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# Effect of Various Cultivation Methods on Cellobiohydrolase Production from *Aspergillus niger*

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***Abstract*— Three different cultivation methods, i.e., shaking-flask culture (SFC), static surface liquid culture (SSLC) and membrane surface liquid culture (MSLC) were used to cultivate the filamentous fungi, *Aspergillus niger* PY11 in order to differentiate its behaviours by different cultivation methods using the same media, by determination of growth profile during fermentation. The dry cell weight, protein concentration, cellobiohydrolase (CBH) activity and residual sugar concentration in SSLC and MSLC were 1.5 to 2-folds than that by SFC. Cultivation of *A. niger* using MSLC was higher than SSLC with maximum biomass concentration, protein concentration and enzyme activity were 0.93 g dry weight/ml medium, 3.49 mg/ml and 36.99 U/ml respectively. MSLC possess the best growth characteristics and was the best cultivation method in production of CBH from *A. niger* PY11.**

***Keywords*— filamentous fungi; *Aspergillus niger*; cellobiohydrolase; membrane-surface liquid culture; static culture.**

## I. INTRODUCTION

Fermentation of filamentous fungi and their applications have been extensively studied, in recent years. In the field of biotechnology, filamentous fungi are widely utilized in the production of heterologous proteins, organics acids, enzymes, pigments in addition to the production of fermented foods and beverages [1]. Production of cellulases from filamentous fungi has attracted a world-wide attention due to the possibility of using this enzyme complex in many industrial applications such as in food, textile and paper industry. The complex mixture of cellulase is a combination of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase / cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) [2].

The fermentation systems of filamentous fungi still encounter many serious challenges and difficulties especially in cultivation methods. Filamentous fungi are generally belonging to absolute aerobe with aerial mycelia and spores and require strong oxygen for growth. Filamentous fungi usually cultivated by solid substrate fermentation (SSF) [3], [4], [5], [6] and submerged fermentation (SMF) [7], [8] to produce cellulase enzyme. In SSF, filamentous fungi naturally grow on solid substrates and are able to thrive on relatively low humidity, extending long, thin, branched threads of mycelium, which form spores when nutrients are scarce. SMF is usually used to cultivate filamentous fungi due to their ease of medium preparation, control of pH and temperature and recovery of products [9]. Until now, various conditions of both cultivation techniques have been studied to improve the production. However, there are many challenges in fermentation techniques. SMF is easier, but the quality of product is not good because the molds may be damaged by shear stress, while the cells in the interior of pellets are apt to go autolysis because of oxygen deficiency [10]. Although SSF can produce higher levels of enzyme, there are restrictions for production in bioreactor, the medium is restricted to natural agricultural products, and the production rate is usually limited by mass transfer inside the solid substrate [1].

A fermentation which statically on the surface facing the air but using liquid medium is proposed to overcome the disadvantages of SSF and SMF. SSLC is a method where the fermentation biomass culture is made to grow on the surface of fermentation media and does not require vigorous shaking or physical agitation due to the facts that fungi are filamentous in nature and agitation may break their mycelia [11]. While MSLC is the fermentation which molds are grown on porous membrane that is in contact with the liquid culture facing the side opposite air [10].

In this study, we cultivated *A. niger* PY11 by SFC, SSLC and MSLC under the same cultivation conditions and measured growth, to differentiate the behaviours of *A. niger* by different cultivation methods in order to understand and quantify advantageous features of MSLC in CBH production.

## II. Materials and Methods

### A. Microorganism and Media

A recombinant *Aspergillus niger* PY11 that carrying *cbh1* gene of *Trichoderma virens* UKM1 was obtained from the Molecular Biology Laboratory, Faculty of Science and Technology, Universiti Kebangsaan Malaysia.

The Complete Media (CM) agar which was used to grow the fungus at 30°C consists of (per litre); 50 ml nitrate salt solution, 5 ml 2.25 M magnesium sulphate solution, 10 g dextrose, 1 g casamino acid, 1 ml vitamin solution and 7.5 g agar. The spore was harvested after 4 days of cultivation by diluting it with sterile distilled water. The required amount of spores of $1x10^8$ per ml in 1% Tween 80 solution, was used as inoculums with CM as the inoculum medium. The spore number was estimated by direct microscopic counting using haemocytometer [12].

