

Ammonium Uptake and Growth Kinetics of *Pseudomonas putida* PGA1

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Received 18 March 2006 / Accepted 20 June 2006

Abstract. Ammonium uptake kinetics of low specific growth rate cultures of *Pseudomonas putida* PGA1, a gram-negative fluorescent pseudomonad, which is an important producer of poly(3-hydroxyalkanoates) (PHA) molecules, were studied. The ammonium uptake follows a first-order kinetics model, indicating that the micro-organism's specific uptake rate of ammonium and its growth would increase as the ammonium ion concentrations become higher. Optimal ammonium uptake and growth rates were observed at 0.1 gL⁻¹ ammonium ion in aqueous medium. It also indicated that the ammonium transport mechanism is probably inducible by ammonium concentration. The limit of ammonium ion level increase before cells stop growing completely is given by the substrate inhibition constant, K_i which equals to 1.2 gL⁻¹ ammonium ion concentration.

Keywords. Ammonium, growth, kinetics, uptake, poly-(3-hydroxyalkanoates)

INTRODUCTION

Poly(3-hydroxyalkanoates) (PHA) are structurally simple macromolecules accumulated as discrete intracellular granules by a number of micro-organisms. They are generally believed to play a role as sink for carbon and reducing equivalents. When nutrient supplies such as nitrogen, oxygen, phosphorus, sulfur or magnesium are imbalanced, it is advantageous for bacteria to store excess carbon intracellularly by polymerizing soluble carbon intermediates into water insoluble molecules like PHA. Through polymerization, cell does not undergo alteration of its osmotic state and at the same time leakage of these valuable compounds out of the cell is prevented (Madison and Huisman, 1999). Wang and Bakken (1998) indicated the possible role of stored PHA in enhancing survival of starvation in soil bacteria.

PHA has similar characteristics as the petrochemical derived plastics (Hocking and Marchessault, 1994). It has a huge potential of being an alternative to the synthetic plastics in numerous applications which is further made attractive by the fact that the PHA is readily biodegradable and naturally assimilated in the environment.

PHA can be divided into two classes: (a) the short-chain-length PHA (PHA_{SCL}) where the length of the monomers' carbon atom is four or five. Typical examples of the PHA_{SCL}

are poly(α -hydroxybutyrate) (PHB) and poly(α -hydroxybutyrate-*co*-valerate) (PHBV). A well known producer of PHA_{SCL} is *Alcaligenes eutrophus*; (b) the medium-chain-length PHA (PHA_{MCL}) contains monomers with the carbon atom length ranging from 6 to 18. This class of PHA is primarily produced by the fluorescent pseudomonads (Huisman *et al.*, 1989).

As the PHA accumulation by bacteria is a response to the imbalance in growth environment, where suitable carbon source is in excess and other nutrient e.g. nitrogen is limiting, this physiological condition can be exploited in the fermentation process to achieve high yields and productivity. Ammonium ion is frequently chosen as the limiting nutrient because growth of microorganisms is more dependent on nitrogen than on other mineral ions and the nitrogen source is assimilated more rapidly compared to other mineral ions (Suzuki *et al.*, 1986).

Ammonium limitation efficiently encouraged PHA_{MCL} accumulation in organism such as *Pseudomonas putida* (80% of total dried biomass) (Kim *et al.*, 1997), *Pseudomonas oleovorans* (33% of total dried biomass) (Hazenberg and Witholt, 1997) and *Pseudomonas* sp. K (57% of total dried biomass) (Suzuki

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et al., 1986), when these organisms were cultivated on octanoate, octane and methanol, respectively. These percentages are 2 to 7 times higher than that obtained from other nutrients limitation, as shown by the data of these workers. Limitation of other nutrients (*e.g.* SO_4^{2-} , Mg^{2+} , Fe^{2+} , *etc.*) did not as efficiently promote PHA accumulation as the ammonium.

Kleiner (1985) showed the transport of nitrogen with respect to ammonium is an energy dependent, carrier specific process. This active transport distinguishing characteristic is the movement of a component, in this case, ammonium against its chemical (or electrochemical) gradient, from regions of low to high concentration. The same author also reported that when even though the ratio of intra-cellular to extra-cellular ammonium can be as high as >3000 times, the organisms can still be observed to be actively transporting ammonium across the membrane into the cell. The rate of this process is believed to be the limiting step which determines the growth rate of some cells.

Since ammonium was the limiting substrate in this study, the kinetics of its uptake is of significant interest. Thus, the uptake of ammonium is expected to have a major influence on the growth of *Pseudomonas putida* PGA1, an important PHA_{MCL} producing micro-organism (Tan *et al.*, 1997). It would be interesting to find out the nature of its uptake kinetics under low specific growth rate or non-growth condition which is an appropriate representation of the PHA accumulation phase. In this study, *P.putida* PGA1 cells with a low specific growth rate was suspended in a phosphate buffer system and analyzed for its ammonium uptake kinetics. Under this non-growing condition, the cells' ammonium uptake process was also challenged with conditions that are known to adversely affect oxidative phosphorylation and ammonium uptake mechanism in bacteria. Based on the data obtained and literature, a possible pattern of growth and ammonium uptake kinetics by the micro-organism as a function of increasing ammonium concentration is hypothesized, and subsequently confirmed by further experimental data. The physiological information obtained would help to further explain and possibly improve PHA_{MCL} production process in *P.putida* PGA1.

