Production of Ethanol by Fed-Batch Fermentation

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ABSTRACT

The production of ethanol, from glucose in batch and fed batch culture, was investigated. In the fed batch culture, the glucose feeding was added into the culture at 16th hour of fermentation. The effects of different glucose concentration feeding rates on ethanol fermentation were investigated for fed batch culture. The $2 gL^{-1}hr^{-1}$ glucose concentration feeding rate was found to give higher ethanol yield (2.47 g ethanol g glucose^{-1}), with respect to substrate consumed as compared to $8 gL^{-1}hr^{-1}$ (0.23 g ethanol g glucose^{-1}) and $4 gL^{-1}hr^{-1}$ (0.20 g ethanol g glucose^{-1}). The ethanol yield with respect to substrate consumed obtained in batch culture was 0.81 g ethanol g glucose^{-1}. The fed batch culture at $2 gL^{-1}hr^{-1}$ glucose concentration feeding rate was proven to be a better fermentation system than the batch culture. The specific growth rate, specific glucose consumption rate and specific ethanol production rate for the fed batch fermentation, at $2 gL^{-1}hr^{-1}$ glucose concentration feeding rate, were $0.065 hr^{-1}$, $1.20 hr^{-1}$ and $0.0009 hr^{-1}$, respectively.

**Keywords: Batch culture, ethanol, fed batch culture, fermentation, glucose feed rate,
*Saccharomyces cerevisiae***

INTRODUCTION

Due to a rapid depletion of the world's petroleum reserves and its rising prices day by day, new sources of hydrocarbons must be found to supply chemical and energy needs (Sitton and Gaddy, 1980; Lee *et al*., 1983). In this context, ethanol fermentation offers promising alternative as it can be produced from various sources of raw materials. In view of increasing importance of ethanol, as an alternative source for chemicals and liquid fuel, a great deal of research interest in ethanol fermentation has been generated in the last two decades (Vega *et al*., 1987; Converti *et al*., 1985). Many different types of processes for ethanol fermentation have been proposed including batch fermentation, continuous fermentation, continuous fermentation with cell recycling, fed-batch and repeated-batch culture (Yoshida *et al.*, 1973). The fed-batch culture with the intermittent addition of glucose and without the removal of fermentation broth is one of the most common methods for the production of ethanol in the industry. One advantage of this process is the reduction of substrate inhibition. A high concentration of sugar in fermentation medium inhibits growth and ethanol production. Other advantages of this process are higher productivity, higher dissolved oxygen in the medium, decreased fermentation time and reduced toxic effects of the medium components, which are present at high concentrations (Stanbury and Whitaker, 1984).

The ethanol production, which involves fed-batch methods and using baker's yeast as biomass is a complex, time-variant, nonlinear process. Baker's yeast, one of the *S. cerevisiae* strains, has been used intensively for the production of single cell protein (SCP for human and animal consumption), and ethanol (industrial and portable alcohol) from fermentable sugar because of its GRAS (Generally Regarded as Safe) status (Solomon *et al*., 1997). This yeast strain can produce a high concentration of ethanol and it is preferred for most ethanol fermentations (Yan *et al*., 2006). Therefore, the aims of this work were to: (a) determine the switching time for fed batch fermentation, (b) investigate the optimum glucose concentration feeding rate of ethanol production by *S. cerevisiae* cells in fed batch fermentation, using glucose as the source of carbon, (c) study the kinetic parameters of the system which include specific growth rate, specific glucose uptake rate, specific ethanol production rate and the production yield of ethanol.

MATERIALS AND METHODS

Micro-organism

S. cerevisiae (Baker's yeast, Mauri-Pan), used throughout this investigation, was maintained at 4°C on agar slants. The compositions of the agar were: 5 gL^{-1} yeast extract, 5 gL^{-1} malt extract, 5 gL^{-1} peptone, 20 gL^{-1} glucose and 20 gL^{-1} agar. The cultures were maintained by sub-culturing every 20 days and the plates were incubated at 30°C for 24 hours.

