ISSN: 0128-7680
Pertanika J. Sci. & Technol. **18 (2)**: 365 – 375 (2010)

Repeated Fed-Batch Cultivation of Nitrogen-Fixing Bacterium, *Bacillus sphaericus* UPMB10, Using Glycerol as the Carbon Source

A.B. Ariff[1*], T.C. Ooi[1], M.S. Halimi[2] and Z.H. Shamsuddin[2]
[1]Department of Bioprocess Technology,
Faculty of Biotechnology and Biomolecular Sciences,
[2] Department of Land Resource Management, Faculty of Agriculture,
Universiti Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[*]E-mail: arbarif@putra.upm.edu.my

ABSTRACT

The exponential fed-batch cultivation of *Bacillus sphaericus* UPMB10 in 2 l stirred tank fermenter was performed by feeding the initial batch culture with 14 g l^{-1} of glycerol according to the algorithm aimed at controlling the specific growth rate (μ) of the bacterium. Very high viable cell count (1.14 x 10^{10} cfu ml^{-1}), which was four times higher as compared to batch cultivation, was achieved in the fed-batch with a controlled μ at 0.4 h^{-1}. In repeated exponential fed-batch cultivation, consisting of four cycles of harvesting and recharging, a final cell concentration of 1.9 x 10^{11} cfu ml^{-1} was obtained at the end of the fourth cycle (46 h). Meanwhile, acetylene reduction of cell samples collected from repeated fed-batch cultivation remained unchanged and was maintained at around 20 nmol C_2H_2 h^{-1} ml^{-1} after prolonged cultivation period, and was comparable to those obtained in batch and exponential fed-batch cultivation. Glycerol could be used as a carbon source for high performance cultivation of *B. sphaericus,* a nitrogen fixing bacterium, in repeated fed-batch cultivation with high cell yield and cell productivity. The productivity (0.68 g l^{-1} h^{-1}) for repeated fed-batch cultivation increased about 6 times compared to that obtained in conventional batch cultivation (0.11 g l^{l} h^{-1}). A innovative method in utilizing glycerol for efficient cultivation of nitrogen fixing bacterium could be beneficial to get more understanding and reference in manipulating the integrated plans for sustainable and profitable biodiesel industry.

Keywords: Nitrogen fixing bacterium, *Bacillus sphaericus,* glycerol, repeated fed-batch cultivation

INTRODUCTION

Glycerol is an attractive carbon substrate for biological conservation because it is available from renewable resources in large amounts and can be utilized by a number of micro-organisms (Lin, 1976). Moreover, glycerol is produced as a surplus by-product in the growing oleochemical industries for production of soaps, fatty acids, waxes, and surfactants. Crude glycerol is also the principal by-product of biodiesel production, which is about 10% of the weight of vegetables oils (Dasari *et al.*, 2005). The usage of low-grade quality glycerol obtained from biodiesel production is a big challenge as this particular glycerol type cannot be used for direct food and cosmetic uses. Thus, several environmentally friendly processes have been proposed from glycerol utilization based on microbial fermentation. For example, mixed culture fermentation of glycerol synthesizes short- and medium-chain polyhydroxylalkonate blends (Koller *et al*., 2005). Glycerol has also been used as a carbon source for the production of nitrogen-fixing bacterium, *Azospirillum brasiliense*

Received: 24 April 2009
Accepted: 15 February 2010
[*]Corresponding Author

(Fallik and Okon, 1996). Meanwhile, nitrogen fixing bacteria have been used for centuries to improve the fertility of soils. The potential and pitfalls of exploiting nitrogen fixing bacteria in agricultural soils as substitute for inorganic fertilizer have been reviewed by Cummings *et al*. (2008). For the preparation of biofertilizer, large-scale production of nitrogen-fixing bacterium is essential before inoculation into suitable solid substrate for composting. The development of a commercially feasible fermentation process for large-scale production involves improvement of yield and overall productivity.

Reduced specific growth rate and yield of *Bacillus shaericus* UPMB10 batch cultivation were observed with increasing glycerol concentration due to reduction in $a_{w,}$ which greatly repressed the growth (Ooi *et al.,* 2008). The effect of glycerol inhibition to growth could be minimized through fed-batch cultivation technique by controlling the concentration in the culture at low levels through regulation of nutrients feed rates. Several feeding strategies, such as constant feeding, intermittent feeding and exponential feeding, could be applied in fed-batch cultivation. The theory and application of fed-batch cultivation have been well-discussed in the literature (e.g. Lee *et al.,* 1999; Ezequiel and Dirk, 2005). The productivity can be increased by extending the life of the fed-batch fermentation and converting it into repeated fed-batch cultivation. In the repeated fed-batch cultivation, a portion of the reactor content is periodically withdrawn and the residual growing culture in the reactor is used as the starting point for further fed-batch process. This ensures the benefit of high inoculum ratio at the time of fresh feed.

