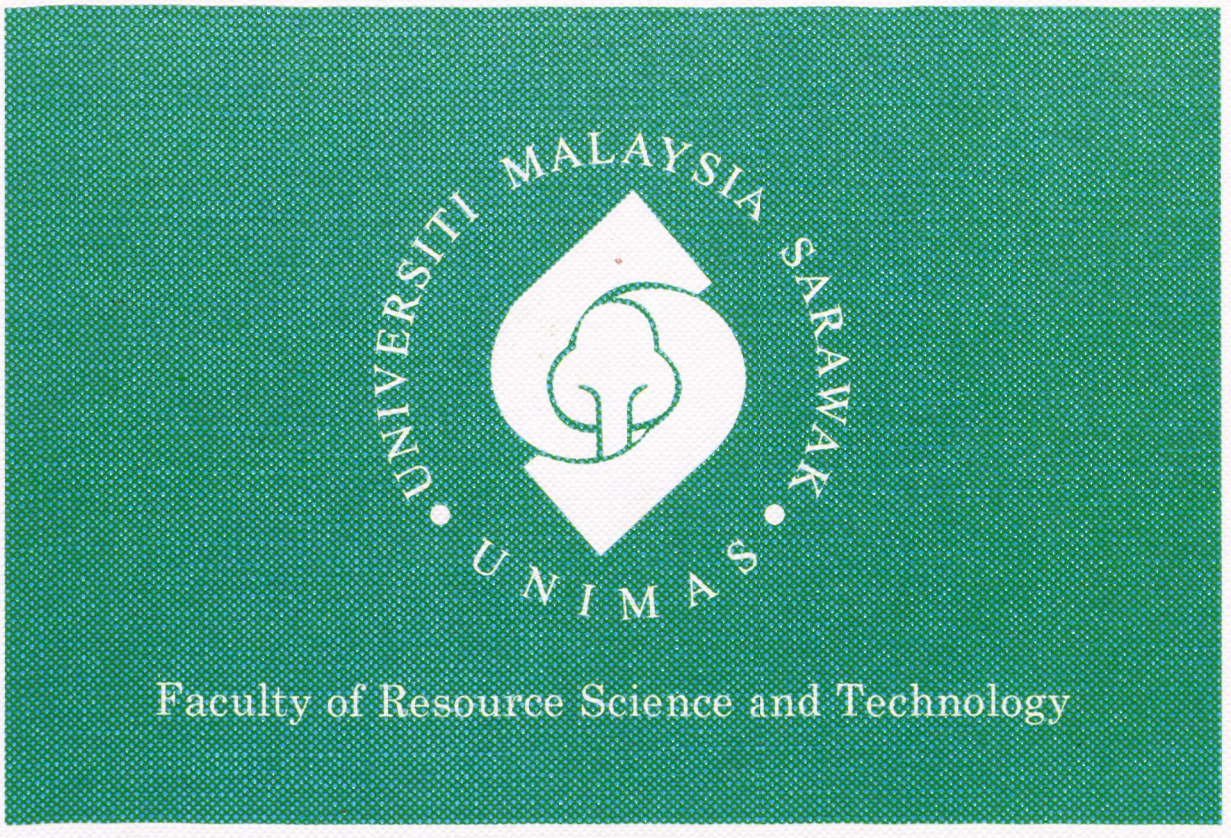

EFFECTS OF CASTOR OIL ON CELLULASE ACTIVITIES DURING FED-BATCH ANAEROBIC FERMENTATION

Nurul Shafikah Binti Che Lit

Bachelor of Science with Honours
(Resource Biotechnology)
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Effects of Castor Oil on Cellulase Activities during Fed-batch Anaerobic Fermentation

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32504

This thesis is submitted in partial fulfillment of the requirements for the degree of Bachelor of Science with Honours (Resource Biotechnology)

