Journal of Agricultural Technology 2010 Vol.6(1):107-115
Available online http://www.ijat-rmutto.com
ISSN 1686-9141

Growth and pyoverdine production kinetics of *Pseudomonas aeruginosa* 7NSK2 in an experimental fermentor

Fallahzadeh, V.*, Ahmadzadeh, M. and Sharifi, R.

Department of plant protection, Faculty of agriculture, University of Tehran, Karaj, Iran.

Fallahzadeh, V., Ahmadzadeh, M. and Sharifi, R. (2010). Growth and pyoverdine production kinetics of *Pseudomonas aeruginosa* 7NSK2 in an experimental fermentor. Journal of Agricultural Technology 6(1): 107-115.

Growth and pyoverdine production kinetics of *Pseudomonas aeruginosa* 7NSK2 under uncontrolled pH condition were studied in an experimental fermentor. Lag phase was partially long in this condition. Production of pyoverdine began from middle exponential phase. Along the fermentation pH decreased and at 28 hour reached 4.56. After the 17 hour production of pyoverdine continued exponentially until 29 hour and then gradually decreased and final amount of it was 137.29 µM. Specific production rate of pyoverdine was 10.21 h^{-1}. The yield of the pyoverdine production was 11.44 µMs pyoverdine (g glucose)$^{-1}$. Growth rate in this fermentation was low and maximum specific growth rate (μ_{max}) was calculated 0.269 h^{-1} and final concentration of biomass at the end of the fermentation reached 4.63 g l^{-1}. Maximum specific consumption rate of glucose ($q_{glucose,\ max}$) was 0.048 h^{-1}. At the end of the fermentation residual glucose was 2.1 gr l^{-1}. Maximum specific consumption rate of ammonium was calculated 0.194 h^{-1}. Consumption of ammonium increased after 10 hour and continued exponentially until 30 hour that its concentration reached near 0 mg/lit.

Key words: *Pseudomonas aeruginosa* 7NSK2, kinetics, pyoverdine, fermentor

Introduction

Current agricultural practices emphasize on environmental sustainability by limiting the use of chemical fertilizer and pesticides. Over the last decades, many studies have reported on natural activity of some fungi and bacteria against pathogens, and this is considered as a very appealing alternative to the use of chemical fungicides (Gerhardson, 2002; Welbaum *et al*., 2004). The rhizosphere microorganisms have exceptional ability to promote the growth of host plant by various mechanisms such as the production of phytohormones (Gaudin *et al*., 1994), siderophores (Burd *et al*., 2000) and solubilization of phosphate (Ryan *et al*., 2008). Also these bacteria have various mechanisms to

*Corresponding author: Vahid fallahzadeh; e-mail: vfm128@gmail.com

suppress plant diseases including production of antibiotics, induction of systemic resistance, efficient root colonization and production of powerful siderophores (O'Sullivan and O'Gara, 1992; Hass and Defago, 2005). Various formulations of these bacteria have been developed in several investigations (Vidhyasekaran and Muthamilan, 1995; Moenne-Loccoz *et al*., 1999) and nowadays there are some efficient commercial products of these agents in the market (Wilson, 1997; Fravel, 2005). Furthermore, there were some reports in literature that purified siderophores exhibited disease suppressive effect similar to the producer strain (Kloepper *et al*., 1980; Neilands and Leong, 1986).

The applications of purified siderophores as bacteriostatic or fungistatic agents in combination with other antibacterial factors will certainly raise great interest in the near future (Raaska *et al.*, 1993). Also there are some reports that these metabolites can be used for removal of heavy metals (Neu *et al*., 2000) from environment or decreasing the toxicity of them to plants (Burd *et al*., 1998, 2000; Rajkumar *et al*., 2006).

There are some reports in literature, purified siderophores exhibit disease suppressive effect similar to the producer strain (Kloepper *et al*., 1980; Neilands and Leong, 1986). One of the best products that recently was developed is a biological product GLUTICID, which is an antifungal product constituted by antimicrobial metabolites such as siderophore pyoverdine and salicylic acid produced by *Pseudomonas aeruginosa* PSS. This product has been very effective against *Paeronospora tabacina* in tobacco culture, *Alternation solani* in tomato and *Pseudoperonospora cubensis* in cucumber. Pyoverdine, the main siderophore of fluorescent pseudomonads, is a yellow-green, very water-soluble molecule and a powerful chelator of ferric iron, which is bound with a stoichiometry of 1:1 (Cox and Adams, 1985; Wendenbaum *et al*., 1983) and a stability constant of approximately 10^{24} M^{-1} at neutral pH (Meyer and Abdallah, 1978; Wendenbaum *et al*., 1983) In spite of these advantages, there is little published data on the growth or siderophore production kinetics of these bacteria. Kinetic parameters are important to better design and operate a bioreactor (Gaden, 2000). In our studies, we used *Pseudomonas fluorescens* 7NSK2, with powerful ability of pyoverdine production. Kinetic data have not been previously published for this strain, Therefore, the objective of this study was to characterize the growth of *P. fluorescens* 7NSK2 to obtain the yield coefficients, the maximum specific growth rate (μ_{max}), the maximum specific consumption rates of glucose ($q_{glucose, max}$), ammonium ($q_{NH_4^+,max}$) and the specific production rates of pyoverdine ($q_{pyoverdin, max}$) in a fermentor. The yield coefficients for biomass and pyoverdine produced from glucose were determined.

