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Growth kinetics of diazotrophic *Bacillus sphaericus* UPMB10 cultured using different types and concentrations of carbon and nitrogen sources

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ABSTRACT

Growth kinetics of newly isolated diazotrophic *Bacillus sphaericus* UPMB10 grown in various carbon (lactate, acetate, glycerol, malate, fructose, xylose and sucrose) and nitrogen (glutamate, yeast extract, arginine, hystadine, glycine, polypeptone, tryptophan, lysine, NH_4Cl and urea) sources was investigated using 2 L stirred tank fermenter. The highest growth was obtained in a medium containing lactate as a carbon source, which gave the highest maximum cell concentration of 2.30 g/L, which is corresponding to maximum viable cell count of 4.60×10^9 cfu/mL. However, the highest cell yield (1.06 g cell/g carbon consumed) was obtained in cultivation using glycerol though slightly lower maximum viable cell count was obtained (3.22×10^9 cfu/mL). In addition, cost for the production of live cell using glycerol was about 15 times lower than the cost using lactate. Growth performance of this bacterium when yeast extract was used as a nitrogen source was comparable to the use of pure amino acid. The medium containing 1.8 g/L glycerol and 2 g/L yeast extract was suggested as optimal for growth of this bacterium, which gave carbon to nitrogen ratio (C/N) of 10:1. The maximum viable cell count obtained in cultivation using optimised medium in 2 L stirred tank fermenter was 3.34×10^9 cfu/mL and the cells maintained its capacity for N_2 fixation at 18 nmol C_2H_2/h.mL.

Keywords: *Bacillus sphaericus*, diazotroph, growth kinetics, nitrogen fixing bacterium

INTRODUCTION

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 fertiliser have been reviewed by Cummings *et al.* (2008). New N_2-fixing bacilli have been isolated from roots of oil palm tree in Malaysia (Shamsuddin *et al.,* 1999). Experiments with ^{15}N fertilizer confirmed that the strain was capable of nitrogen fixation and identified as *Bacillus sphaericus* UPMB10 (Amir, 2001). This newly isolated diazotroph strain has been successfully applied on oil palm and banana crops in Malaysia.

For the preparation of biofertilizer, large-scale production of starter culture or microbial inoculants containing either single pure strain or mixed culture 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. Various methods of optimisation, such as experimental design, mathematical methods and kinetic models can be applied to improve the large scale cultivation process.

A wide variety of bacteria have been known to fix N_2. Other than the capacity for N_2 fixation, these microorganisms represent species from different taxonomic groups with totally different life-styles (Flores-Encarnacion *et al.,* 1999). Therefore, medium development for *B. sphaericus* UPMB10 should be detailed without adaptation from other N_2-fixing bacteria. The main objective of the present study was to investigate the effect of different types of carbon and nitrogen sources on live cell production of *B. sphaericus* UPMB10 in 2 L stirred tank fermenter. Experimental data obtained were analysed using models for calculation of kinetic parameters. The kinetic information may allow better understanding of the cultivation process and may also be used for optimisation.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

B. sphaericus. UPMB10 was obtained from Department of Land Management, Universiti Putra Malaysia. A single colony of *B. sphaericus* UPMB10 was cultured in a 100 mL Erlenmeyer Flask containing nutrient broth (NB). The cells obtained were then resuspended in 15% glycerol (v/v) and kept as stock culture at –80 °C. For inoculum preparation, stock culture was streaked on nutrient agar (NA) slant and incubated at 30 °C overnight. A single colony was then removed from NA slant and inoculated into 100 mL of nutrient broth (NB) in Erlenmeyer Flasks

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(250 mL). Inoculated flasks were shaken vigorously (rotary shaker, 200 rev per min) for 10 h at 30 °C. Optical density of the 10 h culture which was approximately 0.8 (OD of 1.0 corresponds to approximately 1 x 10^9 cfu/mL) was used as standard inoculum.

