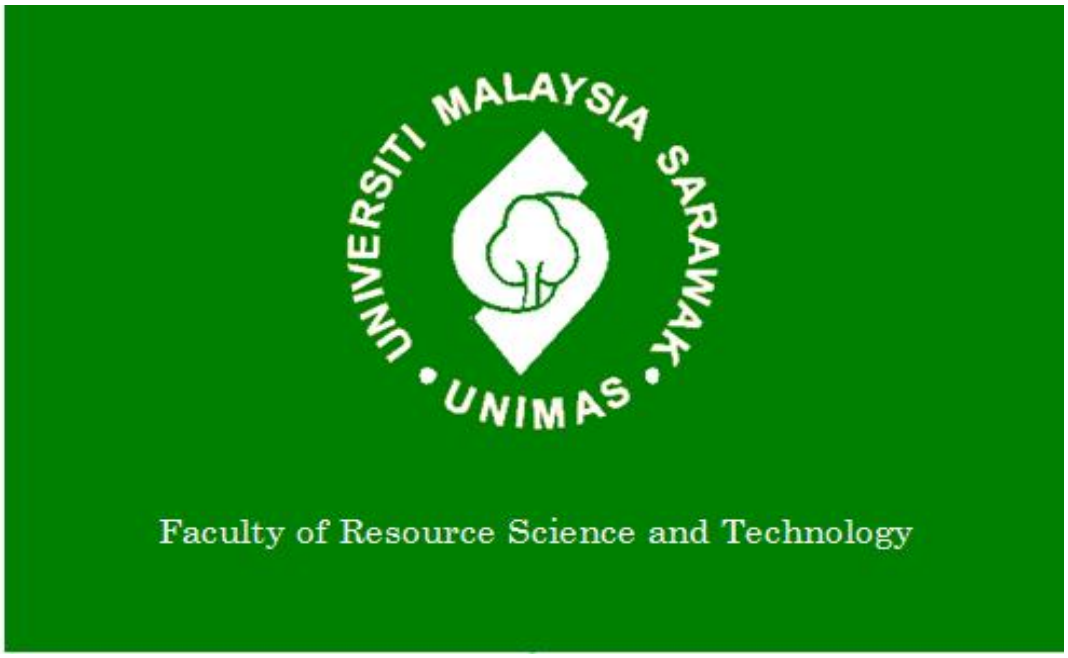

Coculture of Amylolytic Strain and *Saccharomyces cerevisiae* for Ethanol Production

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List of Abbreviations

rpm	Revolutions per minute
ml	milliliter
μm	micrometer
g/L	gram per litre
°C	degree Celsius
h	hour
L	Litre
M	Mole
%	Percentage
CFU	Colony Forming Unit
DCW	Dry cell weight
HCl	Hydrogen chloride
HPLC	High Performance Liquid Chromatograph
NaOH	Sodium Hydroxide
OD	Optical Density

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Coculture of Amylolytic Strain and Saccharomyces cerevisiae for Ethanol Production

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ABSTRACT

The objective of this study was to test the hypothesis if the coculture of an amylolytic strain isolated from local *ragi tapai* and *Saccharomyces cerevisiae* CSI-1 is able to perform ethanol fermentation efficiently. The amylolytic strain isolated from local *ragi tapai* was cultivated in raw sago starch and after 10 h of fermentation was inoculated with a culture of *Saccharomyces cerevisiae*. The results showed that the coculture was able to produce only 1.89 g/L of ethanol in 72 h, while the maximum ethanol production at 84 hour was 2.55g/L. In conclusion, the use of an amylolytic strain and *Saccharomyces cerevisiae* could be a good technique to enhance the ethanol production to reduce the use of enzymes and cost of ethanol production. Further studies are necessaries to enhance the performance of this coculture.

Keywords: Amylolytic strain, *Saccharomyces cerevisiae,* Coculture, ethanol

ABSTRAK

Objektif dalam eksperimen ini ialah untuk menyiasat hipotesis tentang pengkulturan bersama strain amylolytik yang diasingkan daripada ragi tapai tempatan dan Saccharomyces cerevisiae CSI-1 dapat melaksanakan fermentasi etanol dengan cekap. Strain amylolytik diasingkan daripada ragi tapai tempatan akan dikulturkan dalam kanji sago mentah dan dikultur bersama Saccharomyces cerevisiae selepas 10 jam. Keputusan menunjukkan yang pengulturan ini dapat menghasilkan etanol hanya sebanyak 1.89 g/L pada penapaian 72 jam manakala penghasilan maksimum etanol pada 84 jam ialah 2.55g/L. Kesimpulannya, penggunaan starin Amylolytik bersama Saccharomyces cerevisiae boleh digunakan sebagai teknik yang baik untuk meningkatkan penghasilan etanol bagi menggurangkan penggunaan enzim dan kos penghasilan etanol. Kajian lanjutnya perlu dilakukan bagi meningkat prestasi pengkulturan ini.

