



## Research Article

# GROWTH DYNAMICS OF *Pseudomonas putida* (NBAIL-RPF9) UNDER ABIOTIC STRESS CONDITIONS

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**Abstract-** Experiments were conducted to know the growth variations adopted by *Pseudomonas putida* (NBAIL-RPF 9) an abiotic stress tolerant plant growth promoter and biological control agent. Cell growth and survival was monitored under different time periods with two temperature regimes (25° and 45° C) and data on viable cell count was analysed. *P. putida* (NBAIL-RPF9) experienced a delayed lag phase when grown under heat stress as compared to its growth at normal temperature which was complemented by expression of higher whole cell protein concentration. Under heat stress increased protein content was noticed in lag phase. Whole cell protein analysis of *P. putida* (NBAIL-RPF 9) by SDS-PAGE analysis showed expression of proteins ranging between 40KDa to 60KDa which match with molecular weight of major heat stress related proteins like GroEL and DnaK. This supported the fact that various proteins were triggered under stress conditions to ensure its survival.

**Keywords-** *Pseudomonas putida*, heat stress, salt stress, growth curve, exponential phase, protein profile, HSPs, antagonism.

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## Introduction

Soils are exposed to varied environmental changes which impacts the growth of bacteria dwelling in them. The extreme changes challenge bacteria to adapt to these systems through alterations in their metabolic processes. *Pseudomonas* sp. are one among ubiquitous Gram negative soil bacteria that is well studied for its wide range of application in agriculture especially as a biological control agent and also as a plant growth promoter. Fluorescent pseudomonads are considered as the highly diversified group of plant rhizobacteria. Specific strains of fluorescent pseudomonads have been used as biological control agents against phytopathogens due to their ability to produce different pigments, hormones, enzymes, antibiotics and metabolites. These bacteria are known to produce an array of antibiotics such as phenazine, pyocyanin, pyoluteorin, phenazine-1-carboxamide (PCN) [12], phenazine-1-carboxylic acid [19], 2, 4-Diacetylphloroglucinol [3, 9] which has been involved in disease suppression of crop plants. The broad-spectrum antimicrobial activity of DAPG produced by *P. fluorescens* strains recently has drawn great attention in the agriculture due to its ability to suppress a wide range of plant pathogens [18, 5].

It is necessary for *Pseudomonas* sp. to adapt well to changes in growth conditions to perform its beneficial activity. They encompass a wide range of metabolic changes to counteract the detrimental effect of such changes to its normal growth conditions in case of temperature shift, osmolarity variation or chemical stress. The stress urges bacteria to produce stress related proteins which helps them to survive under harsh conditions. A mild shift in temperature hinders the protein folding and aggregation. The disorganization of protein structures are rectified by heat shock related proteins or HSPs which are a super family of evolutionary well conserved, ubiquitous proteins existing in a large variety of organisms [17]. These specific proteins have a significant role in the cell to control the stress response and protect organisms from destructive stress conditions [20]. *P. putida* (NBAIL-RPF9) was identified as an abiotic stress tolerant organism able to withstand 48°C and 1.5M saline. We wanted to find out the growth dynamics of this beneficial microbe under abiotic stress conditions as this information will throw a light on its

capability to survive under adverse conditions. Since soils in India affected by vagaries of temperature and salinity we conducted growth response studies under ambient, high temperature and salt stress conditions.

## Material and Methods

### Bacterial strain and growth conditions

The strains used in the study *Pseudomonas putida* (NBAIL-RPF 9) is an abiotic stress tolerant strain isolated from pigeon pea rhizosphere soil [1] [Table-1]. The bacterium was grown in Luria Bertani (LB) broth overnight at 37°C. Heat stress: The overnight culture was inoculated to 250 ml LB broth with an optical density of 0.5 (exponential phase cells) at 600 nm and incubated under constant shaking (150 rpm) at 28°C. A separate set was maintained at 45°C under constant shaking to study the growth under heat stress. Samples were taken in an hourly interval for two days after starting the experiment. Salt stress: The overnight culture was inoculated to two sets of 250 ml LB broth, one which had regular media composition (constituting 10g/L NaCl corresponding 0.25M NaCl) and other set with additional NaCl to achieve 1M in order to induce salt stress. Both the sets were maintained under constant shaking at 28°C till further studies.

