International Journal of Biological, Life and Agricultural Sciences
ISSN: 2415-6612
Vol:4, No:5, 2010

Process Optimization for Enhanced Production of Cell Biomass and Metabolites of Fluorescent Pseudomonad R81

M.V.R.K Sarma, Krishna Saharan, Lalit Kumar, Ashwani Gautam, Avhijeet Kapoor, Nishant Srivastava, Vikram Sahai and V.S Bisaria*

***Abstract*—**The fluorescent pseudomonad strain R81 is a root colonizing rhizobacteria which promotes the growth of many plants by various mechanisms. Its broth containing siderophore (iron-chelating compound) and 2,4- diacetyl phloroglucinol (DAPG) is used for preparing bioinoculant formulations for agronomical applications. Glycerol was found to be the best carbon source for improved biomass production. Splitting of nitrogen source to NH_4Cl and urea had a stabilizing effect on pH during batch cultivation. L-tryptophan at 0.5 % in the medium increased the siderophore production to 850 mg/l. During batch cultivation of the strain in a bioreactor, a maximum of 4 g/l of dry cell mass, 1.8 g/l of siderophore and 20 mg/l of DAPG was achieved when glycerol was 15 g/l and C/N ratio was maintained at 12.5. In case of intermittent feeding of fresh medium during fed-batch cultivation, the dry cell mass was increased to 25 g/l with improved production of DAPG to 70 mg/l.

***Keywords*—**Batch cultivation, Fed-batch cultivation, fluorescent pseudomonad, Metabolites

I. Introduction

FLUORESCENT pseudomonads are a group of plant growth promoting rhizobacteria (PGPR) which rigorously colonize roots and provide beneficial effects to plant development. The PGPR have been known to directly enhance plant growth by a variety of mechanisms, namely, fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus and synthesis of phytohormones ([1], [2]). PGPR can also indirectly enhance plant growth via suppression of phytopathogens by a variety of mechanisms. One of the mechanisms includes the ability to produce siderophores that chelate iron, making it unavailable to pathogens [3]. Other desirable features for a potent organism are that it should have the ability to synthesize anti-fungal metabolites, such as the antibiotic 2, 4- diacetyl phloroglucinol (DAPG), hydrogen cyanide, and cell-wall lysing enzymes, which suppress the growth of fungal pathogens [4]. An extensive work on the possible role of siderophores in biocontrol of plant diseases has been documented ([5], [6]).

Authors are with the Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, 110016. India (* Corresponding author: Tel. +91 11 2659 1002 Fax: +91 11 2658 2282; e-mail: vbisaria@dbeb.iitd.ac.in,vsbisaria@hotmail.com).

Fluorescent pseudomonad strain R81 is a root-colonizing rhizobacterium which could be potentially used in bio-inoculant formulations due to its plant growth promoting characteristics [7]. Schlegel's synthetic medium has been widely used for cultivating this strain. The objective of the study was to develop a process which produces improved biomass, siderophore and DAPG in submerged fermentation. The studies were directed towards modification of Schlegel's synthetic medium to obviate the need of pH control in batch cultures along with improved siderophore production. A fed-batch technique was also developed to produce high cell density with improved production of siderophore and DAPG simultaneously. The culture broth so obtained could be used for bio-inoculant preparations for agronomical applications.

II. Materials and Methods

Bacterial strains and growth conditions: Fluorescent pseudomonad strain R81 used in the present investigation, was obtained from Dr. A K Sharma of Department of Biological Sciences, GB Pant University of Agriculture and Technology (GBPUAT), Pantnagar, India. It was isolated from the rhizosphere of wheat (variety UP 2338) from Budaun District, Uttar Pradesh, India ([5], [7]). The bacterial culture was maintained as 50% glycerol stocks at –20ºC in King's B medium. All chemicals (extra pure grade) used in this study were obtained from Merck (Mumbai, India).

Growth medium: The Schlegel's medium (SM) was prepared as described by Aragno and Schlegel (1991) [8], omitting solution III (5% $NaHCO_3$). In order to avoid precipitate formation, the three solutions were prepared and sterilized separately by autoclaving and mixed after cooling. Solution I: Na_2HPO_4 (9.0 g/l), KH_2PO_4 (1.5 g/l), NH_4Cl (1.0 g/l), KCl (1.0 g/l), $MgSO_4.7H_2O$ (0.2 g/l) and trace elements solution (1 ml/l). Solution II: Ammonium ferric citrate (50 mg), $CaCl_2.2H_2O$ (100 mg), distilled water 250 ml. Solution III: Glycerol/ Glucose/ Citric acid (10 g), distilled water 100 ml. After cooling 900 ml of solution I, 10 ml of solution II and 90 ml of solution III were mixed to prepare one litre of Schlegel's medium.

