

Scaling-up production of pyocyanin from *Pseudomonas aeruginosa* JY21 as biocontrol agent against certain plant pathogenic fungi

Gaber A. Abo-Zaid¹, Elsayed E. Wagih², Saleh M. Matar¹,
Nader A. Ashmawy², Elsayed E. Hafez³

¹Bioprocess Development Department, Genetic Engineering and Biotechnology Research Institute, City of Scientific Research and Technological Applications; New Borg El-Arab, Alexandria, Egypt. Post Code: 21934 Alex.

²Plant Pathology Department, College of Agriculture, University of Alexandria, El-Shatby, Alexandria, Egypt.

³Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications; New Borg El-Arab, Alexandria, Egypt. Post Code: 21934 Alex.

Abstract: The antagonistic effect of *Pseudomonas aeruginosa* isolate JY21 (GenBank accession number, KF922508) obtained from El-Minoufiya in Egypt, was tested against plant pathogens belonging to different genera, *Alternaria* sp, *Pythium* sp, *Phytophthora infestans*, *Rhizoctonia solani* and *Sclerotium* sp. Batch and fed-batch processes of *P. aeruginosa* isolate JY21 were performed in a 7-L bench-top bioreactor (Bioflow 310, New Brunswick, N J, USA) for maximizing pyocyanin production. The processes were automated through a computer aided data bioprocessing system *BioCommand* multi-process management programme. In a shake flask and batch cultivation, cell mass was increased exponentially over time with a constant specific growth rate of 0.4 h^{-1} and 0.09 h^{-1} , respectively. Pyocyanin concentration reached its highest value of $365 \mu\text{g.ml}^{-1}$ in *death phase*, 84 h post-inoculation in a shake flask but in batch cultivation, pyocyanin concentration reached its highest value of $614 \mu\text{g.ml}^{-1}$ in *death phase*, 81 h post-inoculation. Constant fed-batch cultivation gave higher cell mass and lower pyocyanin concentration of 3.2 g.l^{-1} and $413 \mu\text{g.ml}^{-1}$, respectively than batch cultivation. Pulse feeding strategy was performed to avoid the repression of pyocyanin production after starting of feeding in constant fed-batch cultivation. Pulse feeding strategy improved pyocyanin productivity hence pyocyanin concentration reached its highest value of $676 \mu\text{g.ml}^{-1}$ during the *death phase* at 77 h post-inoculation.

Keywords: Pyocyanin, *Pseudomonas aeruginosa* JY21, biocontrol agent, fed-batch.

Introduction

Phenazines are low-molecular-weight (“secondary”) metabolites including nitrogen-containing heterocyclic pigments synthesized by a limited number of bacterial genera including *Pseudomonas*, *Burkholderia*, *Brevibacterium*, and *Streptomyces* [1, 2, 3]. The major phenazine synthesized by *P. aeruginosa* is pyocyanin (1- hydroxy-5-methyl phenazine) [4]. Almost all phenazines exhibit broad spectrum activity against plant pathogenic bacteria and fungi [5]. In addition to inhibiting fungal pathogens, phenazines play an important

role in microbial competition in rhizosphere, including survival and competence. For example, phenazines produced by *P. aeruginosa* PNA1 (wild type) are involved in the control of root rot of cocoyam caused by *P. myriotylum* [6].

Batch fermentation is the simplest mode of operation, and is often used in the laboratory to obtain substantial quantities of cells or product(s) for further analysis. A batch fermentation is a closed system, where all of the nutrients required for the organism's growth and product formation are contained within the vessel at the start of the fermentation process. After medium sterilization, the organism is inoculated into the vessel and allowed to grow. The fermentation is terminated when one or more of the following has been reached: (i) microbial growth has stopped due to the depletion of the nutrients or the build of toxic compounds; (ii) the concentration of desired product has been achieved [7]. The production of the antibiotic phenazine-1-carboxylic acid (PCA) of *P. fluorescens* 2-79 was evaluated in a batch bioreactor. When pH was maintained at 7, specific PCA production at 25°C could be modulated by the choice of carbon source supplied. PCA accumulation per unit biomass reached 0.31 g.g⁻¹ on glucose, 0.16 g.g⁻¹ on glycerol and xylose, and only 0.09 g.g⁻¹ on fructose. Although nitrogen source was also tested as a variable, and found to have a little influence on culture productivity of PCA under controlled pH [8]. The enhancement of phenazine-1-carboxylic acid (PCA) production was investigated in a 10 L fermentor using a *gacA* chromosomally inactivated mutant *Pseudomonas* sp. M18G. In batch culture, the optimal growth conditions with a two-stage agitation and aeration at 1.72 vvm improved PCA production to 1987 mg.l⁻¹ with 48 h fermentation [9]. *Bacillus subtilis* isolate G-GANA7 was selected from fourteen isolates for batch cultivation in bioreactor and the biomass achieved was 3.2 g.l⁻¹. Inhibitory activity of supernatant against *Alternaria* sp was increased near the end of the *stationary phase*. The activity reached its highest value as indicated by a 1.7 cm perpendicular inhibition distance for the bacterial growth line during the *death phase* [10].