### Growth response and measurements

The samples taken every hour was immediately used to measure optical density at 600 nm recorded using a spectrophotometer (UV VIS spectrophotometer, Shimadzu). During each sampling period, CFU was determined on LB media incubated under 28°C for 48 h.

### Protein analysis of cell contents

Bacterium subjected to both heat and salt stress was extracted for protein contents. Mid log phase cultures of *P. putida* (NBAIL-RPF 9) was inoculated into 250 ml of LB broth and maintained under 28°C under constant shaking at 150 rpm for 24 hours. In case of heat stress induction, 250 ml of LB broth inoculated with overnight culture was incubated at 45°C and salt stress was induced by amending

**Table-1** Attributes of *Pseudomonas putida* (NBAlI-RPF9)

Organism	Abiotic tolerance			Biochemical reaction	Antagonistic against
	Temp	Saline	Osmotic		
<i>Pseudomonas putida</i> (NBAlI-RPF9) – Acc. No. HM439967 Source – Karnataka 15.89° N, 75.26° E	48°C	1.5M NaCl	-103 Mpa	IAA, phosphatase, protease, ACC deaminase, HCN, chitinase, cellulase	<i>Sclerotium rolfisii</i> , <i>Rhizoctonia solani</i> , <i>Fusarium udum</i>

the media with 1M NaCl. The culture grown after 24 hr was centrifuged at 7000 rpm for 10 minutes. The supernatant was discarded and the pellet was suspended in five ml of sodium phosphate buffer (pH 7.0). The pellets were washed for five times with buffer using centrifugation at 7000 rpm for 10 minutes. The pellets were suspended in 1ml of lysis buffer (0.01M Tris HCl, pH 7.4, 1mM EDTA, 8M urea, 10% glycerol, 0.5% tritonX-100, 6% ampholytes, 0.05M DTT and 2mM PMSF) and sonicated in ice 10 times, 30 sec each with a gap of 1 min. The suspension obtained was vortexed well and incubated at -20°C for 1 hr. The crude cell lysate was centrifuged at 13000 rpm for 10 minutes. The soluble proteins in the supernatant were precipitated with two volumes of mixture of TCA: acetone (1:4) containing 20nm DTT at -20°C for 1 hr. The precipitate was washed 3 times with 2 ml of acetone-DTT and air dried. Protein estimation of the pellet resuspended in 1 ml of resuspension buffer [85 mM Tris (pH 7.4) and 0.2% SDS] was carried out using Lowry's method with bovine serum albumin as standard.

#### Whole cell protein profiles of *P. putida* (NBAlI-RPF9)

Electrophoresis was carried out using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 % (w/v) separating gel and 5 % (w/v) stacking gel [13]. The molecular weight of the dissociated proteins was estimated using 20µl of protein marker with molecular weight ranging 3000Da-20500Da (Merck ready to use protein molecular weight marker). 150 µg of cell protein was loaded in wells and electrophoresed (BioRad Mini protean II) in glycine buffer for 3 hours at 50V till bromophenol blue dye (BPB) reached the bottom of the plate. The gel slab was stained with 2% Coomassie brilliant blue R-250 in methanol, acetic acid and water mixture (5:1:5) for 4 hr at room temperature. The gel was further de-stained with same solvent without dye. The gel was observed for protein fractions in gel documentation system (INR Gel documentation system, Israel). The molecular weight of different protein bands separated in expressed by *P. putida* (NBAlI-RPF9) under heat stress when compared to normal growth was analyzed using protein markers.