Fermentation Medium

One litre of production medium was prepared according to the requirement of *S. cerevisiae*, containing 50.0 gL^{-1} glucose, 1.0 gL^{-1} yeast extract, 5.0 gL^{-1} KH$_2$PO$_4$, 2.0 gL^{-1} (NH$_4$)$_2$SO$_4$ and 0.4 gL^{-1} MgSO$_4$7H$_2$O. The medium was sterilized and the pH was adjusted to 5.0.

The Preparation of Inoculums

The micro-organism was cultured in 250 mL Erlenmeyer flasks, containing 100 mL of the medium, which has the same composition as the fermentation medium. The Erlenmeyer flask was incubated at 28°C for 6 hours on a rotary shaker at 200 rpm.

The Fermentation Conditions

(i) Batch Culture

The fermentation was carried out in a 2 litre stirred tank fermentor, with a working volume of 1.5 litres. The 900 mL fermentation medium was inoculated with 100 mL inoculums and the pH was adjusted to 5.0. It was carried out at 250 rpm and temperature of 30°C, with an air flow rate of 1vvm. Samples were taken every 2 hours for the entire fermentation cycle, which was terminated after 42 hours.

(ii) Fed Batch Culture

The substrate was fed continuously into the bioreactor with a peristaltic pump, at a glucose concentration feeding rates of 2gL^{-1}hr^{-1}, 4gL^{-1}hr^{-1} and 8gL^{-1}hr^{-1}. The impeller speed was set at 250 rpm and 30°C, with the air flow rate of 1 vvm for all the runs.

The Analytical Techniques

Fermentation broth was removed from the fermentor and analyzed at a predetermined time interval. Yeast growth was evaluated by spectrophotometric measurements at 260 nm in a spectrophotometer and calibrated against cell dry weight measurements. The concentration of glucose was determined

using the 3, 5 dinitrosalicylic acid (DNS) method (Miller, 1959). Meanwhile, the concentrations of ethanol in the medium were determined by gas chromatography, using a polyethylene glycol column, nitrogen as carrier gas and flame ionization detection with the following conditions: an injection temperature of 250°C, initial oven temperature of 45°C to final temperature of 250°C at a rate of 8°C min^{-1}, a flow rate of carrier gas of 4 mL min^{-1} and an injected volume of 1 μL (Agilent Technologies, 2000).

The Kinetic Parameters

Ethanol yield was calculated with respect to both glucose consumed and biomass generated. The specific growth kinetic, specific substrate uptake rate and specific ethanol production rate were determined using a simulation method based on the MATLAB Programme. A set of system equations was derived from the material balances of cell, sugar and ethanol for the fed batch fermentation:

$$\frac{dX}{dt} = \mu X - \frac{F}{V} X \qquad\qquad (1)$$

$$\frac{dS}{dt} = \frac{F}{V}(S_0 - S) - \nu X \qquad\qquad (2)$$

$$\frac{dP}{dt} = QX - \frac{F}{V} P \qquad\qquad (3)$$

Where X, S and P denote the concentration of cells, glucose, and ethanol, respectively. V is the culture volume, and S_o and F are the sugar concentration and feed rate of the feed medium added to the fermentor, respectively; μ, ν and Q are the specific rates of growth, glucose consumption, and ethanol production, respectively.

RESULTS AND DISCUSSION

Batch Fermentation

In order to conduct the fed batch fermentation, this batch fermentation was carried out to study the trend of cell growth, glucose consumption and ethanol formation. The switching time for the fed batch fermentation was determined from the result of the batch fermentation. Based on the data presented in *Fig. 1*, a typical batch growth phase can be observed, including the following phases: lag phase (0 - 6 hr), exponential growth phase (6 - 30 hr), deceleration phase (30 - 34 hr) and stationary phase (34 - 42 hr).

In the first 6 hours of fermentation, the yeasts adapted themselves to growth conditions. During the exponential phase, the periods of exponential growth were of limited duration due to the depletion of some rate-limiting resources. After 34 hours of fermentation, the growth rate was found to slow down as a result of glucose depletion.