Comparisons between optimal repeated fed-batch cultivation and other cultivation modes have been made for the substrate-inhibited culture systems (Weigand, 1980). Meanwhile, significant improvement in cell productivity was obtained by repeated batch as compared to batch and continuous cultivations. Improvement of the cultivation or fermentation performance, in term of the overall productivity, has been reported for several processes. The productivity of xylitol fermentation by *Candida parapsilosis* in repeated fed-batch was increased by about 40% as compared to the batch fermentation without loss in yield (Furlan *et al.,* 1997). The average productivity of L-sorbose fermentation by *Gluconobacter oxydans* using D-sorbitol as substrate was also improved by the use of repeated fed-batch fermentation (Giridhar and Srivastava, 2001). The production rate of lipase, in the repeated fed-batch fermentation employing *Acinetobacter radioresistens*, was improved by about 3.3 times as compared to the use of fed-batch fermentation (Li *et al.,* 2005). Improvement of biological treatment of pre-treated landfill leachate, using repeated fed-batch technique, has been reported (Kargi and Pamukoglu, 2004), where significant improvement in COD removal was obtained as compared to single-cycle operation.

The objective of this study was to investigate the possibility of using repeated exponential fed batch cultivation technique to improve *Bacillus sphaericus* UPMB10 cultivation using glycerol as a substrate in term of yield ($Y_{x/s}$) and overall cell productivity (P).

MATERIALS AND METHODS

Micro-organism and Inoculum Preparation

The bacterium, *Bacillus sphaericus* UPMB10, was used throughout this study. This bacterium was obtained from the Department of Land Management, Universiti Putra Malaysia. Cell cultures of *B. sphaericus* UPMB10 were suspended in 15% (v/v) glycerol and kept as a stock culture at –80ºC. As for the inoculum preparation, the stock cultures were streaked on nutrient agar (NA) slant and incubated at 30ºC overnight. A single colony was removed from NA slant and inoculated into 100 ml of Nutrient Broth (NB) in 250 ml Erlenmeyer Flasks. The flasks were agitated in a rotary shaker at 200 rpm and incubated at 30ºC for 10 h and used as inoculum for the cultivation. This

inoculum has an optical density of approximately 0.8 measured at 600 nm, which was equivalent to about 1 x 10^7 cfu ml^{-1}.

Media

All cultivations were carried out using a basal medium consisting of 1.4 g l^{-1} yeast extract, 2.8 g l^{-1}, KH_2PO_4, 1.12 g l^{-1} Na_2HPO_4, 0.01 g l^{-1} $CaCl_2.2H_2O$, 0.1 g l^{-1} $MgSO_4.7H_2O$, 0.004 g l^{-1} $MnCl_2$, 0.003 g l^{-1} $FeSO_4.7H_2O$, 0.003 g l^{-1} biotin and 0.03 g l^{-1}thiamine hydrochloride. For the initial batch cultivation, 1.4 g l^{-1} glycerol was added to the basal medium, which was optimal for the growth of *B. sphaericus* UPMB10 (Ooi *et al.,* 2008). High glycerol concentration (14 g l^{-1}) in a basal medium was used as a feeding nutrient for the exponential fed-batch and repeated exponential fed-batch cultivations.

Cultivations

All cultivations of *B. sphaericus* UPMB10 were carried out in a 2 l stirred tank fermenter (Biostat B, B. Braun Germany). The fermenter was equipped with pH, temperature and foam control systems. During the cultivation, agitation speed and aeration rate were maintained at 600 rpm (impeller tip speed = 3.1 m s^{-1}) and 0.5 vvm, respectively. The temperature within the fermenter was controlled at 30°C. A polarographic dissolved oxygen probe (Ingold, Switzerland) was used to measure the dissolved oxygen tension (DOT) levels. In all the fermentation runs, the initial pH of the culture was set at 7.0 and was not controlled during the cultivation. Meanwhile, the level of foaming was controlled by the automated addition of antifoam A (Sigma Chemical Co., St Louis, MO).

Fed-batch cultivation was initiated as a batch with a working volume of 600 ml in a 2 l stirred tank fermenter. The exponential fed–batch fermentation was started after the glycerol in the initial batch had been depleted. The feeding of the sterile medium was carried out exponentially according to equation (1):

$$F_{i(t)} = (\mu^* X_o V_o)\, e^{\mu^* t} / Y_{x/s}\, S_i, \qquad (1)$$

where the kinetic parameter values for all the experiments were as follows: (X_o = 1.6 g l^{-1}, V_o = 0.6 l, $Y_{x/s}$ = 0.98 g cell g substrate^{-1}, S_i = 14.0 g l^{-1}, while μ* was varied according to the requirement of each experiment).

