analysis showed that the correlation value between PKC and estimated fungal dry cell weight based on glucosamine concentration was -0.299, indicating that the estimated fungal dry cell weight based on glucosamine concentration did not reflect the growth of *Aspergillus niger* FTCC 5003 in this system. Ooijkaas *et al.* (1998) reported that glucosamine content was not suitable to be used as biomass indicator for their study because the content of mycelia increases during fungal development was cause by the resistance of chitin to breakdown after fungal death. The chitin accumulated in the

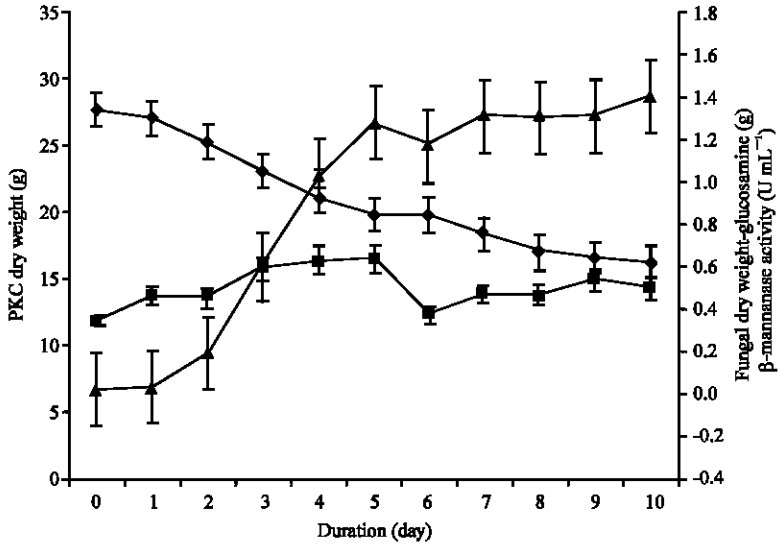

Fig. 4: *Aspergillus niger* FTCC 5003 growth profile in SSF on PKC which estimated fungal dry weight was based on glucosamine concentration (-■-) fungal dry weight, (-◆-) PKC dry weight and (-▲-) β-mannanase activity

Table 2: Statistical analysis for palm kernel cake dry weight in relation to β-mannanase activity and estimated fungal dry cell weight based on protein and glucosamine concentration

PKC in to:	β-mannanase activity	Fungal D_w estimated via protein concentration	Fungal D_w estimated via relation glucosamine concentration
Correlation coefficient	0.970	-0.967	-0.299
R^2	0.941	0.936	0.089

Experimental unit, n = 120

empty ghost hyphae and caused increase of glucosamine content that resulted in the inaccuracy of glucosamine for biomass estimation that do not reflect the growth of *Coniothyrium minitans* in solid-state fermentation.

The relationships between PKC and estimated fungal dry cell weight based on protein concentration and between PKC and beta-mannanase activity well described the growth pattern of *Aspergillus niger* FTCC 5003 in this system. Kinetic study was then performed to establish a model that able to describe and simulate the *Aspergillus niger* FTCC 5003 growth under various conditions. The estimated fungal dry cell weight based on glucosamine concentration was less suitable to described the growth of *Aspergillus niger* FTCC 5003 in SSF using PKC as substrate but the result obtained were still be used in the kinetic and modelling study as comparison parameter to estimate fungal dry cell weight based on protein concentration.

CONCLUSIONS

Correlations between protein and glucosamine contents and the dry cell weight of *A. niger* FTCC 5003 have been developed. These correlations were successfully used in the estimation of growth profile of *A. niger* FTCC 5003 during SSF using PKC. However, the correlation based on protein content gave better accuracy than glucosamine content.

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