OPTIMIZATION OF CARBOXYMETHYLCELLULASE AND POLYOSES PRODUCTION FROM OIL PALM EMPTY FRUIT BUNCH FERMENTATION BY *Trichoderma virens*

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Specially dedicated to My Beloved Mother; Punniah Ahmad, My Siblings; Mohd Ridhwan Hanif, Mohd Saufi Shahril, Mohd Khairul Annuar, Nur Asila Ashikin, Nur Amirah Idayu, all my family and fellow friends.

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ABSTRACT

Optimization of CMCase and polyoses production by *Trichoderma virens* via batch culture fermentation of pretreated oil palm empty fruit bunch (OPEFB) was carried out using statistical software of Design Expert ® version 6.0.4. The General Factorial Design was applied to study the influence of nitrogen sources towards CMCase production. Combination of peptone and ammonium sulphate with C:N 39.2 mM significantly increased CMCase activities, specific activity and total polyoses production. Mixture of peptone and ammonium sulphate was further utilized for screening of significant factors for CMCase production using 2-Level Factorial Design. The significant factors that influenced CMCase production were temperature, pH, fermentation time, concentration of substrate and Tween 80. These significant factors were used for optimization process using central composite design (CCD). The optimal conditions which stimulate the highest CMCase production and its specific activity were 7 days fermentation, 0.4% (v/v) of Tween 80 concentration, 0.1% (w/v) OPEFB concentration, 25^{o}C and initial pH at 5.56. Under those optimum conditions, CMCase production was 0.39 U/mL and specific activity at 0.24 U/mg, indicated the improvement of 1.9 fold as compared to that of non optimized condition. The polyoses production increased for 2.5 fold in comparison with that of non optimized conditions. Various types of polyoses were produced such as cellobiose, maltose, glucose, xylose, galactose, arabinose and mannose.

ABSTRAK

Pengoptimuman penghasilan CMCase dan polioses oleh *Trichoderma virens* melalui fermentasi kultur sesekelompok menggunakan serabut tandan kelapa sawit kosong (TKSK) telah dijalankan menggunakan perisian statistik *Design Expert 6.0.4*. Rekabentuk faktorial umum, telah digunakan untuk mengkaji kesan sumber nitrogen terhadap penghasilan CMCase. Kajian mendapati kombinasi pepton dan ammonium sulfat menunjukkan kesan signifikan terhadap penghasilan CMCase, aktiviti spesifik dan jumlah polioses tertinggi. Kombinasi pepton dan ammonium sulfat digunakan untuk penyaringan faktor signifikan bagi penghasilan CMCase menggunakan rekabentuk faktorial tahap 2. Faktor yang mempengaruhi penghasilan CMCase secara signifikan ialah suhu, pH, masa fermentasi, kepekatan TKSK dan Tween 80. Faktor-faktor signifikan ini telah digunakan dalam proses pengoptimuman menggunakan rekabentuk komposit berpusat. Keadaan optima yang menghasilkan CMCase dan aktiviti spesifik tertinggi adalah pada hari ke 7 fermentasi, 0.4 % (i/i) Tween 80, 0.1% (j/i) TKSK, 25^{o}C dan pada pH 5.56. Penghasilan CMCase pada keadaan optima ialah 0.39 U/mL dan aktiviti spesifik 0.24 U/mg yang menunjukkan peningkatan sebanyak 1.9 kali ganda berbanding dengan keadaan sebelum pengoptimuman. Penghasilan polioses pula menunjukkan peningktan 2.5 kali ganda berbanding dengan keadaan sebelum pengoptimuman. Jenis-jenis polioses yang dihasilkan antaranya sellobiosa, maltosa, glukosa, xilosa, galaktosa, arabinosa dan mannosa.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
OPEFB	Oil Palm Empty Fruit Bunch
PDA	Potato Dextrose Agar
DNS	Dinitrosalicyclic Acid
CMCase	Carboxymethyl Cellulase
FPase	Filter Paper Culture enzyme
HNO_3	Nitric Acid
$(NH_4)_2SO_4$	Ammonium Sulphate
H_2SO_4	Sulphuric Acid
NaOH	Sodium Hydroxide
pNPG	pNPG p-nitrophenyl β-D-glucoside
HPLC	High Performance Liquid Chromatography
RSM	Response Surface Methodology
CCD	Central Composite Design
ANOVA	Analysis of Variance
OFAT	One Factor At One Time
mM	Milimolar

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Research Background

Oil palm empty fruit bunch (OPEFB) is main by-product produce from palm oil mills. In Malaysia, about 15 millions tones of OPEFB were generated annually, and it is believed that this will continuously increase in proportion to the world demand of edible oils (Simarani *et al.,* 2009; Baharuddin *et al.,* 2009). Now, some researchers believe that the dependency of fossil fuel and the environment pollution can be reduced by using the biocatalyst cellulase which derived from the cellulolytic organisms to convert the high content of cellulosic biomass such as OPEFB to the fermentable sugars which can be used to generate other useful products (Lynd *et al.,* 2004).