***Kata Kunci**: Strain Amyloltik, Saccharomyces cerevisiae, Pengkulturan bersama, Etanol,*

1.0 Introduction

Ethanol industry has become one of major industry in the world as solution to fossil fuels shortages. The government and private companies start to grab this opportunity to produce new source of energy. In Malaysia through biofuel policy, the biodiesel research is conducted in order to have alternative energy source as reducing the nation depend on petroleum source that will deplete soon, promoting the demand for palm oil and price stabilisation of palm oil (Nagarajan, 2009). Meanwhile in UNIMAS, the ongoing research on sago to produce ethanol in cheapest way become driven interest for contribution on this study.

Furthermore, major contribute for development of ethanol industry is oil crisis during late 1970 as oil price is tremendously increased which cause dispute among oil producer countries and oil buyer countries. So in order to seek alternative way to tackle this problem in near future, the researchers start to find another source which could be source of new energy as replacement the oil as main source of energy. For example Brazil developed a program called "Proalcohol" which focus on production of Brazilian gasohol by blending the anhydrous ethanol with gasoline (Jacques *et al*, 2003) during late 1970. Meanwhile in United States, ADM (Archer Daniels Midland Company) is corporations which held responsible producing the ethanol from corn. Besides that, the Kyoto protocol also become one of major driven factor in 1990s for resurrection of ethanol production as improvement of environment by lessen air pollution and green house effect. Moreover, the production of ethanol and biodiesel from canola are welcomed by European Union in 2001 as agreement with Kyoto protocol and production of non-polluting renewable fuel based on crop. In addition in Asia, nations such as India, China, Thailand and

Malaysia are concern in producing new alternative fuel due to the impact from increased oil price during late 2007.

Although many countries able to produce the ethanol from agriculture crops, it also have impact in agriculture industry. The corn, sugarcane and cereal are main staple food supply for human consumption and animal feed industry. This have contributes price of agriculture commodities to rise in the world market. Therefore, researchers try to seek another alternative material for ethanol production. So, Sago has become one of alternative source for ethanol production. This is due to higher productivity of sago starch compare to corn, cereal and sugar cane, wheat and tapioca (Bujang, 2000). Furthermore, the sago is not main human dietary food.

Therefore in this study of co-culture of *Saccharomyces cerevisiae* and an amylolytic strain to ethanol, sago has become the main substrate for ethanol fermentation. This study will enable the reduction fermentation time and cost of ethanol production. The basis of the reduction of ethanol production costs are through improvement in feedstock pretreatment, shortening of fermentation time, lowering the enzyme dosages, improving the overall starch hydrolysis and integration saccharification and fermentation (Nikolic *et al.*, 2010). The direct conversion of sago starch into ethanol by co-culture of *Saccharomyces cerevisiae* and the amylolytic strain will improved the method of ethanol fermentation. Furthermore, co-culture will enable faster and more efficient production of ethanol for industry proposes. This is due to co-culture enables the two distinct cells such as the *Saccharomyces cerevisiae* and an amylolytic strain grows together without effect each other life span in the culture which enables the researcher to easily use them for researcher purpose.

The main objective of this study is to test whether the coculture of an amylolytic strain and *Saccharomyces cerevisiae* is able to produce ethanol. Furthermore, the amylolytic strain isolated from local ragi tapai was tested whether it can hydrolyse sago starch. In addition, the coculture of co-culture of *Saccharomyces cerevisiae* and amylolytic strain is tested whether both organisms able to co-exist to perform the ethanol fermentation. The aim of the research is to have an amylolytic strain which produce amylolytic enzymes to produce glucose and then add the ethanologenic *Saccharomyces cerevisiae* to use the sugar to produce ethanol instead of use commercial industrial enzymes for hydrolysis of sago starch.