In a fed-batch process, one or more substrates, nutrients, and/or inducers are fed into the reactor during the process, but the harvesting is still done all at the end of the process. This type of process is used very often since it allows relatively high biomass or product concentration to be achieved. This may not be achievable in batch cultivation because the total amount of substrate needed would be so much that the initial substrate concentration would strongly inhibit the process [11, 12]. Production of phenazine-1-carboxylic acid (PCA) using a *gacA* chromosomally inactivated mutant *Pseudomonas* sp. M18G were achieved in a DO-stat fed-batch system which maintained a constant DO of 20% by a two-pulse glucose feeding strategy. With a total 6.6 g.l⁻¹ glucose feeding, the maximum PCA production of 2597 mg/l was obtained upon 72 h fermentation, resulting in a 44.5% increase in PCA production and 10-fold greater cell numbers as compared to batch culture [9]. The maximum amount (4.12 g.l⁻¹) of rhamnolipid production by using *P. aeruginosa* was achieved when fed batch run was performed with glycerol as feed [13]. Various feeding strategies have been developed to control the nutrient concentration within the optimal range, and have been applied to high cell density culture of several microorganisms such as *Escherichia coli* [14]. The nutrients can be added intermittently at predetermined times. Sometimes, in order to overcome some of effects of a sudden increase of osmolarity, a semi-continuous of nutrient addition is adopted for the subsequent fed-batch culture. Semi-continuous mode means the time of addition and the duration are predetermined [15]. Fed-batch cultivation of *P. aeruginosa* USM-AR2 with pulse feeding strategy using diesel as carbon substrate was carried out at different feeding time interval in bioreactor to increase the rhamnolipid production and productivity. The best feeding mode was recorded to be 12 h-feeding interval which resulted in 12.6 g.l⁻¹ of biomass with 3.13 g.l⁻¹ of rhamnolipid being achieved in shorter time, 48 hours cultivation. The highest biomass and rhamnolipid production, 24.1 g.l⁻¹ and 13.4 g.l⁻¹, respectively, were obtained when multiple substrates were fed (diesel and yeast extract) [16]. The nutrients can also be added at a constant feed rate. When substrate is limited, cells can grow linearly and the specific growth rate continuously decreases. When substrate is in excess, cells can grow exponentially. However, no quasi steady state is achieved [17]. Traditional batch operations suffer from low cell mass and protein productions because a high initial glucose concentration causes substrate inhibition and also product inhibition due to acetate accumulation. An exponential fed-batch strategy was developed to prevent these inhibitions. By dual exponential feeding strategy, a high cell density of *B. subtilis* of 17.6 g.l⁻¹ and a final α -amylase activity of 41.4 U.ml⁻¹ and the overall specific biomass yield of 0.39 g.g⁻¹ were achieved [18]. Exponential fed-batch cultivation of *B. subtilis* isolate G-GANA7, for example, was conducted at the specific growth rate of 0.1 h⁻¹. High cell density of 14.6 g.l⁻¹ was achieved, with an overall specific biomass yield of 0.45 g.g⁻¹. The inhibitory activity of antifungal in supernatant against *Alternaria* sp reached its maximum value of 2.2 cm perpendicular inhibition distance [19].

The objective of this study was to scale-up production of pyocyanin produced by *P. aeruginosa* from lab-scale to bench-top bioreactor using different fermentation strategies and maximizing its inhibitory effect to use as a biocontrol agent.

Materials and methods

Bacterial and fungal isolates

The bacterial isolate used in this study is *P. aeruginosa* isolate JY21 which was isolated from rhizosphere of potato, and was obtained from El-Minoufiya in Egypt. *P. aeruginosa* isolate JY21 was identified according to 16S rRNA gene sequencing with GenBank accession number KF922508. The test fungal pathogens used in this study were kindly provided by the Plant Pathology Department, Faculty of Agriculture, Alexandria University, Egypt. They included *Alternaria* sp isolated from tomato, *Pythium* sp isolated from potato, *P. infestans* isolated from potato, *R. solani* isolated from peanut and *Sclerotium* sp.

In vitro antagonistic effect of *P. aeruginosa* isolate JY21

Antagonistic effect of *P. aeruginosa* isolate JY21 was performed against all fungal pathogens according to Toure method [20]. Data obtained were statistically analyzed using the Statistical Analysis System (SAS).

Shake-flask cultivation

A whole colony of *P. aeruginosa* isolate JY21 was inoculated into a LB medium and cultured overnight at 30°C with constant shaking at 200 rpm. Eight ml of culture was transferred into a 2 L Erlenmeyer flask containing 392 ml of optimized culture medium for pyocyanin production (peptone, 20 g; MgCl₂.6H₂O, 4.2 g; K₂SO₄, 10 g; Glycerol, 20 ml; MgSO₄.7H₂O, 1 g; KCl, 1.25 g and dH₂O up to 1000 ml, pH, 8.2) and cultured overnight at 30°C with constant shaking at 200 rpm. During the time of cultivation, several samples of culture were taken for pyocyanin assay and the inhibitory activity assay of pyocyanin was performed against *Alternaria* sp according to Chitarra method [21]. Cell number was determined by measuring extinction at 650 nm until the beginning of pyocyanin production, 10 ml of cultivated broth was separated overtime to apply the dry test method for biomass determination.

Bioreactor

Batch cultivation was performed in a 7-L bench-top bioreactor (Bioflow 310, New Brunswick, N J, USA) equipped with two 6-bladed disc-turbine impeller and four baffles. The process was automated through a computer aided data bioprocessing system *BioCommand* multi-process management programme. The set points for temperature and pH value were 30°C and 8.2, respectively and pH was controlled by automatic feeding of 2N NaOH and 2N HCl. Compressed air was supplied initially at 0.5-1.0 VVM (air volume per broth volume per minute) through sterile filter. It was manually controlled in parallel with agitation speed (100-400 rpm) to maintain the dissolved oxygen level above 30%. Dissolved oxygen level and pH values were determined on-line with METTLER TOLEDO electrodes. Antifoam A (Sigma) was used for eliminating foaming.