Supervisor: Dr. Micky Vincent

Resource Biotechnology

Department of Molecular Biology

Faculty of Resource Science and Technology

University Malaysia Sarawak

2014

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LIST OF ABBREVIATIONS

CMC	Carboxymethylcellulose
HPLC	High Performance Liquid Chromatography
DNS	Dinitrosalicylic acid
$(C_6H_{10}O_5)_n$	Cellulose
H_2O	Water
$C_6H_{12}0_6$	Glucose
C_2H_5OH	Ethanol
CO_2	Carbon dioxide
NaOH	Sodium hydroxide
FPU	Filter Paper Unit
LB	Luria Broth
YP	Yeast Peptone

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Effects of Castor Oil on Cellulase Activities during Fed-batch Anaerobic Fermentation

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ABSTRACT

The discovery of bioethanol production from anaerobic fermentation has been a great contributor in preventing the massive depletion of the fossil fuels. Not just that, this particular energy source can be generated from renewable carbohydrate sources such as wastepaper via microbial fermentation. For this study, *Saccharomyces cerevisiae* was used as the conversion microorganism and carboxymethylcellulose (CMC) as its substrate material. Enzyme cellulase is also crucial in this study as it convert CMC into glucose. High Performance Liquid Chromatography (HPLC) analysis is chosen as one of the analytical methods to analyse the samples collected. Based on the result obtained, the highest ethanol yield was reported at 50 FPU 2.5% CMC. As for conclusion, the castor oil observed to have effects on the cellulase activities during the fermentation process and it also aids in reducing the ethanol toxicity. In order to obtain a better yield of ethanol, it was suggested that the effects of pH towards the fermentation profile is carried out.

Keywords: anaerobic fermentation, Carboxymethylcellulose (CMC), ethanol, High Performance Liquid Chromatography (HPLC), *Saccharomyces cerevisiae*

ABSTRAK

Penemuan penghasilan bioethanol daripada proses penapaian telah menjadi penyumbang penting dalam usaha menghalang tenaga fossil daripada terus berkurang. Bukan itu sahaja, sumber kuasa ini dapat dihasilkan daripada sumber karbohidrat yang mampu diperbaharui seperti kertas-kertas buangan melalui proses penapaian mikrob. Bagi kajian ini, Saccharomyces cerevisiae telah digunakan sebagai mikroorganisma penukaran dan carboxymethylcellulose (CMC) pula sebagai bahan substrat. Enzim cellulase bertindak sebagai ejen penukaran CMC kepada glukosa. Salah satu kaedah analisa yang digunakan untuk menganalisa sampel-sampel yang terkumpul ialah High Performance Liquid Chromatography (HPLC). Berdasarkan hasil analisa yang diperoleh, hasil etanol yang tertinggi adalah pada 50 FPU 2.5% CMC. Kesimpulannya, minyak kastor terbukti mempunyai impak terhadap aktiviti enzim sepanjang proses penapaian berlangsung. Malah, minyak kastor juga berjaya membantu mengurangkan kadar ketoksidan etanol terhadap mikroorganisma. Bagi memperoleh hasil etanol yang lebih baik, adalah disarankan agar ujian pH terhadap cecair likat penapaian dilaksanakan.

Kata kunci: Carboxymethylcellulose (CMC), etanol, High Performance Liquid Chromatography (HPLC), penapaian anerobik, Saccharomyces cerevisiae

1.0 INTRODUCTION

The successful discovery of bioethanol production from anaerobic fermentation by previous scientists has served as an alternative source to petroleum-derived transportation fuels. This brilliant work is a great contributor in preventing massive depletion of crude oil which eventually may lead to their eternal loss (Balat *et al.*, 2008). Plus, it also aids in reducing the environmental pollutions such as CO_2 emission from the petroleum (Balat *et al.*, 2008). Ethanol can be produce from raw renewable sources such as wastepaper or any other carbohydrate feed stocks, for example lignocellulosic biomass (Alvira *et al.*, 2010). According to Yazdani & Gonzalez (2007), ethanol can be obtained through biological conversion of glucose from cellulose to alcohol during the microbial fermentation. Industrial yeast such as *Saccharomyces cerevisiae* plays a significant role in the fermentation process. The same role also applied to cellulase, an enzyme that is responsible in digesting the feed-stocks into glucose.