Preculture preparation: Pre-cultures were grown overnight from −20 °C glycerol stocks on Schlegel's medium (SM) in baffleless 500 ml Erlenmeyer flasks with 100 ml of working volume. The cultivation was carried out at 28 °C for 20 h in an orbital shaker (Scigenics Biotech, India) at 240 rpm.

Subsequently, 50 ml of this culture was used to inoculate a 5 l bioreactor with 2 l of batch working volume.

Batch and fed-batch cultivation in bioreactor: 5 l bioreactor (Minifors AG, Switzerland) was used for both batch and fed-batch cultivations. Batch cultivations were carried out at 2 l capacity and fed-batch cultures were started from an initial volume of 2 l. The medium composition for batch (Table 1) and fed-batch cultivation varied according to the requirement of the metabolites. For batch cultivation alone, the iron in the form of ammonium ferric citrate (AFC) was used at a level of 100 µg/l to produce high siderophore levels. In case of fed-batch, the initial medium for batch cultivation was amended with 800 µg/l of AFC, 0.058 g/l of $ZnSO_4$ $7H_2O$ and 0.124 g/l of $(NH_4)_6$ Mo_7O_{24} $4H_2O$ for improved production of biomass and DAPG. The feed composition used was glycerol 800 g/l, NH_4Cl 40.4 g/l, urea 30 g/l, and $MgSO_4$ $7H_2O$ 48.4 g/l. During fed-batch phase, 25-35 ml pulses of feed were intermittently added into the bioreactor. The initial air supply and agitation speed were kept at 0.1 vvm and 550 rpm, respectively, to achieve turbulent fluid regime and uniform distribution of air bubbles in the reactor. The level of dissolved oxygen was maintained at 25% by using a double cascade in which the stirrer speed was first increased to a maximum of 700 rpm, followed by a gas mix-mode where the airflow was enriched with pure oxygen to maintain the required level of dissolved oxygen in the culture broth. Antifoam A (Sigma–Aldrich, A5633) was added to a concentration of 20 ppm at the onset of the foam formation and then manually added as required throughout the fermentation.

The measurement and control of the pH, temperature, dissolved oxygen, and agitation speed were performed by a digital measuring and control system of the bioreactor. The pH was maintained at 7.0 and the temperature was controlled at 28 °C. During cultivation the samples were taken at frequent intervals to measure cell density, residual glycerol and other parameters using the methods described below.

Estimation of dry cell weight and siderophore: The cell growth was estimated turbidimetrically using Helios Thermo Spectronic spectrophotometer (Thermo Electron Corporation, USA). The sample was diluted, if required, with 0.2% saline to get cell optical density (OD) in range of 0.1- 0.4 at 600 nm. Dry cell weight (g/l) was estimated by multiplying the OD by the conversion factor of 0.57, which was obtained from standard curves between dry cell weight and OD. The quantification of hydroxamate- type siderophore was done according to Meyer and Abdallah [9]. The siderophore concentration (g/l) was calculated using the expression $O.D_{400nm}$ x MW /ε. The values of extinction coefficient, ε = 16,500 M^{-1} cm^{-1} and of molecular weight, MW = 1500 Da were used.

Estimation of glycerol and glycerol kinase assay: Residual glycerol in the culture broth was estimated calorimetrically according to the method described by Bok and Demain [10]. Glycerol kinase activity was measured spectrophotometrically based on glycerol-dependent formation of NAD^+ at 340 nm in a coupled reaction using pyruvate kinase and lactate dehydrogenase by a method adapted from Hua [11]. The reaction mixture contained 48.4 mg of ATP, 42.7 mg of $MgCl_2$ $.6H_2O$, 4.33 mg of phosphoenolpyruvate, 2.98 mg of NADH, 80 U of crystalline pyruvate kinase, 80 U of crystalline lactate dehydrogenase, and 20 mM potassium phosphate (pH 7.5) to a final volume of 21 ml. To 1.5 ml of this reaction mixture 25 µl of 0.3 M glycerol and 50 µl of test solution were added. This was incubated at 25 ^oC for 3 min.

