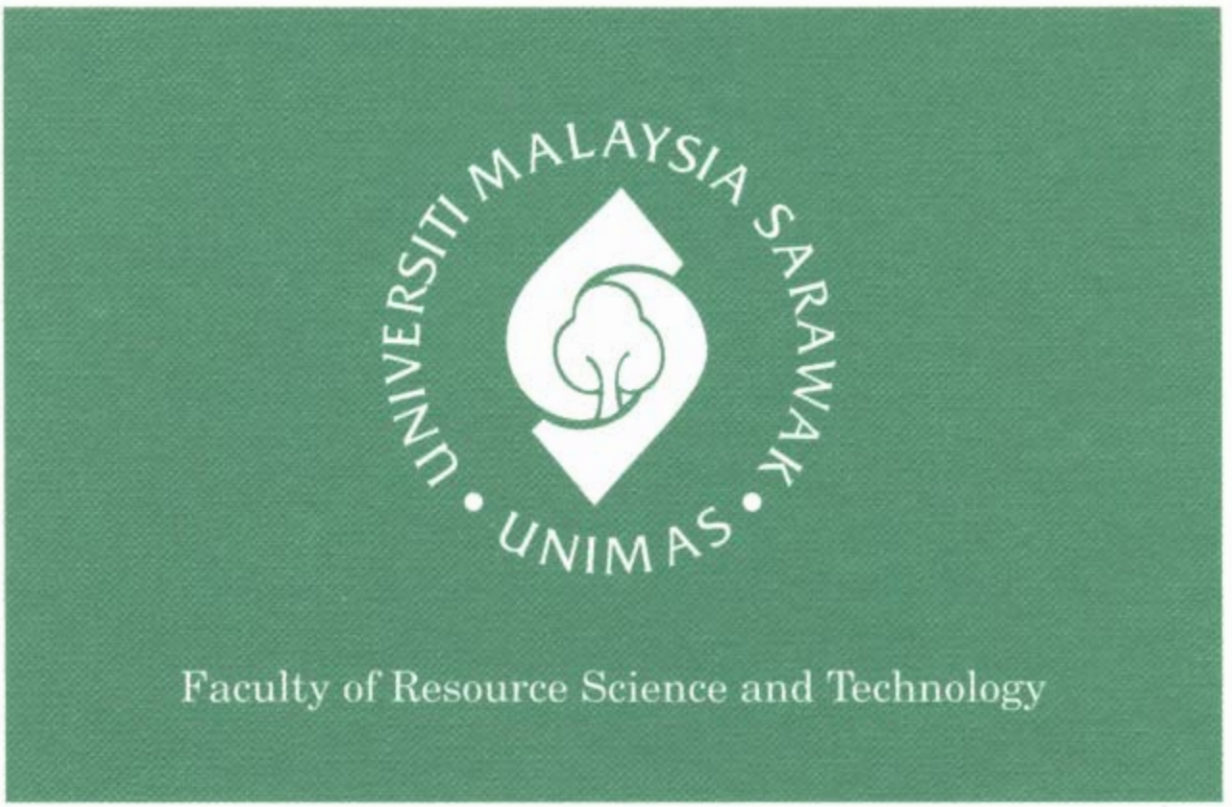

EFFECTS OF DIFFERENT GLUCOSE CONCENTRATIONS DURING FED-BATCH LACTATE FERMENTATION

Siti Hajrah Binti Mohamed Gaus

Bachelor of Science with Honours
(Resource Biotechnology)
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ABSTRACT

Fed-batch lactate fermentation was carried out using *Lactococcus lactis* IO-1 with hydrolysed sago starch (HSS) as the sole carbon source. Four different initial and feed glucose concentrations were studied; 30g/L, 40g/L, 50g/L and 60g/L at pH6 with feeding and harvesting volume of 200mL. Fermentation broth was harvested and fed with fresh media for every 24 hours. Maximum lactate was obtained; 63.71 g/L at 60g/L glucose in 48 hours with lower residual glucose of 0.83g/L. Lower lactate concentration was observed; 13.40g/L, 27.67g/L and 42.50g/L when using lower glucose concentrations; 30g/L, 40g/L and 50g/L respectively in 48 hours. Fed-batch fermentation performed here revealed that fermentation process should be terminated after 48 hours since minimum residual glucose with higher dry cell weight (DCW) and lactate concentration were obtained.