MATERIALS AND METHODS

Microorganism. *Pseudomonas putida* PGA1 strain was obtained from Professor G. Eggink of the Agrotechnological Research Institute, Wageningen, The Netherlands.

Medium composition. Growth medium employed was a mineral salt solution containing the following: 5.74 gL^{-1} K_2HPO_4 , 3.7 gL^{-1} KH_2PO_4 , 0.14 gL^{-1} $\text{NaNH}_4\text{HPO}_4 \cdot \text{H}_2\text{O}$, 1.0 ml trace elements (MT) solution (Table 1) and 10.0 ml

Table 1. Composition of MT solution (Lageveen *et al.* 1988)

Components	Mass (in grams) in 500 mL of 1N HCl
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	1.39
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.99
$\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$	1.41
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.74
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.08
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.14

$0.1 \text{ M MgSO}_4 \cdot 7\text{H}_2\text{O}$. To avoid precipitation during autoclaving, solutions of magnesium salt, trace elements and 100 mM D-glucose or 5.0 gL^{-1} saponified palm kernel oil (SPKO) were sterilized separately before adding to the rest of the medium components.

Biomass concentration. A pre-determined calibration curve relating optical density at 450nm to dried biomass concentration, X (gL^{-1}) was used to rapidly estimate biomass concentration.

Growth and ammonium uptake assay. *P. putida* PGA1 cells grown on two slants of nutrient agar were transferred to the mineral salt medium containing D-glucose as sole carbon and energy source. The cultivation was carried out using Hotech Model 718 orbital shaker incubator. The temperature and agitation speed of the incubator were fixed at 30°C and 250rpm, respectively. Growth was monitored at regular intervals using optical density reading at 450nm by taking out 1.0 ml samples in triplicate. Biomass was aseptically harvested by centrifugation. The cells were washed twice using 0.05M phosphate buffer (pH 7.0). A known amount of biomass (dry weight) i.e. $0.87 (\pm 0.05) \text{ mg}$ was transferred to the Erlenmeyer flasks containing 15.0 ml phosphate buffer with specific D-glucose, ammonium chloride and sodium azide or potassium chloride concentrations (Table 2).

The flasks were incubated in shaking water bath at 30°C for 15 minutes to equalize the temperature before the *P.putida* PGA1 cells were transferred into them. Immediately after the transfer of cells into the buffer, 1.0 mL sample was withdrawn and designated as zero minute sample. Samples were taken every three minutes for the duration of fifteen minutes. The samples were centrifuged at 10,000 rpm for 1 minute and the cell-free supernatants were analyzed for residual ammonium using the spectrophotometric method (Solorzano, 1969). The amount of ammonium taken up by the cells of *P. putida* PGA1 was calculated by subtracting the ammonium amount sampled at different time intervals from the initial ammonium amount at the start of experiment (0 min).

Table 2. Experimental conditions for ammonium uptake assay.

	In a total volume of 15.0 mL 0.05M phosphate buffer (pH 7.0)		
	Control cells	Cells challenged with 20 mM sodium azide	Cells challenged with 78 mM KCl
Solution composition	100mM D-glucose 10 μ M NH ₄ Cl 20 mM sodium azide	100mM D-glucose 10 μ M NH ₄ Cl 78 mM KCl (added 5 min prior to start)	100mM D-glucose 10 μ M NH ₄ Cl (added 5 min prior to start)

Viable cell count. To estimate the viable cells from the assay, biomass pellet were re-suspended using phosphate buffer, serially diluted and spread on nutrient agar plates to determine the number of viable cells at zero and fifteen minutes for each of the three experimental conditions (Table 2). Plates were incubated at 30°C for 36h.

Culture growth and ammonium ion inhibition studies.

A rapid and simple batch experiment using shake-flasks based on different initial ammonium concentrations was carried out, where the effect of ammonium ion concentrations on the specific growth rate (μ) of the cells was studied. In this experiment, highly reduced substrate i.e. fatty acids mixture from saponified palm kernel oil (SPKO) was used as the sole carbon and energy source for growth of *P.putida* PGA1 cells. Each ammonium ion concentration was prepared in triplicate shake-flasks with the SPKO solution (pH 7.0) supplied at 5.0 gL⁻¹ in the mineral salt medium.

