Dual Carbon Fermentation for the Production of Inducible Cellobiohydrolase by Recombinant Aspergillus Niger

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***Abstract*—This study investigates the use of two carbon sources in fermentation for the production of cellobiohydrolase (CBH), an adaptive or inducible enzyme. Selection of carbon source was first done using single substrate cultivation between glucose, maltose and lactose using recombinant *A. niger* PY11. This recombinant microorganism will produce CBH in high quantity, with maltose acting as its main inducer. Glucose and maltose were selected in dual carbon fermentation, as glucose produce highest biomass 0.32g/ml at day 4th and maltose produce 0.42g/ml biomass at day 9th. Cultivation of *A. niger* was done for dual carbon containing sugar glucose and maltose with concentration ratio 1:1. Cultivation with medium containing maltose was done for comparison between single carbon and dual carbon. For dual carbon cultivation, highest biomass form at day 7th at 0.44g/ml while specific enzyme activity reached 10.54 U/g at day 8th. For single substrate, highest biomass production was recorded at day 9th with 0.41g/ml.while specific enzyme activity at day 7th at 4.02 U/g.**

***Index Terms*—cellobiohydrolase (CBH), dual carbon cultivation, recombinant *Aspergillus niger*. static surface liquid culture. (SSCL)**

I. Introduction

Fermentation process using filamentous fungi was broadly investigated in order to find the best suitable condition for mycelium growth at larger scale production. *Aspergillus niger (A. niger)* was recognized as one of the most efficient citric acid producer in industry [1]. With the advance in molecular biology especially in genetic engineering, *A. niger* was now widely used as an expression host for heterologous protein, coupled with its capability to secrete high level of protein to its medium [2]. Intensive researches have been conducted to increase the knowledge related to these filamentous fungi. This includes studies done on population pattern, hyphal morphology, type of fermentation, effect of agitation and glucose concentration [3]. Cultivation technique is also one of the main providers for higher product formation. Study done by [4] shows that CBH enzyme can be produced in high volume by Static Surface Liquid Culture (SSLC). Another alternative is the use of support material for *A. niger* or immobilized cell. It can reduce the viscosities of free filamentous broth and minimize diffusion limitation. Diffusion limitation can cause central autolysis [5]. Research done by [6] stated that immobilization helped to increase production at higher sugar concentration.

Cellulases is a combination of several hydrolytic enzymes that are grouped together to break down cellulose into smaller molecular and are usually abundant in fungi [7]. Cellulase mainly consists of three enzyme:(i)endoglucanases (EG) or 1,4-β-D-glucan-4-glucanohydrolases, (ii)exoglucanases, also known as 1,4-β-D-glucan cellobiohydrolases (CBH), and (iii)β-glucosidases or β-glucoside glucohydrolases [8]. CBH functions to cut and separate the units from the reducing end and non-reducing end of cellulose complex, forming simplex molecule such as cellobiose. This enzyme was usually coded as cbh1 and cbh2 [9].

Inducible enzyme refers to enzyme produced when cells are exposed to certain substance, usually substrate that triggers the formation of specific enzyme [10]. According to [11], cellulase can be considered as inducing enzyme. One of the main inducer for cellulase is cellulose. However, due to insoluble properties of cellulose, yield of cellulase is very low and require longer time. To solve the problem, other possible inducer such as cellobiose was used to induce cellulase [12]. The hydrolytic enzymes of recombinant cellulases in *A. niger* require maltose as an enzyme inducer during enzyme production [13]. Another example of inducer was the use of lactose for cellulase production [14]. Constitutive enzymes are enzyme that constantly produced by the cell under all physiological condition due to their roles in maintaining cell structure while inducible enzyme were produced with presence of its substrate. Using this characteristic, a strategy to yield high level of enzyme in shorter time was used by means of increasing mycelium mass. A high enzyme production was hoped to be achieved when inducer was added to the system as a result of higher mycelium mass. Due to that, the use of

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two carbon sources was suggested. Two carbon sources or dual substrate fermentation had been applied by [15] in production of xylitol. Before xylose can be converted into xylitol, glucose was used for cell growth as volumetric productivity is proportional to cell mass. This method can also reduce conversion time. A study done by [16] also shows that for production of ethanol on cellulosic substrate, growth yield on cellobiose were higher compared to glucose but at slower rates. Due to that, cellobiose and glucose were used together in dual substrate continuous culture where the study also demonstrates how organism preferred the disaccharide for cell growth. For two carbon sources fermentation, the primary carbon source, for example, glucose can be used in *A. niger* cultivation to increase mycelium mass, while the second carbon source, such as maltose or lactose acted as the inducer for production of CBH. This study will investigate the effect of dual substrate fermentation on the production of CBH enzyme by SSCL using recombinant *A.niger*.