Batch cultivation

Batch cultivation was initiated in a bioreactor using a volume of 2940 ml of fresh optimized culture medium for pyocyanin production which was then inoculated with 60 ml of shake flask pre-cultured seeds which had previously been prepared as follows: a single colony of *P. aeruginosa* isolate JY21 was inoculated into a 250 ml Erlenmeyer flask containing 60 ml of the optimized culture medium and cultured overnight at 30°C with constant shaking at 200 rpm. During the time of cultivation, several samples of culture were taken for pyocyanin assay and the inhibitory activity assay of pyocyanin was performed against *Alternaria* sp according to Chitarra method [21]. Cell number was determined by measuring the extinction at 650 nm until the beginning of pyocyanin production, 10 ml of cultivated broth was separated overtime to apply the dry test method for biomass determination. Dry weight was determined from a calibration curve of extinction at 650 nm (E_{650}) of the fermentation broth versus cell dry weight. One unit of E_{650} was found to be equivalent to 0.28 g/l dry cell weight.

Fed-batch cultivation

Fed-batch cultivation was started with batch phase with an initial volume of 2940 ml of fresh optimized culture medium for pyocyanin production inoculated with 60 ml of shake flask pre-cultured seeds. At the end of *exponential phase*, feeding phase was started with addition of the feeding medium (peptone, 300 g; MgCl₂.6H₂O, 42 g; K₂SO₄, 100 g; Glycerol, 300 ml; MgSO₄.7H₂O, 10 g; KCl, 12.5 g and dH₂O up to 1000 ml). The strategy of feeding was studied using two different modes: constant feeding with a feeding rate of 0.3 ml/min [18] and pulse feeding which was started with addition of the feeding medium when the dissolved oxygen level became less than 30% with 10 ml of feeding medium per pulse [16]. During the time of cultivation, several samples of culture were taken for pyocyanin assay and the inhibitory activity assay of pyocyanin was performed against *Alternaria* sp according to Chitarra method [21]. Cell number was determined by measuring extinction at 650 nm until the beginning of pyocyanin production, 10 ml of cultivated broth was separated overtime to apply the dry test method for biomass determination. Dry weight was determined from a calibration curve of extinction at 650 nm (E_{650}) of the fermentation broth versus cell dry weight. One unit of E_{650} was found to be equivalent to 0.28 g.l⁻¹ dry cell weight.

Analytical procedures

Pyocyanin assay

The pyocyanin assay is based on the absorbance of pyocyanin at 520 nm in acidic solution [22]. A 5 ml sample of culture broth was extracted with 3 ml of chloroform and then reextracted into 1 ml of 0.2 N HCl to give a pink to deep red solution. The absorbance of this solution was measured at 520 nm using a spectrophotometer (Pharmacia Biotech, England). Concentrations, expressed as micrograms of pyocyanin produced per milliliter of culture supernatant, were determined by multiplying extinction at 520 nm (E_{520}) by 17.072 [23].

Cell number and biomass assay

Cell number was monitored by measuring extinction of the cell suspension at 650 nm. Dry cell weight was estimated by centrifuging 10 ml sample at 894 g for 10 min and the pellet was re-suspended, washed and centrifuged again as before. Pellets were then dried overnight in a dry-air oven at 80°C [24].

Inhibitory activity assay

Pyocyanin was filter (0.22 µm) sterilized after extraction and tested for antifungal activity. Two wells, 5 mm in diameter were made in each plate of PDA medium using a sterilized cork borer. The wells were then filled with 200 µl of pyocyanin solution. A mycelial plug (5 mm in diameter) of an actively growing culture of the fungus (*Alternaria* sp) to be tested was placed in the centre of plate. Plates were incubated for 7-10 days at 30°C and subsequently, the diameter of the inhibition zone was measured. All experiments were carried out with three replicates each [21].

Results

P. aeruginosa isolate JY21 had antagonistic effect against all tested pathogens, but showed more antagonistic effect against *Alternaria* spp, *P. infestans* and *R. solani* (Fig. 1).

Batch cultivation of *P. aeruginosa* isolate JY21 in shake flask was carried out in 2-L Erlenmeyer flask containing optimized culture medium for pyocyanin production and cultured overnight at 30°C with constant shaking at 200 rpm. Fig. 2 shows biomass, pyocyanin concentration and inhibitory activity of pyocyanin plotted against time. Cells were grown directly after the *lag phase* which lasted for about 2 h. Cell mass was then increased exponentially over time with a constant specific growth rate, μ (h⁻¹) within the *exponential (logarithmic) phase*. During this phase, the specific growth rate of 0.4 h⁻¹ was determined from the logarithmic relationship illustrated in Fig. 4. In this figure, the values of the lnX (values of X were obtained by using the correlation coefficient δ , 0.28, calculated from the linear relationship between dry mass weight and extinction illustrated in Fig. 3) were plotted versus time on a semi-logarithm graph to give a straight line. The biomass achieved was 0.98 g.l⁻¹. Pyocyanin production started to visually appear after 10 h and increased rapidly upon entry into the *stationary phase*. Pyocyanin concentration reached its highest value of 365 µg.ml⁻¹ during the

death phase at 84 h. Also, inhibitory activity of pyocyanin as measured by the vertical inhibition distance against *Alternaria* sp amounted to 1.8 cm in *death phase* at 84 h.