Over accumulation of ethanol during the process may affect yeast severely and it may lower ethanol production (Nagodawithana & Steinkraus, 1976). Thus, to solve this problem, castor oil can be used to absorb the ethanol from the fermentation broth. Castor oil is added at zero hr before fermentation. Since castor oil is insoluble in water, the over accumulated ethanol may mix freely with the castor oil due to differential miscibility. The ethanol/castor oil phase will form the uppermost layer and can be driven off easily by heating to obtain the ethanol (Jenkins, 1999). Consequently, the over accumulation of ethanol is reduced.

Nevertheless, the main issue here is either the addition of castor oil may affect the cellulase activities during anaerobic fermentation or not. As stated before, cellulase is essential in breaking down the cellulose into sugar (Sun & Cheng, 2002). Without glucose, production

of ethanol is arrested. Both castor oil and cellulase are measured as important elements in the production of bioethanol. Accordingly, it is very crucial to observe whether castor oil has any effects on the cellulase activities, given in any amount. Therefore, this study was performed to meet this particular purpose.

1.2 Objectives

The objectives of this study are:

i. To determine the effects of castor oil on cellulase enzyme activities during anaerobic fermentation.

ii. To observe the effects of castor oil on reducing the level of ethanol toxicity towards *Saccharomyces cerevisiae*.

iii. To measure the amount of ethanol produce during anaerobic fermentation in the presence of castor oil.

2.0 LITERATURE REVIEW

2.1 Castor oil

Castor oil is extracted from the castor seed, collected from the castor oil plant, *Ricinus communis* (Akaranta & Anusiem, 1996; Akpan *et al.*, 2006). Castor oil can be obtained from the seed via milling, boiling, pressing or solvent extraction process. *R. communis* belongs to the Euphorbiaceae family, the spurge family of flowering plants (Reeves, 2011). It is a semi-woody large shrub that can grow up to 5 meters tall (Reeves, 2011). It can be found at sandy soil areas, creek banks, along the road shoulders and at the edge of cultivated lands. Even though the plant is indigenous to the Middle East and Africa area, it has been introduced and cultivated at countries with tropical and subtropical climate.

The seed of the castor oil plant is called castor beans which contain ricin, a protein believed to be one of the most extreme toxic to livestock and humans. Apart from that, according to Worbs *et al.* (2011), ricin has gained attention worldwide as a potential biological weapon. However, according to Johnson (2007), the industrial castor oil is safe to be used since ricin is a water-soluble protein which is excluded from being extracted during castor oil production.

In total, the seed contains around 50% of oil. It is known as the only hydroxyl acid based olein. Castor oil has unique nature which it is easily soluble in ethanol but difficult to dissolve in petroleum ether. The usage and applications of castor oil varies according to different fields. For instance, it can be used as organic fertilizers in agriculture. It also acts as lubricants and dyeing agent. Plus, it can be used to separate ethanol from water via differential miscibility (Jenkins, 1999). Therefore, addition of castor oil in the ethanol/water solution allows the ethanol to mix freely with it which insoluble in the water. In most solution, the oil usually accumulates at the top layer, so does the ethanol/castor oil

phase in this case. To obtain the ethanol, the upper layer (ethanol/castor oil phase) will undergo heating process (Jenkins, 1999). The plus point of using castor oil in this separation process is that it can be exploited again and again once the ethanol is extruded. It can be returned to the ethanol/water solution to dissolve more ethanol thus repeating the process. Hence, this process turns into a continuous process. This principle is very useful in order to execute the study of the effects of castor oil on cellulase activities during anaerobic fermentation.

Figure 1: *Ricinus communis* (Source: Kramer, 2010).

Figure 2: Castor seeds (Source: Indo Exports, 2009).