2.0 Literature Review

2.1 Sago

Sarawak is one of primary source of sago palm in Malaysia especially in areas of Oya and Mukah districts. The sago is actually derived from Javanese word that refers to starch containing palm pith. This is due to sago is obtained from various tropical palm stems especially from spongy centre or pith. The scientific name for sago palm is *Metroxylon sagu* (McClatchey *et al*., 2006). The true sago palm belongs to the family Plamae Jussieu, subfamily *Metroxyline Blume* and genus *Metroxylon* Rottoboell (Uhl and Dransfield, 1987). As extremely hardy plant, sago palm can ready thrive in swampy, acidic peat soils, submerged and saline soils as only few other crops can survive in which growing more slowly peat soil than in mineral soil (Flatch 1997).

In Sarawak, thorny *Metroxylon rumphii* Mart and the *thornless Metroxylon sagu* Rottb (Sim, 1985) are two main species of sago available. Therefore, different grade of sago flavour can be produced through using this two different species of sago palm in modern factories especially in Sarawak. The mechanism process involve in extraction of starch from sago palm are debarking, rasping, sieving, settling, washing and drying. Besides that, the factors such as soil conditions and spacing in the plantation will directly influence content of sago starch in palm. According to Bujang and Ahmad (1999), 100-300 kg of starch/palm (dry weight basis) is produced in cultivation and 120 kg of starch/palm (dry weight basis) produced from wild condition while 185 kg starch/palm (dry weight basis) produced in semi wild condition. Furthermore, the source of sago readily available as sago palm always reproduce without

replanting due to young palms will develop from these suckers and these suckers can be developed more before reaching its maturity at about 9 to 11 years (Bujang, 2006). Moreover, the sago plants grow wild and abundantly in acidic peat swamp of Sarawak, Malaysia (D.S.Awg-Adeni *et al*, 2010) which no need special management unlike other agriculture crops.

The Sago starch is use in this study as the starch is second most abundant compound that produced in higher plants after cellulose (Satyanarayana and Kunze, 2009). The function of starch is temporary energy storage of plants that can be accessed at a later time point while cellulose as the structural component of plants. Starch is semi crystalline granules that consist of amylose and amylopectin. The source of starch and the maturity of the crop plant will determine the ratio of amylose and amylopectin but 20 to 25 % of starch is generally amylose. The ratio of amylose and amylopectin content will determine the temperature of starch gelatinization. Apart of that, starch is nontoxic, renewable carbon source and cheap that abundant available in agriculture crops such as corn, cereals, sugarcane, sago and tapioca. Moreover, the storage of starch only requires minimum attention as the starch is not susceptible to spoilage. The starch can be stored as flour in minimum condition such as room temperature.

2.2 Yeast *Saccharomyces cerevisiae*

The "sugar fungus" is Latin language for *Saccharomyces cerevisiae* which utilise by human for thousands of years. The ancient peoples of Sumeria, Babylon, and Ur were documented beer making in their artwork and later by Egyptians who also depicted the baking of leavened bread (Verachtert and De Mot, 1990). Although the fermentation is used globally, it was after independent studies by Cagniard de la Tour, Schawnn and Kutzing that showed the

organism that responsible for fermentation is yeast. Yeast is unicellular, microscopic fungi that reproduce by budding and binary fusion. According to the species, the size of yeast cells varies and diameters can range from 2 to 10 μm. According to Kurtzman, the two major taxonomic classes of yeast are Ascomycotina and basidiomycotina. Moreover there are about 600 known species of yeast and among them the most industrially commercialised is *Saccharomyces cerevisiae* that generally is used for baking and brewing. The shape of genus Saccharomyces is elliptic yeast. *Saccharomyces cerevisiae* is belongs to fungal group of Ascomycetes (Beudeker *et al*, 1990). The genome of *Saccharomyces cerevisiae* is divided up into 16 chromosomes ranging in size between 250 kb and >2500kb. Moreover, *Saccharomyces cerevisiae* will produce daughter cells (buds) through multilateral budding from any part of the external surface of the cell. Besides that, there are many strains within the *Saccharomyces cerevisiae* which show very minor genetic differences.

The yeast has become best model use for fermentation as it has several advantages. First and foremost, yeast is unicellular organism which can be grown easily on defined media which the researcher can complete control over environmental conditions. Another advantage of yeast is through biochemical approaches, the yeast have studied in great detail for its genetic techniques and functions. Furthermore, the life cycle of yeast is short compare to other organisms which made it suitable for fermentation as *Saccharomyces cerevisiae* exists and grows in the haploid life cycle and diploid cellular forms.