OPEFB as lignocellulose biomass is a renewable biomass and low-cost raw materials for the production of high valuable products such as biofuels, biofertilizers, animal feeds and other biochemical products (Howard *et al.,* 2003; Tengerdy and Szakacs, 2003). According to Aziz *et al.,* (2002), OPEFB contains cellulose, hemicellulose and lignin and also other compound such as protein, lipid and ash. All of

the components, lignin is the most recalcitrant to degradation compared to cellulose and hemicellulose. It is because of its highly ordered crystalline structure which composed of several types of aromatic alcohols that preventing penetration of solutions and enzymes (Howard *et al.,* 2003; Juhász *et al.,* 2005). Thus, pretreatment of lignocellulosic materials is necessary to modify the intact structure in order to enhance the enzymatic degradation by removing lignin barrier (Zhou *et al.,* 2009; Lin *et al.,* 2010).

Bioconversion of lignocellulosic biomass to other valuable products is catalyzed by a group of enzymes called cellulase. There are three categories of cellulase enzymes to convert cellulose into soluble sugars, which subsequently fermented to biofuels, include endoglucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). All three enzymes act synergistically to hydrolyse cellulose by creating new unit available sites for each other removing obstacles and relieving product inhibition (Howard *et al.,* 2003; Juhász *et al.,* 2005; Zhou *et al.,* 2009; Jabasingh and ValliNachiyar, 2010). Many microorganisms are shown to produce cellulase. The ability of certain fungal species like *Trichoderma* species, to decompose the lignocellulosic biomass into glucose, which in turn can be converted into valuable chemicals, has made cellulases as one of the most important commodity (Krishna *et al.,* 2000; Howard *et al.,* 2003; Zhou *et al.,* 2009). Thus, the production of enzymes cellulase from fungi has been extensively studied (*Trichoderma* sp. and *Aspergillus* sp.). Significant stability of *Trichoderma virens* to produce cellulases for effective degradation of cellulose from waste materials has been extensively reported by Hamedo and Shamy, 2008 as well as Dayana Amira *et al.,* 2011.

There are several factors that influenced cellulase production by microorganisms such as nutritional sources and culture condition (Krishna *et al.,* 2000; Gorret *et al.,* 2004; Juhasz *et al.,* 2005; Alam *et al.,* 2008; Vintila *et al.,* 2010). Recently, different statistical designs for fermentation condition optimization concerning cellulase

production have been reported, which factorial experiments and response surface methodology (RSM) is included. These statistical methods are very useful tool as it provides statistical models which help in understanding the interactions among the parameters that have been optimized. Furthermore, this statistical designs are required to reduce number of experimental trials to evaluate multiple parameters and their interaction, thereby resulting in saving time, glassware, chemicals and man power (Singh *et al.*, 2009; Zhou *et al.*, 2009; Ma *et al.*, 2008).

1.2 Problem of Statement

Although lignocellulosic biomass (OPEFB) is available in large quantities, the main challenge for commercialization is to reduce the major operating costs of biomass conversion processes, mainly related primarily pretreatment and enzymes requirements. Research in pretreatment mostly focused on developing processes that would result in reduced bioconversion time, high cellulase enzyme production, and/or higher polyoses yields. This pretreatment is important to ensure optimum cellulase production because these enzymes depend on the successful pretreatment that have been done. Thus, several physical, chemical and biological treatments are under evaluation. The resulting composition of the treated material is dependent on the source of the biomass and the type of treatment used (Baharuddin *et al.*, 2009; Sun and Cheng, 2002; Champagne and Li, 2009).