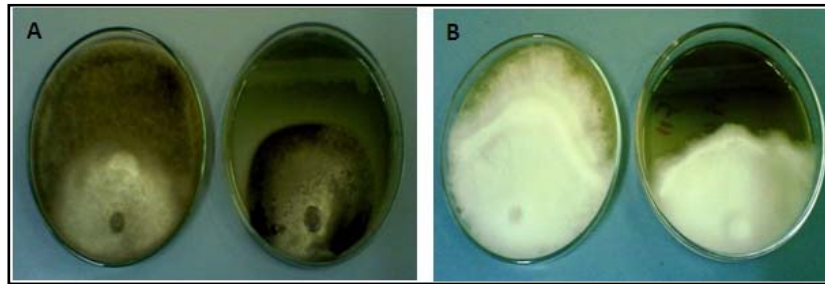


Fig. 1. Antagonistic effect of *Pseudomonas aeruginosa*, isolate JY21 against A, *Alternaria* sp. and B, *Pythium* sp. Plate on the left in each photo is the corresponding control without the antagonist.

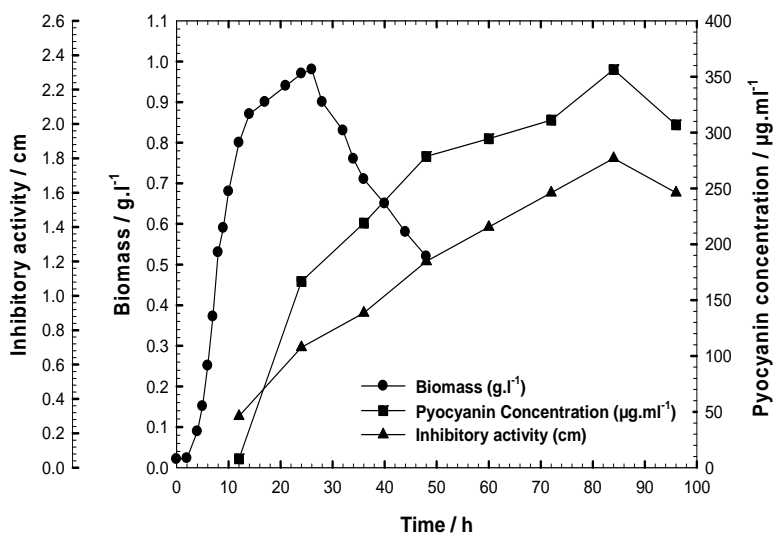


Fig. 2. Biomass, pyocyanin concentration and inhibitory activity of pyocyanin as a function of time for batch cultivation in shake flasks of *Pseudomonas aeruginosa* isolate JY21.

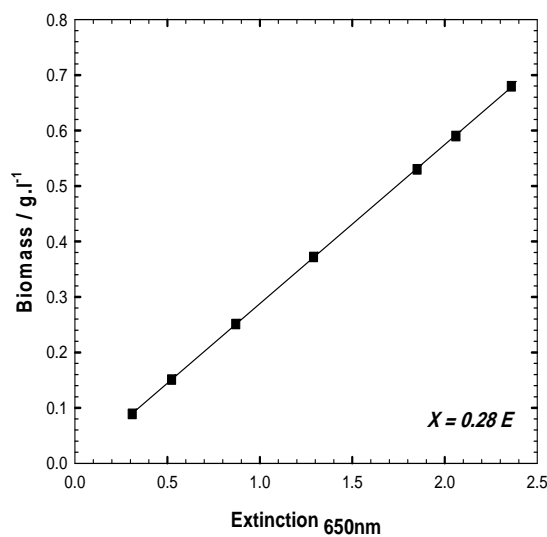


Fig. 3. The relationship between extinction 650nm and biomass (g.l⁻¹) in the exponential growth phase of batch cultivation in shake flask of *Pseudomonas aeruginosa* isolate JY21.

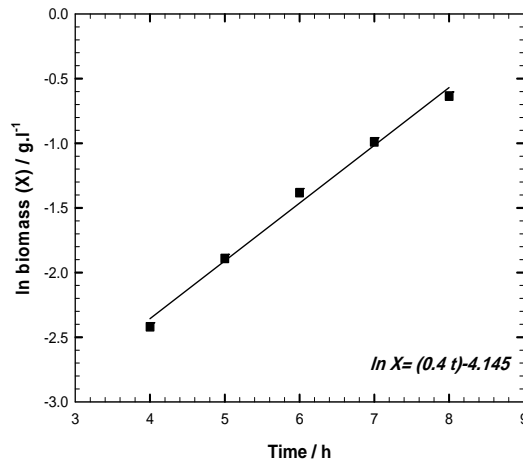


Fig. 4. Ln biomass (X) in g.l⁻¹ as a function of time in the exponential growth phase of batch cultivation in shake flask of *Pseudomonas aeruginosa* isolate JY21.