Materials and methods

Stock culture

P. aeruginosa 7NSK2 kindly provided by M. hofte from Laboratory of Microbial Ecology, State University of Gent. Stock cultures were prepared for storage at -80°C in 1.5 vials by mixing equal volumes of 50 % glycerol and 24-h culture broth (from single colony inoculum, 25ml LB medium, 100ml flask, 130 rpm).

Inoculum preparation

Sample from the stored strain was used to prepare overnight culture in 25 ml of liquid King's B medium and incubated with agitation at 130 rpm and 30º C. Sample of 100 µl (OD_{600}= 0.1) of this medium was inoculated to 500 ml flask containing 50 ml medium like that used in fermentor After 6 hours incubation at mentioned conditions, content of this flask was transferred to fermentor.

Fermentation condition

A 7.5 liter bench-top experimental fermentor (BioFlo 110, New Brunswick Scientific) with a 2 l working volume was operated in batch culture mode. Temperature was controlled at 30°C and the initial pH was adjusted to 6.0. pH was monitored by pH probe (Metter Toledo 405-DPAS-SC-k85/325). Dissolved oxygen were monitored with polarographic DO electrode (Ingold Inpro 6800 series), The DO probe was calibrated at 0%, (obtained by briefly disconnecting the cable), and at 100%, (obtained using 800 rpm agitation and 5L/M [1 vvm] airflow). An agitation cascade was selected in the controller to maintain DO at 25-30% saturation through automatic adjustment of agitation speed. The fermentation medium was glucose simple medium containing: Glucose 12 gl^{-1} K_2HPO_4 6 gl^{-1}, KH_2PO_4 3 gl^{-1}, $MgSO_4.7H_2O$ 0.2 gl^{-1}, ammonium sulphate 1 gl^{-1}, $ZnSO_4$ 125 µM. After the fermentor contents except glucose and $ZnSO_4$, had been sterilized for 25 min at 121°C, a filter-sterilized glucose and $ZnSO_4$ stock solutions, comprising the remaining 12% of the medium volume, was added to the vessel, and the medium pH adjusted to 7 by addition of 2 N H_2SO_4. All glassware in this work acid washed with 0.1 N HCl solution for removal of residual minerals.

Analytical methods

The 5 ml samples of medium from fermentor gathered in intervals and used for estimation. The residual glucose and ammonium and pyoverdine production samples were centrifuged and supernatant used for colorimetric estimation. Glucose and pyoverdine adjustment of supernatant pH to 7 before estimation was used. Biomass concentrations were calculated by using calibration curves of biomass dry weight. The biomass from each sample was retained on 0.2 μm Millipore filters, washed with about 150 ml of distilled water, and then dried at 103°C for 4 h before weighting. Dry weight concentrations were linearly correlated with culture absorbance at 600 nm with correlation coefficient of $r^2 = 0.992$.

Concentration of pyoverdine was calculated using absorption maximum and the molar absorption coefficient (λ_{max}=400 nm and ε= 20000 $M^{-1}cm^{-1}$) according to method of Meyer and Abdallah (1978).

Glucose was determined by Dinitrosalicylic acid colorimetric method (Miller, 1959). The assay reagent used as DNS reagent was prepared by solving 10 g of dinitrosalicylic acid, 2 g phenol, 0.5 g sodium sulfite and 10 g sodium hydroxide in 1 liter water. 3 ml of DNS reagent was added to 3 ml of sample in a lightly capped test tube and heated in boiling water for about 10 min to develop the red-brown color. After cooling in a cold-water bath for 15 min, the absorbance was measured at 575 nm with a spectrophotometer ($T70^+$ UV/VIS, PG instruments). Using a calibration curve, the concentration of glucose was determined.