The exponential fed-batch cultivations were carried out at different required μ* values (i.e. ranging from 0.2 h^{-1} to 0.5 h^{-1}) lower than the maximum specific growth rate (μ_{max}) of B. *sphaericus* UPMB10 (0.53 h^{-1}) for the growth in an optimal concentration of glycerol (Ooi *et al*., 2008). Control of the peristaltic pump was facilitated using autofermenter control system (MFCS)/win software, which is a fermenter supervisory control and data acquisition system (SCADA). This 32-bit PC-based application, using algorithm or a profile inputs, interfaces with a local fermenter control unit for the control of peristaltic pump. The MFCS/win software was also configured for repeated exponential fed-batch cultivation. The fed-batch cultivation was converted into repeated fed-batch mode by rapidly withdrawing three-quarters (1.5 l) of the 2 l culture from the fermenter. Four cycles of culture withdrawal and fresh nutrient medium addition were performed in the repeated exponential fed-batch cultivation experiment.

Analytical Procedure

During the course of cultivation, samples were withdrawn at time intervals for analysis. The sample was centrifuged at 10,000 rpm for 10 min. Cells were washed and re-suspended twice with salt solution for turbidity determination at 600 nm, as well as used for the measurement of dry cell weight

using filtration and oven dry method. The supernatant was used for the chemical analysis. Organic acid such as acetic and lactic acid was measured using high performance liquid chromatography (HPLC) with UV detector (SPD-10A Shimadzu, Japan) at a wavelength of 210 nm. The separation of organic acid was obtained using Biorad aminex HPX-87H cation–exchange resin column (300 x 7.8 mm I.D.) as the stationary phase. The mobile phase was 7 mM H_2SO_4. The flow rate of the mobile phase and column temperature was controlled at 1.0 ml min^{-1} and 50°C, respectively.

Glycerol was determined using a high performance liquid chromatography (HPLC) (ConstaMetric 3000, LDC Analytical, Florida) with a refractive index detector. Sample injection was performed using a sample loop valve equipped with 25 µl loop. The stationary phase was a pre-packed Merck NH_2 column, while the mobile phase was an isocratic mixture of acetonitrile and water (80:20 v/v). The flow rate of mobile phase was 0.7 ml min^{-1} and the reaction was kept at room temperature.

Total nitrogen content in the culture filtrate was quantified using micro-Kjeldahl method. Approximately 1.0 ml of the sample was subjected to Kjeldahl digestion in a 100 ml digestion flask containing concentrated sulphuric acid. The clear digest was analyzed for total N on a Chem Lab autoanalyser. Details of the micro-Kjeldahl and the N determination using an autoanalyser can be found elsewhere (Bremner and Mulvaney, 1982).

Acetylene reduction was estimated for the culture sample at the end of cultivation run. The culture sample was inoculated into 100 ml of nitrogen free broth (NFB) supplemented with 0.05% (w/v) yeast extract in a 250 ml shake flask. Approximately 10 ml of the culture was centrifuged and re-suspended in minimal lactate broth. The re-suspended culture was transferred into a 30 ml McCartney bottles which were sealed with rubber serum stoppers (Thomas Scientific). The McCartney bottles were then flushed with O_2-free nitrogen and shaken for four hours at 30°C. The bottle was injected with 10% acetylene and incubated at 30°C. Periodically (15 min intervals) after incubation, 0.5 ml of gas was tested for the presence of ethylene using gas chromatography (GC) (Autosystem XL, Perkin Elmer) equipped with a flame ionization detector and 1-m Porapak N column. A calibration curve was plotted for each experiment. Rate of N_2 fixation was expressed as the quantity of ethylene accumulated per h.

RESULTS

Batch Cultivation

Fig. 1 shows the typical time course of the batch cultivation of *B. sphaericus* UPMB10 using glycerol as a carbon source. Growth of *B. sphaericus* UPMB10 was found to be very rapid from inoculation to 10 h of cultivation, where lag phase was not observed. During the active growth, glycerol was rapidly consumed for growth. Cells grew to a final cell concentration of 1.62 g l^{-1} or 3.26 x 10^9 cfu ml^{-1} after 14 h with a maximum specific growth rate, μ_{max} of 0.43 h^{-1}. At the end of the fermentation, 0.25 g l^{-1} of glycerol was left unutilized. Dissolved oxygen tension (DOT) level was concomitantly decreased with decreasing glycerol concentration in the culture, indicating that glycerol and oxygen were required for rapid growth. The culture pH was maintained at around pH 7 throughout the cultivation. It was found that the DOT level had dropped to 30% at the end of cultivation, suggesting that the oxygen supply was not limited. Yield ($Y_{x/s}$) and productivity (P) of *B. sphaericus* UPMB10 in batch cultivation were 1.34 g cell g $glycerol^{-1}$ and 0.11 (g $l^{-1}h^{-1}$), respectively.