Keywords: Fed-batch fermentation, *Lactococcus lactis* IO-1, sago starch, glucose concentrations, feed volume

ABSTRAK

Fermentasi separa kelompok telah dijalankan dengan menggunakan Lactococcus lactis *IO-1 dan kanji sagu terhidrolisis sebagai sumber karbon utama. Empat kepekatan awal dan kepekatan penambahan media yang berbeza diuji iaitu 30g/L, 40g/L, 50g/L dan 60g/L dengan isipadu penambahan dan pengekstrakan sebanyak 200mL. Pengekstrakan dan penambahan isipadu media fermentasi dijalankan pada setiap 24 jam. Kepekatan laktat maksimum diperolehi apabila menggunakan kepekatan glukosa 60g/L iaitu sebanyak 63.71g/L dengan baki glukosa yang rendah iaitu 0.83g/L pada masa fermentasi ke 48 jam. Kepekatan laktat yang rendah diperoleh; 13.40g/L, 27.67g/L dan 42.50g/L apabila menggunakan kepekatan glukosa yang rendah; iaitu masing masing pada 30g/L, 40g/L dan 50g/L pada masa 48 jam. Fermentasi separa kelompok yang telah dijalankan ini menunjukkan bahawa proses fermentasi ini boleh dihentikan selepas 48 jam kerana berat sel kering dan kepekatan laktat yang diperoleh adalah tinggi selain baki glukosa yang rendah.*

Kata kunci: *Fermentasi separa kelompok,* Lactococcus lactis *IO-1, kanji sagu, kepekatan glukosa dan isipadu penambahan*

1.O INTRODUCTION

Lactate fermentation is the major energy yielding pathway in many anaerobic bacteria and in animal cells operating under anaerobic or hypoxic (oxygen-deficient) conditions (Becker *et al*, 1996). Nowadays, it has become one of the most important biotechnological process that has large potential of commercial value. The product of lactate fermentation; lactic acid has a wide variety of industrial application, being used as a preservative and acidulates in foods and as a precursor for polymer in polylactic acid.

There are three types of fermentation systems; batch, fed-batch and continuous system. In this research, fed-batch fermentation system is to be used for lactate fermentation utilizing *Lactococcus lactis* IO-1 with enzymatically hydrolyzed sago starch (HSS) as the sole carbon source. Different initial and feed glucose concentrations; 30g/L, 40g/L, 50g/L and 60g/L will be used with 200mL harvest and feed volume.

Previously, some studies on the effects of glucose concentration during batch lactate fermentation showed that lower initial glucose concentration (30g/L) was better to ensure maximum consumption of glucose by *Lactococcus lactis* IO-1 whereby 3.64g/L residual glucose was observed. However, the concentration of lactate was a bit low; 22.28g/L compared to higher initial glucose concentration (60g/L and 90g/L); 32.56g/L and 37.52g/L respectively. For continuous fermentation, previous result showed that using lower initial glucose concentration ; 30g/L with dilution rate of $0.25h^{-1}$, higher lactate yield (0.96g/g) was obtained with 0.33g/L residual glucose in the spent medium (Bujang and Jobli, 2002) . During fed-batch fermentation, previous study using different harvest and feed volume with 60g/L revealed that 200mL of harvest and feed volume was

the best volume that produced 59.38g/L lactate, 5.35g/L of biomass and 3.61g/L residual glucose.