II. Materials and Methods

A. Microorganism

Aspergillus niger PY11 strain CBH1, characterized to produce CBH enzyme was obtained from the Microbiology Laboratory, Faculty of Science and Technology, National University of Malaysia. The strain CBH1 was isolated from *Trichoderma virens* UKM1 and expressed in *A. niger*. Recombinant *A.niger* was constructed by adding vector ANIpCBH that comprises of glucoamylase promoter (GlaP) to drive the expression of CBH1 by addition of maltose. *A. niger* PY11 will secrete CBH1 when maltose was present in the system. In regards to that, maltose will act as the inducer and carbon source for this study.

B. Inoculum Preparation

Complete Media (CM) agar was used to grow *A. niger* spore. CM agar consists of (per litre); nitrate salt solution (50mL), 2.25M $MgSO_4$ (5mL), Hunter Trace Element (1mL), vitamin solution (1mL), dextrose (10g), bactopeptone (1g), yeast extract (1g), casimini acid (1g) and agar (15g). It was cultivated for 5 days at 30^{o}C in growth chamber. The spores were then harvested and kept in saline water containing 0.1% (v/v) Tween 80 and 15g NaCl.

C. Cultivation Method

For enzyme production, Minimal Medium J (MMJ) was used in the fermentation process. MMJ contains (per litre): nitrate salt solution (200mL), Hunter Trace Element (4mL), and carbon source (150g). 20ml of sterile 2.25M $MgSO_4$ will be added to medium after autoclave. The medium will be slightly altered in order to fulfill the objective of this study, where the use of two carbon sources will be utilized. The first stage of this study was done in order to determine the capability of *A. niger* to consume selected carbon source by preparing MMJ containing single carbon source, glucose (MMJ-glucose), lactose (MMJ-lactose), and maltose (MMJ-maltose). The concentration of the all MMJ has been set at 150g/l. Two carbon sources fermentation was done by mixing two carbon sources at the ratio of 1:1, for example 75g/l glucose will be mix together with 75g/l maltose, so that the final concentration of MMJ with two carbon source will be at 150g/l. The MMJ containing glucose and maltose will be address as MMJ-Glumal.

10% (v/v)of *A. niger* ($1x10^5$ spores/ml) spore suspension was transferred into a Erlenmeyer flask containing 250ml of MMJ with respective carbon source. The flask was then incubated at 30^{o}C and 150 rpm in a shaker incubator for 24 hours. 25ml of the culture was then transferred to petri dish with 50mm diameter. All the dishes were then incubated in sterile box at 30^{o}C for 10 days. Wet tissues were placed under the dishes to provide humidity for the fermentation. The fermentation was based on fed batch fermentation, where 1ml of MM J was added to the dishes every day to maintain total volume of medium.

D. Analytical Methods

Biomass concentration was used to determine the cell growth during the fermentation. The analysis was conducted based on method described by [17]. Filter paper Whatman No.4 was dried at 105^{o}C for one hour before allowing it to cool down in desiccator. The filter paper will be weigh first before being used to filter the mycelium from the culture. The mycelium recovered then was dried at 80^{o}C for 24 hours. Final weight of dried mycelium was then recorded.

Protein concentration was determined following on the method described by [18] where bovine serum albumin (BSA) was used as the standard. In this assay, 100 µl dye reagent was mixed together with 100 µl diluted raw enzyme and 10 µl of 0.15M NaCl that act as the buffer. The absorbance was measured at wavelength 595nm after 30 minutes of incubation against the blank of 0.15M NaCl.

Enzyme activity of CBH was determined using p-nitrophenol-celobioside (pNPC) as substrat. One unit of CBH activity (U/mL) was defined as 1mM of p-nitrophenol liberated per ml enzyme per minute under assay condition. The culture filtrate was diluted appropriately. 0.01ml of diluted sample was vortex together with 0.09ml of 1mM pNPC, prepared in acetate buffer at pH3 to ensure proper mixing. The sample was then incubated in water bath at 65^{o}C for 30 minutes. At the end of incubation, 0.1ml of 1M natrium carbonate ($NaCO_3$) was added as stop reaction [19]. The absorbance was measured at wavelength 420nm. All assays were repeated three times and final data was presented as mean of the triplicate experiments.

III. Results and Discussion

A. Single Carbon Fermentation

The first part of the study was to investigate the capability of *A. niger* to consume the carbon source for cell growth. Influence on different type of carbon source on the growth pattern was examined by studying the

morphology of mycelium and concentration of biomass produced. In this experiment, biomass refers to the mycelium and free spores that are produced during fermentation. The experiment was done in 10 days.