Batch cultivation of *P. aeruginosa* isolate JY21 was carried out in 7-L bench top bioreactor (Bioflow 310, New Brunswick, NJ, USA) at a constant temperature of 30°C. Fig. 5 shows biomass, pyocyanin concentration and inhibitory activity of pyocyanin of *P. aeruginosa* isolate JY21 plotted against time. Cells were grown directly after the *lag phase* which lasted for about 2 h. Cell mass was then increased exponentially over time with a constant specific growth rate, μ (h⁻¹) within the *exponential phase*. During this phase, the specific growth rate of 0.09 h⁻¹ was determined from the logarithmic relationship illustrated in Fig. 6. In this figure, the values of the lnX were presented by a straight line on a semi-logarithm plot versus time. The culture was maintained at a higher value of dissolved oxygen which decreased gradually indicating cell growth and substrate consumption from the culture broth. The dissolved oxygen decreased due to increase in O₂ demand for culture growth (Fig. 7). To guarantee a sufficient oxygen supply, oxygen was kept at above 30% by raising the agitation speed. After 5.5 h the dissolved oxygen was increased gradually so that the agitation speed decreased gradually. The biomass achieved was 1.34 g.l⁻¹. Pyocyanin production started to visually appear after 7 h and increased rapidly after entry into the *stationary phase*. Pyocyanin concentration reached its highest value of 614 $\mu\text{g.ml}^{-1}$ in *death phase* at 81 h. Also, inhibitory activity of pyocyanin reached its highest value of 2.4 cm in *death phase* at 84 h.

When fed-batch cultivation of *P. aeruginosa* isolate JY21 was carried out in 7-L bench top bioreactor (Bioflow 310, New Brunswick, NJ, USA) and biomass, pyocyanin concentration and inhibitory activity of pyocyanin were measured as a function of time, the results shown in Fig. 8 were obtained. In the batch phase, the culture grew after the *lag phase*, exponentially with a maximum specific growth rate of 0.09 h⁻¹. Dissolved oxygen decreased gradually during the *exponential (logarithmic) phase* due to increase in O₂ demand for the growing culture. Oxygen was kept at above 30% by controlling motor speed and consequently agitation rate (Fig. 9).

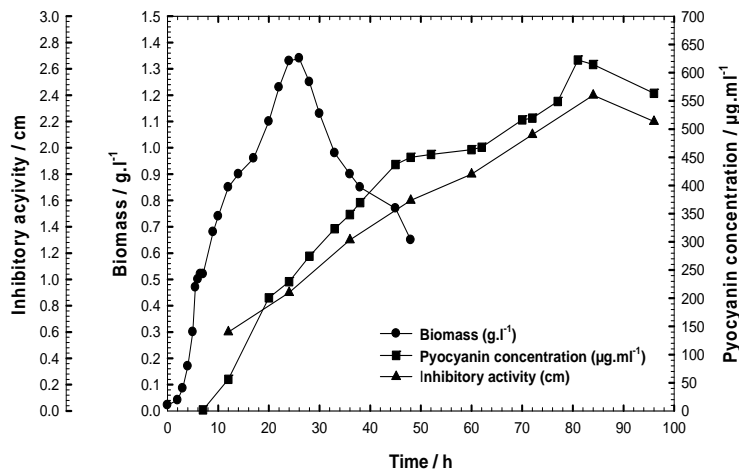


Fig. 5. Biomass, pyocyanin concentration and inhibitory activity of pyocyanin as a function of time for batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

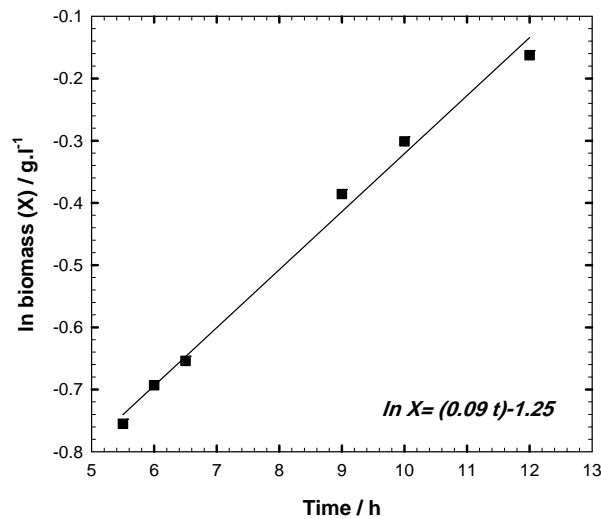


Fig. 6. Ln biomass (X) in g.l⁻¹ as a function of time in the exponential growth phase of batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

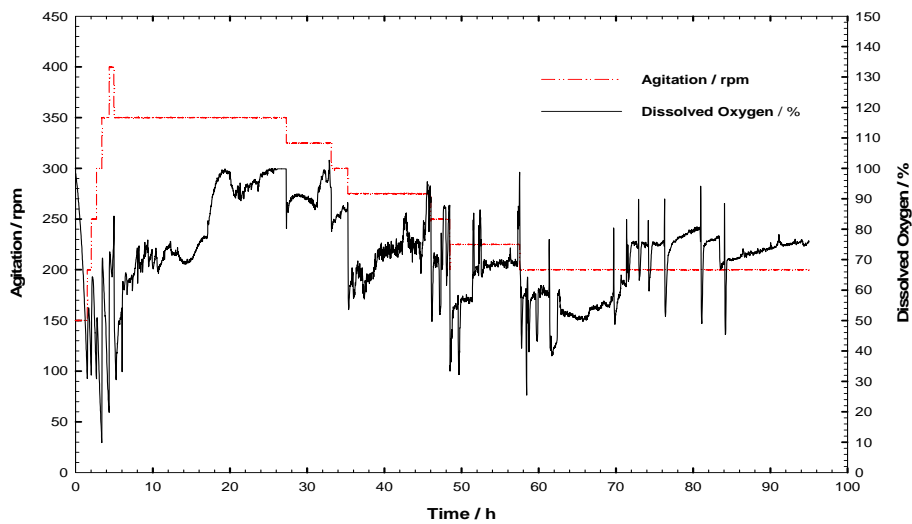


Fig. 7. Agitation and dissolved oxygen as a function of time during batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