Culture Media

All cultivations were carried out using a basal medium consisted of Na_2HPO_4, 2.8 g/L, KH_2PO_4, 1.12 g/L, $CaCl_2.2H_2O$, 0.01 g/L, $MgSO_4.7H_2O$, 0.1 g/L, $MnCl_2$, 0.004 g/L, $FeSO_4.7H_2O$, 0.003 g/L, biotin, 0.003 g/L, thiamine hydrochloride, 0.03 g/L, though the type and concentration of carbon and nitrogen sources were varied according to the needs of each experiment. The effect of lactate, malate, acetate, xylose, fructose, sucrose and glycerol carbon sources on growth of *B. sphaericus* UPMB10 was studied. Sodium (Na)-acetate (5 g/L) was replaced with other carbon sources containing equal concentration of total carbon. Effect of different nitrogen sources such as glutamate, glycine, lysine, arginine, hystadine, tryptophan, yeast extract, polypeptone, ammonium chloride, and urea on growth characteristics of *B. sphaericus* UPMB10 was also studied. All nitrogen sources contained equal amount of total nitrogen as glutamate (2 g/L). In subsequent experiment, the effect of different concentrations of selected carbon and nitrogen sources on growth kinetic of *B. sphaericus* UPMB10 was also investigated.

Cultivation Experiments

All cultivation experiments of *B. sphaericus* UPMB10 were carried out in a 2 L stirred tank fermenter (Biostat B, B. Braun, Germany) using a medium containing predetermined amounts of suitable carbon and nitrogen sources. The fermenter was equipped with pH, temperature, dissolved oxygen tension (DOT) and foam control systems. During the cultivation, agitation speed and aeration rate was maintained at 600 rpm (impeller tip speed = 3.1 m/s) and 0.5 vvm, respectively. The temperature within the fermenter vessel was controlled at 30^oC. A polarographic dissolved oxygen probe (Ingold, Switzerland) was used to measure DOT levels and the value was recorded continuously. In all cultivation experiments, shake flask containing 100 mL of standard was inoculated into 900 mL of medium in the fermenter, which gave an inoculum size of 10 % (v/v). In all cases, pH was uncontrolled and the initial pH was set at 7.0. The level of foaming was controlled by the automatic addition of antifoam A (Sigma Chemical Co., St Louis, MO).

Analytical Procedures

During the cultivation, samples were withdrawn at time intervals for analysis. The sample was centrifuged at 10,000 rpm for 10 min. Cells were washed and resuspended twice with 0.1% NaCl for turbidity determination at 600 nm and was also subsequently used for measurement of dry cell weight (DCW). On the other hand, the supernatant was used for chemical analysis. Organic acid (acetic and lactic acid) was measured using high performance liquid chromatography (HPLC) with UV detector (SPD-10A Shimadzu, Japan) at wavelength of 210 nm.

The separation of organic acid was obtained by using Biorad aminex HPX-87H cation–exchange resin column (300 x 7.8 mm I.D.) as stationary phase. The mobile phase was 7 mM H_2SO_4. The flow rate of mobile phase and column temperature was controlled at 1.0 mL/min and 50 °C, respectively.

Sugar in growth medium was determined using a high performance liquid chromatography (HPLC) (ConstaMetric 3000, LDC Analytical, Florida) with 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. The mobile phase was an isocratic mixture of acetonitrile and water (80:20 v/v). Flow rate of mobile phase was set at 0.7 mL/min and the reaction was kept at room temperature.

Total nitrogen content in culture filtrate was quantified using a micro-Kjeldahl method. A 1.0 mL of 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 Assay

Pre-cultures of N_2-fixing bacterium were grown overnight in nitrogen free broth (NFB) supplemented with 0.05% yeast extract. Approximately 10 mL of culture was centrifuged and re-suspended in minimal lactate broth. The re-suspended culture was transferred into a 30 mL McCartney bottles sealed with rubber serum stoppers (Thomas Scientific). The McCartney bottle 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 ionisation 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.

Mathematical Methods

Kinetic and Modeling

The following simplified batch fermentation kinetic models for cell growth and substrate consumption based on logistic equation, which have been described elsewhere [1], were used to evaluate the growth kinetics of *B. sphaericus* UPMB10,

Cell growth:

$$dX/dt = [\mu_{max}(1-X/X_{max}) \qquad (1)$$

Substrate consumption:

$$-dS/dt = \alpha(dX/dt) + \beta X \qquad (2)$$

Where X is the cell concentration (g/L), X_{max} is the maximum cell concentration (g/L), μ_{max} is maximum specific growth rate (1/h), S is the substrate concentration (g/L), α is growth associated constant for substrate consumption (g substrate/ g cell), β is the non-growth associated constant for substrate consumption (g substrate/g cell/h) and t is the fermentation time (h).