For fermentation process that use fungal as microorganism, the morphology of the mycelium plays important role [20]. It can provide ease in filtration and also affecting cell performance rate in term of production of product and byproduct. MMJ-glucose and MMJ-maltose each produce mycelium that branched together forming single thick clump. For MMJ-lactose, there is no mycelium formed, only free spores. However, after day 5th, the clump of mycelium for MMJ-glucose starts to break into smaller pellets due to the effect of decreasing glucose concentration. At early stage of fermentation, nutrient intake will focus at the middle of the clump. As the concentration of nutrient decrease, mycelium breaks into smaller pellet to cover more surface area [21]. This can be solved by using fed batch fermentation to maintain the volume and concentration of medium throughout the fermentation.

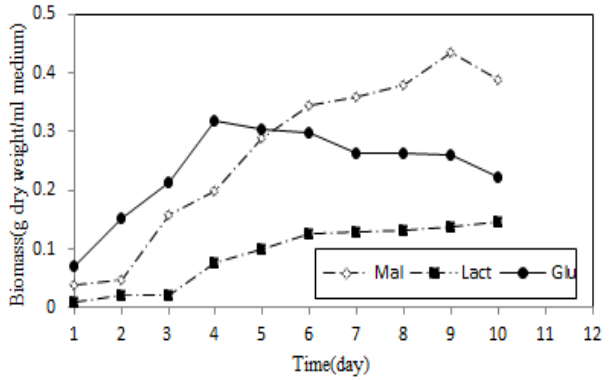

Figure 1. Growth profile of dry cell weight for glucose, lactose and maltose.

Fig. 1 shows the growth profile for the three types of carbon sources. For each medium, highest dry biomass concentration was achieved at day 9th by MMJ-maltose at 0.45g/ml, while MMJ-glucose record highest biomass concentration with 0.32 g/ml at day 4. For MMJ- lactose, there is only slight increase for the overall 10 days fermentation, with day 10th recorded highest mass at 0.15 g/ml. It is observed that the difference in biomass production was affected by the number of carbon molecule of the feed and type of bond between the molecules. MMJ-maltose need longer time to achieve highest biomass production compared to MMJ-glucose because the glucose is more favorable by *A. niger*. Sugar from polysaccharide group has low effectiveness for fermentation by *A. niger* because the hydrolysis process to simple sugar form consume longer time [22]. This could not cover the high cell metabolism rate during fermentation. Glucose is the simplest form for molecule, thus can freely transported into cell for biological process. [23] also stated on how the monosaccharide was easily detected by the lysing mycelium. Maltose with glycosidic bond (1-4) broke down into two glucose by glucoamylase enzyme, produced by *A. niger* [24]. However, glucoamilase does not react with lactose bond causing low biomass production as no carbon source can be absorbed by the cell. Fig. 1 also shows the degrading value of dry biomass for MMJ-glucose and MMJ-maltose after reaching their peak value, caused by autolysis. Autolysis can be found occurred during the last few days of cultivation [25]. Autolysis comes from many factors, usually due to material imbalance cause by lack of nutrients [26]. It can also due to self-toxicity caused by accumulating metabolites [27].

B. Two Carbon Fermentation

Lactose was not used in two carbon fermentation as it does not provide any increase in mycelium growth. Medium MMJ-glumal, a combination of glucose and maltose was used for two carbon fermentation. The time course study of two carbon fermentation was conducted to monitor the formation of biomass, protein concentration, enzyme activity and specific enzyme activity. Fermentation using MMJ-maltose was done as comparison between the use of two substrates and single substrate. Studies on *A. niger* conclude that morphology and product formation was affected by initial glucose concentration [28]. Fed batch fermentation method was done using SSLC in order to avoid mycelium from breaking into smaller pellets.

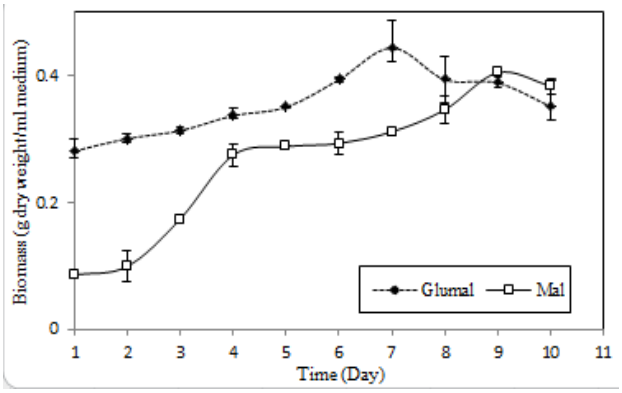

Figure 2. Dry Biomass of MMJ-Glumal and MMJ-maltose.