The fermentation medium is Minimal Medium J (MMJ) [13] containing (per litre): 20 ml 2.25 M magnesium sulphate solutions, 200 ml nitrate salt solution, 4 ml Hunter's Trace Element solution and 150 g maltose. The medium was sterilized at 121°C for 20 minutes and the pH of medium was adjusted to certain pH before starting off the fermentation.

### B. Cultivation Methods

SFC was started by inoculating 10 % (v/v) spore suspension *A. niger* ($1x10^8$ spores/ml) in 250-ml conical flasks containing 50 ml MMJ on an incubator shaker with 120 rpm for 10 days. The temperature in the incubator shaker was controlled at 30°C. SSLC and MSLC were conducted in 150 mm-diameter of petri dishes using the same spore concentration, volume of inoculums and medium as used in SFC. A cotton net with same diameter with petri dishes which acted as membrane was used on the surface of MMJ in MSLC. The dishes of SSLC and MSLC were incubated statically at 30°C in a sterile fermentation box with wet cotton cloth were placed at the bottom of the box to maintain humid environment during cultivation.

For each SFC, SSLC and MSLC cultivation were done simultaneously, and one of the cultures was withdrawn for analysis at appropriate times during the course of cultivation. The mycelium was separated from the fermentation broth through Whatman No. 1 filter paper for biomass determination. The filtrates were kept further analysis and spectrophotometric assay.

### B. Analytical Methods

The growth of the molds was estimated from the dry cell weight of the mycelia. After filtration of the broth, mycelia recovered were dried at 80°C for 24 hours to a constant weight, and the weight was measured.

Protein assay was performed using method described by [14] and bovine serum albumin (BSA) was used as a standard. 1 ml of Bradford reagent was added to 100 µl of sample and the absorbance was measured after incubation for 30 minutes at wavelength 595 nm.

The concentration of total reducing sugars of the culture broth was measured by the dinitrosalicylic acid (DNS) test as described by [15]. A 2-ml volume sample was mixed with 1 ml DNS reagent. The yellow colour of reagent changed to brown after being heated on the heating block at 100°C for 15 minutes. Changes in the absorbance were measured using spectrophotometer at 540 nm.

The culture filtrate, which was diluted appropriately, was analyzed to determine CBH activity in pH 3.5 acetate buffer by a spectrophotometric method using p-Nitrophenol Cellobioside (p-NPC) as a substrate [16]. In a typical analysis, a 0.5 ml aliquot of the sample, diluted appropriately with 0.1 M acetate buffer, was mixed with 1.5 ml p-NPC and the reaction mixture incubated at 37°C for 15 minutes. One unit of enzyme activity was defined as the amount of enzyme required to release 1 µmol of p-nitrophenol from the substrate at pH 3.5 and 37°C. All assays were carried out in triplicate and the results were presented as mean of the triplicate experiments.

## III. Result and Discussion

Influence of mode of fermentation on CBH production was examined by studying the growth pattern of cultivated *A. niger* using SFC, SSLC and MSLC. The growth profile and fermentation process was monitored for as dried cell weight, protein concentrations, enzyme activity and residual maltose remained in the medium. The growth profile was studied to monitor the production pattern of CBH in order to elucidate the differences of its behaviors by different cultivation methods. Figure 1, 2, 3 and 4 show the typical time courses of *A. niger* in SFC, SSLC and MSLC.

Figure 1 shows the comparison of cell growth using the three different cultivation methods applied. The changes in the cell growth showed a similar tendency for both SSLC and MSLC. Dry cell weight (DCW) increased earlier in SFC than SSLC and MSLC. The maximum DCW was obtained from MSLC after 8 days of fermentation with 0.93 g dry weight/ml medium. DCW increased in the early stage and then tended to plateau after maximum value obtained.

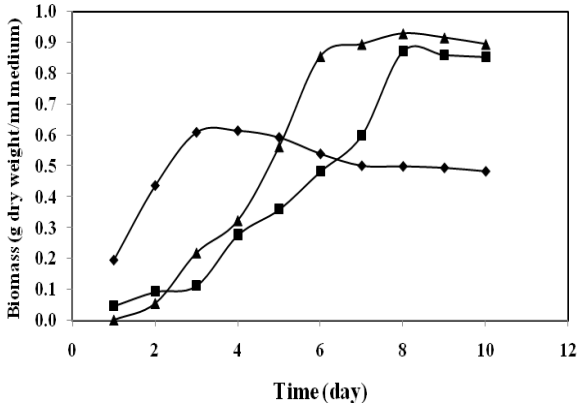


Fig. 1 Growth profile of dry cell weight during CBH production from *A.niger* at 30°C.
Symbols: ♦, SFC; ■, SSLC; ▲, MSLC.