In order to eliminate the lag period upon cells transfer to the medium with the respective ammonium concentrations, inoculum was prepared using the same medium composition with 0.4 gL⁻¹ ammonium and 10.0 gL⁻¹ SPKO. Three flasks containing the inocula were incubated for 24h. Cells were aseptically harvested by centrifugation and washed twice with saline solution to get rid of the residual ammonium. Cells pellet from the three flasks was mixed and re-suspended in the mineral medium minus the ammonium and SPKO. This cells suspension was used to inoculate the flasks with different ammonium ion concentrations. The ammonium ion concentrations used were (gL⁻¹): 0.008, 0.04, 0.08, 0.24, 0.40, 0.56 and 0.80 with 5.0 gL⁻¹ SPKO. The starting optical density of the culture at 450nm was approximately 0.1206 (\pm 0.001) units. Samples were withdrawn at regular intervals and centrifuged. The collected cells were washed twice in saline prior to optical density determination at 450nm with saline as blanks. If necessary, appropriate dilution of the cells with saline was performed so that the optical density reading recorded is within the linear range of pre-determined calibration.

Overall specific growth rate, μ (h⁻¹) was calculated according to Bitar and Underhill (1990), described in equation (1):

$$\mu = (\ln OD450_{t_1} - \ln OD450_{t_2}) / (t_2 - t_1) \quad (1)$$

where OD450_{t₁} being the optical density at 450nm at time t₁, OD450_{t₂} is the optical density at 450nm at time t₂, and t₂-t₁ is the sampling time interval (h).

Data analysis and numerical calculations. Statistics and calculations for kinetics data were performed using MATLAB 6.1 software (The MathWorks Inc.).

RESULTS

Preparation of low specific growth rate culture of *P.putida* PGA1 in D-glucose as sole carbon and energy source.

The aim of this experiment was to determine whether *P. putida* PGA1 biomass with a low overall specific growth rate can be obtained with D-glucose as the sole carbon and energy source. The idea was to use this culture with low specific growth rate for the ammonium uptake experiments. Growth plot of *P. putida* PGA1 cultivated on 100 mM D-glucose is shown in Figure 1(A) and (B). It appears from the plot that a relatively low overall specific growth rate (0.043 \pm 0.024 h⁻¹) can be obtained from cultivation period of 0-32 h.

Ammonium uptake experiments. PHA accumulation phase in many micro-organisms is characterized by the non-growth or low specific growth rate condition. Thus, the uptake experiment has to be carried out under condition that does not permit growth using a population of cells with low specific growth rate; and must be carried out within shortest assay time possible, before massive cell death occurs. In addition, the inoculum with low specific growth rate should come from a culture that is actually growing very slowly, possibly on its less preferred substrate like glucose as shown by the previous result. The culture should not come from the cells population within the stationary phase; although this phase is characterized by the organism's low specific growth rate, its physiology is very different.

In this procedure, approximately $0.87 (\pm 0.05)$ mg (dry weight mass) of *P. putida* PGA1 whole cells were transferred to a 50 mL Erlenmeyer flasks containing 15 mL of buffer solution with components as outlined in Materials and Methods section. Non-growth condition was achieved through the use of phosphate buffer solution that contained 100 mM D-glucose and $10 \mu\text{M NH}_4\text{Cl}$, which provides the ammonium-limited condition. Since the inoculum was cultivated on 100mM D-glucose, it was expected that upon transfer to the phosphate buffer solution, the cells can immediately use the glucose as energy source to remain viable without adaptation period. The whole assay procedure was limited to 15 minutes to minimize the risk of massive cells death due to starvation. Assuming that majority of cells population remained viable throughout the assay period, the whole cells suspended in phosphate buffer was subjected to three conditions i.e. control; cells challenged with 20 mM sodium azide; and cells challenged with 78 mM KCl.

The concentrations of sodium azide and KCl used in this study were based on the study of methylammonium uptake (an analogue of ammonium) by Jahns *et al.* (1986), using *Alcaligenes eutrophus* H16, another PHA-producer organism. They found that 20 mM sodium azide and 78 mM KCl were sufficient to affect the uptake process for comparative study with respect to control. The phosphate buffer formulation used in this study was according to the same publication.

The results of the ammonium uptake by low specific growth rate *P. putida* PGA1 cultures are shown in Figure 2. The numerical values with the corresponding standard deviations are listed in Table 3.

The amount of viable cells at the start (0 min) and end (15 min) of the experiment is shown in Table 4. The count indicated that the majority of the *P. putida* PGA1 cells population remained viable throughout the duration of the ammonium uptake assay. From Figure 2, when compared to control cells, it is clear that ammonium uptake process of the *P. putida* PGA1 cells was affected to some extent by the presence of 20 mM sodium azide and badly affected with 78 mM KCl present in the buffer solution.