Those previous results showed that by the end of experiments, lower initial glucose has a potential to yield high lactate concentration and minimum residual glucose in batch and continuous fermentation. Therefore, this research should be proceed using fed-batch fermentation in order to find the most appropriate initial and feed glucose concentration that still produce high lactate but will reduce residual glucose as minimal as possible.

The main objective of this research is to identify the effects of feeding lower different glucose concentrations (30g/L, 40g/L, and 50g/L) compared to the commonly used glucose concentration; 60g/L during fed-batch lactate fermentation. Harvest and feed volume of 200mL which was the best volume obtained from the previous study will be used in this study. The effects of feeding different concentrations of glucose will be identified based on the production of lactate, biomass formation and residual of glucose by the end of fermentation. Instead of the above objective, this study also aims to investigate whether this fed-batch fermentation should be terminated after 48 hours.

2.0 LITERATURE REVIEW

2.1 Lactic acid

Lactic acid; $CH_3CHOHCOOH$ (2-hydroxypropanoic acid, 2-hydroxypropionic acid) is a chemical compound that plays a role in several biochemical process. In solution, it can lose a proton from the COOH (carboxyl group), turning into lactate ion $CH_3CHOHCOO^-$. Its molecular weight is 90.08g/mol with heating and melting point at 122°C and 17°C respectively. This colorless liquid organic hydroxyl acid that miscible with water or ethanol; is a weak acid, non-corrosive and a good solvent which can be produced either by microbial fermentation or chemical synthesis. In fermentation, lactate is a secondary metabolite that produced during stationary phase of *Lactococcus lactis* IO-1 growth.

Nowadays, lactate shows a great potential as the main commodity for future usage since the production of lactate via anaerobic fermentation can reduce carbon dioxide that can cause green house effects in atmosphere (Ishizaki, 1997). Lactic acid was first commercially produced by Charles E. Avery at Littleton, Massachusetts, USA in 1881 (Vickroy,1985). It is produced commercially for use in pharmaceuticals and foods, in leather tanning and textile dyeing, for cosmetics especially for the correction of scars and wrinkles and in making plastics, solvents, inks and lacquers.

Within the last decade, lactate is widely used as a precursor in polylactic acid (PLA) synthesis to produce biodegradable plastic (Bujang *et al.,* 2001). Recently, there has been an increased interest in lactic acid production of polylactic acid, a polymer used as specialty medical and environmental-friendly biodegradable plastics, which substitute for synthetic plastics derived from petroleum feedstock (Datta *et al.,* 1995). PLA is 100%

degradable, non-allergenic and has been approved by the US Food and Drug Administration (USFDA), which may make it useful for certain food or health applications. The plastic can also be made with various physical properties and degradation rates, making it extremely versatile.

Lactic acid has a pleasant taste. It does not create any foreign element in food, which is a great merit from health point of view. Lactic acid is also used in dairy products such as in the production of cheese, margarine and yoghurt powder. It is also widely used in preserving fruits like firmness of apple slices processing and also inhabitates discolouration of fruits and some vegetables. The use of buffered lactic acid improves the taste and flavour of many beverages, such as soft drinks, mineral water and carbonated fruit juices. It is also useful in processing of meal in sauces for canned fish to improve the taste and flavour and also to relieve the unpleasant amine flavour from fish meal for incorporation into bread.

2.2 *Lactococcus lactis* IO-1

Lactic acid bacteria (LAB) species are applied in human and animal foodstuffs for preservation, flavour enhancement, and some of them are useful for probiotic purposes, and therefore become available to the gastrointestinal tract (Carr *et al.*,2002). Lactic acid bacteria can be divided into two subspecies; *lactis* and *cremonis.*

Lactococcus lactis was isolated in the middle of May 1986 from water in drain pit of a kitchen sink in Fukuoka-shi, Japan. These bacteria are Gram-positive cocci, catalase negative, homofermentative, non-sporulating, and non-motile, that group in pairs and short chains. They are ovoid coccus in shape, 0.8-0.9 μm width and 1.1-1.2μm length (Ishizaki *et al.*, 1990). *Lactococcus lactis* can grow in both anaerobic and macroaerophilic condition (Wood & Halzapel, 1995). The ideal pH for glucose consumption of these bacteria is pH6.0 while the optimum temperature for their growth is 37°C. However, they still can grow at 10°C-45°C temperature range (Ishizaki and Ohta, 1989). These bacteria are also tolerant in 6.5% of NaCl. They have a fermentative metabolism and as expected for lactic acid bacteria, they produce copious amounts of lactic acid.

