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Cell Growth and Metabolites Produced by Fluorescent Pseudomonad R62 in Modified Chemically Defined Medium

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***Abstract*—**Chemically defined Schlegel's medium was modified to improve production of cell growth and other metabolites that are produced by fluorescent pseudomonad R62 strain. The modified medium does not require pH control as pH changes are kept within ± 0.2 units of the initial pH 7.1 during fermentation. The siderophore production was optimized for the fluorescent pseudomonad strain in the modified medium containing 1% glycerol as a major carbon source supplemented with 0.05% succinic acid and 0.5% L-tryptophan. Indole-3 acetic acid (IAA) production was higher when L-tryptophan was used at 0.5%. The 2,4- diacetylphloroglucinol (DAPG) was higher with amended three trace elements in medium. The optimized medium produced 2.28 g/l of dry cell mass and 900 mg/l of siderophore at the end of 36 h cultivation, while the production levels of IAA and DAPG were 65 mg/l and 81 mg/l respectively at the end of 48 h cultivation.

***Keywords*—**Fluorescent pseudomonad, Fermentation, Metabolites production, PGPR.

I. INTRODUCTION

During document the last decade, there has been an increased interest in plant growth promoting rhizobacteria (PGPR), particularly pseudomonads, which are used as bioinoculants for plant protection against fungal and bacterial parasites as well as plant nutrition helpers [1],[2]. Fluorescent pseudomonads secrete siderophores (high affinity iron-chelating compounds), which allow them to take up highly insoluble Fe^{+3} [3]. Such bacterial siderophores are compatible with plant's iron uptake systems so that these bacteria improve plant iron nutrition and have been shown to inhibit root pathogens by depriving them of iron [4]. An extensive work on the possible role of siderophores in biocontrol of plant diseases has been documented [5], [6]. Fluorescent pseudomonad strain R62 is root-colonizing rhizobacteria and could be potentially used in bio-inoculant formulations due to their plant growth promoting characteristics [7].

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Although complex media like King's B [8] support their growth, they also enhance the risk of contaminants' proliferation during storage of the formulated product owing to the presence of unconsumed nutrients in them. Therefore, a chemically defined medium containing the cells and other metabolites are highly desirable. Therefore, the development of a chemically defined medium which does not allow significant variations in pH and stimulates cell growth and production of other metabolites are needed. The results of such a study are described here.

II. MATERIAL AND METHODS

A. Bacterial conditions

Fluorescent pseudomonad strain R62 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. This strain was isolated from the rhizosphere of wheat (variety UP 2338) from Budaun District, Uttar Pradesh, India [6],[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). The pseudomonad R62 was grown in baffle-less 500 ml Erlenmeyer shake flasks containing 100 ml Schlegel's medium (SM) [9].

B. Pre-selection of suitable carbon sources with Biolog metabolic profiles

Initial BIOLOG GN2 Micro Plate™ (Hayward, California) experiments were performed in order to identify the substrates preferred by the strain for their growth on 95 different carbon sources. The strain was cultivated overnight in 15 ml nutrient broth medium (Bio life™) in 50 ml flasks under stirring. After centrifugation of the cultures (6700 g for 5 min), the bacterial pellets were washed twice in 1.5 ml sterile 0.85% NaCl and then re-suspended in 1.5 ml of this solution. This suspension was then further diluted in 0.85% NaCl to an optical density (1 cm path, 600 nm) between 0.21 and 0.28. Each well of Biolog GN2 Micro Plate™ received 150 µl of the suspension. The MicroPlates were incubated at 30°C. The growth responses were read after 24 and 48 h of incubation with the Micro Plate reader (ASYS Hitech GmbH, Austria). The optical density of each suspension in the wells was measured at 600 nm. Different carbon sources that could be used by R62 was determined from these data.

C. Growth medium for cultures

The Schlegel's medium (SM) was prepared as described in [9], 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^{-1}). 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. Copyright Form.

D. 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 factors of 0.51, which were obtained from standard curves between dry cell weight and OD. The quantification of hydroxamate- type siderophore was done according to [10]. 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.

E. Glycerol kinase assay

Glycerol kinase activity was measured according to [2].

F. Estimation of Indole-3 Acetic acid

Bacterial culture supernatant (1 ml) was added to 2 ml Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ plus 50 ml of 35% (v/v) $HClO_4$) and the contents were incubated at 30°C for 30 min. The absorbance was read at 530 nm and the concentrations are quantified from standard curve of IAA (0–25 µg/ml).The colorimetric assay described in [11] was used to measure IAA production in vitro by bacteria.

