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Preliminary Study of Cellobiohydrolase Production from Aspergillus niger Using Static Liquid Culture Fermentation

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Abstract: This study was aimed to investigate the production of recombinant cellobiohydrolase (CBH) in static liquid culture (SLC) fermentation using newly developed recombinant of *A. niger*. Different environmental parameters and culture conditions were used to attain good cultivation of *A. niger* in SLC fermentation and highest level of CBH production. The fermentation results were evaluated from CBH production, total biomass, total protein and the residual main carbon source, maltose after 9 days of cultivation. The CBH production was found effectively enhanced under optimized cultivation temperature, initial pH, inoculums size and pre-shaked inoculums. Maximum biomass, protein concentration and enzyme activity were determined as 1.5 g dry weight/ml medium, 3.5 mg/ml and 40 U/ml respectively. The results also show that SLC could enhance production of the recombinant CBH.

Key words: *Aspergillus niger*; cellobiohydrolase production; pre-shaked inoculums; static liquid culture.

INTRODUCTION

Production of cellulases and their properties have been extensively studied during recent years. Cellulases are among the industrially important hydrolytic enzymes which break down cellulose, the main component of the cell walls, into β-glucose. They are widely used in food, feed, textile, pulp and paper industries (Nakari and Pentilla, 1995) and important in the economical production of bioethanol, single cell proteins and other chemicals. The microbial conversion of cellulosic / lignocellulosic biomass into useful fermentable products is a complex process involving a combined action of three enzymes namely endoglucanase (EC 3.2.1.4), exoglucanase / cellobiohydrolase (CBH) (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21) (Erikson and Patterson, 1975). The development of microbial strains, media composition and process control have all contributed to achievements of high levels of extra cellular accumulation of cellulases for subsequent applications in industrial processes (Gosh, 1987).

The fermentation system for filamentous fungi presents special challenges in optimization and scale-up because of the variation of fungal morphological form compared to many unicellular microbes (Wang, *et al.*, 2005). Fungal morphology has distinct effect on the rheological nature of a fermentation broth. Filamentous fungi usually cultivated by solid-substrate culture using water insoluble products such as steamed rice and wheat bran to secrete various metabolites and enzymes. Some heterologous proteins have shown some improvement to produce at much higher levels by varying the cultivation technique instead of using common submerged liquid fermentation (Imanaka, *et al*., 2010).

Static liquid culture (SLC) is a method where the fermentation biomass culture is made to grow on the surface of fermentation media, SLC does not require vigorous shaking or physical agitation due to the facts that *fungi* are filamentous in nature and agitation may break their mycelia (Jacob and Prema, 2006). Although a relatively long period is required to complete fermentation, for large scale operation of this technique, the factory investment is not costly and product quality of targeted protein is good (Nanda, *et al*., 2001). SLC fermentation is an alternative process to submerged fermentation due to its low production cost and labor requirements. Some essential criteria such as less process sensitivity to short interruptions such as breakdown in aeration, expenses for equipment, consumption of electrical energy, and higher yield and productivity, showed that SLC is more superior to submerged fermentation (Darouneh, *et al*., 2009).

According to (Ray, *et al.,* 2007), regardless of the fermentation process that is used to grow cells, it is necessary to monitor and control parameters including inoculums volume, pH, temperature, incubation period etc. Changes in one of these parameters can have a dramatic effect on the yield of cells and the stability of protein products. The high rate of metabolism supports the critical period of metabolite production. Converti and

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Dominiguez (Converti and Domininguez, 2001) suggested that the phenomenon limiting CBH production could be enzymatic reaction/s under varying fermentation conditions as observed for other enzymes.

In this study, the SLC of *A. niger* was used to produce recombinant cellobiohydrolase (CBH) using maltose as carbon source. Different environmental parameters such as cultivation temperature and initial pH, and fermentation conditions such as inoculums size and pre-shaked inoculums were investigated to determine optimum data for maximum CBH production.

MATERIALS AND METHODS

Microorganism:

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. *A. niger* was selected as an expression host since this type of organisms has the ability to secrete high level of proteins into the culture medium. *A. niger* is categorized a Generally Regarded as Safe (GRAS) microorganism in the food and food processing industries (Wang, *et al.*, 2005).

Inoculum Preparation:

The fungus was periodically subcultured and maintained on Complete Media (CM) agar. The CM agar 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, was used to grow the fungus spore in 30^{o}C. 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, were used as inoculums with CM as the inoculums medium. The spores' number was estimated by direct microscopic counting using haemocytometer (Haddad and Lindegren, 1953).