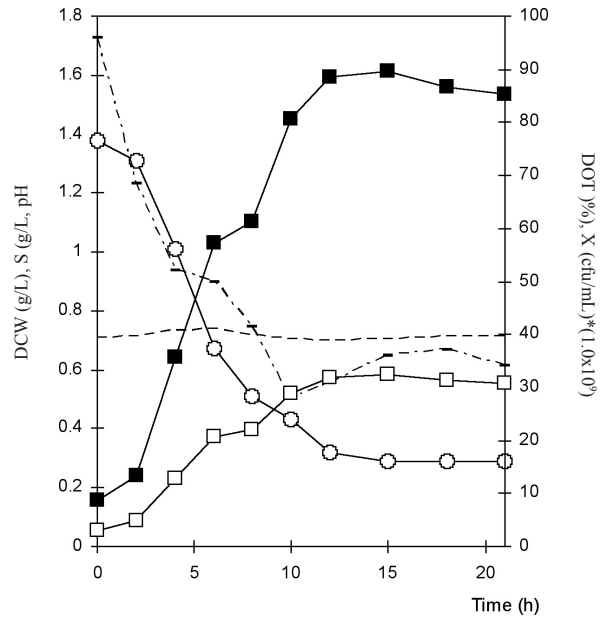

Fig. 1: The time course of batch fermentation using glycerol as the carbon source in a 2 l stirred tank fermenter. Symbols represent (◆) cell count, X (cfu ml^{-1} x 10^{9}); (……) dissolved oxygen tension, DOT (%); (◊) pH/10; (■) dry cell weight, DCW (g l^{-1}); (○) glycerol concentration, S (g l^{-1})

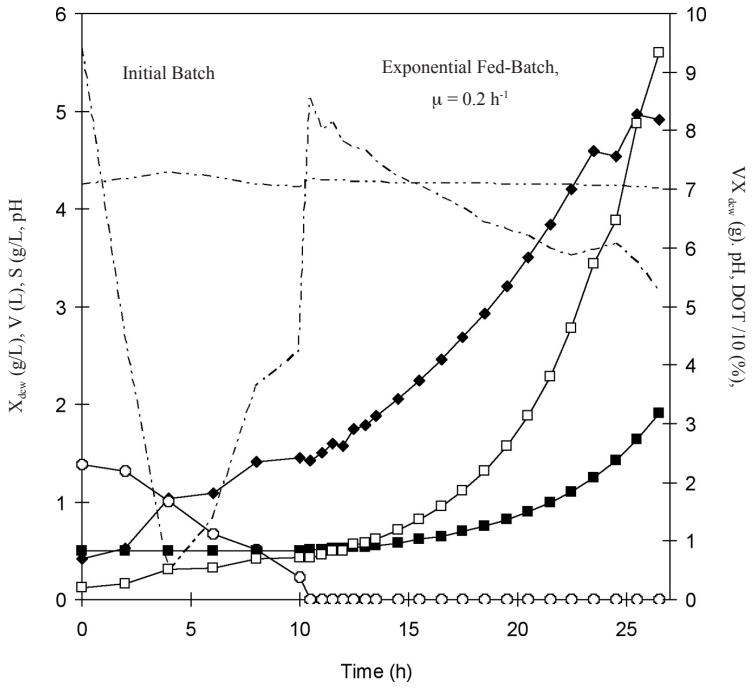

Fig. 2: The time course of exponential fed-batch fermentation at μ = 0.2 h^{-1} using glycerol as the carbon source in a 2 l fermenter. Symbols represent (◆) X; (O) S; (■) V; (□) VX; (----) pH; (__) % DOT/10. Arrow indicates the start of feeding for the fed-batch fermentation

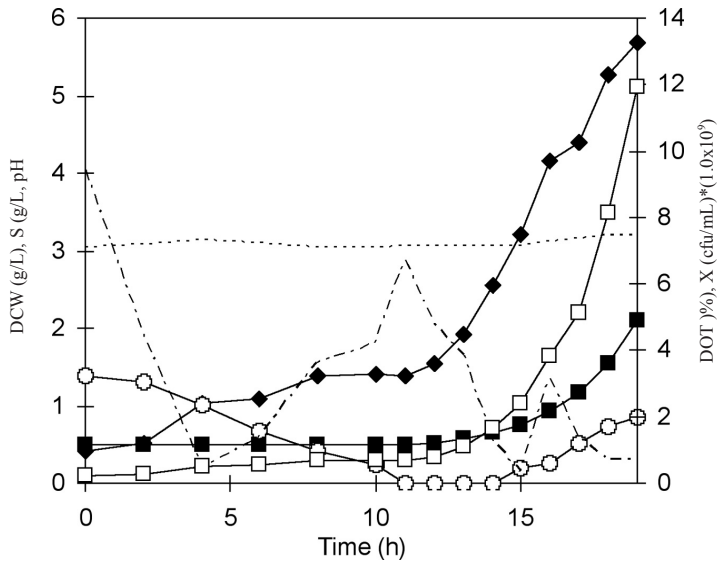

Fig. 3: The time course of the exponential fed-batch fermentation at μ = 0.40 h^{-1} using glycerol as the carbon source in a 2 l stirred tank fermenter. Airflow was increased to 4 L min^{-1} at 900 rpm after 17 h. Symbols represent (◆) DCW; (O) S; (■) V; (□) VX; (----) pH; (__ _) % DOT/10. Arrow indicates the start of feeding for the fed-batch fermentation