Based on *Fig. 1*, the concentration of glucose is shown to remain almost constant for the first 6 hours. The concentration of glucose was then decreased as expected during the fermentation, coinciding with an increase in the production of cell and ethanol. This is due to the cells consuming the glucose in the system to increase the growth of cell and the production of ethanol. The feeding of the substrate was initiated when most of the substrates have been consumed and the growth of
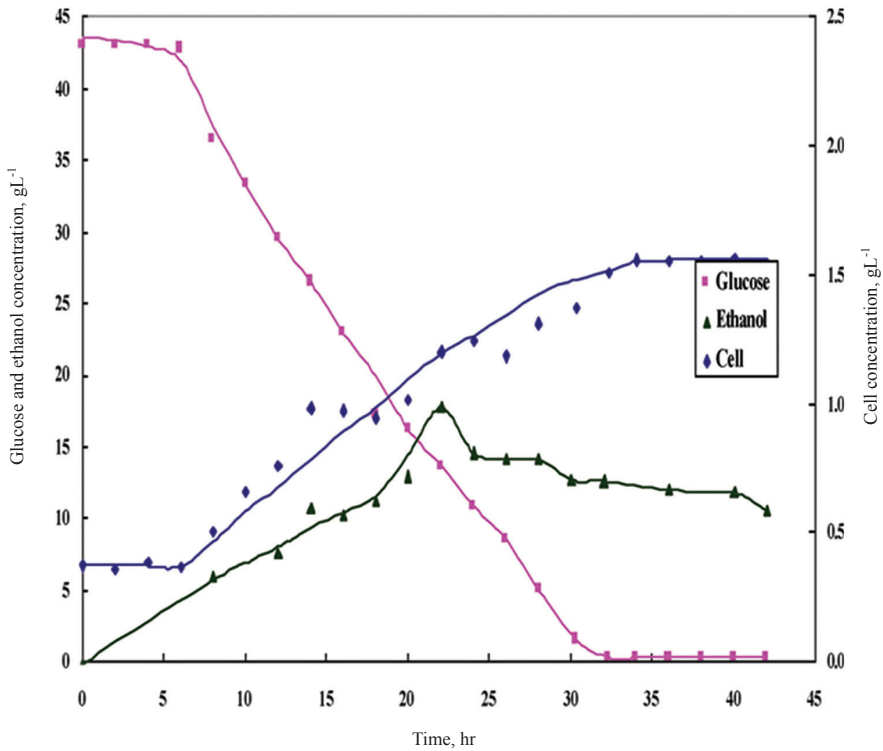
Fig. 1: Fermentation of S. cerevisiae cells during ethanol production from glucose in batch culture

yeast was in the exponential phase. It could also be observed that the system glucose was depleted after 34 hours.

The concentration of ethanol was found to increase rapidly during the first 20 hours of fermentation. It started to decrease only after achieving a maximum concentration of 18 gL^{-1} at 22 hours of fermentation. The ethanol might have been used as a carbon source by the yeast for its growth after the 22nd hours, when the concentration of glucose started to deplete (Bauchop and Elsden, 1960; Coppella and Dhurjati, 1989). By comparing the cell and the concentrations of ethanol, it can be classified as a growth-associated product in which the product is produced simultaneously with the cell growth.

Fig. 1 indicates that the optimum switching time from batch to fed batch fermentation is between 14th -18th hr. Therefore, the feeding of substrate was decided to start at 16th hours of fermentation cycle for all the fed-batch systems.