The kinetic models (equations 1 to 2) were fitted to the experimental data by non-linear regression with a Marquadt algorithm using MATLAB computer software. The model parameter values were first evaluated by solving equations 1 to 2 and then the computer program was used as a search method to minimize the sum of squares of the differences between the predicted and measured values. The predicted values were then used to simulate the profiles of cell, substrate and product concentrations during the fermentation. In order to determine whether the deviations between the experimental and calculated data are significant or not-significant, statistical analysis (unpaired t-test) was also carried out.

Doubling time, t_d (h) is described by equation (3),

$$t_d = \ln 2 / \mu_{max} \qquad (3)$$

The yield, $Y_{x/s}$, is determined by measuring the maximum cell concentration attained and total amount of substrate consumed during the fermentation,

$$Y_{x/s} = (X_{max} - X_i) / (S_i - S_o) \qquad (4)$$

Where X_i is the initial cell concentration (g/L), S_i is the initial substrate concentration (g/L) and S_o is the residual substrate concentration at the end of fermentation (g/L).

Overall productivity, P, (g/L/h) was determined by measuring the total amount of biomass formed over a period of cultivation time,

$$P = (X_{max} - X_i) / t \qquad (5)$$

Statistical Analysis

The statistical significance of the cells produced during various treatments was analysed using the SAS program (SAS, Institute Inc, 1988). Treatments were arranged according to the complete randomised design (CRD). The least significant difference (LSD) multiple comparison method was used to compare the difference between treatment means.

RESULTS AND DISCUSSION

Time Course of Batch Cultivation

Typical time course of batch cultivation of *B. sphaericus* UPMB10 using glycerol as a carbon source is shown in Figure 1, which also shows the fitness of the calculated data according to equations 1 and 2 to the experimental data. Growth of *B. sphaericus* UPMB10 was 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. Dissolved oxygen tension 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 only slightly reduced from pH 5 to around pH 4.5 at the end of cultivation. From T test analysis, the deviations between the calculated and experimental data of cell growth and glycerol in the culture are not significant at a significance probability of 5%. This result suggests that the proposed models based on logistic equations are sufficient to describe growth of *B. sphaericus* UPMB10 and glycerol consumption. The proposed models are also sufficient to model growth of *B. sphaericus* UPMB10 in various carbon and nitrogen sources, where the kinetic parameter values such as μ_{max} and X_{max} were calculated through the modeling exercise.

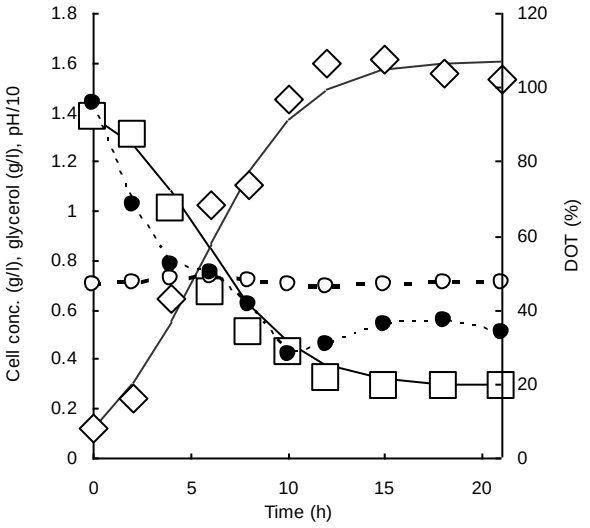

Figure 1: Time course of batch cultivation of *B. sphaericus*. UPMB10 in 2 L stirred tank fermenter using glycerol as a carbon source, also showing the comparison of calculated data to the experimental data. (♦) cell concentration; (■) glycerol concentration; (●) dissolved oxygen tension (%); (O) culture pH; (_____) calculated data of cell and glycerol concentrations according to the proposed models (Equations 1 and 2).