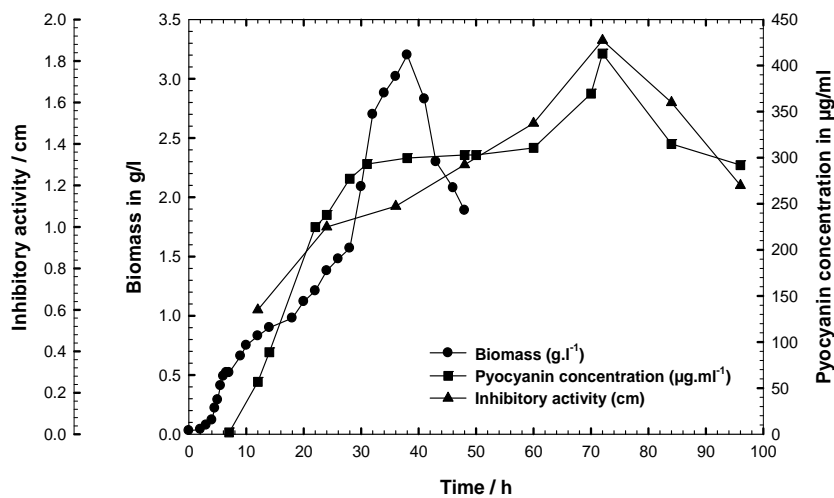


Fig. 8. Biomass, pyocyanin concentration and inhibitory activity of pyocyanin as a function of time for constant fed batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

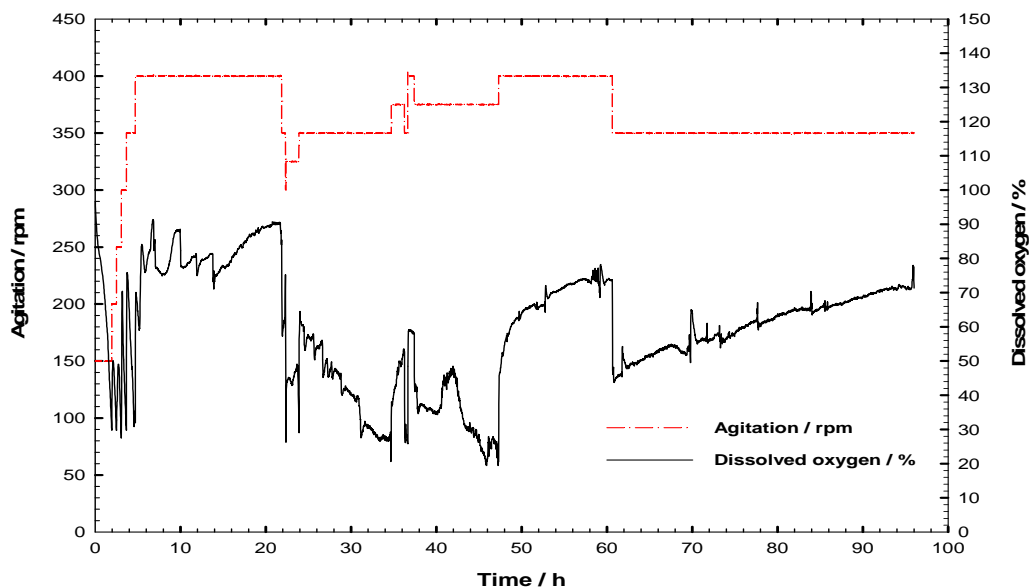


Fig. 9. Agitation and dissolved oxygen as a function of time during constant fed-batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

After 5.5 h, dissolved oxygen was increased gradually until the feeding medium was added. At this point dissolved oxygen decreased gradually therefore agitation speed was increased gradually. Biomass was used as an indication of ending the batch phase. After 24 h and in the end of the *exponential phase*, the fed-batch phase was started with addition of the feeding medium using constant feeding strategy with a feeding rate of $0.3 \text{ ml}\cdot\text{min}^{-1}$. Cells were grown linearly after starting the feed stream with a specific growth rate of 0.07 h^{-1} . In the end of fed-batch phase at 48 h, dissolved oxygen was increased secondly. The biomass achieved was $3.2 \text{ g}\cdot\text{l}^{-1}$. Pyocyanin production started to visually appear after 7 h and increased rapidly after the end of the *exponential phase*. In batch phase, rapid increase in the level of pyocyanin produced was observed but through feeding phase, slow increase in pyocyanin concentration was evident. After ending of fed-batch phase, pyocyanin concentration increased gradually but did not reach the highest value achieved in batch cultivation. While pyocyanin concentration achieved a value of $413 \mu\text{g}\cdot\text{ml}^{-1}$ in *death phase* at 72 h, inhibitory activity of pyocyanin reached its highest value of 1.9 cm in the same phase at the same time.