Fed Batch Fermentation

(i) Comparison of the Cell Concentration

Fig. 2 shows that the cell concentration generally remained almost constant for about 5 hours and it increased gradually throughout the fermentation. In general, there was no stationary phase observed
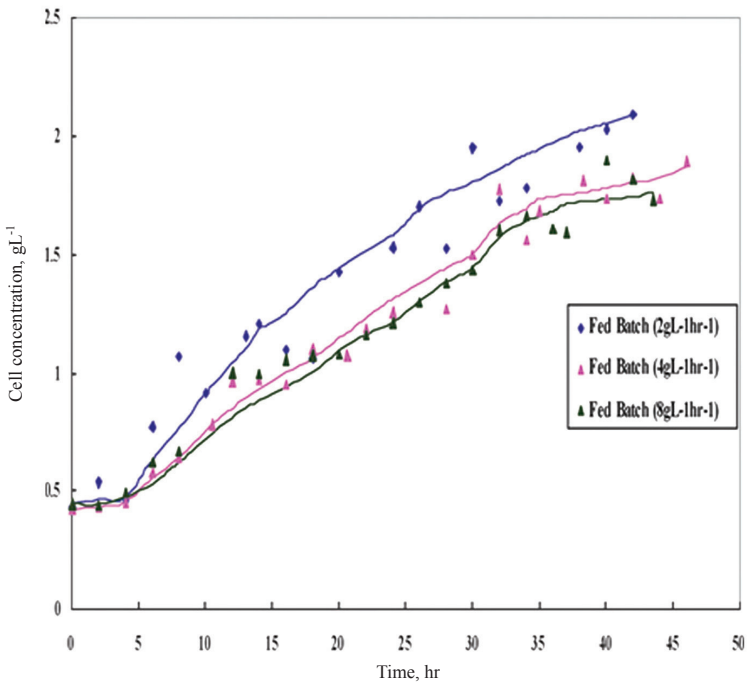
*Fig. 2: Comparison of cell concentration at different glucose
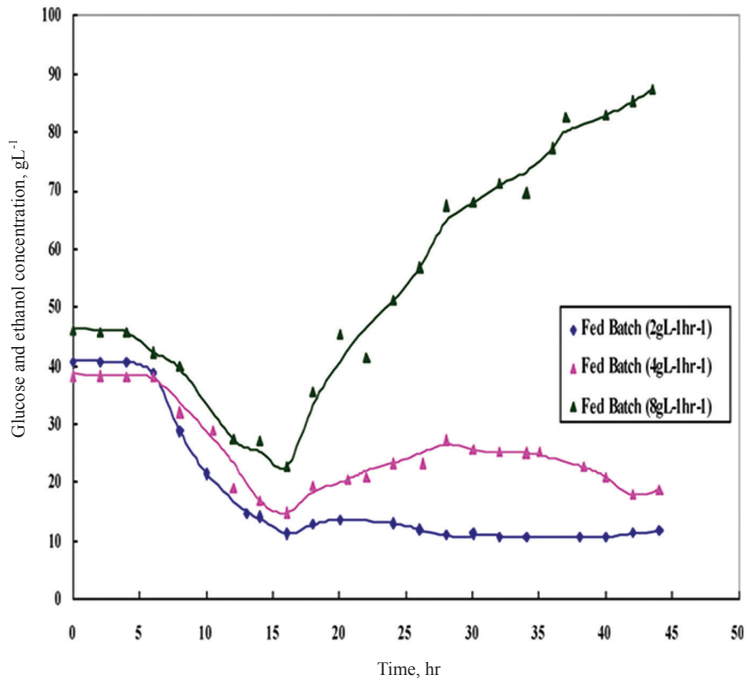concentration feeding rate for fed batch fermentation*

*Fig. 3: Glucose concentration with time at different glucose
concentration feeding rates for the fed batch fermentation*

for the fed batch culture, at all the glucose concentration feeding rates. Comparatively, $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate gave a better cell concentration than $4 \text{ gL}^{-1}\text{hr}^{-1}$ and $8 \text{ gL}^{-1}\text{hr}^{-1}$. This was probably due to the $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate which had provided a better growth environment to the yeast, so that the yeast cells could divide more rapidly.