NH_4^+ was determined by the phenol-hypochlorite method. A 40-μl sample was mixed with 2.5 ml of reagent-1 (10 g of phenol and 50 mg of sodium nitroprusside in 1 l of distilled water) and 2.5 ml of reagent-2 (5 g of NaOH and 8.4 ml of sodium hypochlorite in 1 l of distilled water), and left for 30 min at room temperature. The reaction was slowed in a cold-water bath for 5 min, and the absorbance was measured at 630 nm within 30 min. The NH_4^+ concentration was determined from a calibration curve. Glucose and NH_4^+ assays were done in duplicate and the average reported.

Calculation of the kinetic parameters

The experimental estimation of the parameters was performed using the following equations:

Maximum specific-growth rate (μ_{max}) (h^{-1}) was calculated by linear regression from plots of lnX νs t:

$$\frac{dX}{dt} = \mu_{\max} X$$

A maximum specific consumption rate of glucose ($q_{glucose,\ max}$) was calculated by linear regression from plots of lnS vs t when S > S´:

$$\frac{dS}{dt} = -q_{glucose,\max} S$$

Maximum specific consumption rate of ammonium ($q_{NH_4^+,\max}$) was calculated by linear regression from plots of lnN vs t when N > N´:

$$\frac{dN}{dt} = -q_{NH_4^+,\max} N$$

Maximum specific production rate of pyoverdin ($q_{pyoverdin,\ max}$) was calculated by linear regression from plots of lnP vs t:

$$\frac{dP}{dt} = q_{pyoverdine,\max} P$$

Cell yield ($Y_{X/S}$) and ($Y_{X/N}$) was calculated by linear regression from plots of $X–X_0$ vs $S-S_0$ and $N-N_0$:

$$X - X_0 = -Y_{X/S}(S - S_0),\ X - X_0 = -Y_{X/N}(N - N_0)$$

Product yield ($Y_{P/S}$) and ($Y_{P/N}$) was calculated by linear regression from plots of $P - P_0$ vs $S - S_0$ and $N - N_0$

$$P - P_0 = -Y_{P/S}(S - S_0),\ P - P = -Y_{P/N}(N - N_0)$$

Where t is the time (h), P the pyoverdin production (µM/lit), X the biomass concentration (g /lit), S the residual glucose concentration (g /lit), and N is the residual ammonium (g /lit) concentration.

Results and discussion

Despite medium used as seed culture was taken from the vessel medium and pH and temperature of these two steps was similar, bacteria after inoculation to fermentor exhibited partly a long lag phase (Fig. 2). This phenomenon may be occurred as a result of difference in dissolved oxygen of flask and fermentor steps. After this phase, bacteria entered to exponential phase and growth continued steadily. In normal conditions secondary

metabolism starts during the deceleration and stationary phases but it is now obvious that the culture conditions may be manipulated to induce secondary metabolism during logarithmic growth, for example by selection of carbon source type or concentration of it or other factors as also reported by Stanbury *et al*. (1997). For this propose we used a medium with high concentration of phosphate, then the rate of the growth in this work was low but production of pyoverdine started that from middle exponential phase and green florescent color of this metabolite was obviously in showed fermentor but amount of it was not detectable before 17 hour. This high concentration of phosphate was important role for production of pyoverdine. Indeed concentration of phosphate is one of the most important factor for regulating the secondary metabolism in the microorganisms; for example, in bacteria there is a two-component PhoR-PhoP system that regulates the biosynthesis of antibiotics (Juan, 2004; Sola-Landa *et al*., 2003). In most cases high concentration of this factor had negative effect on secondary metabolism (Stanbury *et al*., 1997) but its effect on pyoverdine production is positive.

Other factors that regulate the production of pyoverdine were pH and temperature of medium. In previous work, the production of this metabolite increased when pH decreased, as in pH 5.6 was high (unpublished data). The best temperature was 30°C. and primary pH of medium were adjusted to 7. Along the fermentation, pH decreased to 4.56 at 28 hour. The growth was decreased at that time but production of pyoverdine continued (Fig. 2). Acidification of medium in this condition was as a result of glucose metabolism and removal of NH_4^+ by bacteria from NH_3SO_4 and released of SO_4^{2-} ion to the medium. The production of pyoverdine continued exponentially after 17 hour until 29 hour and then gradually decreased and final amount of it was 137.29 µM. Specific production rate of pyoverdine ($q_{pyoverdin,\ max}$) was 10.21 h^{-1}. Drastic decrease of pH at 29 h had negative effect on pyoverdine production and after this time production of this metabolite was reduced. The yield of the pyoverdine production respect to glucose consume was 11.44 µMs pyoverdine (g glucose)$^{-1}$.