G. Estimation of DAPG

The pseudomonad strain R62 was cultivated in shake flask, the harvested broth was centrifuged and the supernatant was pooled. The pH of the supernatant was adjusted to 2.0 by adding 4N HCl and the samples were extracted twice with equal volume 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). 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) of the standard antibiotic (Toronto Research Chemicals Inc, Canada).

III. RESULTS AND DISCUSSION

A. Screening of suitable carbon source

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 (having no carbon source) were considered as positive for the respective substrates. The preliminary analysis of the growth requirements of showed that this strain is prototrophic. Considering that growth rate is a function of pH, which means if pH digressions from initial value to a large extent the biomass levels will be significantly reduced. Therefore, need was felt to to find the best source of carbon that leads to minimal digression of pH during fermentation, the strain was grown on different carbon sources, namely, glucose, glycerol, citrate and succinate at 1% level. Here, all the medium components were retained at the same level as in Schlegel's medium except for the carbon source. The maximum growth OD of 4.4 was obtained when glycerol was used as carbon source for R62 at the end of 36 h. The pH deviations from the initial value were more in citric acid and glucose medium, and the least in glycerol medium. In the case of citric acid, pH increased from initial value of 6.9 to 9.01 at the end of 36 h (Table I). Similarly, when glucose was used as carbon source the pH decreased to 4.30-4.50 and in case of succinate, the pH increased to 8.7. Whenever the pH digressed to a large extent from the initial value, it had an adverse effect on cell growth. With glycerol, the pH digression was the least (1.2 units change from initial pH of 6.9) and the cell OD was the highest amongst all the carbon sources at the end of fermentation. Therefore, glycerol was chosen as the C-source for cell mass production of fluorescent pseudomonad strain R62.

TABLE I EFFECT OF VARIOUS CARBON SOURCES ON GROWTH AND PH DURING CULTIVATION OF FLUORESCENT PSEUDOMONAD STRAINS R62

C- source	[a]Growth OD at 600nm	ΔpH
Glycerol	4.4	-1.2
Glucose	2.8	-2.6
Citrate	1.4	+2.1
Succinate	1.6	+1.8

B. Effect of iron on cell growth and siderophore production

The SM contains 4000 µg/l ammonium ferric citrate (AFC) as the iron source. In addition, some iron comes as impurities (~130 µg/l basal level) present in SM components. Some researchers removed these traces of iron by complexing it with

8-hydroxyquinoline [10], [12]. Here in the current study deferration of the medium was not adopted as it is uneconomical for large scale preparations. The effect of added AFC over the basal level was studied on siderophores production by the pseudomonads. The added AFC exerted a negative effect on siderophore production, and thus an inverse relation between added Fe^{+3} and siderophore production was observed (Fig 1).

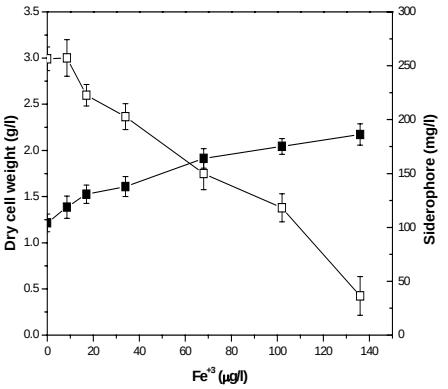


Fig. 1 Effect of concentration of iron (µg/l) Fe III (Ammonium ferric citrate), on dry cell mass and siderophore production after 24 h in shake flask. Dry cell weight (■), siderophore (mg/l) (□) for strains R62. The y-bars show the mean ± S.E (n=2).

C. Effect of splitting nitrogen source on pH stabilization

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 [8], 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 [1]. 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. When 34% of the nitrogen requirement was 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 and 1.3 times increment in siderophore production to 530 mg/l was obtained. The growth, however, remained the same in the two media. It was thus obvious that splitting of nitrogen source played a significant role in stabilizing the pH during fermentation and siderophore production (data not shown).

D. Effect of co-subtracts as supplements on growth production

It was observed that the growth of R62 on 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 rates for both the strain. Increasing the succinate concentration beyond 0.05% did not give any further reduction in lag time and also the increment in growth rate was insignificant. The glycerol kinase activity measured at the end of 24 h showed that the addition the succinic acid (SA) and citric acid (CA) in the medium at supplemental level increased the glycerol kinase activity by about 15 times for both the strain when compared to their respective controls. At 0.05% citrate supplementation in the medium, the pH increased to 7.50 at the end of 36 h for both the strain.

The second advantage of SA supplementation, in addition to enhancement of specific growth rate (table II), was that the downward trend in pH following inoculation was arrested due to concomitant rise in pH by consumption of succinate [10]. Therefore, the rest of the experiments were carried out in succinate-supplemented medium.