Exponential Fed-batch Cultivation

A typical time course of exponential fed-batch cultivation of *B. sphaericus* UPMB10 fed with glycerol is shown in *Figs. 2* and *3*. In the fed-batch cultivation operated at μ* of 0.2 h^{-1}, the residual glycerol in the culture remained at 0 g l^{-1}. The growth was found to have increased exponentially, according to the feeding rate of glycerol, reached the maximum dry cell weight (X_{dcw}) of 4.96 g l^{-1} and corresponded to 9.43 x 10^9 cfu m^{-1}. The DOT level was maintained at very high levels (60-80% saturation), suggesting that the oxygen supply was not limited. On the other hand, residual glycerol in the culture was only maintained at 0 g l^{-1} up to 14 h for the fed-batch cultivation operated at μ* of 0.4 h^{-1}. Beyond that, the residual glycerol concentration was gradually increased with cultivation time and reached a value of 0.82 g l^{-1} at the end of cultivation, indicating that the glycerol feeding rate was higher than the consumption rate. However, the dry cell weight was increased exponentially throughout the fed-batch phase and reached the maximum concentration of 5.67 g l^{-1}. The DOT level was found to drop drastically to around 10% saturation at the later phase of fed-batch, suggesting that the cultivation might be oxygen limited. Similar to the batch, the culture pH in all the fed-batch fermentations was maintained at around pH 7, indicating that pH did not influence the performance of cultivation.

The performance of the fed-batch cultivation of *B. sphaericus* UPMB10, fed with glycerol and controlled at different specific growth rates (μ*), is summarized in Table 1. The final X_{dcw} obtained at the end of cultivation was slightly increased by increasing μ* up to 0.4 h^{-1}. On the contrary, a significant reduction in X_{dcw} was observed in the fed-batch operated at μ* of 0.5 h^{-1}. In this cultivation run, the residual glycerol concentration gradually increased in the fed-batch phase and reached a value of 1.21 g l^{-1}. In addition, the DOT levels dropped to very low levels (i.e. around 5% saturation) during the fed-batch phase (data not shown). High residual glycerol concentration and low DOT levels in the culture were the reason for the reduction in the growth of *B. sphaericus* UPMB10 cultivated in the fed-batch mode at higher μ* (0.5 h^{-1}).

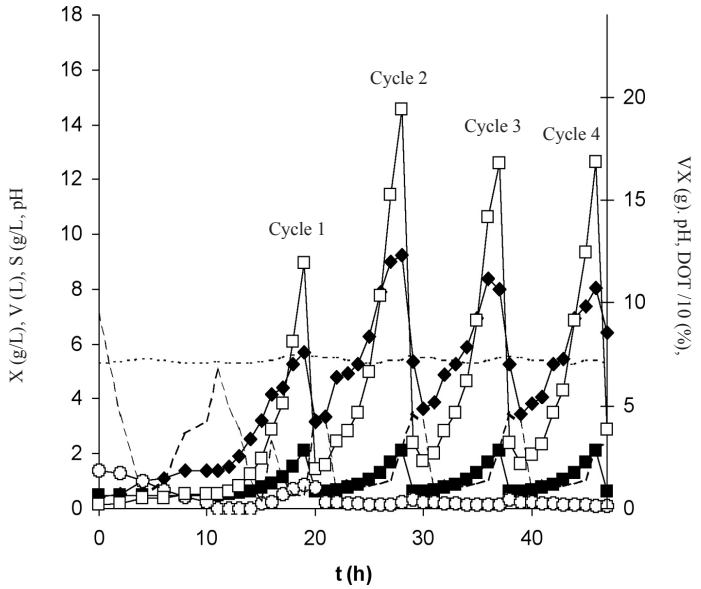

Fig. 4: Repeated exponential fed-batch culture of Bacillus sp operated at $\mu = 0.4\ h^{-1}$*. Symbols represent (◆) X; (O) S; (■) V; (□) VX; (----) pH; (__ _) %DOT/10. Arrow indicates the start of feeding for the fed-batch fermentation at each new cycle*

The actual specific growth rate during the fed-batch cultivation was calculated by plotting the logarithmic value of the total cell concentration (ln [VX_{dcw}]) against time (t). The slope of the straight line is the actual specific growth rate of the bacterium during the cultivation. Table 1 also presents a comparison between the required μ* and the actual μ calculated from the experimental data. In all cases, the actual μ during fed-batch cultivation was closed to the required μ*, except for μ* of 0.5 h^{-1}. Meanwhile, the actual μ for fed-batch operated at μ* of 0.5 h^{-1} was significantly lower and the value was similar to the value of μ_{max} for *B. sphaericus* UPMB10.