Fermentation Medium:

The SLC fermentation medium is Minimal Medium J (MMJ) (Kafer, 1977). Each litre contains: 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^{o}C for 20 minutes and the pH of medium was adjusted to certain pH before starting off the fermentation.

SLC Fermentation:

A. niger at 1x10^{8} spores was cultivated in 90 mm-diameter of petri dishes. These dishes were incubated at 30^{o}C in a sterile fermentation box with wet cotton cloth were placed at the bottom of the box to maintain humid environment during cultivation.

Analytical Methods:

The mycelium was separated from the fermentation broth through Whatman No. 1 filter paper for biomass determination. The filtrates were kept further analysis and spectrophotometrically assay (Deshpande, *et al.,*1984). 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^{o}C for 24 hours to a constant weight, and the weight was measured.

Protein assay was performed using method described by Bradford (Bradford, 1976) and bovine serum albumin (BSA) was used as a standard. 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 using glucose as a standard (Miller, 1959). 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 substrate (Deshpande, *et al.,* 1984). 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^{o}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 4.0 and 37^{o}C. All assays were carried out in triplicate and the results were presented as mean of the triplicate experiments.

RESULTS AND DISCUSSION

Time Course of CBH Production:

The time course study to monitor the production pattern of CBH and to determine the period were conducted for maximum value to achieve in order to optimize the parameters and conditions of fermentation. The cultures were grown in the time course studies to determine the minimum time for induction, other kinetic parameters and biosynthesis of enzyme and the cell mass (Hanif, *et al.,* 2004). The growth pattern of cultivated *A. niger* was

observed and fermentation process was monitored for as dried cell weight, protein concentration, enzyme activity, pH and residual maltose remained in the medium. The fermentation was carried out for 12 days to investigate the CBH production. Several petri-dish cultures were done simultaneously and one culture was taken at appropriate times for analysis during the course of cultivation.

The results of SLC fermentation of $1x10^8$ spores in petri dishes at 30°C and initial pH 6.5 was presented in Figure 1. The maximum CBH production levels were reached after 9 days with maximum activity was 40.1 U/ml although maximum protein concentration of 3.3 mg/ml occurred earlier at 7th days of the fermentation. The growth of the molds was estimated from the dry cell weight of the mycelia. Mycelia formed on the surfaces of medium could easily be removed. The maximum dried biomass produced was 0.87 g dry weight/ml medium on day 9, while the doubling time of the growth was 6.5 days.

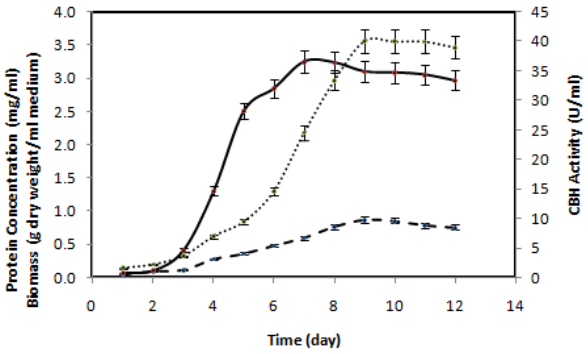

Fig. 1: Growth profile of protein concentration (—), biomass (---), and CBH activity (···) during CBH production from *A. niger* at 30°C. The error bars represent the averages of three samples.

From Figure 2, pH value decrease slightly from pH 6.5 to pH 5.3 at the beginning of fermentation until 5th day and increased afterwards to pH 8 until the end of fermentation. This was probably associated with the accumulation of organic acids during fermentation (Lin and Chen, 2004). The results appeared to be a positive correlation between the increasing pH and activities of CBH (Lin and Chen, 2004). The increasing rate of pH was maximum on day 6 could be due to more nitrogenous wastes being produced as indicated by the high utilization maltose rate of as suitable carbon source by the fungi and eventually led to the rapid increase of pH (White, *et al.*, 2002). The residual sugar was totally depleted by the 12th day fermentation and at that time the biomass started to reduce as it approaching to deceleration growth of less food available.

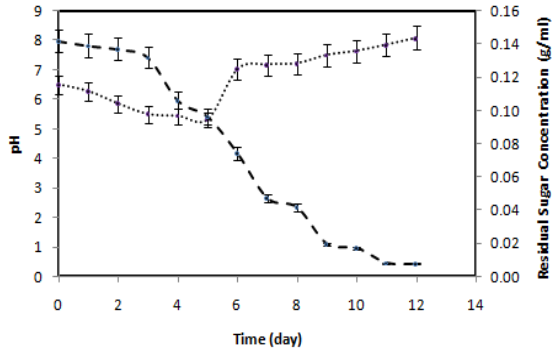

Fig. 2: Changes of pH (···) and residual sugar concentration (---) during CBH production from *A. niger* at 30°C. The error bars represent averages of three samples.