Apart of that, *Saccharomyces cerevisiae* is highly specialised for converting certain sugar to ethanol and carbon dioxide which made it uniquely suited for producing alcoholic drinks and for raising bread (Zimmermann and Entian, 1997). Furthermore, *Saccharomyces cerevisiae* is choose in the winemaking process due to several desirable characteristics. They are alcohol tolerant which their ability to ferment to dryness and in the presence of relatively high concentration of alcohol, sulphur dioxide tolerance, ability to carry out even fermentation, ability to ferment at low temperatures that allow retaining of fruit characters and low acetic acid formation, produces clean ferments with desirable characters and low volatile acidity.

2.3 Amylolytic Strain from *Ragi Tapai*

Ragi tapai is dry-starter culture that can be produce from a mixture of rice flour, spices and water or sugar cane juice or extract (Azmi *et al*, 2010). Moulds (*Rhizopus oryzae, Amylomyces rouxii, Mucor sp.* and *Candida utilis*) and yeasts (*Saccharomyces cerevisiae, Saccharomycopsis fibuliger* and *Endomycopsis burtonii*) are mainly found in the ragi tapai. In every sample of dry starter, one or two of bacteria of types of cocci were present with at least one yeast and one *Mucoraceous* mold (Heseltine *et al*., 1988). In this study, the amylolytic strain is isolated from local *ragi tapai* which produces amylase enzymes. The amylase enzyme could be produced economically by this microorganism (Nigam and Singh, 1995) which can be use to easily hydrolysed raw starch to fermentable sugars such as glucose.

Besides that, according to Nurachman *et al* (2010) and Jamai *et al* (2007) amylase, amyloglucoamylase and pulluanase are enzymes needed for hydrolysing of starch. The linear α-D-(1, 4)-glucan of amylose and branched amylopectin are two main polymers of starch

components that need to be hydrolysed into glucose (fermentable sugars) for ethanol fermentation by ethanolic yeasts. Therefore the amylolytic strains will produce amylase enzyme for hydrolysis of raw starch into glucose. It is also known that the *Saccharomyces cerevisiae* unable to produce ethanol from direct conversion of starch due to lack of the starch decomposing enzyme.

The three stage process involve in conversion of starch for conventional method that are liquefaction of starch by α-amylase , saccharification of liquefied starch by enzymes to sugars followed by fermentation using *Saccharomyces cerevisiae* (Saiffuddin and Hussain., 2011). Therefore, the liquefaction and saccharification of starch that used the industrial enzymes such as Termamyl SC and Dextrozyme will add to the overall the cost of ethanol production. So according to Somda *et al* (2011) and Nadir *et al* (2009), the effective method for direct fermentation of starch is simultaneous saccharification of starch with an amylolytic yeast or mould and fermentation of saccharified starch by distillers yeast. A multistage process of simultaneous saccharification and fermentation for conversion of starch into ethanol can be carried out in one reactor and *Saccharomyces cerevisiae* can use to simultaneously fermented glucose that produced during the saccharification to ethanol (Verma *et al*., 2000).

2.4 Co-culture

In recent years, a new method is introduced which combines both hydrolysis and fermentation of starch in a single-step process and referred to as simultaneous saccharification and fermentation (Ado *et al.*, 2009). The interaction of two different cell types is useful in understanding the co-culture. In the same vessel, co-culturing of synergistic relationships

between two organisms is culture together which would faster the rate of reaction and reduce the amount of time in the fermentation. In order to maximize the ethanol yield from starch, the co-culture of amylolytic and sugar fermenting organisms will eliminate enzymatic saccharification process. The co-culture of microorganisms will allow the ethanol production to be efficient and economical method due to lesser environment cost. The use of commercial enzyme such as glucoamylase that used for saccharification contributes to increase the price of the ethanol production (Manikadan & Viruthagiri, 2010). A study conducted (Abourized & Reddy, 1986) shows that co-culture between *Saccharomyces cerevisiae* (ethanolic yeast) with an *Aspegillus* species in a starch medium would prevent accumulation of inhibitory concentrations of reducing sugar and enhancement of the amylolytic activity , the amount of starch metabolize and the total ethanol yield. The fermentation using coculture must set under low aeration and low agitation conditions to allow ethanol production (Kobayashi, 2004).