Various types of microorganisms have been used for lactic acid fermentation such as *Lactobacillus casei* subspecies *rhamnosus* (Senthuran *et al,* 1997), *Lactobacillus rhamnosus* NRRL B445 (Xavier *et al,* 1995) and *Lactococus lactis* ssp. *Lactis* ATCC 19435 (Akerberg *et al.,* 1989) other than *Lactococcus lactis* IO-1. *Lactococcus lactis* IO-1 is to be used in this research because of its special characteristic that can convert more than 90 % of the glucose to lactic acid by utilizing 1 % of glucose in inoculum (Ishizaki *et al.*, 1990). Besides that, *Lactococcus lactis* IO-1 produces lactic acid as major

metabolic product from glucose and no other significant lower fatty acid (Ishizaki *et al.*, 1990). Another important criteria of this strain is it produces solely L-homolactic acid , that would not produce carbon dioxide and can widely used for large scale lactate production (Ishizaki *et al.*, 1993).

2.3 Sago

Sago palm (*Metroxylon sagu*) derived from Palmae famili and also known as 'mulong' tree in local. It is wild in swamp forests of eastern Indonesia and New Guinea and may have originated in the Moluccas (Davis, 1986). This palm was then taken to Malaysia, Indonesia and some islands of Polynesia (Yen, 1973). This crop has become an excellent target for development in utilizing sago starch for lactate fermentation as an alternative source of sole carbon due to its ability to grow with minimum care in peat swamps, where other cash crops failed to proliferate.

In Malaysia, more than 90% of all sago planting-areas are found in the state of Sarawak, whereby about 75% of Sarawak is occupied with sago plantation, mostly founding in the areas of Mukah, Igan and Oya , Dalat, districts of Sibu Division, and Pusa, Saratok districts of Sri Aman division. The total area cultivated with crop is estimated to be about 20 000 ha with the total annual production estimated to be around 90,000 tonnes (Bujang *et al.*,2000).

Sarawak is the major exporter of sago products, with an annual export of over RM25 millions for the state. The total amount of sago starch exported in 2000 from the state of Sarawak was 61,000 tonnes. At a very low price of US$0.15/kg, this crop still manage to procure a total income of US$9.15 million for the state (Dept of Statistic, 2001).

Raw material such as whole-wheat flour (Akerberg *et.al.*,1986), whey (Roy *et al,*1986), barley starch (Linko and Javanainen,1996) and sago starch (Ishizaki *et al*,1999) have been used as the carbon source in lactic acid fermentation. However in this study, sago starch is to be used as the sole carbon source since it is the highest among all grains and cereals; almost three times greater than corn, rice and wheat, and about 17 times than

tapioca (Ishizaki, 1997). Sago starch is fermentable by most microorganisms, whereby it is easily hydrolysed into glucose by fungal or bacterial amylase and this can be further converted into lactic acid by anaerobic bacteria or ethanol by yeast (Bujang and Ahmad, 2000).

The potential of the sago industry can be discussed from three view points which are as foodstuff to avoid famine caused by the increased in human population, raw material for the manufacturing of food additives, sugars and flavouring and also a potentially recyclable source of energy through conversion of starch to biofuel (Bujang and Ahmad, 2000). In Malaysia, sago starch is mainly utilized for the purpose of food production, including manufacture of vermicelli, bread, crackers, and other traditional foods. It is widely used in the production of flavour enhancer, Monosodium Glutamate (MSG), and the production of syrup with high fructose and glucose content.