Beside, enzyme production cost is the most critical part of producing products from lignocellulosic materials. The final target of the whole research is to produce economically acceptable enzymatic conversion of cellulosic biomass to glucose for fermentation to ethanol or other products. Hydrolysis of these polymers releases a

mixture of neutral sugars including glucose, xylose, mannose, galactose, and arabinose. Cost-effective hydrolysis is an important goal in the search for a feasible enzymatic conversion process for lignocellulose materials. Due to the crystalline structure of cellulose, as well as its complex structural organization, lignocelluloses are difficult to break down. Cellulase enzyme has been used for the bioconversion of lignocellulosics to these useful products. Many fungi such as *Aspergillus sp* and *Trichoderma sp* produce enzymes that enable them to break down polysaccharides and proteins into sugars and amino acids that can be assimilated easily (Lynd *et al.,* 2002).

In this research, *Trichoderma* species, especially *Trichoderma virens* was used for cellulase production during degradation of pretreated OPEFB. The medium optimization was carried out in shake flask culture. The optimization of fermentation conditions is a major problem in the development of economically feasible bioprocess. The cellulase production can be optimized with the help of statistical methodologies. First, categorical factors (General Factorial Design) are studied to determine the types of nitrogen sources suitable for optimizing fermentation process. Using 2-Level factorial Design method six physical factors or parameters which are concentration of OPEFB, concentration of Tween 80, temperature, inoculum size, pH and time incubation are screened. Then, insignificant ones are eliminated in order to obtain smaller and manageable set of factors. The remaining factors are optimized by Response Surface Methodology (RSM). RSM is a set of statistically design experiments, building models, evaluating the effects of factors and searching for the optimum conditions, has been used successfully in the optimization of bioprocess (Hao *et al.,* 2006; Ma *et al.,* 2008; Alam *et al.,* 2008; Zhou *et al.,* 2009).

1.3 Objectives of the Research

The objectives of this research are:

1) To determine the effect of nitrogen source on carboxymethylcellulase production during fermentation of pretreated OPEFB by *Trichoderma virens* using General Factorial Design.

2) To screen the significant factors influencing carboxymethylcellulase and polyoses production during fermentation of pretreated OPEFB using 2-Level Factorial Design.

3) To optimize of carboxymethlcellulase and polyoses production using Response Surface Methodology (RSM) from fermentation of pretreated OPEFB.

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APPENDIX A

Spore count using Haemocytometer

Total spore can be easily and rapidly determine using haemocytometer. However, the process can be relatively inaccurate if sample forms clumps or aggregates. Haemocytometer slides have a series of grids etched on it (Scragg, 1991). Samples were introduced beneath the cover slip and the number of spores is measured with the aid of phase contrast microscope. The total number of spores counted under the grid is multiplied by the volume of the grid giving total spore number per ml.

Reagents

a) 1% (v/v) Tween 80
b) 70% (w/v) Ethanol

Procedures

1. 5 mL of 1% (v/v) Tween 80 added to the 7 days cultured PDA plate. The spores harvested by hockey stick with gently scratch the surface of the agar. The solution collected and transfer to a 50 mL centrifuge tube.
2. The centrifuge tube was centrifuged for 20 minutes at 4°C and 4000rpm.
3. The supernatant discarded and the pellet resuspended with 20 mL of water. The mixture vortex under minimum speed.
4. Next, 1 mL of the mixture was sucked out and mixed with 9 mL of sterilized distilled water. The mixture was vortex under minimum speed.
5. 1 mL of mixture from step 4 was sucked out and mixed with 9 mL of sterilized distilled water. The mixture was vortex under minimum speed.

6. Draw out 10 μL of mixture from step 5 and inject into the sample introduction point of the hemocytometer. However, the cover glass and lens of microscope must be clean with 70% (w/v) ethanol before performing any injection or counting.
7. The injected spores must evenly distribute and must not have any leakage for the injection. The hemocytometer placed on the microscope stage and observe under 400X magnification (40X objective lens).
8. The spores only are counted with located on the centre and 4 corners square of the grid (labeled with X).
9. The procedure of calculating spores under hemocytometer (step 6 to 8) were repeated for three times and the number of spores would be calculated according the calculation below. Next, the serial dilution would be conducted by using the second equation.
10. The diluted spores suspension was transfer to fermentation medium according to the inoculums size (10% (v/v)).

Calculations:

1. Each large square gives a volume of 10^{-4} ml. Figure A below shows the haemocytometer grids and calculations.
2. Spores/ml = total spores per large squares x dilution factor (df) x 10^{4}
3. Dilution factor is obtained from spore suspension. For example, if spore is diluted as 1/10, thus the dilution factor is 10.