Effects of Incubation Temperature and Initial pH:

It is known that temperature is one of the most critical parameters that have to be controlled during fermentation process. The effect of incubation temperature on CBH production is shown in Figure 4 and was examined at initial pH 6. The samples were harvested after 9 days of fermentation for analysis. Five temperatures in the range 24-36°C were tested. From Figure 4, similar pattern of enzyme production profiles were exhibited at all different incubation temperatures used. The excretion rates of CBH were about the same during the first 7 days and slowly reduce thereafter. The CBH production achieved maximum level 38.1 U/ml after 9 days at fermentation temperature of 30°C. Different incubation temperatures used in the fermentation process had showed

no great influence on CBH activity with maximum activity (U/ml): 23.9, 33.1, 35.2 and 32.0 at 24°C, 28°C, 32°C and 36°C respectively. This possibly due to the narrow range of incubation temperatures used (Lee, *et al.*, 2011).

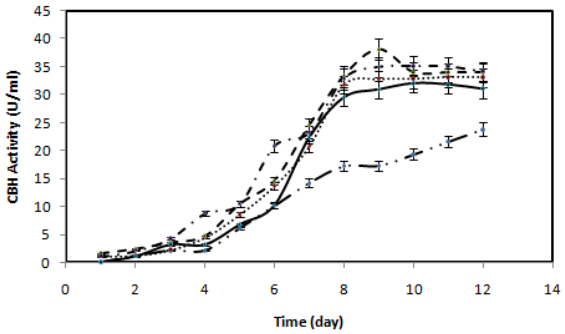

Fig. 3: Effects of incubation temperature (24°C (-··-), 28°C (···), 30°C (---), 32°C (-·-) and 36°C (—)) on CBH production from *A. niger*. The error bars represent the averages of three samples.

The initial pH affected the production of CBH. The effects of initial pH of medium on CBH activity shown in Figure 3 were studied at incubation temperature 30°C and in the range of pH 4-8. The samples were harvested after 9 days of fermentation for analysis. Maximum activity of CBH occurred at initial pH 6 with 35.1 U/ml and CBH activity was slowly declined when the initial was pH 7. Enzyme activity was lower when pH of the medium more acidic or more basic. The instability of this enzyme at very low or very high pH value was generally denatured at extreme pH value (Steiner, *et al.*, 1994)

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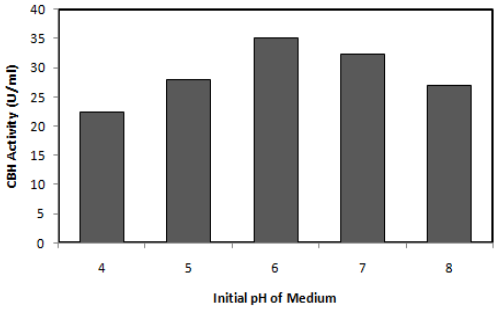

Fig. 4: Activity of CBH produced by *A. niger* at 30°C with varying initial pH. Data presented were the averages of three samples.

Effects of Pre-Shaked Inoculums:

Pre-shaked inoculum was prepared by agitating the initial inoculated fermentation broth for 2 days in a conical flask at 150 rpm and 30°C before pouring into petri dishes for cultivation process. Figure 5 shows the effects of using pre-shaked inoculums for production of CBH. Two sets of experiment were carried out to be compared; SLC using pre-shaked inoculums and SLC without using pre-shaked inoculums. The maximum CBH production levels (37.1 U/ml) were reached 10 days fermentation using pre-shaked inoculums. Agitation increased the amount of dissolved oxygen and dispersion of macromolecules in the inoculums and medium. It might, therefore, have contributed to the greater growth and better enzyme production.

Effect of Inoculums Size:

Previous investigations by (Lee, *et al.,* 2004;Park, *et al.*, 2001) shown that inoculums size influenced the mycelia growth and production. Reference (Abd-Aziz, *et al.*, 2008) also reported that the inoculums size was one of the important factors influenced production. CBH production by *A. niger* increased steadily when the inoculums size was increased as shown in Figure 6. Optimum CBH produced when the medium received 8 % (v/v) of inoculums with maximum enzyme activity 32.7 U/ml and a further increased of inoculums up to 12 % (v/v) showed decreasing trend. A great quantity of inoculums enhances the production. However, (Assamoi, *et al*., 2008) said it is not the best solution to improve the production. Indeed, with a low quantity of inoculums, the entire medium will be colonized but tardily, and the level of the production would be equivalent to production obtained with a great quantity of inoculums in the same conditions (Assamoi, *et al*., 2008).