Our observation agreed well with the data obtained by Jahns *et al.* (1986), where they found that potassium caused the reduction of the methylammonium (an analogue of ammonium) gradient from 102 to 34 in *A. eutrophus* H16, presumably due to the electrogenic influx of potassium causing a decrease of its membrane potential. Thus, it is highly likely that membrane potential is also involved in the uptake mechanism of ammonium ions in *P. putida* PGA1. Exposure of the culture to sodium azide resulted only in a slight decrease in the amount of ammonium taken up by the biomass as compared to control. Sodium azide is a known inhibitor of the respiratory chain; its mode of action is by interfering with the function of electron carrier at the terminal oxidase and prevents its oxidation and reduction (Cunningham and

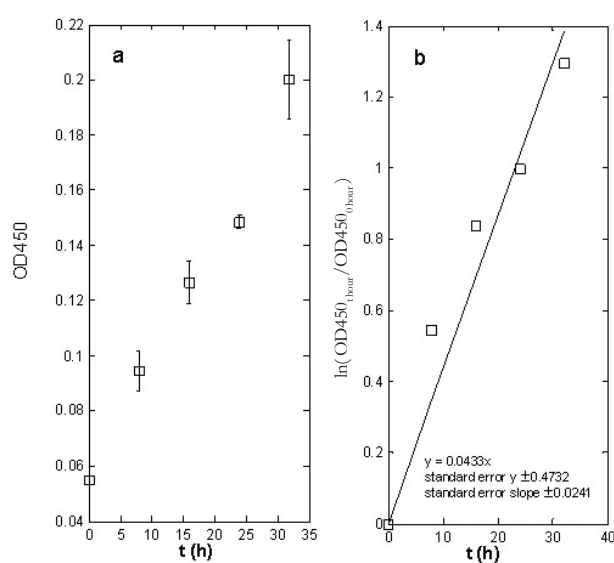


Figure 1. Growth profile of *P. putida* PGA1 in 100 mM D-glucose (a); and determination of overall specific growth rate of the culture (b). (OD450: optical density at 450nm)

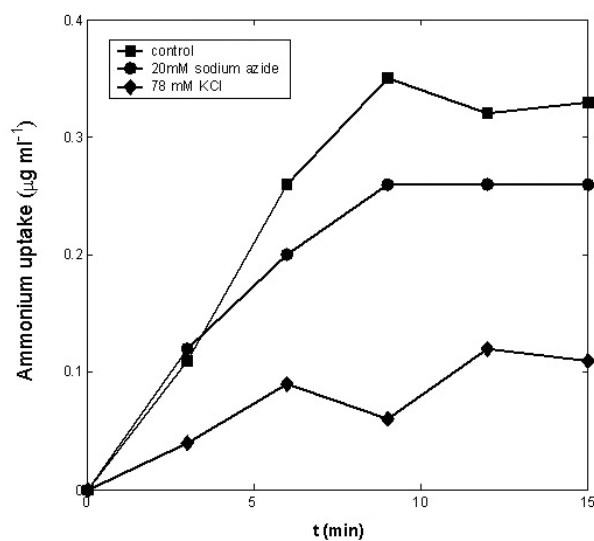


Figure 2. Amount of ammonium taken up by the *P. putida* PGA1 biomass suspended in phosphate buffer against incubation time.

Williams, 1995; Terpe *et al.*, 1999). This caused a stop to both the electron flow and ATP synthesis. From the results obtained, it is clear that ammonium uptake mechanism of *P. putida* PGA1 was not very sensitive to the disruption of cellular oxidative phosphorylation by sodium azide. This is in contrast to the results of Jahns *et al.* (1986), where at 20 mM sodium azide, a major reduction from 100% to 8% was

Table 3. Numerical values of ammonium uptake experiment (for Figure 2).

t (min)	Amount of ammonium taken up ($\mu\text{g/mL}$)		
	Control	Challenged with 20 mM sodium azide	Challenged with 78 mM KCl
0	0.0	0.0	0.0
3	0.11 \pm 0.06	0.12 \pm 0.01	0.04 \pm 0.02
6	0.26 \pm 0.01	0.20 \pm 0.04	0.09 \pm 0.08
9	0.35 \pm 0.07	0.26 \pm 0.04	0.06 \pm 0.01
12	0.32 \pm 0.04	0.26 \pm 0.06	0.12 \pm 0.01
15	0.33 \pm 0.03	0.26 \pm 0.04	0.11 \pm 0.04

Table 4. Viable cell count for control cells, cells exposed to sodium azide and cells exposed to KCl.

t (min)	Colony forming unit (CFU/mL) $\times 10^5$		
	Control	20 mM sodium azide	78 mM KCl
0	221	218	216
15	166	155	156

observed in the relative methylammonium uptake gradient of *A. entrophus* H16.

Kinetics of the ammonium uptake by viable *P. putida* PGA1 under non-growth condition. Since no further uptake of ammonium was observed after 10 minutes (Figure 2), only four data points were available for each treatment to be used in the subsequent model fitting exercise. The data from Table 3 was fitted to a first-order model *via* a plot of natural logarithm of residual ammonium against incubation time to determine specific rate constant of ammonium uptake (min^{-1}), which can be determined directly from the slope of the line (Figure 3a-c). As shown in Figure 3(a) for the control cells, the ammonium uptake process of viable *P. putida* PGA1 under non-growth condition follows a first-order reaction. This indicated that the ammonium uptake rate would be higher as the ammonium ion concentrations increase. The specific rate constant of ammonium uptake by the whole cells suspended in phosphate buffer alone was estimated to be 0.0262 (± 0.0059) min^{-1} .