Effect of Carbon Source

Growth of *B. sphaericus* UPMB10 was greatly influenced by the different carbon sources used in medium formulation (Table 1). The highest growth, with a final cell concentration of 2.30 g/L with corresponding viable count of 4.60 x 10^9 cfu/mL was obtained when lactate was used as a carbon source. This gave the cell yield ($Y_{x/s}$) value of 1.05 g/g, calculated as dry cell weight produced divided by the amount of carbon source consumed. The second preferred carbon source for growth of *B. sphaericus* UPMB10, which produced final cell concentration of 2.26 g/L, was acetate. Although the final cell concentration obtained in cultivation using glycerol was about 30% lower than growth in lactate, the cell yield ($Y_{x/s}$) (1.06 g/g) was slightly higher than that obtained in cultivation using lactate. In addition, μ_{max} for growth in glycerol was significantly higher than growth in lactate and the value was comparable to growth on malate and fructose.

Growth performance of *B. sphaericus* UPMB10 on malate was relatively poor, resulting in relatively low final cell concentration. This is in contrast to a number of free-living N_2 fixers, which normally grew well on malate. Among these N_2 fixers are *Azospirillum brasilense* SP7 (Zhulin *et al*., 1996), *Klebsiella pneumoniae* (Jack, 1998) and *Bacillus* spp. (Wahab and El-Sharuony, 1979). Poor growth was also observed in medium containing fructose, xylose and sucrose. In general, higher final cell concentration (X_{max}) was produced in cultivation using organic acids as compared to sugars. This could be due to the fact that *B. sphaericus* UPMB10 requires low oxygen concentration for nitrogen fixation and has evolved preference towards partially oxidized carbon source.

B. sphaericus UPMB10 was able to utilize a number of carbon sources. The highest carbon consumption was observed in cultivation using organic acid, lactate and malate, as a carbon source. During the cultivation, about 50% of the organic acid was used within 13 h. The rate of consumption and amount of fructose, glycerol, xylose and acetate consumed during cultivation was slightly lower than for lactate and malate. The amount of sucrose consumed by *B. sphaericus* UPMB10 was very low, in which, only about 15% of the total sucrose added to the medium was consumed at the end of cultivation (24 h). This is in contrast to the growth characteristics of acid-tolerant nitrogen fixing bacterium, *Saccharobacter nitrocaptans*, where the best growth occurred at high sucrose concentrations (10%) (Cavalcante and Dobereiner, 1988). In addition, nitrogenous activity of *Bacillus* spp. was greatly enhanced with added sucrose in medium (Li *et al.,* 1992). For acetate, malate and xylose the amount of carbon utilized did not reflect the concentration of biomass produced. This could be due to the production of by products such as exopolysaccharides or bacteriocins.

It is interesting to note that glycerol provided a better alternative carbon source for growth of *B. sphaericus* UPMB10. It was more economical to use glycerol as compared to organic acid because the cost per kg of cells produced was about 16 times lower as compared to lactate and 5 times lower as compared to acetate (Table 1).

Effect of Glycerol Concentration

Table 2 shows the effect of different concentrations of glycerol on growth of *B. sphaericus* UPMB10. Growth of this nitrogen fixing bacterium was significantly increased with increasing glycerol concentration from 0.46 g/L to 1.38 g/L. The maximum cell concentration attained in cultivation using 1.38 g/L glycerol was 1.51 g/L, which is corresponding to viable cell count of 3.02 x 10^9 cfu/mL. However, the use of higher glycerol concentration up to 2.30 g/L did not improve further growth of *B. sphaericus* UPMB10. The X_{max} and maximum viable cell count for growth in 1.38 g/L to 2.30 g/L glycerol were not significantly different. Cell yield ($Y_{x/s}$) was decreased with increasing glycerol concentration because glycerol was not only consumed as a source of carbon but also to balance the high osmotic pressure when glycerol concentration was high. Reduced μ_{max} was observed at higher glycerol concentration, which might be due to catabolite repression by glycerol. Complete utilization of glycerol was observed when 0.46 g/L and 0.92 g/L glycerol was used but at higher concentration of glycerol about 0.14 g/L residual glycerol was still remained unconsumed in the culture. The highest productivity (0.089 g/L.h) for the cultivation of *B. sphaericus* UPMB10 was obtained at 1.38 g/L glycerol.

Effect of Nitrogen Source

Table 3 shows growth performance of *B. sphaericus* UPMB10 in different types of amino acid and other forms of nitrogen (N) sources. Maximum cell concentration obtained with glutamate was significantly ($P<0.05$) higher than other N sources. The maximum cell concentration obtained with glutamate was 1.64 g/L. In addition, the highest μ_{max} (0.3 h^{-1}) which is corresponding to a doubling time (t_d) of 2.31 h was obtained during the cultivation in glutamate. Nitrogen (N_2) fixing bacteria such as *Azospirilum lipoferum* and *Azospirillum amazonens*e have been reported to grow on glutamate as the sole nitrogen source (Hartman *et al*., 1988).