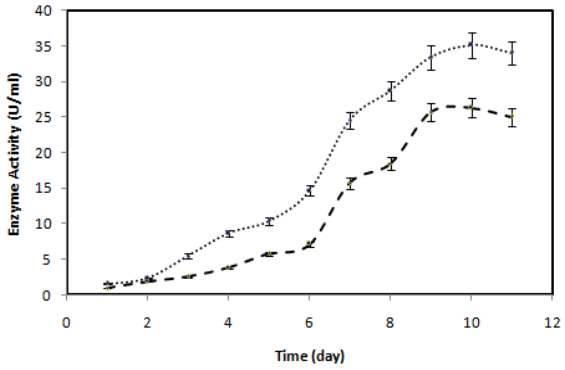

Fig. 5: Effects of pre-shaked inoculums on CBH production from *A. niger* at 30°C. (---) represents SLC fermentation without pre-shaked inoculums and (···) represents SLC fermentation using pre-shaked inoculums. The error bars represent the averages of three samples.

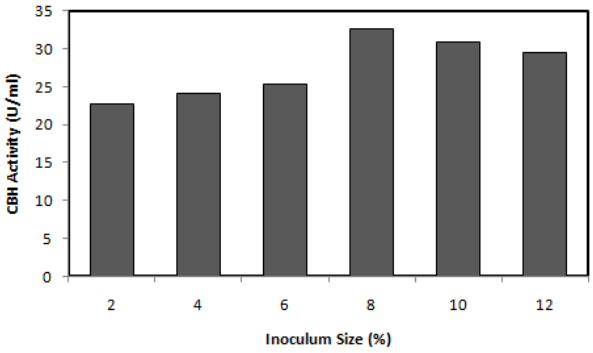

Fig. 6: Activity of CBH produced by *A. niger* at 30°C with varying inoculums size. Data presented were the averages of three samples.

SLC for CBH Production in Petri Dishes and Conical Flasks:

The optimized conditions achieved from each experiment were applied in conical flasks and petri dishes. *A. niger* PY11 was grown in liquid cultures for 11 days, at initial pH 6, incubation temperature 30°C, and using pre-shaked 8% (v/v) inoculums. Figure 7 shows the enzyme activity increased regularly starting at day 3 and reached the highest level after 8-9 days of culture in both experiment. The average of maximum CBH activities were obtained after 9 days, (32.1 U/ml) in conical flasks and (40.2 U/ml) in petri dishes. Limitations of aeration and surface area for cultivation were probably the reasons for lower enzyme activity and longer fermentation time to reach maximum value in the shake flask study. As suggested by (Lee, *et al.*, 2011), one of the possible solutions would consist to increase the cultural surface by increasing the dimension of reaction surface in the reactor.

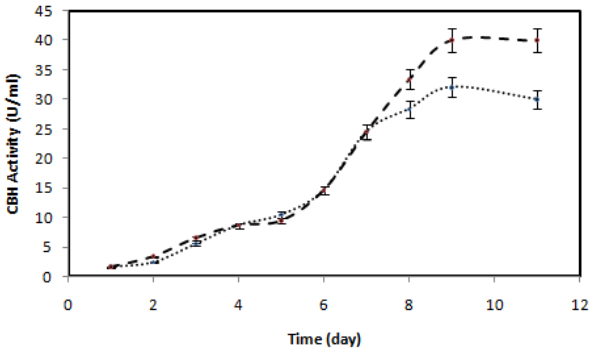

Fig. 7: Activity of CBH produced by *A. niger* at 30°C in conical flasks (···) and in petri dishes (---). The error bars represent the averages of three samples.

Conclusion:

The present work has established the potential of SLC fermentation for CBH production using maltose as carbon source. The growth pattern of cultivated *A. niger* for CBH production was monitored for as dried cell weight, protein concentration, enzyme activity, pH and residual maltose remained in the medium. The optimal paramenters for production of CBH from *A. niger* was proposed at 30^{o}C and initial pH 6, and using 8% (v/v) pre-shaked inoculums. The maximum biomass, protein concentration and enzyme activity obtained using optimal fermentation conditions were 1.5 g dry weight/ml medium, 3.5 mg/ml and 40 U/ml respectively.

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