When the cells were challenged with sodium azide, the specific ammonium uptake rate decreased only slightly to 0.0224 (± 0.0048) min^{-1} , which is not significantly different from the uptake rate of the culture suspended in phosphate buffer alone (Table 5). Furthermore, it still exhibited a good fit to first-order type of reaction (Figure 3(b)). On the other hand, when the cells were challenged with 78 mM KCl, the ammonium uptake process was badly affected with the specific rate constant estimated from the slope of the fitted line was 0.0094 (± 0.0021) min^{-1} . This was nearly 64% less compared to the uptake rate constant for control cells (Table 5).

From the ammonium uptake experiments, it can be shown that the ammonium uptake of *P. putida* PGA1 under ammonium-limited, non-growth condition showed a substrate concentration dependent kinetics. Thus, it is very likely that *P. putida* PGA1 biomass during active PHA accumulation phase where the growth rate is very low would be exhibiting a similar behavior. This also indicates that *P. putida* PGA1 has a high affinity for ammonium ions under ammonium-limited, and very low- or non- growth condition; which normally coincides with an active PHA accumulation. Hence, it can be said that the PHA biosynthesis process itself is characterized by a specific need for the presence of ammonium ions albeit in low concentrations

Effect of increasing ammonium ion concentrations on the specific growth rate (μ). The calculated μ at different ammonium ion concentrations shown in Figure 4 indicated that μ increased with the increase in ammonium ion concentrations and reached its maximum value around 0.1 gL^{-1} of ammonium ion concentration. This observation is in agreement with the earlier conclusion for ammonium uptake by low specific growth rate culture of *P. putida* PGA1. However, when the ammonium ion concentrations were further increased, μ started to decline indicating inhibition by high substrate concentration.

Similar results on the inhibitory effect of high ammonium ion concentration towards bacterial growth were reported previously. Suzuki *et al.* (1986) tested six types of ammonium salt compounds (NH_4Cl , $\text{NaNH}_4\text{HPO}_4 \cdot 4\text{H}_2\text{O}$, $\text{NH}_4\text{H}_2\text{PO}_4$, $(\text{NH}_4)_2\text{HPO}_4$, NH_4HCO_3 and NH_4NO_3) for the growth of *Pseudomonas* sp. K on methanol. In each case, cell growth was

Table 5. Estimated specific rate constant of ammonium uptake for control cells, cells exposed to sodium azide and cells exposed to KCl.

	Specific rate constant (min ⁻¹)(=slope)	Standard error of the slope(±)	Coefficient of correlation	Reduction of rate constant compared to control cells (%)
Control	0.0262	0.0059	0.8989	-
20 mM sodium azide	0.0224	0.0048	0.9174	15
78 mM KCl	0.0094	0.0021	0.9014	64

optimal at 0.2 gL⁻¹ NH₄⁺, and was severely inhibited when NH₄⁺ exceeded 1.0 gL⁻¹, and they attributed the decline in growth to the toxic effect of excess ammonium ion on the microorganism.

In the present study, the increasing μ values were obtained between 0.04-0.10 gL⁻¹ ammonium ion concentrations and this is in agreement with the reported values by Mulchandani *et al.* (1989), for *Alcaligenes eutrophus* ATCC17697. They observed μ was the highest when ammonium ion (in sulfate salt form) as limiting substrate was between 0.08-0.11 gL⁻¹.

Belfares *et al.* (1995) reported that in their shake-flask experiments using glucose as a carbon source, increasing amount of the ammonium ion concentrations caused a linear inhibition of *Alcaligenes eutrophus* DSM545 growth above a threshold concentration of 0.4 gL⁻¹. The value of μ obtained in this study also decreased in an almost similar manner with increasing ammonium ion concentrations above 0.2 gL⁻¹ (Figure 4). Beaulieu *et al.* (1995) also reported that significantly less biomass of *Alcaligenes eutrophus* DSM545 was obtained on 3% (w/v) glucose when initial ammonium ion concentrations were increased above 0.5gL⁻¹, indicating that growth was probably impaired at higher ammonium ion concentrations.

Thus, it is clear that the presence high concentration of ammonium ion in aqueous medium could significantly impaired microbial growth although the tolerance limit appears to be dependent upon microbial species and cultivation environment.

Growth model and its kinetic parameters estimation.