Yeast extract also significantly supported good growth of *B. sphaericus* UPMB10. The X_{max} obtained was comparable to that obtained in cultivation using glutamate. The cell yield ($Y_{x/s}$) and overall productivity (P) values for cultivation using yeast extract were higher as compared to those obtained in glutamate. It is important to note that cost of nitrogen per kg of cells produced from cultivation using yeast extract was five times lower than glutamate. Yeast extract has often been employed as the nitrogen source for the cultivation of N_2-fixing *Bacillus* spp. (Wahab and El-Sharouny, 1979; Li *et al.,* 1992), *Azotobacter diazotrophicus* (Bagwell, 1998) and *Azospirillum* (Bagwell, 1998). Yeast extract was also required to enhance nitrogeneous activity of *Bacillus* sp., though thiamine and biotin have more significant effect (Li *et al.,* 1992).

Inorganic nitrogen source was not as good as organic nitrogen for growth of *B. sphaericus* UPMB10. It is interesting to note that ammonium chloride (NH_4Cl) contributed to a higher maximum cell concentration (0.86 g/L) as compared to cultivation using urea (0.28 g/L). However, both ammonium chloride and urea were only marginally utilized by *B. sphaericus* UPMB10.

Table 1: Effect of different carbon sources on growth characteristics of *B. sphaericus* UPMB10

Carbon source	Maximum cell concentration, X_{max} (g/L)	Maximum Viable cell count (cfu/mL)	μ_{max} (h^{-1})	Carbon consumed during cultivation (%)	Cell yield (g cell/g carbon source)	Overall Productivity (g/L/h)	Cost of carbon per kg of cell produced (USD)
Lactate (4.26 g/L)	2.30 ^{a}	4.60 x 10^{9} ^{a}	0.212 ^{e}	63.2 ^{a}	1.05 ^{ab}	0.104 ^{a}	48.68
Acetate (3.74 g/L)	2.26 ^{b}	4.32 x 10^{9} ^{b}	0.344 ^{bc}	54.1 ^{b}	0.98 ^{ab}	0.102 ^{a}	14.47
Glycerol (5.13 g/L)	1.61 ^{c}	3.22 x 10^{9} ^{c}	0.344 ^{bc}	40.6 ^{c}	1.06 ^{a}	0.056 ^{b}	3.00
Malate (4.82 g/L)	1.16 ^{d}	2.32 x 10^{9} ^{d}	0.357 ^{b}	64.6 ^{a}	0.35 ^{d}	0.026 ^{d}	131.53
Fructose (4.52 g/L)	1.05 ^{e}	2.10 x 10^{9} ^{e}	0.330 ^{c}	37.6 ^{d}	0.58 ^{c}	0.036 ^{c}	78.16
Xylose (4.52 g/L)	1.05 ^{e}	2.10 x 10^{9} ^{e}	0.431 ^{a}	29.8 ^{e}	0.78 ^{b}	0.036 ^{c}	14,738.68
Sucrose (4.24 g/L)	0.71 ^{f}	1.42 x 10^{9} ^{f}	0.297 ^{d}	15.8 ^{f}	1.06 ^{a}	0.018 ^{e}	10.43

$^{a-f}$ means with the same letter are not significantly different.
μ_{max} (h^{-1}) is calculated from the modelling exercise according to equations 1 and 2.
The amount of each carbon source used contained approximately 1.8 g/L of total C.