#### Antagonistic activity of *P. putida* (NBAlI-RPF9) against *Sclerotium rolfisii*

The antagonism of NBAlI RPF 9 strain was determined using dual culture assay. Major wilt pathogen, *Sclerotium rolfisii* was used for the study. Freshly grown overnight cultures of the test bacterium was grown in Kings' B agar medium. Five day old cultures of fungal pathogens grown on potato dextrose agar (PDA) were used for the study. NBAlI RPF 9 was streaked length wise on one corner of plate and 5 mm diameter of circular plug from an actively growing fungal culture was placed on the surface of fresh PDA medium. The inhibition of the fungal growth was determined after 5 days incubation in room temperature for all the fungal pathogens. Fungal pathogens inoculated without bacterial streak was considered as control. Inhibition was expressed as percentage of inhibition growth of the fungi caused by strain. The radial fungal growth in the direction of the antagonist in both the control and the plates with bacteria was measured. The percentage of inhibition of pathogens was measured with the formula, [Eq-1]

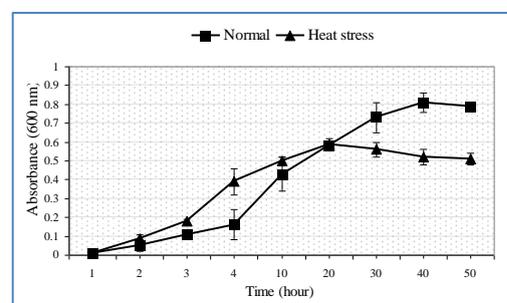
$$\left[ \frac{\text{radial growth of pathogen in control plate} - \text{radial growth of pathogen in treatment}}{\text{radial growth of pathogen in control plate}} \right] \times 100 \text{ [16].}$$

## Results and Discussion

### Growth response of *Pseudomonas putida* (NBAlI-RPF9) under induced stress condition.

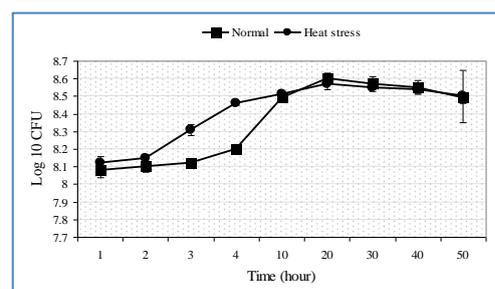
The present studies unraveled the fact that *P. putida* (NBAlI-RPF9) survived stress conditions with varied response to stress induced which was evident in their growth cycle studies. This indicates that bacteria adapt themselves to altering environment through lot of metabolic processes to help them cop under stress.

The initial studies revealed that *P. putida* (NBAlI-RPF9) was able to survive in high temperatures up to 45° C and salt stress up to 1M (NaCl induced) [1]. Experiments were conducted to understand the growth pattern adopted by the bacterium under both stress which was investigated with parameters like turbidity and cell number grown under heat and salt stress conditions. *Pseudomonas putida* (NBAlI-RPF9) grown at 28°C showed one hour more of lag phase compared to two hours of lag phase growth under 45°C. However, the exponential phase extended up to 40 hours when grown at 28°C as compared to short exponential phase of just 20 hours when cultured at 45°C [Fig-1]. This shows that bacteria are not killed whereas their growth was impaired under stress condition. This was evident through the graph plotted with values corresponding OD and CFU count. Highest OD was recorded as 0.81 recording 8.55 log growth of *P. putida* (NBAlI-RPF9) after 30 hours followed by slight decline in initial stationary phase when grown under 28 °C.