Following the observation from previous experiment, it is hypothesized that the growth of the organism could be described using a substrate-inhibition form. The model equation chosen to describe growth took the form of Andrew's equation

$$\mu = \mu_{\max} \cdot S / (K_s + S + S^2/K_i) \text{ with } K_i \gg K_s \quad (2)$$

where μ_{\max} is the maximum specific growth rate (h⁻¹), S is the limiting substrate i.e. ammonium (gL⁻¹), K_s is the substrate saturation constant (gL⁻¹), and K_i is the substrate inhibition

constant (gL⁻¹). Equation (2) is a modification of Monod growth expression which took into account the inhibitory effect of certain substrates towards microbial growth when present at high concentrations.

For comparison, the Monod model would also be fitted to the experimental data, which is in the form of

$$\mu = \mu_{\max} \cdot S / (K_s + S) \quad (3)$$

The equations (2) and (3) were fitted using the data from shake-flasks experiment. The values of kinetic parameters for equation (2) (μ_{\max} , K_s, K_i) and equation (3) (μ_{\max} , K_s) were estimated by fitting each model to the experimental data using a nonlinear least squares optimization technique by iterative methods that start with an initial guess of the unknown parameters. MATLAB 6.1 software was used for this purpose which uses the Gauss-Newton algorithm with Levenberg-Marquardt modifications for global convergence. Similar method was used successfully by Mulchandani *et al.* (1989) to estimate kinetic parameters for growth expression (μ) of PHB-producing *Alcaligenes eutrophus* ATCC 17697, which was subsequently used in modeling its growth and polymer production. In modeling the growth and PHB production by *A.eutrophus* DSM545, Belfares *et al.* (1995) estimated parameters of the specific growth rate expression using batch experiments with different initial ammonium concentrations, which also showed inhibition by ammonium at high concentration. The graphical fitting of each models to the experimental data are shown in Figure 4.

The estimated parameters value returned by the fitting function is shown in Table 6 for both models. The experimental data showed a satisfactory fit to the Andrews model with the residual sum of squares of 0.0003 as compared to Monod's, where the residual sum of squares was 0.0026. This was also evident from visual inspection of the plots in Figure 4. Hence, growth expression of Andrews form was chosen to describe the organism's growth. The parameter K_i indicated that growth would completely have stopped when ammonium ion concentration in the medium is above 1.2 gL⁻¹.

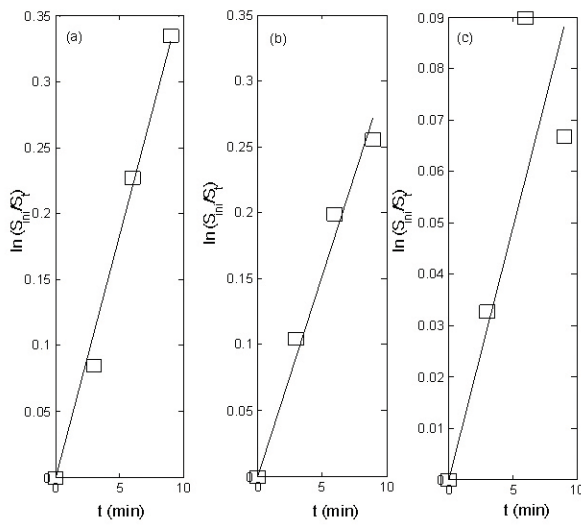


Figure 3. First-order ammonium uptake plots for (a) control cells; (b) cells exposed to 20mM sodium azide and (c) cells exposed to 78 mM KCl. (S_{ini} : initial ammonium concentration; S_t : ammonium concentration at time t)

Specific ammonium uptake rate (Q_{amm}) of *P. putida* PGA1 cells at different initial ammonium ion concentrations.

The volumetric rate of ammonium uptake, dS/dt ($\text{mgL}^{-1}\text{h}^{-1}$) was calculated using equation (4):

$$dS/dt = \mu \cdot X \cdot (1/Y_{X/S}) \quad (4)$$

where μ is specific growth rate (h^{-1}), $Y_{X/S}$ is stoichiometric yield of biomass from limiting substrate ammonium utilized ($\text{mg biomass mg}^{-1}$ ammonium) and X is biomass concentration (mgL^{-1}). The μ and X values were obtained from the previous experiment on the effect of ammonium concentration on the specific growth rate (μ).

Generally, it is difficult to obtain a reproducibly consistent and accurate experimental $Y_{X/S}$ value from batch data due to physiological and cultivation conditions variation. Thus, in this study, the $Y_{X/S}$ parameter was estimated using stoichiometric relationship. The $Y_{X/S}$ was defined as 'a mole of ammonium substrate (S) consumed to produce specific mole of biomass (X)'. To facilitate calculations, the carbon substrate SPKO was represented by lauric acid ($\text{C}_{12}\text{H}_{24}\text{O}_2$) as suggested by Gunstone *et al.* (1994). The calculation is described as follows: in aerobic fermentations, the growth yield per available electron in O_2 molecules ($Y_{X/e}$) is approximately 3.14 ± 0.11 gram dried cells/electron when NH_3 is used as the nitrogen source (Shuler and Kargi, 1992). The number of available electrons per O_2 molecule is four. When the number of O_2 molecules per mole of substrate consumed is known, the growth yield coefficient, $Y_{X/S}$ can be calculated. The