The cells produced from all the fed-batch cultivations have almost similar N_2 fixation capacity ranging from 17.4 to 21.4 nmol C_2H_2 h^{-1} ml^{-1}.

Repeated Fed–batch Cultivation

The time course of the repeated exponential fed-batch cultivation of *B. sphaericus* UPMB10 fed with glycerol, conducted for four cycles, is shown in *Fig. 3*. The culture withdrawal and feeding of fresh nutrient to start the new cycle of fed-batch cultivation was done after the maximum volume of the fermenter (2 l) had been reached, which was at 11 h, 20 h, 29 h, and 38 h. For all the cycles, μ* was set at 0.4 h^{-1}, and the actual μ obtained in all cycles was very closed to 0.4 h^{-1}. The final dry cell weight (X_{dcw}) and cell number obtained at the end of cycles 1, 2, 3, and 4 was 5.67 g l^{-1} (1.09 x 10^{10} cfu ml^{-1}), 9.22 g l^{-1} (1.80 x 10^{10} cfu ml^{-1}), 8.35 g l^{-1} (1.62 x 10^{10} cfu ml^{-1}) and 8.01 g l^{-1} (1.55 x 10^{10} cfu ml^{-1}), respectively. The values were not significantly different for cycles 2, 3 and 4, but it was slightly lower for cycle 1. This result suggests that *B. sphaericus* UPMB10 has increased the adaptation to growth in glycerol after the initial batch and the first cycle of fed-batch phases. The efficiency to grow in glycerol was also maintained up to the fourth cycle of the repeated batch process. In addition, the ability of the cells harvested at the end of each cycle was maintained at

TABLE 1
The growth performance of *Bacillus sphaericus* UPMB10 in the exponential fed-batch fermentation operated at different required specific growth rates (μ*)

Required specific growth rate, μ* (h^{-1})	Actual specific growth rate obtained during the fermentation, μ (h^{-1})	Residual glycerol in the culture in fed-batch phase (g/L)	Cell number, X_{cfu} (cfu ml^{-1} x 10^9)	Dry cell weight, X_{dcw} (g l^{-1})	Total dry cell weight, VX_{dcw} (g)	Overall cell productivity, P (g l^{-1} h^{-1})	Acetylene reduction rate (nmolC_2H_2 h^{-1} ml^{-1})
0.20	0.18	Maintained at 0.00	9.43±0.08	4.96±0.51	9.34	0.19	17.4±1.2
0.25	0.21	Maintained at 0.12	9.69±0.11	4.85±0.38	9.68	0.23	19.8±1.3
0.30	0.31	Maintained at 0.18	10.6±0.25	5.3±0.62	10.52	0.25	18.3±0.4
0.40	0.39	Gradually increased to a final concentration of 0.82	11.4±0.24	5.67±0.52	11.9	0.29	21.4±1.5
0.50	0.42	Gradually increased to a final concentration of 1.21	8.78±0.12	4.39±0.31	6.67	0.25	19.5±2.1

± standard deviation of triplicates

around 20.8 nmol C_2H_2 h^{-1} ml^{-1}. Similarly, the pH of the culture was also maintained at around pH 7 throughout the repeated fed-batch cultivation process.

A comparison of the performance of *B. sphaericus* UPMB10 in the different modes of cultivation is shown in Table 2. The final dry cell weight obtained in the repeated fed-batch cultivation (7.81 g l^{-1}) was about 1.4 and 4.8 times higher than those obtained in the fed-batch and batch cultivations. However, the cell yield for the repeated batch (0.66 g.cell g.glycerol^{-1}) was 2 times lower than the batch, but comparable to the fed-batch cultivation. In term of cell productivity, the values for the repeated fed-batch (0.68 g l^{-1} h^{-1}) was 2.3 and 6.2 higher than those obtained by the fed-batch and batch fermentation. The ability of *B. sphaericus* UPMB10 cells, harvested from the different modes of cultivation, to fix nitrogen was almost similar with the value ranging from 18.5 to 21.4 nmol C_2H_2 h^{-1} ml^{-1}.

DISCUSSION

Microbial fermentation is an important technology for the conversion of renewable sources to valuable products. It can be obtained by microbial fermentation of glycerol, a by-product from biodiesel industry. Glycerol is hygroscopic, which absorbs water from the air. Thus, it will reduce water activity (a_w) in a submerged culture employing glycerol as a substrate. Meanwhile, reduction in water activity led to a disruption of cell tugor pressure and membrane tension, reduced enzyme activity and protein stability, deterioration in integrity and stability of the membranes as well as nucleic acids (Brown, 1990). A number of micro-organisms responded to low-a_w environment by accumulating low-molecular-weight compatible solutes, such as amino acids, amino acid derivatives, trehalose, and polyols (Csonka and Hanson, 1991; Liu *et al*., 1998).