2.2 Ethanol

Ethanol is an alcohol-based fuel that is produced through fermentation of glucose. It is a renewable energy source which acts as the alternative energy for the limited crude oil. In the United States, ethanol is broadly use as a partial gasoline replacement. About 4,540 million liters of ethanol are consumed annually by the United States transportation sector which account for 1% of total consumption of gasoline. The gasoline fuels contain up to 10% ethanol by volume (Sun & Cheng, 2002). For instance, E85, a mixture of 85% ethanol and 15% gasoline is the fuel used in flexible fuel vehicles that are specifically designed to run on gasoline, E85 and any blend of the two (LeGendre *et al.*, 2009). Ethanol production in the United States is expected to increase as a result from Energy Independence and Security Act of 2007, signed on December 19, 2007 which advances the necessities for renewable fuel use up to 36 billion gallons by 2022 (LeGendre *et al.*, 2009).

Figure 3: Biofuel pump at the gas station (Source: U.S. Department of Energy, 2012).

2.3 Cellulase

Cellulase is an industrially important enzyme that hydrolyses cellulose to monomeric sugar. Cellulose degradation into glucose by cellulase involves three steps; adsorption of cellulase onto the surface of cellulose, degradation of cellulose into glucose and desorption of cellulase (Sun & Cheng, 2002). Cellulase will not be affected during the hydrolysis process. Hence, it can be used repeatedly after being recovered from the process. According to Sun & Cheng (2002), cellulase is needed as the biocatalysts in order to break down the cellulose content to its intermediate product, glucose which later will be converted to ethanol via anaerobic fermentation as stated below:

i. Overall Cellulose Hydrolysis to Glucose

(Glucose inhibits the reaction)

$$(C_6H_{10}O_5)_n + (H_2O)_n \xrightarrow{\text{Cellulase}} nC_6H_{12}O_6$$

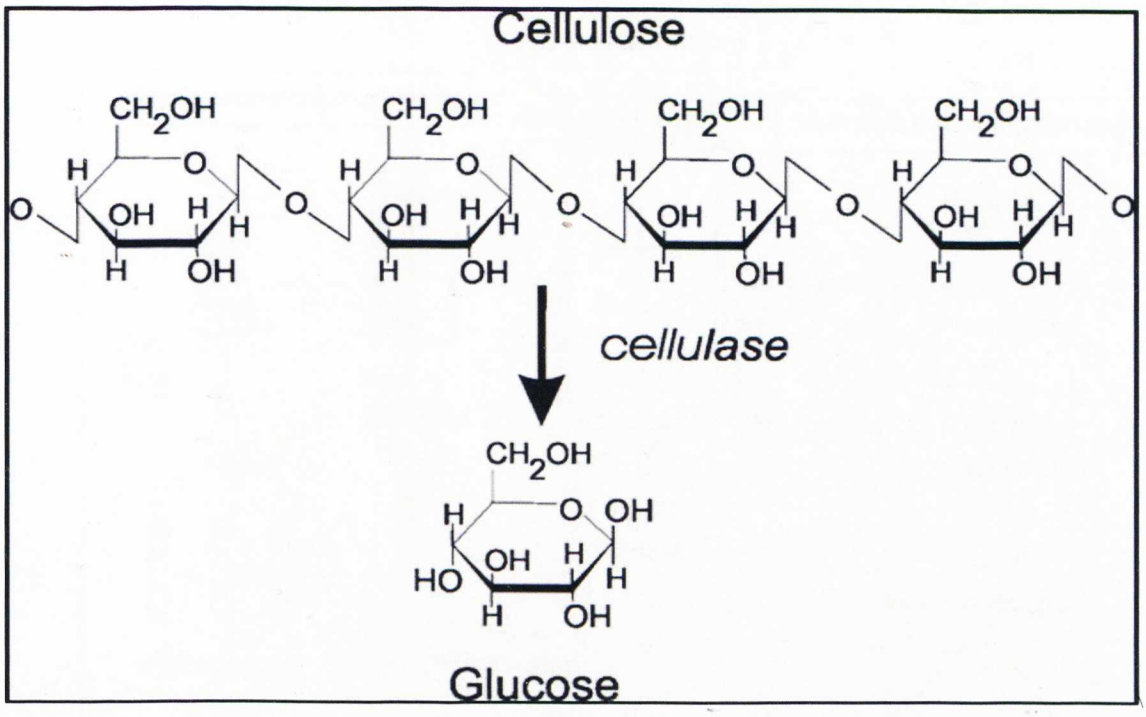

Figure 4: Cellulose digestion into glucose by cellulase enzyme (Source: Held, 2013).