TABLE I MEDIUM COMPOSITION FOR CULTIVATION OF FLUORESCENT PSEUDOMONAD R81

Component	Batch
Na_2 HPO_4(g/l)	4.56
KH_2 PO_4 (g/l)	2.82
Glycerol (g/l)	15.0
Ammonium ferric citrate (AFC) (µg/l)	100
NH_4Cl (g/l)	0.76
Urea (g/l)	0.56
KCl (g/l)	0.50
TE-Solution (ml/l)	0.47
$MgSO_4.7H_2$ O (g/l)	0.91
L-tryptophan (g/l)	1.75

Estimation of DAPG: The pseudomonad strain R81 was cultivated in bioreactor, the harvested broth was centrifuged and the supernatant was collected. The pH of the supernatant was adjusted to 2.0 by adding 4N HCl and the samples were extracted twice with equal volumes of ethyl acetate. The extracts, which contained DAPG, was evaporated to dryness *in vacuo*. The extracted crude antibiotic was dissolved in methanol and stored in -20 ^oC for further purification and analysis. Purification was performed by column chromatography on a silica gel C- 200 (22 mm x 200 mm) (Himedia, India). The crude extract dissolved in methanol was re-dissolved in 20 mL of ethyl acetate and applied to the column. After washing with 50 mL of ethyl acetate, the column was eluted with 300 mL of toluene - acetone (4:1 v v^{-1}). Five milliliter fractions were collected and examined for the presence of the antibiotic by thin-layer chromatography in UV detection chamber against the standard antibiotic. Camag HPTLC instrument (Anchrom, Mumbai) was used for quantitative analysis of the antibiotic. The concentration of the antibiotic in the sample was estimated by using a standard curve between the peak area and the concentration (10-80 mg L^{-1}) of the standard antibiotic (Toronto Research Chemicals Inc, Canada).

III. Results and Discussion

Effect of media components and additives on growth of and siderophore production by pseudomonad R81: Succinate, citrate, glucose and glycerol were found to be good substrates from the BIOLOG GN2 Micro Plate™ experiments (data not shown). The values of more than 160% of the control were considered as positive for the respective substrates. To find the best source of carbon that leads to minimal digression of pH during fermentation, the strain R81 was grown on different carbon sources, namely, glucose, glycerol, citrate and succinate at 1% level (Table 2). Here, all the medium components were retained at the same level as in Schlegel's medium except for the carbon source. The maximum dry cell weight of 2.7 g/l was obtained when glycerol was used as carbon source at the end of 36 h.

TABLE II EFFECT OF VARIOUS CARBON SOURCES ON GROWTH AND PH DURING CULTIVATION OF FLUORESCENT PSEUDOMONAD STRAIN R81

	Fluorescent pseudomonad R81	
C- source	DCW[a]	ΔpH[b]
Glycerol	2.7	-1.2
Glucose	2.1	-2.4
Citrate	1.6	+1.9
Succinate	1.9	+1.8

Each value is expressed as mean ± S.E (n =3).
[a] The dry cell weight, DCW (g l^{-1}) was measured at the end of 36 h of cultivation
[b] The values indicate the deviation from the initial pH of 6.9 measured at the end of 36 h of cultivation.

It was observed that the growth of R81on glycerol as a carbon source was slow with a prolonged lag phase of 5-6 h. Studies were, therefore, carried out to see if the addition of citrate or succinate, at supplemental level, could stimulate growth rate and minimize pH variations. As shown in Table 3, supplementation of the medium with succinate resulted in shorter lag period as well as higher specific growth rate for the strain in comparison to citrate supplementation. However, the specific growth rates in both the cases was higher than the control (without supplementation). These higher growth rates were substantiated by measurement of glycerol kinase (GK) activity, an enzyme required for initial metabolism on glycerol. The GK activity measured at the end of 24 h showed that the addition the succinic acid (SA) or citric acid (CA) in the medium at supplemental level increased the glycerol kinase activity by about 15 times in the strain when compared to control.

The responses on dry cell weight, siderophore production and pH on supplementation of with 0.05 % succinate and citrate are given in fig 1. The maximum level of siderophore produced in SA and CA supplemented medium were 421 mg/l and 80 mg/l respectively. Thus there was increased production of siderophore in SA supplemented medium.