2.5 Batch Fermentation

Batch fermentation process can be defined as the process begins with the inoculation and end with retrieval of the product happens inside a single fermenter with no intermediate steps. The batch fermentation is a closed system and nothing is added apart from oxygen (in the case of aerobic microorganisms), an antifoam agent and acid or base to control the pH (Rastogi, 2007). As a result of the metabolism of the cells, the composition of the culture, the metabolite concentration and the biomass concentration are change constantly. There typical phases of the growth are generally four (lag phase, log phase, stationary phase and death phase) which observed after the inoculation of a sterile nutrient solution with microorganisms and cultivation under physiological condition.

The advantage conferred to batch fermentation is a complete utilization of the substrates due to there is no wastage or waste away of the broth .Moreover the risk of contamination or cell mutation is reduced as the growth period is shorter and low maintenance and low capital investment compared to other fermentation system. The batch fermentation is more flexibility to be interchangeable with different products or biological systems. Batch fermentation has higher raw material convertibility levels due to it is a controlled growth system. The production of biomass, primary metabolites and secondary metabolites are mainly use the batch fermentation in fermentation industries.

2.6 Ethanol as bio-fuel

One industrial importance of the ethanol production is its use as bio-ethanol. As excellent alternative fuel to fossil fuels, bio-ethanol can use either as pure fuel with high efficiency and performance or as a gasoline additive. A variety of plant-derived raw material such as sago, corn, wheat, sugarcane and rapeseed can use to produce the bio-ethanol through fermentation. It is easy transported as it is in liquid state and use to increase octane rating of the fuel by blended it with gasoline. The advantage of bio-ethanol are less polluting than gasoline, less toxic compare to gasoline, secure renewable energy to due to can be produce through fermentation , has higher octane number and higher heat vaporization than gasoline.

Although the bio-ethanol produce from starchy material which meet with demand from the market due to cost effective but it also contribute to disadvantages. The main disadvantage is the starch material is use as animal feed and human needs such as corn is one of staple food in

Africa. This will contribute to increase of commodity price in global market and insufficient of starch material to meet the demand of fuel ethanol industry.

Moreover, the substitute material such as sago starch can be use for ethanol production because it is not staple food nor animal feed. The sago starch is will very suitable for ethanol production as it have more starch content compare to other starch material. As the Malaysian Government is showing interest in bio-fuel industry, many bio-industrial companies try to grab the chance to produce bio-fuel which able to sustain demand from market.

3.0 Materials and Methods

3.1 Sago

Sago starch flour was obtained from Herdsen Sago Mill, Pusa, Sarawak.

3.2 Microorganism

The amylolytic strain isolated from local *ragi tapai* and the yeast *Saccharomyces cerevisiae* CSI-1 (JCM 15097) were obtained from Bio-Fuel R&D lab of the Faculty Resource Science and Technology. The stock culture for *Saccharomyces cerevisiae* CSI-1 (JCM 15097) and amylolytic strain are kept at -84°C in agar slant containing 20 g/l glucose, 5 g/l yeast extract and 1.5% Agar. At room temperature, 1 ml of frozen *Saccharomyces cerevisiae* CSI-1 (JCM 15097) and amylolytic strain are thawed and refreshed in 10 ml culture consists of 5 g/L yeast extract and 20 g/L glucose respectively. At 32°C, the broth culture was incubated for 9 h and every two weeks, the subculture was performed.

3.3 The inoculums preparation

The media for inoculums were prepared for *Saccharomyces cerevisiae* and amylolytic strain. 10 g/L of glucose and 5 g/L of yeast extract were weighted and dissolved into 100ml of distilled water in two Durham tube respectively. Then, the two Durham tube were autoclaved at 120°C at 20 minutes. After that, 10ml of *Saccharomyces cerevisiae* and amylolytic strain were

inoculated separately into Durham tube. The culture of *Saccharomyces cerevisiae* and amylolytic strain were placed in incubator for 24 hours at room temperature.

3.4 Fermentation medium

The fermentation medium was prepared by weighting 10% (w/v) of sago flour that dissolved in 1000ml of distilled water. Then, the mixture undergoes process of starch gelatinisation by heating the slurry at 60°C for 5 minutes by using hot plate stirrer. Then, the temperature of the mixture are increased into 70°C and mixed for 1 hour to allow the starch to dissolve in water. After that, the pH of the mixture was adjusted to pH 5.0-6.0. Then, the mixture was transferred into 2L conical flask and 5 g/L of yeast extract was added into the media as nitrogen source for growth promoter. The fermentation media then was autoclaved at 121°C at 20 minutes.