ii. Bioconversion of Glucose to Ethanol

$$C_6H_{12}O_6 \xrightarrow{\text{Microorganisms}} 2C_2H_5OH + 2CO_2$$

2.4 Fed-batch anaerobic fermentation

Anaerobic fermentation is a natural process and used in the production of bioethanol. It is a metabolism process in the absence of oxygen where an organism breaks down organic matter such as starch or sugars into an alcohol or an acid. For example, yeast executes fermentation by breaking down sugars into ethanol and carbon dioxide while bacteria perform fermentation by producing lactic acid from carbohydrates. According to Cheng *et al.* (2009), advantages of using this method is that it yield higher productivity and reduce substrate inhibition to the production of the final product which is the ethanol. Thus, it is considered as one of the most common method for the production on bioethanol in the industry.

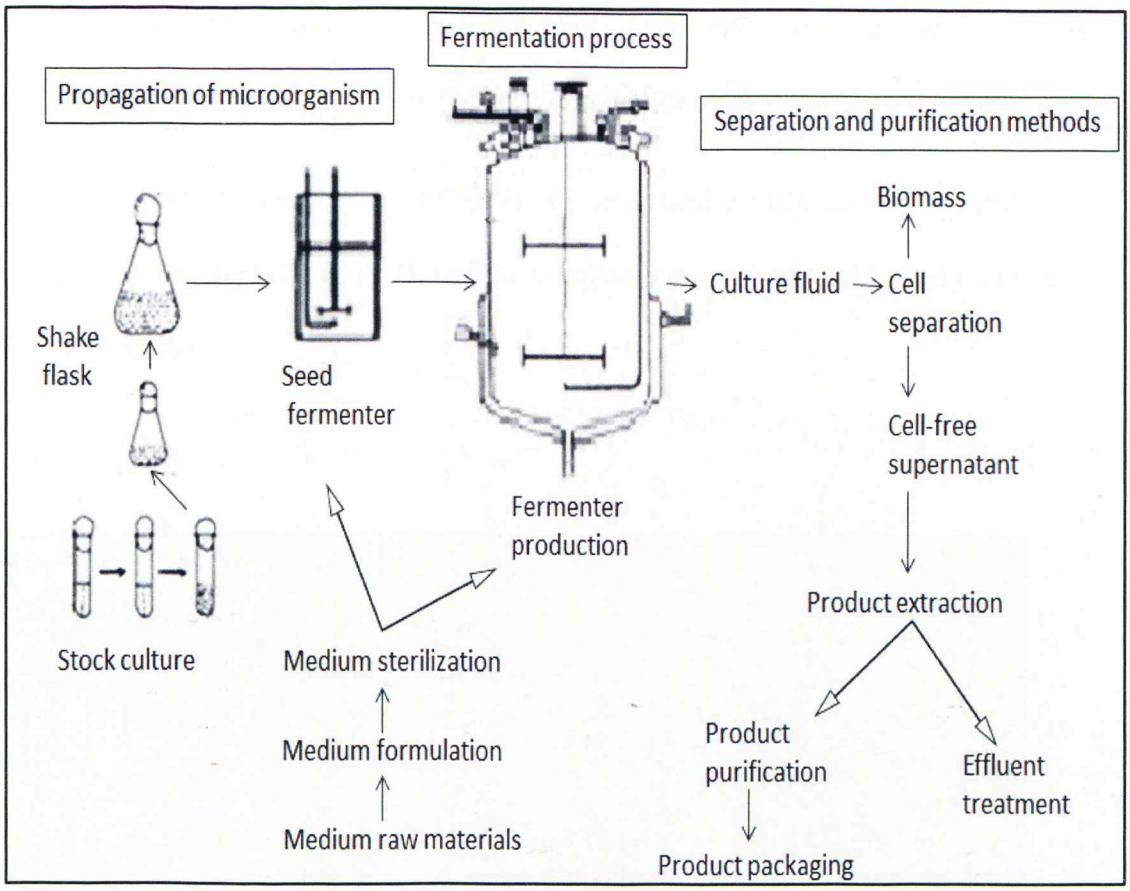

Figure 5: Schematic drawing of general fermentation process (Source: Díaz-Montaño, 2013).