It also might be attributed to the osmotic effect contributed by the high glucose concentrations, resulting in slower proliferation of yeast cells (Thomas *et al.,* 1992). The higher concentration of substrate was also found to affect the pH, viscosity and the activity of the medium. Long hour exposure to high substrate concentration might also cause catabolic repression. The changing of the environment had somehow affected the growth rate of the cell, whereby the cell viability decreased as the sugar concentration increased during the fermentation of ethanol. This trend is in agreement with the report by Thomas and Ingledew (1992).

(ii) Comparison of the Glucose Concentration

From *Fig. 3*, glucose concentration is observed to follow the same pattern as in the batch fermentation before the fed batch fermentation was started. At $8 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate, the concentration of glucose was found to increase exponentially throughout the fermentation. This indicates that the glucose feeding rate is much greater than the glucose consumption rate. Consequently, there is an excess in the glucose left in the medium and it becomes a waste for the system.

Fig. 3 also indicates that the concentration of glucose at $4 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate is generally higher than the glucose concentration at $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate, but less than the glucose concentration at $8 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate. At $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate, the concentration of glucose was found to increase slightly and remain constant at about 10 gL^{-1} after 28 hours of fermentation. This was due to the amount of glucose fed into the system had been fully consumed by the yeast. It could be seen that the $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate gave the best glucose consumption.

(iii) Comparison of Ethanol Concentration

Fig. 4 shows that the overall ethanol concentration, at $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate, is higher than $4 \text{ gL}^{-1}\text{hr}^{-1}$ and $8 \text{ gL}^{-1}\text{hr}^{-1}$ in the fed batch culture. The glucose feeding rate of $2 \text{ gL}^{-1}\text{hr}^{-1}$ was indicated to produce the ethanol concentration up to the maximum 17gL^{-1}. The concentration of ethanol remained almost constant for about 14 hours before it started to decrease at 32nd hours. The decrease in the concentration of ethanol was probably due to the oxidation of ethanol to acetic acid and other components (Mian *et al.,* 1973).

For both the $4 \text{ gL}^{-1}\text{hr}^{-1}$ and $8 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rates, the pattern of ethanol formation was similar but the $4 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate produced more ethanol as compared to that at $8 \text{ gL}^{-1}\text{hr}^{-1}$. The glucose concentration feeding rate at $8 \text{ gL}^{-1}\text{hr}^{-1}$ gave the lowest concentration of ethanol. This might be due to the catabolic repression and glucose overflow metabolism. When the glucose concentration exceeded a critical value, it caused excretion of ethanol.

(iv) Fed Batch Culture at $2 \text{ gL}^{-1}\text{hr}^{-1}$ Glucose Concentration Feeding Rate

The fed batch fermentation conducted at $2 \text{ gL}^{-1}\text{hr}^{-1}$ glucose concentration feeding rate was found to produce the highest ethanol production. The trend of the cell growth, glucose consumption and ethanol formation are shown in *Fig. 5*.
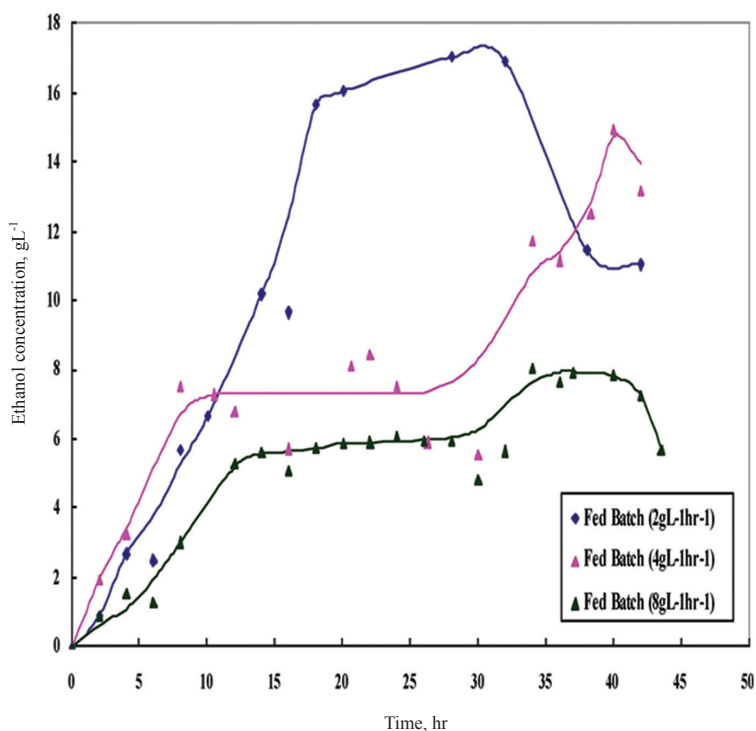
*Fig. 4: Ethanol concentration at different glucose concentration
feeding rates for the fed batch fermentation*