3.5 Coculture fermentation

2 L of conical flask was used to perform coculture of batch fermentation. 1 L was used as the working total volume of fermentation. The temperature set at 30 °C and rate of agitation was fixed at 200rpm. Then, 5% or 10ml (w/v) of amylolytic strain was added in conical flask and fermentation was carried out for 48 hours in conical flask under "limited aerobic" condition and sample was taken every 12 hours for 48 hours to test glucose production. After 48 hours, 5% or 10 ml (w/v) of *Saccharomyces cerevisiae* was added in fermenter flask for ethanol production

and fermentation was continue carried out and sample was taken every 6 hours for next 24 hours to test for ethanol production. The entire procedure of fermentation was repeated for first 60 hours for amylolytic strain fermentation added in the media for glucose production and then *Saccharomyces cerevisiae* was added for ethanol production next for additional 24 hours. The whole process for fermentation was carried out for amylolytic strain and *Saccharomyces cerevisiae* for 72 hours and 84 hours respectively.

3.6 Analytical method

The total samples collected after 72 hours of coculture fermentation were 10 samples and 11 samples for 84 hours of coculture fermentation

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3.6.1 Ethanol analysis using HPLC

HPLC (high performance liquid chromatography) system was used to analysis the production of ethanol and glucose in the culture broth. A Shimadzu (Kyoto, Japan) chromatographic system that consists of Shimadzu LC-20AT (four pumps) and Shimadzu RID-10A Refractive Index Detector were used to perform the HPLC analysis. The chromatographic separation was performed, by a Biorad Aminex column (7.5 mm x 150 mm) at 60°C. At flow rate of 0.60 ml/min, 0.005 M of H_2SO_4 was used as a mobile phase. In the column, the sample injected was exactly 25 μL and ethanol standard also used as sample detection with same amount of condition. The use of ethanol standard for estimate of ethanol equivalent values gives a standard curve.

3.6.2 Determination Optical Density (OD) of sample

The certain OD was obtained by diluting the samples with sterile distilled water. The optical density (OD) of sample was analysed by using Shimadzu UV-Vis Spectrophotometer UV Mini-1240 at wavelength set at 600nm. The blank sample that collected from fermentation medium before adding of inoculums was used as blank.

3.6.3 Determination of DCW

In 50ml centrifuge tube, 10ml of the broth sample will be conducted for determination of dried cell weight. The sample were be centrifuged at 5000 rpm for 10 minutes,. After that, the supernatant from the tube was collected for ethanol and glucose concentration analysis by HPLC and add 10 ml of 0.2 HCl into the tube. Next, again resuspended and centrifuged the cells for another 5000 rpm at 10 minutes. This process was performed twice. Then, the supernatant was discarded, and the pellet was the resuspended using distilled water into the tube and filter with the filter membrane (0.45 μm, in diameter, cellulose). Later, the cells were dry in a oven for three days at 60 °C until the weight will be constant.

For determination of DCW:-

DCW (g/L) $=\frac{(A+B)-A}{M}$x1000

Where:

A=Weight of dried filter (g)

B= Weight of Cells (g)

C=Volume of sample (l)

3.6.4 The analysis of Colony Forming Unit

The colony forming unit (CFU) was analysis by using haemocytometer Hirschmann EM Techcolour (Rouge, 2002). The slide of haemocytometer was washed with distilled water and wiped with ethanol. Meanwhile, the dilution samples was prepared by using 20 µL of samples and 20 µL of Methylene Blue respectively and mixed in an Eppendrof tube. This mixed sample was left for 5 minutes. Then 10 µL of the mix was pipette into both side of haemocytometer counting camber and covered with cover slip. After that, the haemocytometer slide was viewed under 200x magnification of microscope for cell counting. A colony forming unit (CFU) versus optical density (OD) standard graph is produced.

3.6.5 The glucose analysis using Dinitrosalicylic acid (DNS) method

DNS method was used to determine reducing sugar in fermented broth (Miller 1959). The OD reading of mixture samples was done by using Shimadzu UV-Via Spectrometer UV Mini-1240 at 600nm. A standard glucose curve was used to calculate the concentration of glucose in the sample.

3.6.6 Analysis of data

The statically analysis of data was done by using Microsoft Excel 2007.