

**STUDIES ON GROWTH, METABOLITES SYNTHESIS  
AND AGRONOMICAL APPLICATIONS OF  
FLUORESCENT PSEUDOMONAD R81**

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AND AGRONOMICAL APPLICATIONS OF  
FLUORESCENT PSEUDOMONAD R81**

by

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*in fulfillment of requirement of the degree of*

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## **CERTIFICATE**

This is to certify that the thesis entitled “**Studies on Growth, Metabolites Synthesis and Agronomical Applications of Fluorescent Pseudomonad R81**” being submitted by **Mr. M.V.R.K Sarma** to the Indian Institute of Technology, Delhi for the award of the degree of ‘Doctor of Philosophy’, is a record of the bonafide research work carried out by him, which has been prepared under our supervision in conformity with the rules and regulations of the “Indian Institute of Technology, Delhi”. The research reports and the results presented in this thesis have not been submitted for any degree or diploma in any other University or Institute.

**Prof. V. S. Bisaria**

**Dr. Vikram Sahai**

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## Abstract

The strain R81 of a fluorescent pseudomonad, used in the current study, has certain plant growth promoting characteristics, which are of agronomic interest. It has been characterized for rigorous plant root colonization, phosphate solubilization, and for the production of siderophore (an iron-chelator), indole-3 acetic acid (IAA, a plant hormone), 1- aminocyclopropane-1-carboxylate deaminase (which regulates ethylene levels in plants), and 2,4-diacetylphloroglucinol (DAPG, an antifungal compound) making the strain a potential plant growth promoting rhizobacteria (PGPR) for agronomical purposes. The overall objective of the thesis work was to produce maximal amounts of cells, siderophore, and DAPG in the culture broth, so as to prepare effective bio-inoculant formulations for their applications in selected crops of *Triticum aestivum*, *Solanum lycopersicum* and *Vigna mungo*. In this study, a synthetic growth medium was developed and later optimized for the production of biomass and siderophore in shake flask batch cultivations as the first objective. Various nutritional parameters and culture conditions were investigated in this research. The nutritional requirements for simultaneous production of siderophore and biomass were optimized using genetic algorithm. As a second objective, the organism was cultivated for enhanced production of DAPG and improved biomass synthesis in fed-batch mode in a 5-L bioreactor. Various feeding strategies to achieve high cell density cultivation of pseudomonad R81 were investigated. As a final objective, the appropriate harvested broth of R81 was used for making carrier-based powder formulations and enumeration of its efficacy on host plants.

Amongst various carbon sources (glucose, glycerol, succinate and citrate) used for cultivation of pseudomonad R81, glycerol at 10 g/L resulted in maximum dry cell weight of 2.7 g/L with minimal pH deviations from the initial value during growth in shake flask. The supplementation of the medium with 0.5 g/L of succinate resulted in shorter lag period of 3 h in comparison to 6 h in un-supplemented medium. Succinate supplementation also improved the activity of glycerol kinase enzyme by 10-fold and thereby improved the specific growth rate to  $0.3 \text{ h}^{-1}$  in batch cultivation. The major medium component influencing the siderophore production was found to be iron in the form of ammonium ferric citrate (AFC). Splitting of total nitrogen source to  $\text{NH}_4\text{Cl}$  (0.32 g/L) and urea (0.35 g/L) had a stabilizing effect on pH during shake flask cultivation. At 2 g/L of L-tryptophan a maximum of 18.26 mg/L of IAA was synthesized by the strain R81 with minimal pH digressions during cultivation. It was observed that supplementation of the medium with L-tryptophan had a substantial positive effect on siderophore production, which increased by 1.55-fold (to 760 mg/l) on 0.2% supplementation.

The pseudomonad R81 culture broth containing  $1.5\text{-}2.0 \times 10^{10}$  cfu/mL along with siderophore and DAPG was used for the preparation of bio-inoculant formulations. Talcum powder (TP), aluminium silicate (AS), and vermiculite (VC) were used as carrier powders for preparing bio-inoculant formulations. The initial counts of bacteria were approx.  $2.5 \times 10^9$  cfu/g bio-inoculant formulation, and this formulation was used for coating the seeds for agronomical purposes and also for enumerating shelf life of the formulation. The presence of 10 mg DAPG and 54.6 mg siderophore per gram of non-sterile talcum powder was able to reduce the contaminants' initial

count of  $1.0 \times 10^6$  cfu/g to acceptable limit ( $<1.0 \times 10^4$ ) in 22 days when such carriers were stored at 28 °C.

A total of 10 medium components were optimized within 80 experiments for simultaneous production of siderophore and biomass using genetic algorithm (GA). The application of GA was well suited for determination of optimum concentration levels of the medium constituents for a bi-objective function. A high siderophore concentration of 1.9 g/L and cell mass concentration of 2.8 g/L was achieved in the optimized medium. GA was able to increase the siderophore concentration by 2.8-fold when compared to RSM-based optimization. Further, the batch fermentation of the GA-optimized medium in 14 L bioreactor without pH control produced 2.2 g/L siderophore in 36 h, the highest reported so far. GA was also successfully used to estimate the kinetic parameters of the mathematical models of the batch fermentation. The specific growth rate, yield and maintenance co-efficient were found to be 0.22  $\text{h}^{-1}$ , 0.30 g/g and 0.06 g/(g.h) respectively in 14 L batch cultivation.