Table 2: Effect of different glycerol concentrations on growth characteristics of *B. sphaericus* UPMB10

Glycerol concentration (g/L)	X_{max} (g/L)	Viable cell concentration (cfu/mL)	μ_{max} (1/h)	Glycerol consumed during cultivation (%)	Cell yield, (g cell/g carbon source)	Overall productivity (g/L/h)
0.46	1.01 ^{c}	2.02 x 10^{9} ^{c}	0.37 ^{b}	100.0 ^{a}	2.26 ^{a}	0.058 ^{e}
0.92	1.24 ^{b}	2.48 x 10^{9} ^{b}	0.42 ^{a}	100.0 ^{a}	1.39 ^{b}	0.072 ^{d}
1.38	1.51 ^{a}	3.02 x 10^{9} ^{a}	0.30 ^{c}	89.8 ^{b}	1.37 ^{b}	0.089 ^{a}
1.82	1.55 ^{a}	3.10 x 10^{9} ^{a}	0.27 ^{cd}	92.3 ^{b}	0.88 ^{c}	0.082 ^{b}
2.30	1.52 ^{a}	3.04 x 10^{9} ^{a}	0.26 ^{d}	93.9 ^{b}	0.58 ^{d}	0.079 ^{c}

$^{a-d}$ means with the same letter are not significantly different.
μ_{max} (h^{-1}) is calculated from the modelling exercise according to equations 1 and 2.

Table 3: Effect of different nitrogen sources on growth characteristics of *B. sphaericus* UPMB10

Nitrogen Sources	X_{max} (g/L)	Viable cell concentration (cfu/mL)	μ_{max} (h^{-1})	Nitrogen utilized during cultivation (%)	Cell yield (g cell/g N)	Overall productivity (g/L/h)	Cost of nitrogen per kg of cell produced (USD)
Glutamate (2.10 g/L)	1.64 [a]	3.28 x 10^9 [a]	0.30 [a]	75.5 [a]	1.03 [i]	0.074 [c]	85.58
Yeast extract (1.30 g/L)	1.62 [b]	3.24 x 10^9 [b]	0.29 [a]	34.8 [c]	3.61 [e]	0.087 [b]	17.29
Arginine (0.60 g/L)	1.46 [c]	2.92 x 10^9 [c]	0.19 [e]	62.7 [b]	3.89 [h]	0.065 [a]	97.77
Hystadine (0.75 g/L	1.46 [c]	2.92 x 10^9 [c]	0.10 [c]	24.2 [b]	7.98 [d]	0.086 [d]	38.22
Glycine (1.1 g/L)	1.38 [d]	2.76 x 10^9 [d]	0.11 [e]	63.7 [e]	1.99 [a]	0.093 [b]	27.28
Polypeptone (1.45 g/L)	1.21 [e]	2.42 x 10^9 [e]	0.12 [d]	30.1 [d]	2.80 [f]	0.062 [d]	34.92
Tryptophan (1.60 g/L)	1.06 [f]	2.12 x 10^9 [f]	0.23 [d]	30.8 [d]	21.3 [h]	0.042 [f]	5.73
Lysine (0.68 g/L)	1.03 [f]	2.06 x 10^9 [f]	0.18 [b]	62.5 [b]	2.42 [g]	0.055 [e]	44.17
NH_4Cl (0.75 g/L)	0.86 [g]	1.72 x 10^9 [g]	0.12 [f]	18.8 [f]	6.13 [b]	0.035 [g]	2.35
Urea (0.45 g/L)	0.28 [h]	0.56 x 10^9 [h]	0.13 [g]	12.8 [g]	4.80 [c]	0.010 [h]	2.52

[a-h] means with the same letter are not significantly different.
μ_{max} (h^{-1}) is calculated from the modelling exercise according to equations 1 and 2.
The amount of each nitrogen source used contained approximately 0.13 g/L of total N.

Table 4: Effect of different yeast extract concentration on growth characteristics of *B. sphaericus* UPMB10

Yeast extract Concentration (g/L)	**X_{max} (g/L)**	**Viable cell concentration (cfu/mL)**	**μ_{max} (h^{-1})**	**Nitrogen utilized during cultivation (%)**	**Cell yield (g cell/ g N source)**	**Overall Productivity (g/L/h)**
0.5	0.50 ^{d}	1.00 x 10^{9} ^{d}	0.22 ^{c}	38.5 ^{b}	2.55 ^{a}	0.022 ^{d}
1.0	0.79 ^{c}	1.58 x 10^{9} ^{c}	0.20 ^{c}	42.3 ^{a}	1.49 ^{c}	0.038 ^{c}
1.5	1.16 ^{b}	2.32 x 10^{9} ^{b}	0.27 ^{b}	40.5 ^{ab}	1.77 ^{b}	0.068 ^{b}
2.0	1.67 ^{a}	3.34 x 10^{9} ^{a}	0.28 ^{b}	41.0 ^{ab}	2.51 ^{a}	0.089 ^{a}
2.5	1.65 ^{a}	3.30 x 10^{9} ^{a}	0.33 ^{a}	42.8 ^{a}	1.11 ^{e}	0.087 ^{a}

$^{a-d}$ means with the same letter are not significantly different.
μ_{max} (h^{-1}) is calculated from the modelling exercise according to equations 1 and 2.