The results obtained from the application of the pulse feeding strategy are summarized in Fig. 10 which shows the alteration in biomass, pyocyanin concentration and inhibitory activity of pyocyanin of *P. aeruginosa* isolate JY21 plotted as a function of time. The culture grew, after the *lag phase*, exponentially with a maximum specific growth rate of 0.09 h^{-1} . The culture was maintained at a higher value of dissolved oxygen which decreased gradually indicating cell growth and substrate consumption from the culture broth. Compressed air was controlled manually in parallel with agitation speed (100-400 rpm) to maintain the dissolved oxygen concentration at above 30% (Fig. 11).

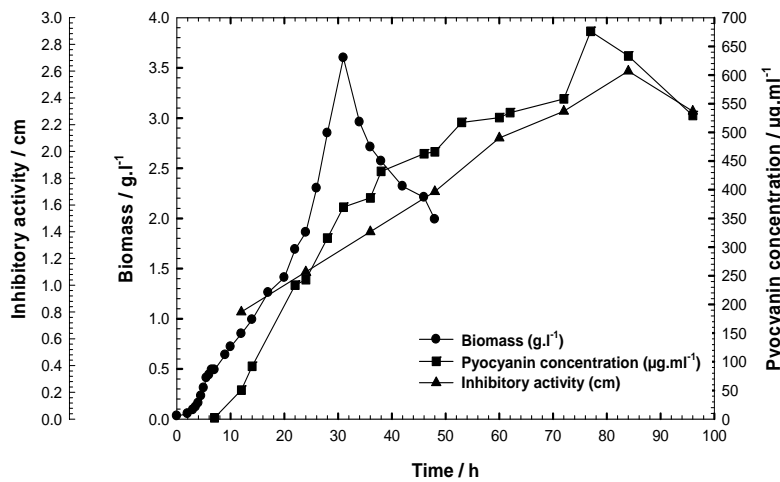


Fig. 10. Biomass, pyocyanin concentration and inhibitory activity of pyocyanin as a function of time for pulse fed batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

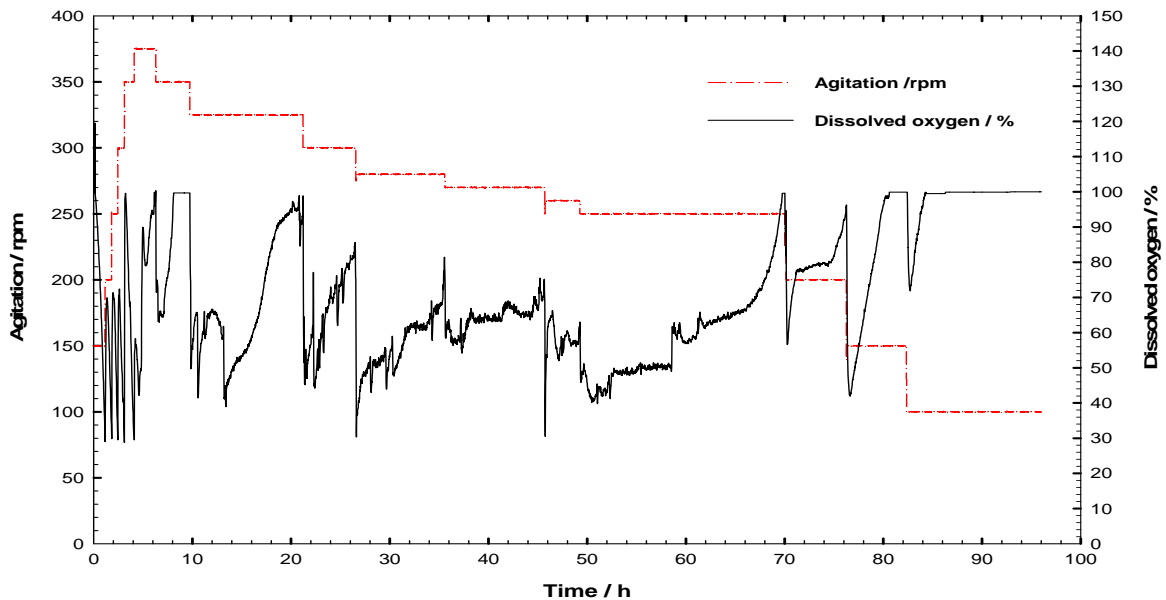


Fig. 11. Agitation and dissolved oxygen as a function of time during pulse fed-batch cultivation of *Pseudomonas aeruginosa* isolate JY21.

Pulse feeding was started before pyocyanin production with the addition of the feeding medium in five pulses with each being given when the decrease of the dissolved oxygen went down to 30%. After 5.5 h the dissolved oxygen increased gradually and for this the agitation speed was consequently decreased. The biomass achieved was 3.6 g.l⁻¹. Pyocyanin production started to visually appear after 7 h and increased rapidly after the end of the *exponential phase*. Pyocyanin concentration reached its highest value of 676 µg.ml⁻¹ in *death phase* at 77 h. Also, inhibitory activity of pyocyanin reached its highest value of 2.6 cm in *death phase* too at 84 h.

Discussion

Metabolic activities of cells are regulated at various levels both inside and outside the cell. These metabolic activities are extremely sensitive to a set of variables of which certain nutritional elements, O₂, pH and the ambient temperature are of most important. Because of this multi-level complex regulation, it was very important, from an engineering point of view, to study and understand the nutritional and environmental factors

affecting cell metabolism. Strategies of bioprocessing were carried out and controlled through a method of growth in shake flasks. Monitoring the optimal performance of the upstream processing via on-line and off-line sensors in the two basic types of cultivation (batch, and high cell density, HCD, fed batch) was useful in this respect.