A number of fermentation processes for the production of chemicals and other valuable products utilizing glycerol as substrate have been developed. Among other, *Klebsiella pneumonia* can be used to ferment glycerol to 1,3-propanediol (Xiu *et al.,* 2004). Meanwhile, Lee *et al*. (2001) reported the production of succinic acid by fermentation of glycerol using *Anaerobiospirillum succiniciproducens*. The production of hydrogen and ethanol from glycerol using *Enterobacter aerogens* HU-101 has also been reported by Ito *et al.* (2005). Polyhydroxyalkonoates can also be produced by a highly osmophilic micro-organism which utilizes glycerol as a substrate (Koller *et al*., 2005). In all the cases, high glycerol concentrations showed the inhibition effect to growth of the micro-organism employed in the fermentation. This means that the batch culture employing high initial concentration of glycerol is not suitable for high yield and productivity process. Improvement of 1,3-propanediol production was achieved using continuous process, whereby a very low residual glycerol concentration was maintained in the culture (Xiu *et al*., 2004). The use of continuous process enables the dilution of glycerol with a synthetic medium to increase the rate of glycerol utilization, which in turn, increased hydrogen and ethanol production (Ito *et al.,* 2005).

The inhibition effect of glycerol to growth of *B. sphaericus* UPMB10 in the batch culture has been reported in the researchers' previous study (Ooi *et al.,* 2008). The optimum glycerol concentration for the growth of this bacterium was very low, i.e. 1.8 g l^{-1}. The use of batch culture for commercial production of *B. sphaericus* UPMB10 cells for subsequent use as starter culture in composting may not be economically viable, since large fermenter shall be used for large production. In this study, the cultivation performance of *B. sphaericus* UPMB10 could significantly be improved using fed-batch fermentation, where glycerol in the culture was maintained at a very low concentration to avoid inhibition effect to growth.

Using the repeated fed-batch cultivation or fermentation technique, more volume of substrate could be processed and not limited by the maximum working volume of the fermenter. An infinite

number of cycles of the culture withdrawal and an addition of fresh nutrient could be performed in the repeated fed-batch fermentation, provided that contamination did not occur. The repeated fed-batch cultivation of *B. sphaericus* UPMB10, which was conducted up to four cycles, could be used for further improvement of cell production, which was about six times higher than batch cultivation. The repeated fed-batch fermentation of xylitol by *Candida parapsilosis* was successfully conducted up to four cycles with a slight reduction in yield (Furlan *et al.,* 1997). Four cycles of the culture withdrawal were also achieved in the repeated fed-batch sorbose fermentation by *Gluconobacter oxydans* (Giridhar and Srivastava, 2001). On the other hand, efficient lipase production by *Acinetobacter radioresistens* in the repeated fed-batch fermentation was successfully conducted up to six cycles (Li *et al.,* 2005). By converting the batch or fed-batch into repeated fed-batch fermentation, the non-productive downtime involving cleaning, sterilization, and filling could therefore be eliminated. Thus, the overall productivity of the process could be increased and the production cost could significantly be reduced. It is also important to state that the nitrogen fixing capacity of the harvested cells, measured as acetylene reduction assay (ARA), remained unchanged and was maintained at around 20 nmolC_2H_2 h^{-1} ml^{-1} after the prolonged cultivation in the repeated fed-batch process. Innovative utilization of glycerol, for the production of valuable product as reported here, could be beneficial to get more understanding and reference in manipulating the integrated plans for sustainable and profitable biodiesel industry.

CONCLUSIONS

The results gathered in this study have demonstrated that the use of repeated exponential fed-batch cultivation greatly improved the utilization of glycerol for the production of *B. sphaericus* cell, nitrogen fixing bacterium. The viable cell count (1.14 x 10^{10} cfu ml^{-1}), obtained in the exponential fed-batch cultivation with controlled μ at 0.4 h^{-1}, was four times higher than the batch cultivation. The final cell concentration of *B. sphaericus* was further improved (1.9 x 10^{11} cfu ml^{-1}) in the repeated exponential fed-batch cultivation consisting of four cycles of harvesting and recharging for a period of 46 h. The overall cell productivity (0.68 g l^{-1} h^{-1}) for the repeated exponential fed-batch cultivation was increased about 6 times compared to the one obtained in the conventional batch cultivation (0.11 g l^{-1} h^{-1}), but more or less the same yields (0.66 g.cell g.glycerol^{-1}). The ability of cell to reduce acetylene after repeated and prolonged cultivation cycles was maintained at around 20 nmol C_2H_2 h^{-1} ml^{-1}, which was comparable to those obtained in the batch and exponential fed-batch cultivations.