2.5 High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography (HPLC) is a form of liquid chromatography (Tissue, 2000). HPLC is used to separate a mixture of compounds that dissolved in solution with the intention of identify, quantify and purify the individual components in the selected mixture.

The production of ethanol as biofuel via anaerobic fermentation will result in the production of fermentation broth with complex mixture of various products and byproducts such as cell debris and nutrients. Therefore, the broths need to be monitored to ensure optimized quantity and quality of ethanol yields. HPLC is use to analyse the broth based on three key parameters; amount of ethanol yields, amount of fermentable sugars such as

maltose and glucose in the broth, and concentration of the unwanted byproducts such as lactic acid and glycerol generated during the fermentation process (Hall & Reuter, 2007).

As stated in the report by Hall & Reuter (2007), the analytical conditions can be optimized to produce the shortest analysis time. Hence, all components can be quantitatively analysed in less than 10 minutes.

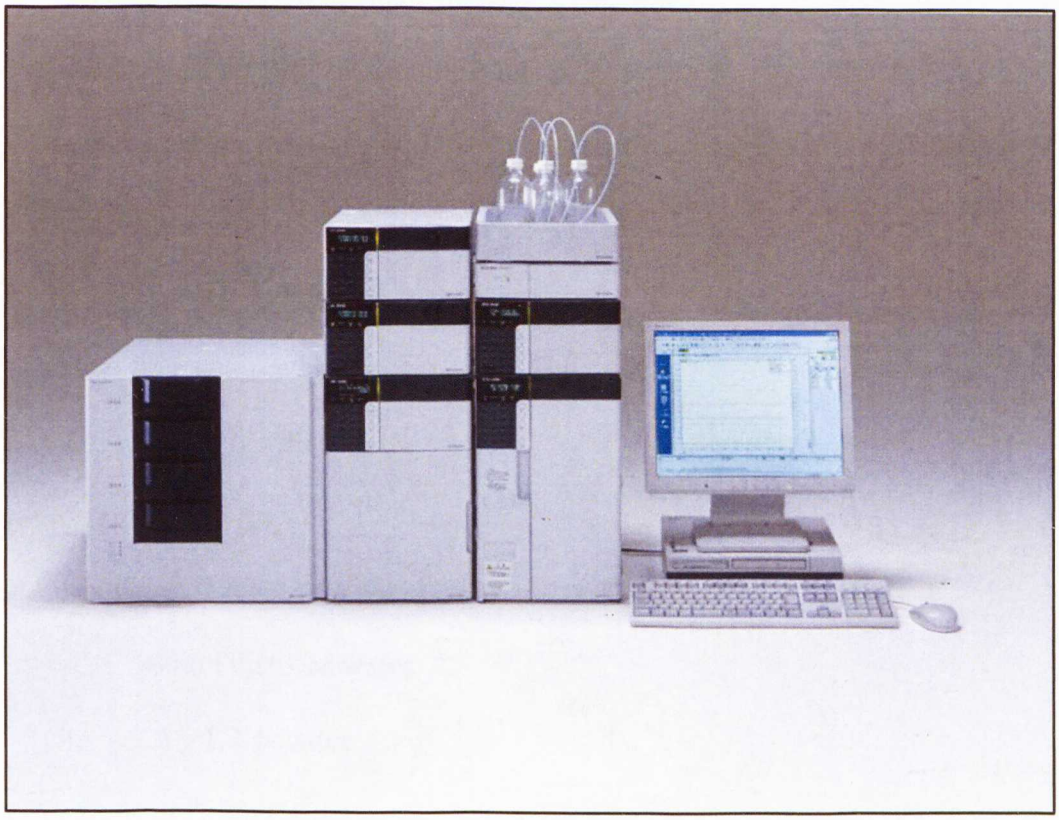

Figure 6: HPLC system (Source: Shimadzu Europa GmbH, 2010).