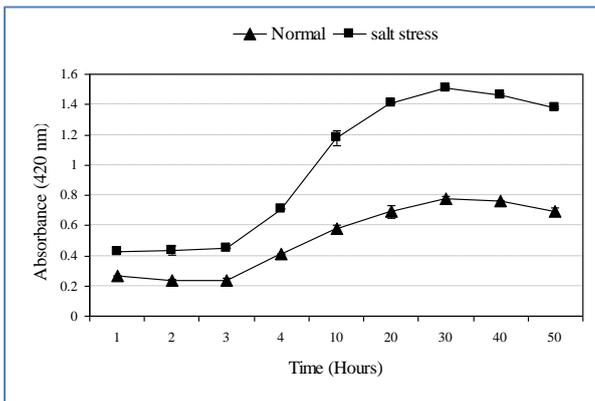


**Fig-1** Graph representing the growth pattern of *P. putida* (NBAlI-RPF 9) maintained under normal (28±2° C) and heat stress (45° C).

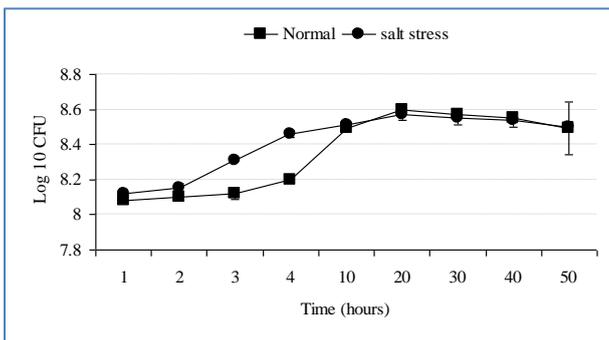
Despite the short exponential growth, cell number was higher under heat stress in comparison to its growth under ambient temperature [Fig-1 and 2]. There was rapid cell growth between 2 to 4 hr when under heat stress (log 8.1 to 8.5) but under normal temperature the growth was gradual (log 8.1 to 8.2). We observed that NaCl mediated salt stress did not impact the cell growth initially, however under salt stress the exponential growth of the bacterium was reduced to 20 hour unlike 30 hours under normal conditions [Fig-3]. The data was complemented by the cell count in both conditions which showed higher CFU of 8.78 after 20 hours which started gradually decreasing later on [Fig-4], whereas under normal conditions highest cell count was observed after 30 hours (8.7). [7] studied that exposure of exponentially growing *P. fluorescens* P7 cells to slight increase in temperature of 36°C for 2 hr from its optimal temperature resulted in death of 99% of cells. In a nutshell, the studies revealed that bacteria were not killed during stress but rather suffered damage and modulated their protein expression level to survive under any such changes in their growth conditions.



**Fig-2** Graph representing the cell count (log numbers/mL) of *Pseudomonas putida* (NBAlI-RPF 9) maintained under normal (28±2° C) and heat stress (45° C).



**Fig-3 Graph representing the growth pattern of *P. putida* (NBAlI-RPF 9) maintained under regular LB media and salt stress induced (1M NaCl).**

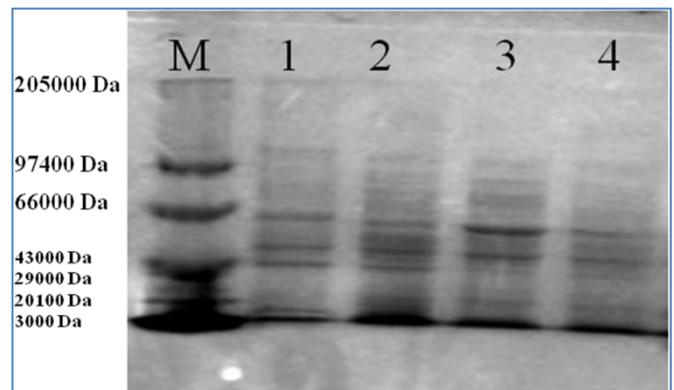


**Fig-4 Graph representing the cell count (log numbers/mL) of *Pseudomonas putida* (NBAlI-RPF 9) maintained under regular LB media and salt stress induced (1M NaCl).**