One of the ingredients of the medium in this study was zinc. In most reports this mineral increased the production of the pyoverdine (Rossbach *et al.*, 2000). One reason for this phenomenon may be correlated to effect of it on iron uptake. Because in poor iron conditions production, pyoverdine was stimulated. Then, it included that the medium with zinc but not iron reached for low growth rate. As mentioned above, growth rate in this fermentation was low and maximum specific-growth rate (μ_{max}) was calculated 0.269 h^{-1} and final concentration of biomass at the end of the fermentation reached 4.63 g l^{-1}. Then

the yield of the biomass formation ($Y_{x/s}$) was 0.385 gram biomass (g glucose) $^{-1}$ and 4.63 gram biomass (g nitrogen)$^{-1}$.

Consumption rate of glucose in first 10 hours was too low but after this time the rate of consumption gradually increased. Maximum specific consumption rate of glucose ($q_{glucose, max}$) was 0.048 h^{-1}. This low $q_{glucose, max}$ was logical because of low rate of growth. At the end of the fermentation residual glucose was 2.1 gr l^{-1}, so it was not consumed completely. Despite at 29 hour growth was stopped but consumption of glucose was continued with low specific consumption rate (Fig. 2). After that time, glucose applied for other usages other than growth like production of various metabolites and providing energy for proton pumps for adjustment of cytoplasm pH as stated by James (2000).

The glucose and consumption of ammonium increased after 10 hour and continued exponentially until 30 hour that its concentration reached near 0 mg/lit (Fig. 2). Despite there was no ammonium in the medium after this time but production of pyoverdine was not stopped. Then bacterium used other compounds (maybe organic compounds that produced with bacterium) as nitrogen source for its activities. Maximum specific consumption rate of ammonium was calculated 0.194 h^{1-}.

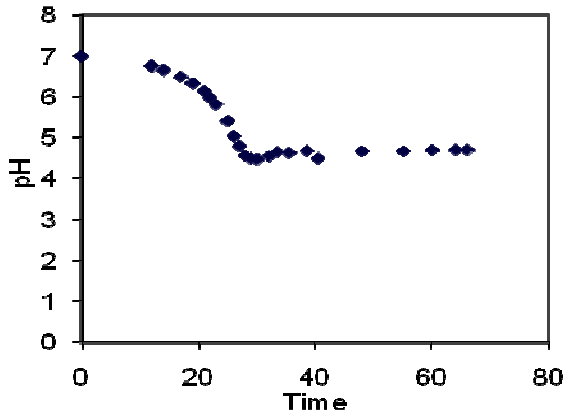

Fig. 1. pH changes of medium during growth of *P. aeruginosa* 7NSK2.

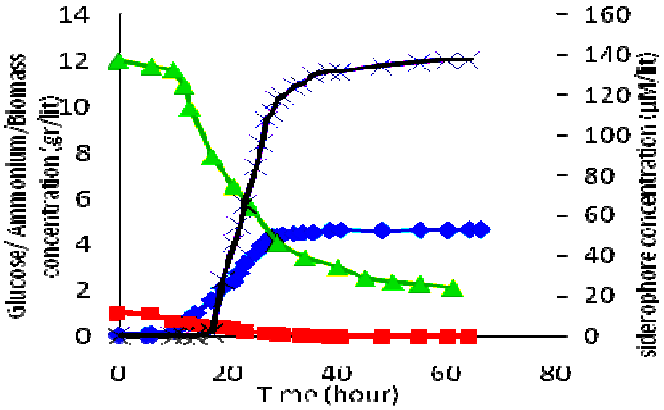

Fig. 2. Changes in the concentrations of cell dry mass (♦), pyoverdin production (×), glucose consumption (▲) and ammonium consumption (■) during the growth of *P. aeruginosa* 7NSK2 under constant temperature of 30°C and 25-30% of dissolved oxygen at the uncontrolled pH condition.

Acknowledgments

The authors thank J. Ramsay (Chemical Engineering Queen's University) for technical assistance and M. Hofte (Laboratory of Microbial Ecology, State University of Gent) for kindly providing 7NSK2. This work was partially supported by research Grant from the Biological Control Research Center which we gratefully acknowledged.

References

Burd, G.I., Dixon, D.G. and Glick, B.R. (1998). A plant growth promoting bacterium that decreases nickel toxicity in seedlings. Applied Environmental Microbiology 64: 3663-3668.

Burd, G.I., Dixon, D.G. and Glick, B.R. (2000). Plant growth promoting bacteria that decreases heavy metal toxicity in plants. Canadian Journal of Microbiology 46: 237-2.

Cox, C.D. and Adams, P. (1985). Siderophore activity of pyoverdine for *Pseudomonas aeruginosa*. Infection and Immunity 48: 130-138.