Fig. 2 shows the comparision for dry biomass concentration between MMJ-glumal and MMJ-maltose. For MMJ-maltose, highest biomass concentration was achieved at day 9th with 0.41g/ml. For MMJ-glumal, highest biomass concentration was achieved at day 7th with 0.44g/ml. Eventhough the initial concentration of both medium is the same, the number of sugar molecules in MMJ-glumal is higher than MMJ-maltose, making the biomass concentration of MMJ-glumal to be higher compared to MMJ-maltose. The result also according to [29] which stated that the combination of two sugars with 50% concentration can increase product yield by mutant strain.

Fig. 3 shows the comparison for protein concentration and enzyme activity between MMJ-glumal and MMJ-maltose. MMJ-maltose recorded an increasing value of protein concentration with peak value at day 8th with 8.82 g/ml before the value starting to decrease. Protein concentration for MMJ-glumal was slightly increasing every day with highest concentration at day 8th with 3.42g/ml. The graphs for enzyme activity for both

medium were almost in similar pattern. Both medium achieved highest enzyme activity at day 8th where MMJ-glumal recorded the value 33.23 U/ml and MMJ-maltose was 35.53 U/ml. Enzyme activities for CBH from MMJ-glumal shows increasing value after 3rd day. However, the use of maltose as carbon source produce long lag phase in enzyme production. This is due to the property of maltose that require glucoamylase enzyme so that maltose can be converted into glucose for cell metabolism. First 72 hours (3 days) was crucial for every fermentation by *A. niger* because it is the time when high cell metabolism occur in order to build more cell mass [30]. This also proven by the overall protein concentration from MMJ-maltose was higher than compared to MMJ-glumal but the enzyme activity for CBH from both mediums is almost equal. Specific enzyme activity for MMJ-glumal at 10.54 U/g was higher than MMJ-maltose, whose specific enzyme activity was at 4.02 U/g.

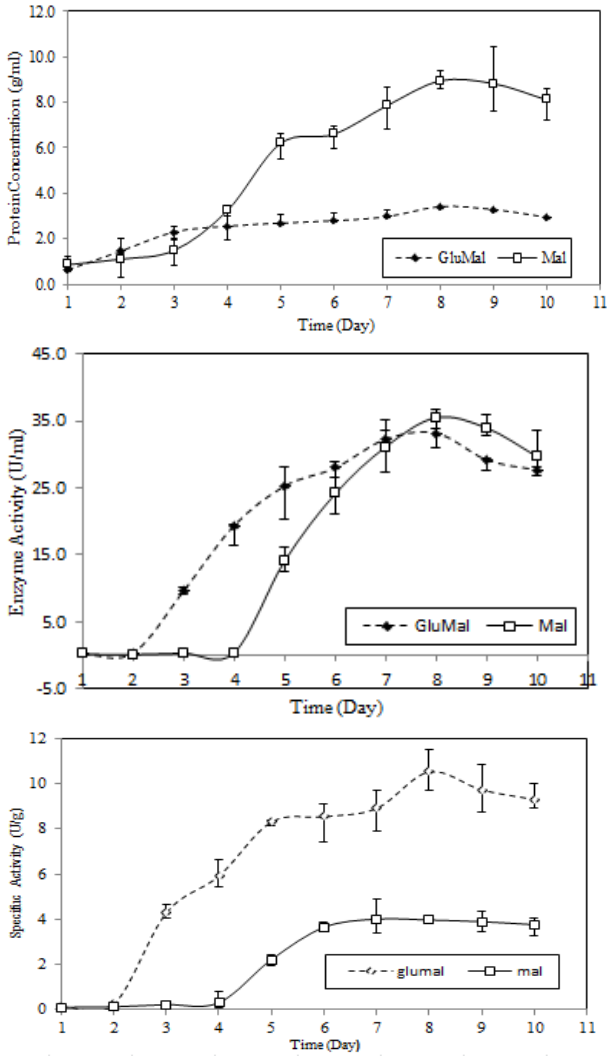

Figure 3. Growth profile of (a) protein concentration,(b) enzyme activity and (c) specific enzyme activity by A. niger using medium MMJ-glumal and MMJ-maltose

IV. Conclusion

For production of CBH, the fermentation methods of using SSCL and fed-batch have been proven to help increase enzyme production. The use of maltose as carbon source has also proven to be successful. However, dual substrate using glucose and maltose can help to increase productivity by minimizing the fermentation time and protein contaminant. The highest specific enzyme activity was also found from MMJ-glumal.

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