Other uses of sago starch are in the wood industry as an extender for urea formaldehyde adhesives (Sumadiwangsa *et al.*, 1987) and also in the petroleum industry to replace hydroxymethyl cellulose to control fluid loss (Issham *et al.*, 1995). The latest discovery was, the potential used of sago starch in the production of paper glue without toxin (Bujang and Ahmad, 2000). Besides, sago waste pith or fibrous residue, known locally as 'hampas' has also been studied for animal feed (Horigome *et al.*1990), partical board (Haryanto *et al.*1991) and mushroom cultivation (Vikineswary, 1997). Undoubtedly, sago industry has a very bright future as there is high demand for sago based products.

2.4 Fed-batch Fermentation System

Fed-batch culture can be defined as a culture with a continuous nutrient supply and can be operated in two modes; one with variable volume and the other with constant volume (Scragg, 1991). It is a fermentation system which may be considered to be intermediate between batch and continuous process (Walker and Gingold, 1993).

In fixed volume fed-batch, the limiting substrate is fed without diluting the culture, whereas in variable volume fed-batch, the volume changes with the fermentation time due to the substrate feed. In all fed-batch system, part of the broth containing the metabolites is extracted at the end of each test run and fed with exactly the same volume of the fresh medium. The nutrient is added in several closes, to ensure that there is not too much of the nutrient present in the fermenter at any time. If too much of a nutrient is present, it may inhibit the growth of the cells. By adding the nutrient a little bit at the time, the reaction can proceed at high rate of production without getting overloaded.

This system is usually superior to conventional batch operation and changing concentration of the broth will affects the yield or productivity of desired metabolite (Yamane, 1995). It is useful in achieving high concentration of cells for a relative large span of time. Fed-batch process are commonly used in industrial fermentations, for example, for the production of baker's yeast, some enzymes, antibiotics, growth hormones, microbial cells, vitamins, amino acids and other organic acids.

The earliest example of the commercial use of fed-batch culture is the production of baker's yeast in 1915 (Walker and Gingold, 1993). The penicillin fermentation provides a very good example of the use of fed-batch culture for the production of a secondary

metabolite (Hersbach *et al.*, 1984). Besides, fed-batch cultures also have been tested with a number of fermentations such as cephalosporin, and β-amylase (Scragg, 1991).

3.0 MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Sago starch

Commercial food grade sago starch powder is obtained from local supermarket. Samples will be kept free of moisture in an airtight container.

3.1.2 Microorganism

Lactococcus lactis IO-1 (Japanese Collection Of Microorganism, JCM 7638) is used in this research. The prepared stock culture is kept in glycerol at ultra low temperature of -80°C. The stock culture is revived in Thioglycolate (TGC) medium without Dextrose (Difco, USA) and incubated for 18 hours at 37°C. This can be stored for up to 2 weeks at 4°C in the same medium prior to use.

3.1.3 Enzyme for hydrolysis

The enzymes used for sago starch hydrolysis are Termamyl-120L (thermostable amylase from *Bacillus licheniforms*, 120 KNU/g) and Dextrozyme (a mixture of glucoamylase from *Aspergillus niger* and pullulanase from *Bacillus acidopullulyticus*, 225 AGU/mL) supplied by Novo Nordisk.

3.1.4 Culture Media

Thioglycolate (TGC) without dextrose is used to culture *Lactococcus lactis* IO-1. The fermentation and feed medium consisted of 30g/L, 40g/L, 50g/L and 60g/L glucose, 5g/L yeast extract, 5g/L sodium chloride and 5g/L polypeptone. The same medium with 10g/L

glucose is used for inoculum preparation. A 10% (volume/volume) inoculum is used for all fermentation trials.

3.1.5 Bioreactor

Fermentation is conducted in a 2L benchtop fermentor (Biostat-B, B Braun, Germany) with a working volume of 1L.