The concentration of ethanol decreased at 32nd hours, although the glucose was fed into the system continuously. The maximum production of ethanol achieved was about 17 gL^{-1}. The yeast cells grew exponentially after 6 hours until the end of the fermentation cycle and no stationary phase was observed. It was found that the concentration of glucose had remained almost constant after 28 hours of fermentation. The added glucose was sufficient and it provided an optimum environment for the cell growth.

In comparison with the batch culture (*Fig. 1*), ethanol was produced constantly for a longer period in the fed batch culture at 2 $gL^{-1}hr^{-1}$ glucose concentration feeding rate. The batch culture produced the maximum ethanol at a single point, but it could not maintain the production due to the depletion of glucose.

Kinetic Parameters

The specific growth rate, glucose consumption rate, production rate and the yield coefficient are commonly adopted to assess microbial performance. The specific cell growth rate, specific glucose uptake rate and specific ethanol production rate, at three different glucose concentration feeding rates, were determined using the modelling method. The MATLAB program was also employed to solve the three ordinary differential equations derived from material balance of cell, glucose and ethanol, with the data obtained from the experiments. Table 1 shows all the kinetic parameters for both the batch and fed batch fermentations. The volume change of the fermentation medium was relatively small and it was assumed to be negligible in the system.
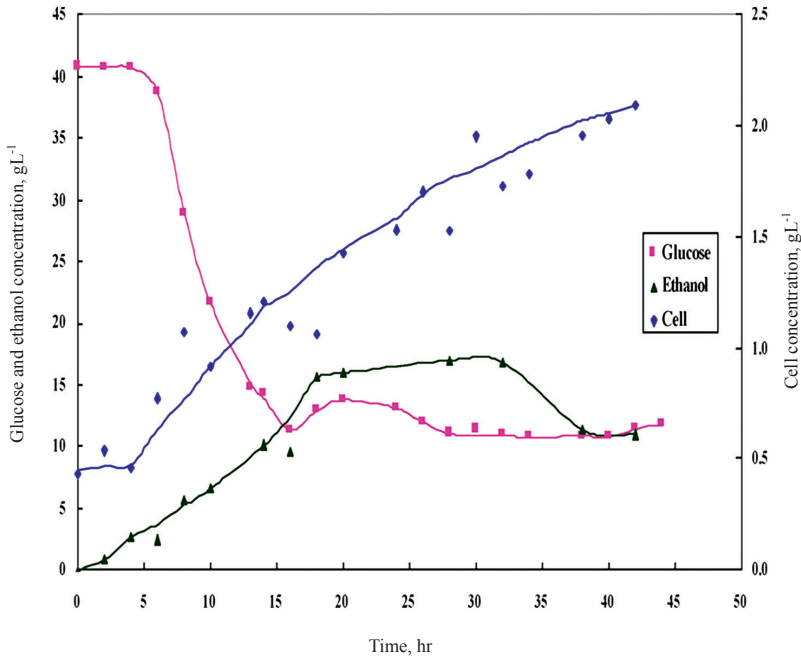
Pertanika J. Sci. & Technol. Vol. 17 (2) 2009