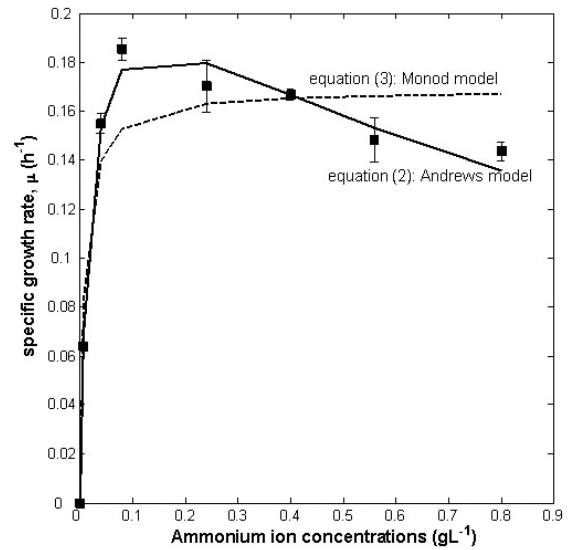
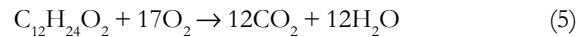
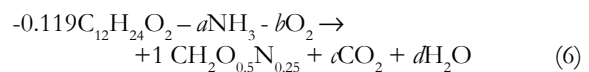


Figure 4. Fitting of Andrews and Monod growth models to the specific growth data of *P. putida* PGA1 culture as a function of ammonium ion concentrations.

stoichiometry of aerobic catabolism of lauric acid was as follows:



The total number of available electrons in 1 mole of lauric acid is 68. The cellular yield per available electron is $68(3.14) = 213.52$ g dried cells / mol lauric acid or $Y_{R/C} = 1.07$ g dried cells / g lauric acid. This was also equivalent to 8.373 C-mole biomass/mole lauric acid. One mole of PHA-free biomass (X) is defined as the amount containing 1 gram atom of carbon. Next, an expression of aerobic growth of organism on lauric acid using NH_4^+ as N-source, where only the production of X , CO_2 and H_2O were considered:



(**Note:** Biomass has per definition coefficient +1, Consumption -, production +)

Taking the empirical formula for X as $\text{CH}_2\text{O}_{0.5}\text{N}_{0.25}$ (Atkinson and Mavituna, 1991) with molecular weight (MW) 25.5, NH_3 MW 17.0 and lauric acid MW 200.3, the macro-chemical balance was calculated. By definition the stoichiometric coefficient of biomass is usually +1. It was possible to calculate the 4 coefficients above if only the $Y_{R/C}$ is known. The unknown coefficients (a, b, c, d) are from 4-elemental conservation laws.

$$\text{C-conservation } -0.119*12+1+c = 0 \quad (7)$$

$$\text{H- } " \quad -0.119*24-3a+2+2d = 0 \quad (8)$$

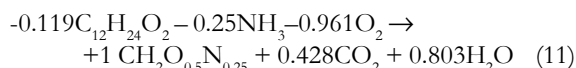
$$\text{O- } " \quad -0.119*2-2b+0.5+2c+d = 0 \quad (9)$$

$$\text{N- } " \quad -a+0.25 = 0 \quad (10)$$

Solving the equations gave

$$a = 0.25, b = 0.961, c = 0.428, d = 0.803$$

Hence,



If biomass (X) composition were constant during growth, the maximum yield coefficient for the growth of the PHA-free biomass, X on limiting NH_4^+ , $Y_{X/S} = 4$ mole/mole (5.67 g R / g NH_4^+).

Following the calculation of dS/dt , specific ammonium uptake rate, Q_{amm} (mg ammonium mg^{-1} biomass h^{-1}) by *P. putida* PGA1 cells was simply obtained by normalizing dS/dt with biomass concentration, X estimated for each of the initial ammonium ion concentrations. The relationship between Q_{amm} and initial ammonium ion concentrations is shown in Fig.5. When the initial ammonium ion concentrations were increased, this was followed by the faster uptake rate of ammonium. However, beyond 0.1 gL^{-1} ammonium ion concentration, steady decline of the uptake rate was observed. This indicated that a high ammonium ion concentration reduced the specific ammonium uptake rate of *P. putida* PGA1, possibly through substrate-inhibition mechanism resulting in decreasing growth rate of the culture.