Fravel, D. 2005. Commercialization and implementation of biocontrol. Ann. Rev. Phytopath. 43: 337–359.

Gaden, E.L.J. (2000). Fermentation process kinetics. Biotechnology Bioenginiering 67: 413–419.

Gaudin, V., Vrian, T. and Jouanin, L. (1994). Bacterial genes modifying hormonal balances in plants. Plant Physiology Biochemistry 32: 11–29.

Gerhardson, B. (2002). Biological substitutes for pesticides. Trends in Biotechnology 20: 338–343.

Hass, D. and Defago, G. (2005). biological control of soil-borne pathogens by fluorescent pseudomonads. *Nature Reviews Microbiology.* doi:10.1038/nrmicro1129.

James, M. Jay. 2000. Modern food microbiology. Sixth Edition. An Aspen Publication.

Juan, F.M. (2004). Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. Journal of bacteriology 186: 5197-5201.

Kloepper, J.W., Leong, J., Teintze, M. and Schroth, M.N. (1980). Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286: 885-886.

Meyer, J.M. and Abdallah, M.A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. Journal of General Microbiology 107: 319-328.

Meyer, J.M., Mock, M. and Abdallah, M.A. (1979). Effect of iron on the protein composition of the outer membrane of fluorescent pseudomonads. FEMS Microbiology Letter 5: 395-398.

Miller, G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar, Analitical Chemistry 31: 426.

Moenne-Loccoz, Y., Naughton, M., Higgins P., Powell, J.,O`Connor, B. and O`Gara F. (1999). Effect of inoculums preparation and formulation on survival and biocontrol efficacy of *Pseudomonas fluorescens* F113. Journal of Applied Microbiology 86: 108-116.

Neilands, J.B., and Leong, S.A. (1986). Siderophores in relation to plant growth and disease. Annual Review of Plant Physiology 37: 187-208.

Neu P.M., Matonic H.J., Ruggiero E.C., and Scott L.B. (2000). Structural Characterization of a Plutonium(IV) Siderophore Complex: Single-Crystal Structure of Pu-Desferrioxamine E. Angewandte Chemie International Edition 39: 1442–1444.

O'Sullivan, D.J. and O'Gara, F., 1992. Traits of fluorescent Pseudomonas spp. involved in suppression of plant root pathogens. Microbiol. Rev. 56: 662–676.

Raaska, L., Viikari, L. and Mattila-Sandholm, T. (1993) Detection of siderophores in growing cultures of *Pseudomonas* spp. J Ind Microbiology 11: 181–186.

Rajkumar M., Nagendran R., Wang L. J.K., Zoo H.L. and Kim, S. (2006). Influence of plant growth promoting bacteria and Cr^{6+} on the growth of Indian mustard. Chemosphere 62: 741-748.

Rossbach, S., Wilson, T.L., Kukuk, M.L. and Carty, H.A. (2000). Elevated zinc induces siderophore biosynthesis genes and a zntA-like gene in *Pseudomonas fluorescens*. FEMS Microbiology Letters 191: 61-70.

Ryan, P.R., Germaine, K., Franks, A., Ryan, J.D. and Dowling, N.D. (2008). Bacterial endophytes: recent developments and applications. FEMS Microbiology Letter 278: 1-9.

Sola-Landa, A., Moura, R.S. and Martin, J.F. (2003). The two-component PhoR-PhoP system controls both primary metabolism and secondary metabolite biosynthesis in *Streptomyces lividans*. Proceedings of the National Academy of Sciences 100: 6133–6138.

Stanbury, P.F., Whitaker, A. and Hall, S.J. (1997). Principles of fermentation technology. Aditya Books, New Delhi, India.

Vidhyasekaran, P. and Muthamilan, M. (1995). Development of formulations of *Pseudomonas fluorescens* for control of chickpea wilt. Plant Disease 79: 782-786.

Welbaum, G., Sturz, A.V., Dong, Z. and Nowak, J. (2004). Fertilizing soil microorganisms to improve productivity of agroecosystems. Critical Reviews in Plant Sciences 23: 175–193.

Wendenbaum, S., Demange, P., Dell, A., Meyer, J.M. and Abdallah, M.A. (1983). The structure of pyoverdin Pa, the siderophore of *Pseudomonas aeruginosa*. Tetrahedron Letter 24: 4877-4880.

Wilson, M. (1997) Biocontrol of aerial plant diseases in agriculture and horticulture: current approaches and future prospects. J Ind Microbiol Biotechnol 19:188–191

.

(Received 22 April 2009; accepted 29 October 2009)