TABLE III EFFECT OF SUCCINIC ACID (SA) AND CITRIC ACID (CA) SUPPLEMENTATION ON SPECIFIC GROWTH RATE AND LAG PERIOD FOR THE GROWTH, BIOMASS, AND SIDEROPHORE PRODUCTION OF FLUORESCENT PSEUDOMONAD STRAIN R81

Treatment	Specific growth rate (h^{-1})	Lag period (h)
Without SA/CA	0.28	6
With SA	0.35	3
With CA	0.34	4

Specific growth rate was calculated using logistic equation.
Each value is expressed as mean ± S.E (n =2).

The pH increased to 7.50 from initial pH 7.0 in case of CA supplementation, whereas the pH remained between 6.7-7.0 in case of SA supplementation. Thus SA was more suitable than CA as a supplement to the medium for siderophore production as well as to contain the pH variations during growth of pseudomonad R81. In SM the only nitrogen source is ammonium chloride, whose metabolism causes a decrease in pH with glycerol as the carbon source. Since the production of siderophore depended largely on the pH of the medium [12], it was very important to maintain the pH of the fermentation broth between 7.0-7.5, which is the optimum for siderophore production in fluorescent pseudomonad [13]. Therefore a second nitrogen source, which can cause an increase in pH of the culture medium on its metabolism, was used. The urea metabolism is known to increase the pH due to hydrolysis of urea into ammonium ion intracellularly. Various combinations of urea and ammonium chloride were, therefore, used keeping the nitrogen content constant at 0.245 N g/l.

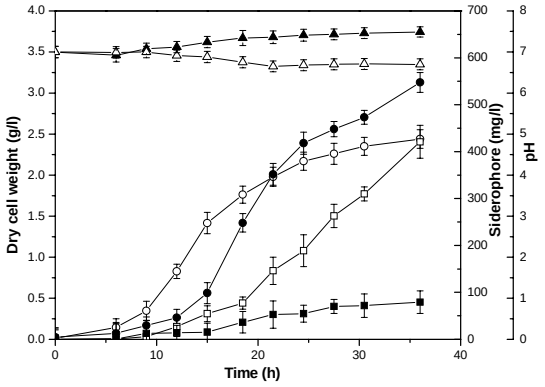

Fig. 1: Effect of supplementation of 0.05% succinic acid (SA) or citric acid (CA) in Schlegel's medium on growth and siderophore production by R81 strain; DCW (○), siderophore (□), pH (Δ) for SA; DCW (●), siderophore (■), pH (▲) for CA. The y-bars show the mean ± S.E (n=2).

When 34% of the nitrogen requirement were met by ammonium chloride (0.32 g/l) and the remaining from urea (0.35 g/l), it was observed that the pH remained fairly constant within ± 0.2 of the initial value. This medium is useful for cell mass production in shake flasks where pH control is not possible, and in fermenters without the need of pH control.

An increase in siderophore level is reported by amending the medium with aromatic amino acids as some of the amino acids are constituents of the siderophores [13]. The effect of supplementation of L-tryptophan at different levels in the SM was, therefore, studied on production of siderophore by R81 strain. It was observed that supplementation of the medium with L-tryptophan had a substantial positive effect on siderophore production, which increased by 3.27 fold (to 850 mg/l) on 0.5% supplementation. The modified medium has been termed as Modified Schlegel's Medium (MSM) and has been used in bioreactor studies.

Batch cultivation in 5l bioreactor: The cultivation of fluorescent pseudomonad R81 was carried in 5 l bioreactor containing 2 l of MSM as described in materials and methods. The batch culture was carried out with an objective to produce higher yield of siderophore. Increased iron levels usually increase the biomass yield and lower the siderophore production [9]. Therefore the medium used here contained very low iron, 100 µg/l of AFC as mentioned in materials and methods. Under these operating conditions, a specific growth rate of 0.3 h^{-1}, maximum dry cell weight of 4 g/l and siderophore of 1.5 g/l was achieved at the end of 36 h (Fig 2). The pH remained fairly stable till the end of harvest without requirement of external pH control.

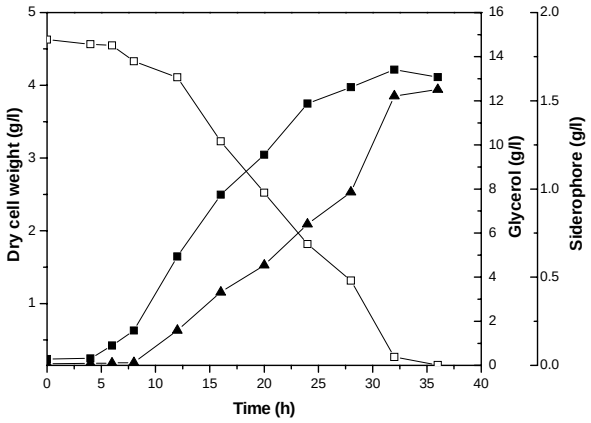

Fig. 2: Growth, siderophore production and glycerol consumption profiles of fluorescent pseudomonad R81 in 5 l bioreactor during cultivation in modified Schlegel's medium. Dry cell weight (■), Glycerol (□), Siderophore g/l (▲).