DISCUSSION AND CONCLUSION

The kinetics of ammonium uptake is of the first-order type, indicating that the specific uptake rate of ammonium and growth would increase as the ammonium ion concentrations increase. However, it is suggested that growth and ammonium uptake rates would reach a maximum and then decrease with the increase in aqueous ammonium ion concentrations due to some possible inhibition mechanism. Since ammonium ion is an ionic species which cannot diffuse freely through the hydrophobic cell membrane, involvement of a specific, inducible and active protein transporter in the uptake mechanism is highly likely. Higher level of ammonium ion present necessitates the synthesis of more protein carrier and resulting in faster uptake rate by the organism. Kleiner (1985) reported that the ammonium uptake in bacteria under ammonium-limited condition is an active process using specific carrier. This carrier is synthesized under ammonium-

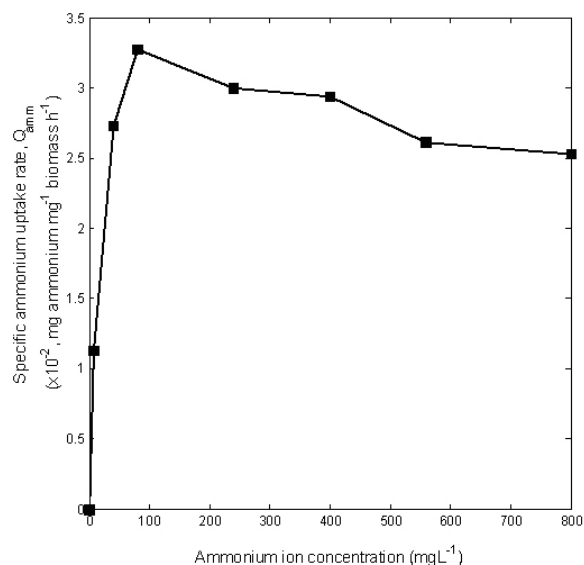


Figure 5. Specific ammonium uptake rate (Q_{amm}) of *P. putida* PGA1 culture as a function of ammonium ion concentrations (mgL^{-1}).

limited condition and its synthesis is repressed when ammonium concentration is excess in the medium. This active ammonium uptake *via* a specific transporter under deficiency condition depends on membrane potential as a driving force (Kleiner, 1985, Russell and Cook, 1995, Burkovski, 2003). The proposed mechanism of concentration inducible carrier-dependent uptake for transporting ammonium ion into the cells could explain the observed results in this study. However, it is not known at this stage whether the inhibition mechanism which led to the reduced growth rate of the *P. putida* PGA1 culture is due to the repression of more carrier protein synthesis or lowered rate of ammonium uptake *via* this protein.

Belfares *et al.* (1995) observed that the growth of *Alcaligenes eutrophus* DSM 545 started to decline when the ammonium concentration exceeded 3.0 gL^{-1} , with glucose as carbon substrate. Cell growth stopped completely when the concentration of ammonium in the reactor reached 9.0 gL^{-1} . In this work, optimal growth and ammonium uptake rates by *P. putida* PGA1 were observed approximately at 0.1 gL^{-1} ammonium. The limit of ammonium ion level increase before cells stop growing completely is given by the substrate inhibition constant, i.e. $K_i = 1.2$ gL^{-1} ammonium ion concentration. It is suggested that the intrinsic characteristics of specific bacterial species and the prevailing growth environment could play an interacting role with respect to the differences in the growth rate and observed limit of ammonium concentration tolerance reported here as compared to other published works. In practice, this information is valuable as it set limit on the range of ammonium ion concentration level that can be included batch-

Table 6. Estimated parameters value of Andrews and Monod growth models.

Model	Parameters	Parameter values	95% confidence intervals for the fitted values
Andrews	μ_{\max} (h ⁻¹)	0.2299	0.1920-0.2678
	K_s (gL ⁻¹)	0.0186	0.0089-0.0283
	K_i (gL ⁻¹)	1.1938	0.4989-1.8888
Monod	μ_{\max} (h ⁻¹)	0.1687	0.1431-0.1943
	K_s (gL ⁻¹)	0.0083	-0.0015-0.0181

wise, fed into and/or controlled inside the bioreactor. An important physiological implication is that at maximum uptake rate, the limiting step of bacterial ammonium utilization could be at the rate of incorporation of ammonium into the biomass and/or at the biochemical pathways level, which are responsible for its metabolism.

The ammonium uptake process of *P.putida* PGA1 under the specified condition does not seem to be sensitive to the disruption of cellular aerobic process indicating that the requirement for oxidative phosphorylation is not mandatory in the uptake process itself. Hence, it is suggested that a brief anoxic condition due to strong gradient of molecular oxygen diffusion should not greatly impact the ammonium uptake process of *P.putida* PGA1. The presence of such physical gradient is not an uncommon phenomenon for many biotechnological processes.

It is concluded that ammonium ion concentration in the aqueous medium should be controlled at ~ 0.1 gL⁻¹ for optimal growth and ammonium uptake by *P.putida* PGA1. The uptake process is not affected by the disruption of cellular oxidative phosphorylation but is significantly impaired under the condition that caused reduction of uptake potential e.g. presence of excess potassium.

ACKNOWLEDGEMENT

This research was funded by the University of Malaya (grant no. Vot F0156/2001A).

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