Glycerol, as carbon source, and  $\text{NH}_4\text{Cl}$ , as nitrogen source were best suited for DAPG production. The effect of trace metal ions on production of DAPG was significant and ions such as  $\text{NH}_4\text{Mo}^{+2}$  and  $\text{Zn}^{+2}$  improved the DAPG production in shake flask cultivation. From batch studies in shake flask, a maximum of 4 g/L of dry cell weight and 20 mg/L of DAPG were obtained using R81. Maximum specific growth of 0.29  $\text{h}^{-1}$  was achieved when initial glycerol concentration was 15 g/L, which corresponded to C/N ratio of 11.7. An increase in glycerol concentration from 15 g/L to 36 g/L led to a drop in the value of the maximum specific growth rate from 0.29 to 0.22  $\text{h}^{-1}$ . Open-loop and automated feedback substrate feeding strategies were developed and

used to obtain high cell densities of pseudomonad R81 to enhance the production of antifungal compound DAPG in fed-batch cultivations. The conventional exponential feeding strategy resulted in increased glycerol accumulation at the end of cultivation when the predetermined specific growth rate ( $\mu$ ) was set at 0.10 or 0.20 h<sup>-1</sup> ( $< \mu_{\max} = 0.29 \text{ h}^{-1}$ ). In case of intermittent- manual feeding using DO as feedback signal, the fed-batch cultivation resulted in 0.25 g/(L.h) biomass productivity and 70 mg/L of DAPG. Automated feeding strategies using dissolved oxygen (DO) and pH as feedback signals resulted in minimal to zero accumulation of glycerol. In case of DO-based feeding strategy the average biomass productivity of 0.28 g/(L.h) was obtained. Using pH- based feeding strategy, the average biomass productivity was increased to a maximum of 0.68 g/(L.h) and the DAPG accumulated to 342 mg/L for R81 strain. The specific growth rate in pH- based feeding increased 3.65-fold compared to DO-based feeding.

The shelf-life of talcum powder (TP) and vermiculite (VC)- based formulations of R81 were analyzed using accelerated shelf life testing (ASLT) with TP and VC. Using ASLT- based model prediction, VC- based bio-inoculant formulations were found to have higher shelf-life of 350 days in comparison to 220 days when TP was used as carrier for bio-inoculants, when formulations were stored at 28 °C. It was also found that storage temperature upto 45 °C had lesser effect on viability of cells in VC-based formulations in comparison to TP. The real time survivability of R81 in TP-, VC- and aluminium silicate (AS)- based formulations were enumerated at 28 °C. The shelf-life of the strain in sterile powder- based formulations was found to be above  $1.0 \times 10^9$  cfu/ g carrier in TP- and VC- based bio-inoculant formulations and above  $1.0 \times 10^7$  cfu/g carrier in case of AS- based bio-inoculant formulations after a period of one

year. The pH of TP and VC- based formulations remained fairly constant at 7.5 and 7.2 respectively, during one year of storage, whereas in the AS- based formulations the pH increased to 8.5.

TP- and AS- based bio-inoculant formulations of strain R81 were used in field-experiments to enumerate its efficacy on *Triticum aestivum*. Both sterile and non-sterile carriers were used to prepare the bio-inoculants so as to test the effect of their sterility status on growth responses of *T. aestivum*. It was observed that both sterile and non-sterile carriers performed equally well, proving the earlier result that the broth containing appropriate levels of siderophore and DAPG would lower the contaminant counts in non-sterile carriers to acceptable levels, and retains the efficacy of the formulation. There was an increase of 1.15- and 1.20-fold in the grain yield of wheat when sterile and non-sterile TP- based R81 bio-inoculants were used respectively.

Glasshouse experiments in *Solanum lycopersicum* resulted in 4.5- and 3.4-fold increase in dry root weight, and 11.6- and 6.9-fold increase in dry shoot weight with TP- and VC- based formulation of R81, respectively. Based on these results, the TP-based bio-inoculant was adjudged as more compatible carrier for tomato seeds. Field trial experiments ascertained the glass house results with considerable amount of increase in plant growth responses. The R81 treatment performed consistently well in field conditions with an increase of 2.4-, 2.1- and 2.6-fold increase in dry root weight, shoot weight, and fruit yield, respectively. There was also significant increase in nitrogen (N), phosphorus (P) and potassium (K) content of the plants in the R81 treated *Solanum lycopersicum* plants. The fluorescent pseudomonad R81 also acted as

biocontrol agents, as its bio-inoculant formulation could significantly control the incidence of wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* in *Solanum lycopersicum* plants.

In case of *Vigna mungo*, under controlled glasshouse experiments, 2.4- and 4.1-fold increases in dry root weight, and 2.7- and 3.3-fold increases in dry shoot weight were observed with TP- and VC- based formulations of R81 in comparison to control treatment. Even in field experiments R81 treatment resulted in significant increase in growth responses of the plant. There were 1.6-, 1.6-, 1.7- and 3.8- fold increase in dry root weight, dry shoot weight, number of nodules and number of pods, in comparison to the control.

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