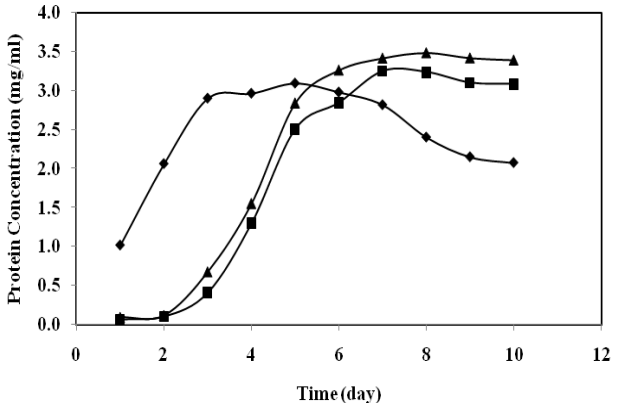


Fig. 2 Growth profile of protein concentration during CBH production from *A.niger* at 30°C.
Symbols: ♦, SFC; ■, SSLC; ▲, MSLC.

The protein secretion levels clearly showed in figure 2 that SSLC and MSLC achieved higher maximum protein concentration compared to SFC. This indicates that the amounts of protein secreted in SSLC and MSLC were slightly higher than that in SFC. The maximum protein concentration was around 3.5 mg/ml after 8 days of cultivation in MSLC.

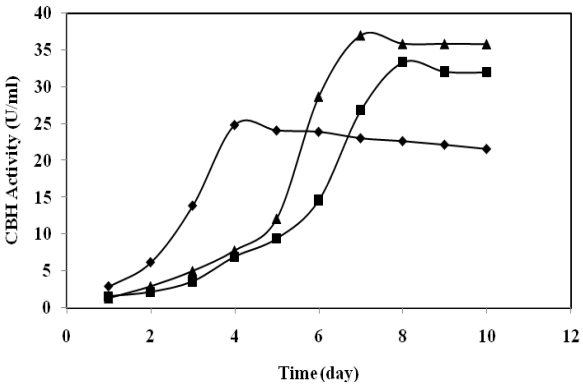


Fig. 3 Growth profile of enzyme activity during CBH production from *A.niger* at 30°C.
Symbols: ♦, SFC; ■, SSLC; ▲, MSLC.

CBH enzyme profile was examined under SFC, SSLC and MSLC for 10 days at 30°C. Enzyme activity was maximized after 4th day of fermentation in SFC, earlier than SSLC and MSLC which took 7-8 days to reach the peaks. The highest enzyme yield was registered with MSLC with the activity was 36.99 U/ml and almost two-fold when compared to SFC. In MSLC and SSLC, static fermentation was found to be better than shaking mode applied in SFC. This observation may be attributed that agitation may break the mycelia of filamentous fungi.

Figure 4 shows the profile of residual sugar concentration during CBH production. The maltose concentration decreased rapidly in the early stage of cultivation and reached zero at the later stage in every cultivation method. Maltose was consumed earlier in SFC compared to SSLC and MSLC. But, in MSLC maltose concentration decreased rapidly and approached zero after 4th day of cultivation.

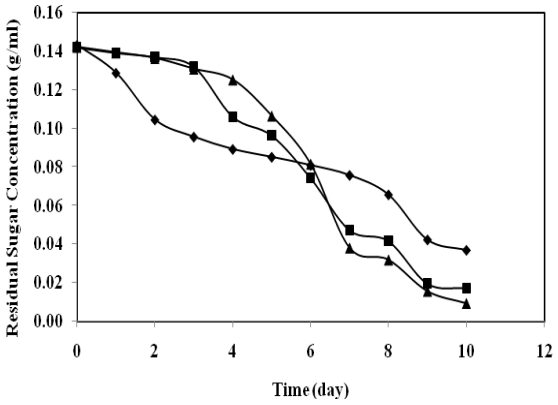


Fig. 4 Growth profile of residual sugar concentration during CBH production from *A.niger* at 30°C.
Symbols: ♦, SFC; ■, SSLC; ▲, MSLC.

## IV. Conclusion

The production of CBH from *A. niger* was higher in MSLC in comparison with SFC and SSLC. MSLC is an energy-saving process, since the amounts of energy required for agitation and aeration is negligible and downstream processing is simple because the medium is not contaminated with cells.

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