3.2 METHODS

3.2.1 Reviving of *Lactococcis Lactis* IO-1

The stock culture is revived in Thioglycolate (TGC) medium without Dextrose (Difco, USA) and incubated for 18 hours at 37°C. This can be stored for up to 2 weeks at 4°C in the same medium prior to use.

3.2.2 Enzymatic Hydrolysis of Sago Starch

200g of sago starch powder is suspended in 1000mL of distilled water. The pH is adjusted to pH6.5. Liquefaction process is carried out by adding 0.5μL/g of Termamyl-120L and 6mg of ion Ca^{+} to the slurry, stirred with strong magnetic stirrer and hold at 90-100°C or 2 hours after which starch gelatinization occurs. After 2 hours, saccharification process is carried out by adding 0.65 μL/g of Dextrozyme at pH 4.5 and incubated at 60°C for further 2-4 hours. Details of this process have been reported earlier (Bujang *et al.*, 1999).

3.2.3 Fed-batch Fermentation System

Fermentation is conducted in a 2L benchtop fermentor (Biostat-B, B Braun, Germany) with a working volume of 1L using 60g/L initial glucose. Temperature and pH are controlled at 37°C and pH6.0 respectively with an agitation rate of 500 rpm. Peristaltic pump is connected to the bioreactor to harvest the fermentation broth for sampling. After 24 hours, 200mL of the fermentation broth will be extracted and the same volume of fresh medium with the same glucose concentration as initial is introduced into the

fermenter. The experiment is later repeated using 30g/L, 40g/L and 50g/L glucose concentration.

3.2.4 Sampling

Samples (20mL) are extracted for every 24 hours until 96 hours, following which trial is terminated. Samples are kept at 4°C prior to analyses.

3.2.5 Analytical Techniques

3.2.5.1 Determination of Biomass by Dry Cell Weight (DCW)

Samples (20mL each time) are centrifuged (KUBOTA) in graduated centrifuged tubes at 6000 rpm for 15 minutes at 4°C. The supernatant is discarded and kept at 4°C for reducing sugar and lactate analyses. The cells are suspended using distilled water and recentrifuged again at 6000 rpm for 15 minutes at 4°C following which the supernatant is discarded. Then, the pellet is dried at 70°C for 24 hours or until the weight is constant. After drying, the graduated centrifuged tube is reweighed and the dry cell weight (DCW) determined as follows:

$$\text{DCW (g/L)} = \frac{[\text{Wt. of centrifuged tube + cells}]\text{g} - [\text{Wt. centrifuged tube}]\text{g} \times 10^3}{\text{Sample volume (mL)}}$$

3.2.5.2 Reducing Sugar Analysis

Reducing sugar is analyzed using the Dinitrosalicylic Acid (DNS) method (Miller, 1959). DNS reagent is prepared according to the percentage of each chemical: 1% DNS powder, 0.2% Phenol, 0.05% Sodium sulphite and 1% Sodium hydroxide. 1% Sodium hydroxide

is dissolved in distilled water and stirred with a strong magnetic stirrer. After that, 1% DNS powder and 0.2% Phenol are dissolved into the NaOH solution. Prepared DNS reagent is kept in reagent bottle to avoid crystallization or any chemical reactions. Meanwhile, 0.05% Sodium sulphite is added to this prior to use.

3.2.5.3 Determination of Lactate

Lactate is analyzed using High Performance Liquid Chromatography (Water 2487), a method modified and developed in Biochemistry Laboratory of UNIMAS. 20µL of the samples is injected into the system (detection temperature 60°C, flow rate 0.6mL/min). 1mM H_2SO_4 (filtered and degassed on Whatman 0.45µm membrane filter) is used as the mobile phase. Concentration of lactic acid against known concentration of 99.9% pure L(+)-lactic acid (Supelco, USA).