Fed-batch cultivation (Intermittent feeding):

High cell density culture is an attractive means of achieving high biomass and product concentrations. However, researchers could not obtain high cell density (greater than 10 g/l) with batch cultures alone, because the cells suffer from substrate inhibition and catabolite repression. It is well documented that catabolites acid by-products accumulate in the fermentation broth during the batch culture, and they will inhibit cell growth ([14], [15]). To achieve high cell density of pseudomonad R81, fed-batch cultivation with intermittent feeding strategy was adopted in the present study. The DO-signal was used for intermittent addition of feed into the bioreactor with a pulse. When glycerol was almost completely consumed at 35th hour of batch cultivation, pulse of feed (as described in materials and methods) was fed to the bioreactor. During cultivation, glycerol was fed in pulses whenever DO level reached 80 % of the saturation value. The DO- signal was essentially used as an indirect signal indicating the exhaustion of carbon source. A total of 5 pulses amounting to 70 g of glycerol was added to the bioreactor from 35 h to 85 h of cultivation. After 100 h of cultivation time, a maximum of 25 g/L of dry cell weight, 70 mg/l of DAPG was achieved (Fig 3). Siderophore production was, however, low as high concentration of AFC was used in the fed-batch cultivation. The objective of fed-batch process was to produce high cell density culture of fluorescent pseudomonad R81 with improved the production of DAPG.

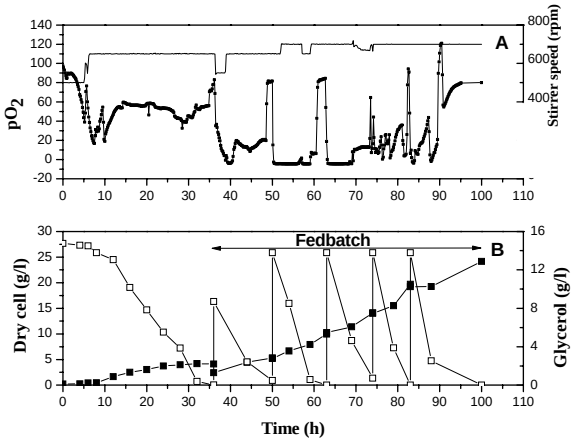

Fig. 3 (A) Dissolved oxygen (pO_2) and rpm, and (B) dry cell weight (■) and residual glycerol (□) profiles during fed-batch cultivation of pseudomonad R81 in MSM.

Here, using intermittent feeding, 6.25 fold increment was observed in dry cell weight and 3.5 fold increase in DAPG when compared to batch data (Table 4). However, siderophore production was compromised as higher cell densities require higher concentration of iron in the medium which lowered the siderophore production. For agronomical applications, the cells of pseudomonad R81 are primarily used. However, the presence of the siderophores and DAPG increases the effectiveness of the bioinoculant. Thus depending on the requirement of the siderophores and DAPG, the concentration of AFC in the medium could be varied so as to obtain optimum levels of cell, siderophores and DAPG in the bioinoculant formulations.

TABLE IV EFFECT OF MODE OF BIOREACTOR OPERATION ON SPECIFIC GROWTH RATE, DRY CELL WEIGHT, SIDEROPHORE AND DAPG PRODUCTION IN FLUORESCENT PSEUDOMONAD R81

Bioreactor mode	Specific growth rate, μ (h^{-1})	Dry cell weight (g/l)	Siderophore (mg/l)	DAPG (mg/l)
Batch	0.30	4.0	1500	20
Fedbatch (Pulse feeding)	0.24	25.0	170	70

IV. CONCLUSIONS

The Schlegel's medium was modified by splitting the nitrogen source to NH_4Cl and urea for stabilization of pH during cultivation of strain R81 in shake flask. The medium was supplemented with 0.05% of succinic acid and amended with 0.5 % of L-tryptophan for improved production biomass and siderophore. Batch and fed-batch processes have been established for improved production of biomass, siderophore and DAPG during cultivation of fluorescent pseudomonad R81. The process was established to produce these metabolites and biomass in a way to achieve desired levels of these metabolites based on the change in medium components and also the mode of operating the bioreactor.