Effect of Yeast Extract Concentration

Growth of *B. sphaericus* UPMB10 was significantly influenced by the concentration of yeast extract in the medium (Table 4). The X_{max} was significantly increased with increasing yeast extract concentration up to 2.0 g/L. The X_{max} obtained at 2.0 g/L yeast extract was 1.67 g/L which is corresponding to maximum viable count of 3.34 x 10^9 cfu/mL. Further increased in yeast extract concentration up to 2.5 g/L did not significantly increase the cultivation performance of *B. sphaericus* UPMB10.

Significant reduction in cell yield was observed in cultivation with yeast extract concentration lower than 2.0 g/L. Maximum growth rate (μ_{max}) of the bacterium, as calculated from modelling exercise, was increased significantly with increasing yeast extract concentration. The μ_{max} of 0.331 h^{-1}which is equal to t_d of 2.1 h was achieved in cultivation using 2.0 g/L of yeast extract.

At 2.0 g/L of yeast extract, which was optimal for good growth of *B. sphaericus* UPMB10, about 0.637 g/L of yeast extract was utilized. At all concentrations of yeast extract investigated, complete utilization of nitrogen source was not observed. In all cases, only about 40% of yeast extract added was utilized. The possible explanation for this is due to the fact that yeast extract used in this study only consist 45% amino acid. During the cultivation, *B. sphaericus* UPMB10 may only used the amino acids for growth while other protein in yeast extract was not utilized.

Cultivation Performance using Optimized Medium

The medium consisted of 1.38 g/L glycerol as the carbon source and 2.0 g/L yeast extract as the nitrogen source can be suggested as optimal for the cultivation of *B. sphaericus* UPMB10. The C/N ratio for this optimized medium is 10:1. The time course for the cultivation of this nitrogen fixing bacterium in 2 L stirred tank fermenter using this optimized medium is shown in Figure 1. The calculated data according to the kinetic models for cell growth and substrate consumption based on logistic model (Equations 1 and 2) fitted well to the experimental data, suggesting that growth is not substrate dependent. In this cultivation run, the maximum cell concentration (X_{max}) and maximum viable cell count obtained was 1.67 g/L and 3.34 x 10^9 cfu/mL, respectively. According to logistic model, growth is at the maximum specific growth rate (μ_{max}), decreased with increasing growth and the μ_{max} became zero when growth reached a stationary phase.

The cell biomass produced using this optimized medium has more or less the same N_2-fixing rate (ARA) of 18 nmol C_2H_2/h/mL than those obtained in cultivation using organic acid (acetate). It is interesting to note that both growth and nitrogenous activity of *B. sphaericus* UPM B10 occurred in aerobic condition. This is in contrast to the characteristics of aerobic *Bacillus* sp. reported by Li *et al.* (1992), where anaerobic condition was required for nitrogenous activity. Recently, nitrogen-fixing *Bacillus* spp. have been enumerated, isolated and identified at seedling stage in rhizosphere of rice (Khan *et al.,* 2008). However, details characteristics of these nitrogen-fixing bacteria are not reported.

CONCLUSIONS

Growth of newly isolated diazotrophic *Bacillus sphaericus* UPMB10 was greatly influenced by the carbon and nitrogen sources supply into the cultivation medium. The highest cell yield (1.06 g cell/g carbon consumed) was obtained in cultivation using glycerol though slightly lower maximum viable cell count was obtained (3.22 x 10^9 cfu/mL) as compared to that obtained in cultivation using lactate (4.60 x 10^9 cfu/mL). The use of yeast extract as a nitrogen source for the cultivation of this bacterium gave the highest growth and the cultivation performance was comparable to cultivation using pure amino acids. The maximum viable cell count obtained in cultivation using optimised medium containing 1.8 g/L glycerol and 2 g/L yeast extract in 2 L stirred tank fermenter was 3.34 x 10^9 cfu/mL and the cells maintained its capacity for N_2 fixation at 18 nmol C_2H_2/h/mL.

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