TABLE II EFFECT OF SUCCINIC ACID (SA) SUPPLEMENTATION ON SPECIFIC GROWTH RATE AND LAG PERIOD FOR THE GROWTH, BIOMASS,SIDEROPHORE AND DAPG PRODUCTION OF FLUORESCENT PSEUDOMONAD STRAIN R62

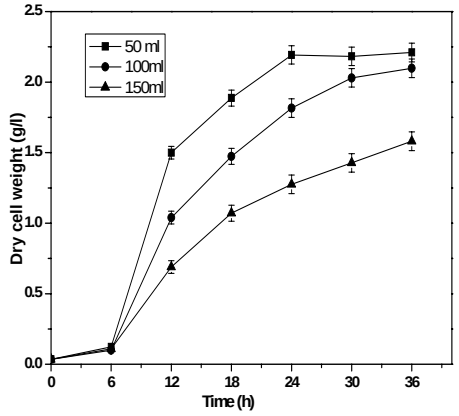
Treatment	Specific growth rate (h^{-1})	Lag period (h)	DCW (g/l)	Siderophore (mg/l)
Without SA	0.31	5	4	450
With SA	0.37	3	7	900

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

E. Effect of aeration on siderophore production

The effect of aeration on growth and siderophore production by fluorescent Pseudomonads strain R62 was tested by growing the culture in three 500ml flasks containing 50 ml, 100 ml and 150 ml of working inoculum media respectively. It was observed that the pH decreased drastically with decreasing aeration (data not shown). Similarly growth and siderophore production increased linearly with increasing aeration and were highest when 50 ml working media was used (Fig 2). These studies indicate that the cell growth and siderophore production are dependent on the DO level of the culture.

(a)



(b)

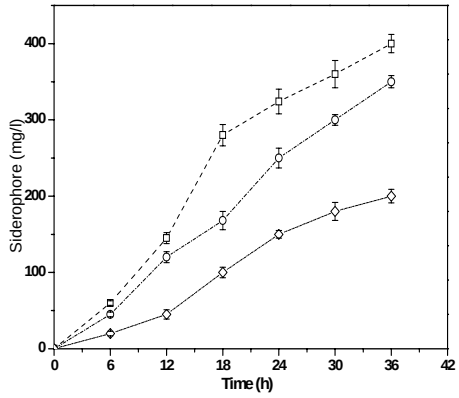


Fig. 2 Effect of aeration on (a) dry cell weight (dcw), and (b) Siderophore Concentration measured at different time intervals after inoculation in modified Schelgel's medium with 1% glycerol and 0.95 g/l ammonium chloride and 0.05% succinic acid.

F. Effect of L- tryptophan on IAA production

Fluorescent pseudomonad R62 was capable of producing IAA, but the amounts of IAA varied with various concentration of L-tryptophan and amendment of tryptophan with various time intervals in the production medium. Therefore various concentrations of L- tryptophan (range 0 – 0.5%) were selected. It was observed that supplementation of medium with L-tryptophan had a substantial positive effect on IAA production, which increased by 9.6 fold (IAA 65.1 mg/l) on 0.5% supplementation respectively. In the absence of tryptophan supplements, the strain R62 produced very low levels of IAA (Fig 3). The production of IAA started simultaneously with the culture growth and the L-tryptophan had no effect on growth during cultivation. The stationary phase of growth was reached after 30 h, but the production of IAA increased gradually up to 48 h, and then remained constant.

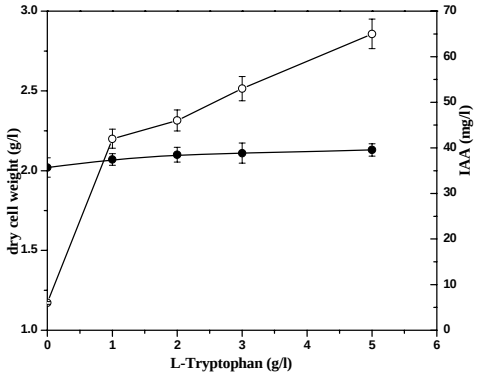


Fig. 3 Effect of L-tryptophan (concentration, g/l) on growth (dcw (g/l), (-●-) and IAA production (mg/l) (-○-) by the fluorescent pseudomonad R62 on production. Growth and IAA production were measured after 48 h of incubation in MSM medium.

G. Influence of L-tryptophan on siderophore production

It was observed that supplementation of medium with L-tryptophan had a substantial positive effect on siderophore production, which increased by 2.25 fold (to 900 mg/l) of R62 on 0.5% supplementation (table III). The supplementation of the medium beyond 0.5% L-tryptophan did not result in significant increase of the siderophores concentration. Thus the SM medium was selectively optimized for production of the siderophore and dry cell mass in shake flask with fairly constant pH and it was termed as modified Schlegel's medium (MSM).