When *P. aeruginosa* isolate JY21 was cultivated in shake flask, the *lag phase* was characterized by a negligible increase in the biomass concentration which lasted for about 2 h. The *exponential phase* was, however, characterized by a sharp exponential increase in the biomass concentration, as cell mass increased exponentially over time with a constant specific growth rate of 0.4 h^{-1} . At the *stationary phase*, the growth rate was zero (no cell division) and this was probably due to the depletion of substrate and accumulation of inhibitory metabolites such as acetic acid. Finally during the death phase, cell growth had a negative value. The highest biomass achieved was 0.98 g.l^{-1} while pyocyanin started to be detectable 10 h post-inoculation and increased rapidly upon entry into the *stationary phase*. Pyocyanin concentration reached its highest value of $365 \mu\text{g.ml}^{-1}$ in *death phase* at 84 h suggesting that its production is stress-related. This was supported by the fact that the inhibitory activity of pyocyanin reached its highest value of 1.8 cm as a perpendicular radial inhibitory distance during the *death phase*, 84 h post-inoculation.

In batch cultivation, Cells grew quickly after the *lag phase* which lasted for about 2 h. Cell mass was then increased exponentially over time with a constant specific growth rate of 0.09 h^{-1} within the *exponential phase* as compared to a specific growth rate of 0.4 h^{-1} in shake flask. By controlling the growth rate at less than approximately 0.1 h^{-1} the repression of pyocyanin production could remarkably be overcome [25]. An inverse relationship between growth rate and pyocyanin production was established, with a decrease in growth rate resulting in increased pyocyanin levels. Pyocyanin production started 7 h post-inoculation while in the shake flask method the corresponding figure was 10 h post-inoculation. In the *exponential phase*, dissolved oxygen decreased due to the increase in O_2 demand for growing the culture but upon 5.5 h the dissolved oxygen was increased gradually. Pyocyanin increased rapidly upon the entry into the *stationary phase* that suffers from low nutrients concentration. Pyocyanin production appeared to be regulated by the energy status of the cell which would be lowered under conditions of low nutrient concentration, resulting in a decrease in growth rate and an increase in the level of pyocyanin production [25]. This was shown here where pyocyanin concentration reached its highest value of $614 \mu\text{g.ml}^{-1}$ during the *death phase* at 81 h post-inoculation with an inhibitory activity of pyocyanin reached its highest value of 2.4 cm as a perpendicular radial inhibitory distance at 84 h and a biomass of 1.34 g.l^{-1} .

Fed-batch fermentation strategy is known to increase the overall biomass and give high concentration of products as compared to batch fermentation through minimizing substrate inhibition and accumulation of inhibitory by-products such as acetic acid and propionic acid which is the most harmful by-product for *B. subtilis* [26]. Accumulation of acetic acid is known to be a function of many factors including anaerobic condition, high partial pressure of carbon dioxide, high glucose concentration and/or high specific growth rate [14].

Although high cell density culture is an attractive means of achieving high concentration of final product(s), researchers cannot obtain high cell density (greater than 10 g.l^{-1}) with batch culture alone, because cells suffer from substrate inhibition and by-products inhibition. Catabolic acid by-products would accumulate in the fermentation broth during batch culturing causing inhibition of cell growth and production of the target compound(s) [27].

Constant fed-batch cultivation in contrast to batch cultivation gave a higher cell mass, lower pyocyanin concentration and lower inhibitory activity of pyocyanin. Cell mass, pyocyanin concentration and inhibitory activity of pyocyanin in constant fed-batch cultivation were 3.2 g.l^{-1} , $413 \mu\text{g.ml}^{-1}$ and 1.9 cm, respectively. In the batch phase, rapid increase in the level of pyocyanin production but through the feeding phase, slow increase in pyocyanin concentration was reported. When fed-batch phase was ended, pyocyanin concentration increased gradually but did not reach the highest value achieved in batch cultivation. Repression of pyocyanin may be attributed to high nutrient concentration in the feeding stream, when nutrients are readily available, the energy generating capacity of the cell increases resulting in an increase in growth rate and repression of pyocyanin [25]. After 5.5 h post-inoculation dissolved oxygen increased gradually until the start of the feeding medium, where dissolved oxygen decreased gradually but in the end of fed-batch phase at 48 h, dissolved oxygen increased again. In the light of these results, one is tempted to suggest that an increase in the level of

pyocyanin is likely to be associated with an increase in the dissolved oxygen value but repression of pyocyanin is linked with a decrease in the level of the dissolved oxygen.

Pulse feeding strategy was performed to avoid the repression of pyocyanin production after starting of feeding in constant fed-batch cultivation. Pulse feeding was performed in five pulses before production of pyocyanin, one pulse at a time in response to the decrease in dissolved oxygen to 30%. Pulse feeding strategy improves an overall biomass and productivity of pyocyanin. The biomass achieved was 3.6 g.l⁻¹ and pyocyanin concentration reached its highest value of 676 µg.ml⁻¹ during the *death phase* at 77 h post-inoculation. Also, inhibitory activity of pyocyanin reached its highest value as indicated by a perpendicular radial inhibitory distance of 2.6 cm during the *death phase*, 84 h post-inoculation.

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