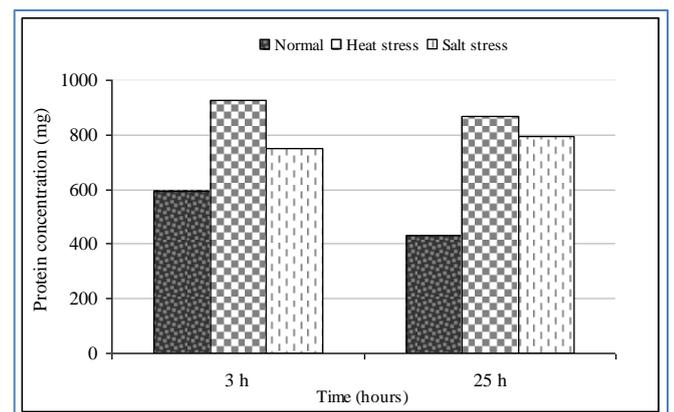
**Accumulation of proteins in stressed cells of *P. putida* (NBAlI- RPF9)**

Based on the initial results proteins were collected from lag phase and exponential phase of *P. putida* (NBAlI- RPF9) to understand the protein profile under normal and stressed condition. Increased accumulation of proteins was noticed in both stressed condition when compared to its normal growth conditions. The protein estimation revealed that cells produced higher concentration of proteins recording 928 mg, 750 mg as compared to 592.1 mg in lag phase of heat stressed, salt stressed and cells grown in ambient condition respectively. Similarly even in mid exponential phase of *P. putida* grown at all the three growth conditions showed that concentration of proteins accumulated in stress conditions were higher than compared to normal condition [Fig-5]. Bacteria accumulated stress related proteins, chaperones which mediated their survival under stressful conditions. The increased concentration of protein indicates preparation of the bacterial cells to survive under harsh growth conditions. These proteins constitute a cocktail of deleterious or the ones which enable the bacteria to thrive under stress. It is evident that stress induces varied reaction in cell, some of which will break down the cell metabolism and its normal growth pattern which can be related through the growth cycle graph. *Pseudomonas* sp. maintained cell homeostasis by accumulation of compatible solutes viz., trehalose, glutamate, mannitol, K<sup>+</sup> ions and proline [10, 6]. The protein estimation revealed that cell accumulated almost two fold protein under both heat and salt stress condition when compared to their normal growth conditions [Fig-5]. The data clearly shows that though the initial concentration was same to begin with the count reduced under stress during the course of growth cycle. On contrary, protein concentration of stressed cells was almost two fold compared to cells grown under normal conditions. This could be due to accumulation of stress related proteins released to counter the damage to cell under stress. *P. mendocina* accumulated osmoprotectants glycine betaine when cells were grown under high osmolarity [15]. Higher concentration of proteins was expressed under exponential growth which exhibits the survival mechanism of bacteria adopted in growth stages. *Pseudomonas* sp. DJ-12 cells subjected to mild treatments of stress such as exposure to biphenyl, ethanol and heat showed production of several stress shock proteins including DnaK and

GroEL in adapted cells [14]. Similar mechanism may be adapted by the isolates tested in our experiment. The SDS PAGE analysis of the proteins extracted showed expression of proteins ranging from 3 KDa to 205 KDa. Band intensity of approximately 97.4 KDa, 66 KDa, 43 KDa and 3000 Da was observed in both normal and stressed conditions of growth [Fig-6]. Increased expression of proteins localized slightly below 66KDa was noticed at mid exponential phase of heat treated cells. Bands of approximately 43 KDa was high in lag phase at both 28 °C and 45 °C whereas not observed in exponential phase. *Burkholderia* sp. resisted cytotoxicity of 2,4 D by induction of 43 KDa DnaK and 41 KDa GroEL protein which was characterized by SDS PAGE and 2D PAGE [4]. The band intensity of approximately 66 KDa was higher in mid exponential phase of heat stressed cells. A strong expression of heat shock proteins of molecular weight 45-66 KDa was observed in membrane fractions of heat subjected *E. coli* cells when deduced by western blotting of SDS PAGE protein fractions [21]. Experimental studies carried out by [8] explained the molecular chaperone, ClpB played integral role in cell survival. The studies showed that null mutant gene for ClpB in *P. putida* showed higher protein aggregation and temperature sensitiveness. The faint protein bands were found in between 66 KDa and 97.4 KDa which could be of protein essential for cell growth.