405

*Fig. 5: Cell growth, glucose consumption and ethanol formation profile of the
fed batch fermentation at 2gL^{-1}hr^{-1} glucose concentration feeding rate*

TABLE 1
Kinetic parameters for the batch and fed batch fermentations

Kinetic Parameter	Batch Fermentation	Fed Batch Fermentation		
		2gL^{-1}hr^{-1}	4gL^{-1}hr^{-1}	8gL^{-1}hr^{-1}
Yield, $Y_{P/X}$ (g ethanol g cell^{-1})	21.49 (22 hr)	24.75 (20 hr)	12.50 (20 hr)	9.28 (20 hr)
Yield, $Y_{P/S}$ (g ethanol g glucose^{-1})	0.81 (22 hr)	2.47 (18 hr)	0.23 (20 hr)	0.20 (18 hr)
Specific cell growth rate, μ (hr^{-1})	0.0613	0.0645	0.0341	0.0313
Specific glucose uptake rate, ν (hr^{-1})	1.183	1.1953	1.4452	1.6133
Specific ethanol production rate, Q (hr^{-1})	0.0006	0.0009	0.0008	0.0005

Table 1 also illustrates that a lower glucose concentration feeding rate correlated with a higher product yield. The fed batch fermentation at $2gL^{-1}hr^{-1}$ glucose concentration feeding rate gave the highest product yield. However, the product yield at $4gL^{-1}hr^{-1}$ and $8gL^{-1}hr^{-1}$ glucose concentration feeding rate was much lower than the batch fermentation. It seemed that the carbon fraction channelling to ethanol synthesis was saturated when the glucose concentration was higher. This indicated that the higher glucose concentration feeding rate did not enhance the production of ethanol. It was due to the substrate inhibition at a higher glucose concentration in the system. The highest product yield for the fermentation was achieved in the range of 18th - 22nd hr.

The decrease in the specific growth rate suggested the inhibition of cell growth by increasing the glucose concentration feeding rate. The decrease in the specific growth rates showed that the rate of biosynthesis was lower at a high glucose concentration, as maintenance coefficient was increased at high glucose concentrations. Thus, it could be concluded that the fed batch fermentation is most suitable to be operated at $2gL^{-1}hr^{-1}$ glucose concentration feeding rate for this study.

The fed batch culture at $2 gL^{-1}hr^{-1}$ glucose concentration feeding rate also supported a higher ethanol yield, specific growth rate, specific consumption rate and specific ethanol formation rate as compared to the batch culture. It was shown that an increase in the concentration feeding rate resulted in a significant decrease in the fermentation efficiency. The decreased efficiency encountered with the highest concentration treatment was probably due to the osmotic effects. Roukas *et al.* (1991) reported that above the critical substrate, the decrease of water activity and the onset of plasmolysis could cause a decrease in the rate of fermentation and the production of ethanol. These results clearly showed that the substrate concentration feeding rate had a significant effect on the kinetic parameter of *S. cerevisiae*. Carine *et al.* (2006) studied the minimization of glycerol production during the high performance fed-batch ethanol fermentation process in *S. cerevisiae* and found that a product yield of 0.34 g g^{-1}, maximum specific growth rate of 0.62 g $g^{-1}hr^{-1}$ were obtained when the fermentor was fed with a sterile concentrated glucose solution of 700 g L^{-1}.

CONCLUSIONS

In this work, the best switching time from the batch to fed batch fermentation was determined at 16th hour. The $2gL^{-1}hr^{-1}$ glucose concentration feeding rate in the fed batch fermentation achieved the best ethanol production as compared to $4gL^{-1}hr^{-1}$ and $8gL^{-1}hr^{-1}$ glucose concentration feeding rates. The maximum product yield was achieved at 18th - 22nd hr of fermentation, and the ethanol production was maintained at 14 g L^{-1} from 16th - 32nd of fermentation time. The fed batch culture at $2 gL^{-1}hr^{-1}$ glucose concentration feeding rate was a better fermentation system than the batch culture as the fed batch culture supported a higher yield of ethanol.

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