TABLE III EFFECT OF L-TRYPTOPHAN SUPPLEMENTATION OF IN SCHLEGEL'S MEDIUM ON SIDEROPHORE PRODUCTION BY FLUORESCENT PSEUDOMONAD STRAINS R62

L-Tryptophan (%)	Siderophore (mg/l)	pH digression
0	401±40	0.0
0.1	682±60	+0.25±0.01
0.2	733±75	+0.30±0.02
0.3	857±86	+0.60±0.05
0.5	900±87	+0.75±0.04

All data (Siderophore and pH digression from initial value of 7.1) are taken after 36 h of fermentation. Each value is expressed as mean ± standard deviations of three experiments.

H. Effect of metal ions on DAPG production

On the basis of medium optimization, trace elements were selected for enhanced DAPG production. Therefore three emended trace elements Zn^{+2}, Mn^{+2} and MoO_4^{-2} were used at 83 µM, 42 µM and 135 µM respectively as extra with their basal concentration in the medium. DAPG is produce in late stationary phase, but the extra amounts of these three elements

were not inhibiting the growth. This experiment was performed in triplicate in shake flasks. The average concentration of DAPG in the broth was 81mg/l from triplicate experiments in shake flask. Fig 4 shows, dry cell production, DAPG production with emended three trace elements, and DAPG production in basal medium.However, this level of DAPG production, obtained using optimized trace elements amendment was 9 folds higher than the non-optimized synthetic Schlegel's medium on basal concentration of trace elements.

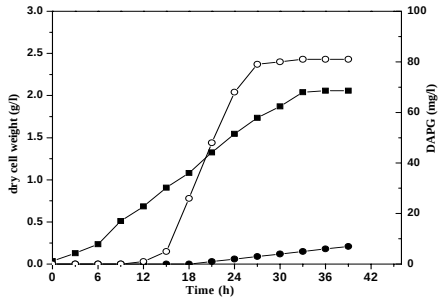


Fig. 4 Effect of basal trace concentration and and amended three trace elemnets concentration on DAPG production by the fluorescent pseudomonad R62 in MSM medium. Dry cell weight g/l (-■-), DAPG mg/l production in basal medium (-●-), DAPG mg/l production in amended trace metal ions (-○-).

IV. CONCLUSION

A synthetic medium is advantageous over the complex medium in many instances, for example in monitoring of a particular component of the medium during growth of a culture. The Schlegel's synthetic medium used in the present investigation, has been widely used for culturing pseudomonads. As the culture broth is used in preparing bioinoculants' formulations, which are later used in agronomical applications the growth of bacteria in a complex medium always gives room for contamination when the formulation is under storage. A synthetic medium with a suitable carbon source is always advantageous in such cases. The uptake of the medium components causes variations in pH during growth of the culture. In case of a simple synthetic medium where all components are well defined, the residual concentration profile of key ingredients along with pH profiling would give insight as to which component's influx is causing the pH aberrations. The pH digression beyond 7.5 is not desirable for the pseudomonads as siderophore production is highly dependent on pH of culture broth [10], and also extensive pH digressions cause estimation problems for siderophore [5].

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REFERENCES

[1] E. María and D.M.E. de Villegas, "Biotechnological production of siderophores," in *Microbial Siderophores*, vol.12, A. Varma, S. Chincholkar, Eds. Berlin, Heidelberg: Springer Verlag, 2007, pp. 219-231.

[2] 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.

[3] D.M.E de Villegas, P. Villa, and A. Frias, "Evaluation of the siderophores production by *Pseudomonas aeruginosa* PSS," *Rev. Latinoam. Microbiol*, vol. 44, pp.112–117, Sep. 2002.

[4] T. Leisinger, and L.Margrafet, "Secondary metabolites of the fluorescent pseudomonads," *Microbiol Rev*, vol. 43, pp. 422-442, Sep. 1979.

[5] 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.

[6] 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.

[7] D. Roesti, R. Gaur, B.N. Johri, G. Imfeld, S. Sharma, K. Kwaljeet, and M. Aragno, "Plant growth stage, fertilizer management and bioinoculation 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] 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.

[9] 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.

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

[11] S.A. Gordon and R.P. Weber, "Colorimetric estimation of indole-acetic acid," *Plant Physiol*, vol. 26, pp. 192-195, Jan. 1951.

[12] B. Schwyn and J.B. Neilands,"Universal Chemical Assay for the detection and determination of siderophores," *Anal Biochem*, vol. 140, pp. 47-56, Jan. 1987.