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3.0 MATERIALS AND METHODS

3.1 Materials and apparatus

1. Carboxymethylcellulose (CMC) (BDH, England).
2. *Saccharomyces cerevisiae* (ATCC 24859).
3. Cellulase (Accellerase, Genencor, USA).
4. Castor oil (SAFC, Aldrich Chemical, USA).
5. Filter paper (No. 1, Whatman, USA).
6. Autoclave (Model 25X-2, Foundry Inc., USA).
7. Incubator shaker (innova 4000, New Brunswick Scientific Co. Inc., USA).
8. 1 M Citrate buffer
    i. 44 g citric acid
    ii. 86 g sodium citrate
    iii. 500 ml distilled water
9. Glycerol stock
10. Luria Broth (LB)
    i. 100 ml distilled water
    ii. 2.5 g LB powder
11. Yeast Malt Broth (YMB)
    i. 100 ml distilled water
    ii. 2.14 g YMB powder
12. Yeast Peptone Broth (YP Broth)
    i. 98.75 ml / 97.5 ml 0.05 M citrate buffer
    ii. 3 g bacterial peptone
    iii. 1.5 g yeast extract
    iv. 1.25 g / 2.5 g CMC

13. DNS reagent

    i. 708 ml distilled water

    ii. 5.3 g 3,5 Dinitrosalicylic acid

    iii. 9.9 g Sodium hydroxide

    iv. 153 g Sodium potassium tartrate (Rochelle salts)

    v. 3.8 ml Phenol (melt at 50 ^{o}C)

    vi. 4.2 g Sodium metabisulfite

14. High Performance Liquid Chromatography (HPLC) system (LC-20A, Shimadzu, Japan).

15. Spectrometer (SP 880, Metertech Inc., Republic of China).

3.2 Methodology

3.2.1 Preparation of inoculum

Inoculum chosen for this study is the *Saccharomyces cerevisiae* (ATCC 24859). For this study, there were two types of yeast culture medium used, Luria broth (LB) and Yeast malt broth (YMB). For the first four fermentations without castor oil (25 FPU enzyme 2.5% CMC, 25 FPU enzyme 5.0% CMC, 50 FPU enzyme 2.5% CMC and 50 FPU enzyme 5.0% CMC), LB was used as the culture medium. To prepare the medium, 100 ml of distilled water was mixed with 2.5 g of LB powder in 250 ml conical flask. Then, the medium was autoclaved. Meanwhile, one vial of *Saccharomyces cerevisiae* (ATCC 24859) was thawed. The vial content was poured into the medium inside the laminar flow hood to avoid contamination. Lastly, the culture was left to culture overnight on the incubator shaker. The quantity of the culture decreases after few observations. Hence, for the last four fermentations with castor oil (25 FPU enzyme 2.5% CMC, 25 FPU enzyme 5.0% CMC, 50 FPU enzyme 2.5% CMC and 50 FPU enzyme 5.0% CMC), YMB was chosen to replace LB as the culture medium. The above steps were executed except when using YMB, 100 ml of distilled water was mixed with 2.14 g of YMB powder.

3.2.2 Preparation of fermentation broth

The yeast peptone broth (YP broth) for 2.5% CMC substrate was prepared by mixing 3 g of bacterial peptone with 1.5 g yeast extract and 1.25 g of CMC in 98.75 ml of 0.05 M citrate buffer. Meanwhile, for 5.0% CMC substrate, 2.5 g of CMC was mixed with 3 g of bacterial peptone and 1.5 g yeast extract in 97.5 ml 0.05 M citrate buffer. This broth was prepared in duplicate. Then, the harvested yeast pellet from previous culture medium was transferred into the broth. Next, cellulase enzyme with desired volume was pipetted into the broth. As for fermentations with castor oil, 15 ml of castor oil was added.