ACKNOWLEDGMENT

This research project has been implemented with financial support of the Swiss Agency for Development and Cooperation, Government of Switzerland and the Department of Biotechnology, Government of India under the Indo-Swiss Collaboration in Biotechnology. The publication does not constitute the endorsement by the Governments of Switzerland and India.

REFERENCES

[1] A. Sharma, B.N. Johri, A.K. Sharma, B.R. Glick, "Plant growth-promoting bacterium pseudomonas sp. strain GRP3 influences iron acquisition in mung bean (Vigna radiata L. Wilzeck)," *Soil. Biol. Biochem*, vol. 35, pp 887-894, Mar. 2003.

[2] A.R. Podile, and G.K. Kishore, "Plant Growth Promoting Rhizobacteria," in *Plant-associated bacteria,* 1st ed. S.S. Gnanamanickam, Ed, Netherlands, Springer, 2006, pp 195–230.

[3] J. Leong, J, "Siderophores: their biochemistry and possible role in the biocontrol of plant pathogen," *Ann. Rev. Phytopathol,* vol. 24, pp 187-209, 1986.

[4] C. Voisard, C. Keel, D. Hass, and G. Defago, "Cyanide production by *Pseudomonas fluorescens* suppress helps black root rot of tobacco under gnotobiotic conditions," *EMBO J,* vol. 8, pp 351–358. 1989.

[5] R. Gaur, S. Noam, S. Kawaljeet, B.N. Johri, P. Rossi, and M. Aragno, "Diacetylphloroglucinol producing pseudomonads do not influence AM fungi in wheat rhizosphere," *Curr. Sci*, vol. 86**,** pp 453-457. Feb. 2004.

[6] D.J. O'Sullivan, and F. O'Gara, "Traits of fluorescent pseudomonas sp. involved in suppression of plant root pathogens," *Microbiol. Rev*, vol. 56, pp 662-676, Dec.1992.

[7] D. Roesti, R. Gaur, B.N. Johri, G. Imfeld, S. Sharma, K. Kwaljeet, and M. Aragno, "Plant growth stage, fertilizer management and bio-inoculation of arbuscular mycorrhizal fungi and plant growth promoting rhizobacteria affect the rhizobacterial community structure in rain-fed wheat fields," *Soil. Biol. Biochem,* vol. 38**,** pp 1111-1120, Jan. 2006.

[8] M. Aragno, and H.G. Schlegel, "The mesophilic hydrogen-oxidizing (Knallgas) bacteria," in *The Prokaryotes*, A. Balows, H.G. Trüper, M. Dworkin, W. Harder, and K.H. Schleifer KH, Eds. Berlin Heidelberg New York: Springer Verlag, 1991, pp. 344-384.

[9] J.M. Meyer, and M.A. Abdallah, "The florescent pigment of *pseudomonas fluorescens* biosynthesis, purification and physical-chemical properties," *J Gen Microbiol*, vol. 107**,** pp 319-328, April 1978.

[10] S.H. Bok, and A.L. Demain, "An improved colorimetric assay for polyols," *Anal. Biochem*, vol. 81, pp.18–20, March 1977.

[11] S.H. Hua, T. Yoshida, Y. Meng, T. Kabashima, K. Ito, Y. Nishiya, Y. Kawamura, and T. Yoshimoto, "Purification and characterization of thermostable glycerol kinase from *Thermus flavus*," *J. Ferment. Bioeng*, vol. 83**,** pp 328-332, Jan. 1997.

[12] D. Rachid, and B. Ahmed, "Effect of iron and growth inhibitors on siderophores production by *Pseudomonas fluorescens*" *African J. Biotechnol*, vol. 4**,** pp 697-702, July 2005.

[13] E. María, and Díaz de Villegas "Biotechnological production of siderophores," in *Microbial Siderophores,* Series: Soil Biology, Vol. 12, A. Varma, and S. Chincholkar, Eds, Berlin, Heidelberg: Springer Verlag, 2007, pp. 219-231.

[14] T. Yamane, and S. Shimizu, "Fed-batch techniques in microbial processes," *Adv. Biochem. Eng*, vol. 30, pp 147-194. 1984.

[15] L. Yee, and H.W. Blanch, "Recombinant trypsin production in high cell density fed-batch cultures in *Escherichia coli*," *Biotechnol. Bioeng,* vol. 41, pp 781-790, April 1993.