4.0 RESULTS

Four different initial and feed glucose concentrations (30g/L, 40g/L, 50g/L and 60g/L) have been performed for fed-batch lactate fermentation with 200mL volume of feeding and harvesting. Every fermentation process was operated for 96 hours and samplings were performed every 24 hours.

4.1 Fed-Batch Fermentation at 30g/L Glucose

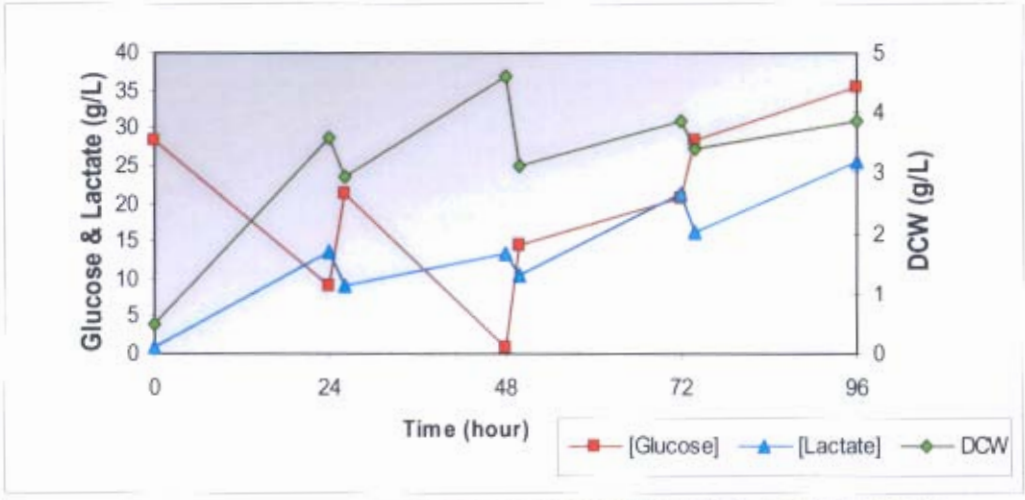

Figure 1: Fed-Batch Lactate Fermentation Utilizing 30g/L of Glucose Concentration

The starting glucose concentration was 30g/L (approximately 28.39g/L upon sampling at 0 hour). After 24 hours, glucose concentration declined to 9.16g/L, a consumption of 67.74%. The dry cell weight (DCW) increased rapidly from 0.48g/L at 0 hour to 3.59g/L and 13.49g/L lactate was produced, at 24 hours showing that most of the nutrient in fermentation media was used for cell growth. The maximum cell growth was 4.60g/L occurred at 48 hours of fermentation with low residual glucose, 0.81g/L. However, lactate production was low during 48 hours at 13.40g/L. This demonstrated that most of the nutrient in fermentation media was used for biomass formation rather

than producing lactate. After 48 hours, the overall fermentation process showed the residual glucose began to increase and the cell density tend to decrease, probably due to cell wash-out.