REFERENCES

Bremner, J.M. and Mulvaney, C.S. (1982). Nitrogen-total in: Method of soil analysis. In A.L. Pace (Ed.), *Agronomic Monograph No.9* (pp 595-624). American Society Agronomic, Madison, Winsconsin, USA.

Brown, A.D. (1990). *Microbial Water Stress Physiology*. Chichester, UK: John Wiley & Son.

Cummings, S.P., Humphry, D.R., Santos, S.R., Andrews, M. and James, E.K. (2008). The potential and pitfalls of exploiting nitrogen fixing bacteria in agricultural soils as a substitute for inorganic fertiliser. *Environmental Biotechnology, 2,* 1-10.

Csonka, L.N. and Hanson, A.D. (1991). Prokaryotic osmoregulation: Genetics and physiology. *Annual Review in Microbiology, 45,* 569-606.

Dasari, M.A., Kiatsimkul, P.P., Sutterlin, W.R. and Suppes, G.J. (2005). Low-pressure hydrogenesis of glycerol to propylene glycol. *Applied Catalysis A: General*, *28,* 225-231.

Ezequiel, F.L. and Dirk, W.B. (2005). Estimation of optimal feeding strategies for fed-batch bioprocesses. *Bioprocess and Biosystem Engineering, 27,* 255-262.

Fallik, E. and Okon, Y. (1996). Inoculants of *Azospirillum brasilense*: Biomass production, survival and growth promotion of *Setaria Italica* and *Zea Mays*. *Soil Biological Biochemistry, 28,* 121-126.

Furlan, S.A., Delia-Dupuy, M.L. and Strehaiano, P. (1997). Xylitol production in repeated fed-batch cultivation. *World Journal of Microbiology and Biotechnology, 13,* 591-591.

Giridhar, R. and Srivastava, A.K. (2001). Repeated fed-batch sorbose fermentation by *Gluconobacter oxydans*. *Chemical Biochemical Engineering Quartely, 15,* 127-129.

Ito, T., Nakashimada, Y., Senba, K., Matsui, T. and Nishio, N. (2005). Hydrogen and ethanol production from glycerol-containing wastes discharged after biodiesel manufacturing process. *Journal of Bioscience and Bioengineering, 100,* 260-265.

Kargi, F. and Pamukoglu, M.Y. (2004). Repeated fed-batch biological treatment of pre-treated landfill leachate by powdered activated carbon addition. *Enzyme and Microbial Technology, 34,* 422-428.

Koller, M., Bona, R., Braunegg, G., Hermann, C., Horvat, O., Kroutil, M., Martinz, J., Neto, J., Pereira, L. and Varilla, P. (2005). Production of polyhydroxylalkanoates from agricultural waste and surplus materials. *Biomacromolecules, 6,* 561-565.

Lee, J., Lee, S.Y., Park, S. and Middleberg, P.J. (1999). Control of fed-batch fermentation. *Biotechnology Advances, 17,* 29-48.

Lee, P.C., Lee, W.G., Lee, S.Y. and Chang, H.N. (2001). Succinic acid production with reduced by-product formation in the fermentation of *Anaerobiospirillum succiniciproducens* using glycerol as a carbon. *Biotechnology and Bioengineering, 72,* 41-48.

Li, C.Y., Chen, S.J., Cheng, C.Y. and Chen, T.L. (2005). Production of *Acinetobacter radioresistens* lipase with repeated fed-batch culture. *Biochemical Engineering Journal, 25,* 195-199.

Lin, E.C.C. (1976). Glycerol dissimilation and its regulation in bacteria. *Annual Review in Microbiology, 30,* 535-578.

Liu, S.Q., Asmundson, R.V., Gopal, P.K., Holland, R. and Crow, V.L. (1998). Influence of reduced water activity on lactose metabolism by *Lactococcus lactis subsp. cremoris* at different pH values. *Applied and Environmental Microbioogy, 64,* 2111-2116.

Ooi, T.C., Ariff, A.B., Halimi, M.S. and Shamsuddin, Z.H. (2008). Growth kinetics of diazotrophic Bacillus sphaericus UPMB10 cultured using different types and concentrations of carbon and nitrogen sources. *Malaysia Journal of Microbiology, 4,* 15-25.

Weigand, W.A. (1980). Maximum cell productivity by repeated fed-batch culture for constant yield case. *Biotechnology and Bioengineering, 23,* 249-266.

Xiu, Z.L., Song, B.H., Wang, Z.T., Sun, L.H., Feng, E.M. and Zeng, A.P. (2004). Optimization of dissimilation of glycerol to 1,3-propanediol by *Klebsiella pneumoniae* in one and two-stage anaerobic cultures. *Biochemical Engineering Journal, 19,* 189-197.