4.2 Fed-Batch Fermentation at 40g/L Glucose

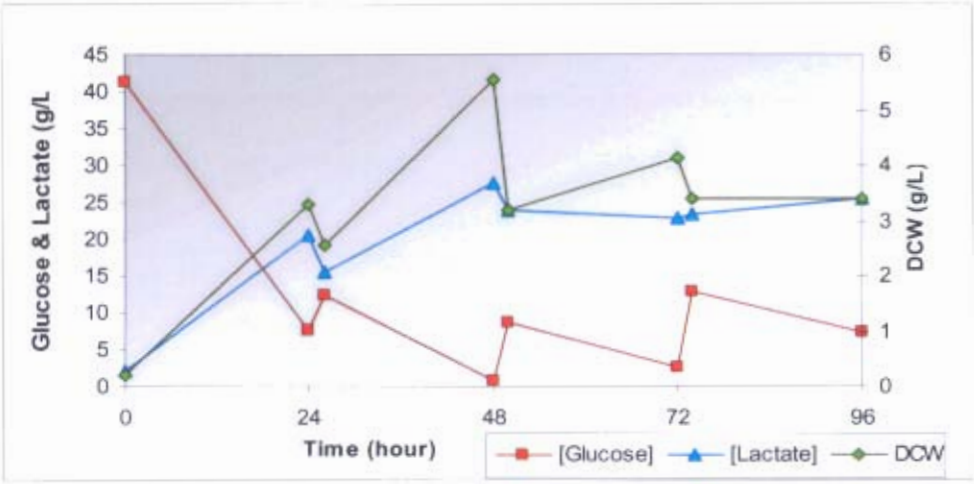

Figure 2: Fed-Batch Lactate Fermentation Utilizing 40g/L of Glucose Concentration

The initial glucose was 40g/L (approximately 41.38g/L when sample was taken at 0 hour). For the first 24 hours of fermentation, glucose concentration was declined to 7.61g/L, a consumption of 81.61% of glucose. After 48 hours of fermentation, maximum concentration of biomass was obtained at 5.54g/L with low residual glucose was observed, 0.74g/L. Lactate showed higher concentration; 27.67g/L after 48 hours. This result indicated that the nutrient from the fermentation broth was used simultaneously to enhance cell growth and increase lactate production. The residual glucose started to increase after 48 hours due to the decreasing concentration of cells in the fermentation broth. During terminating time of fermentation; 96 hours, lactate production is 25.41g/L with dry cell weight at 3.39g/L. Residual glucose was 7.39g/L showing that fermentation

process can still proceed to enhance the consumption of glucose. However the cell concentration declined after 48 hours.

4.3 Fed-Batch Fermentation at 50g/L Glucose

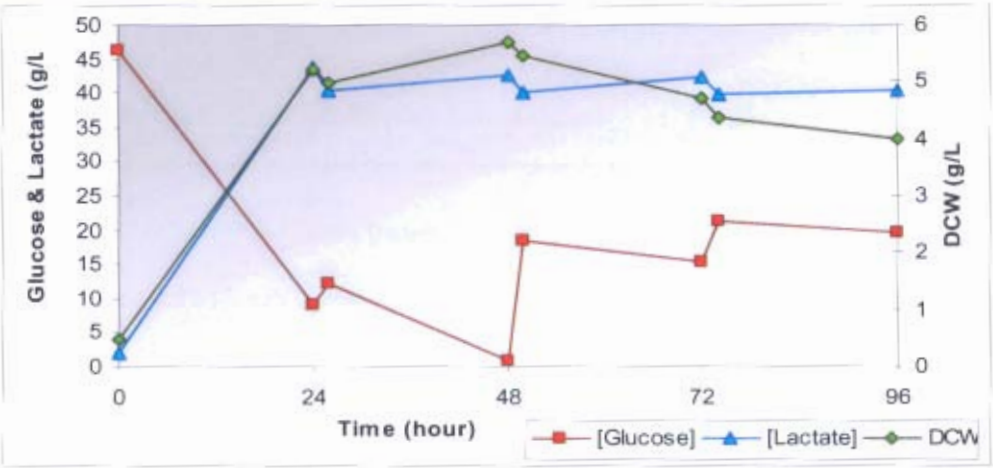

Figure 3: Fed-Batch Lactate Fermentation Utilizing 50g/L of Glucose Concentration

Figure 3 shows that the initial glucose was 50g/L (approximately 46.34 g/L upon sampling). The glucose was drastically decreased after 24 hours; a consumption of 37.19g/L (80.25%). At the same time, the biomass and lactate concentration were extremely increased with the amount of 5.20g/L and 43.62g/L respectively. Meanwhile, after 48 hours of fermentation, the maximum concentration of dry cell weight was obtained with minimum residual glucose; 5.70g/L and 0.74g/L respectively. The concentration of lactate was also high at this stage; 42.50g/L. This revealed that most of the glucose was converted into lactate with lower residual glucose in the fermentation broth. Later, the cells concentration started to decrease due to sampling thus increasing the residual glucose.