**Fig-5 Protein profile pattern of *P. putida* (NBAlI-RPF9) obtained from SDS PAGE subjected to growth under two different temperature conditions (28 °C and 45 °C). M – Protein marker (3 kDa to 205 kDa), Lane 1 – lag phase heat stressed culture, Lane 2 – lag phase normally grown culture, Lane 3 – Mid exponential phase heat stressed culture, Lane 4 – Mid exponential phase normally grown culture.**

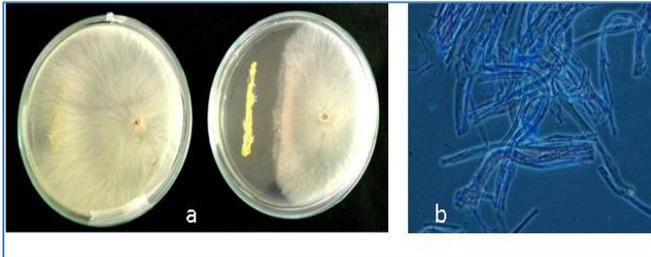


**Fig-6 Difference between whole cell protein content of lag phase culture (3 h) and exponential phase cultures (25 h) of *P. putida* (NBAlI RPF9) during normal, heat stressed and salt stress conditions.**

**Antagonistic activity of *P. putida* (NBAlI RPF 9) against *Sclerotium rolfisii***

It was very well evident that *P. putida* (NBAlI RPF 9) inhibited the growth of wilt pathogen, *S. rolfisii* under dual culture assay. The strain exhibited 65% inhibition of pathogen [Fig-7a] Fungal growth suppression could be due to production of antimicrobial metabolites like DAPG and PLT [22, 1]. The microscopic analysis of fungal mycelium from region of inhibition showed disintegration of mycelium,

hyphal distortion due to the interaction with bacterial metabolites [Fig-7] [16]. The *Pseudomonas* strains have been proved to be effective when used directly or even when their cell free filtrates are effective in inhibition of major pathogens of agricultural crops. [11] exhibited disease mortality of groundnut plant, reduced cell wall degrading enzymes and reduced incidence of stem rot pathogen *S. rolfsii* even when cell free filtrates of *P. aeruginosa* GSE18 and GSE19 strains were used.



**Fig-7** Dual culture assay (a) showing inhibition of *Sclerotium rolfsii* growth in presence of *P. putida* (NBAIL-RPF9) in right side plate whereas complete growth observed in plate without bacterial streak, (b) phase contrast microscopic observation showing hyphal distortion in mycelium near inhibition zone.

### Conclusion

Currently, the biocontrol methods are concentrated in understanding how to leverage the efficacy of antagonistic strains. The study was helpful in elucidating stress tolerance mechanisms adopted by *P. putida* (NBAIL-RPF9). The experiments also provided a platform for future proteomic studies. Studying the microbial response to environmental cues will validate selection of suitable strain for improved results in plant growth.

### Application of research

The findings in the research were valuable in identifying a potential strain of PGPR having biocontrol activity. The isolates efficiency to survive and perform in abiotic stress conditions yielded in a prospect of developing a biocontrol strain suitable for applications in agricultural crops thereafter promoting microbial products.

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### Author contribution

All author equally contributed.

**Author statement:** All authors have read, reviewed, agreed and approved the final manuscript.

### Abbreviations:

NBAIL- National Bureau of Agriculturally Important Insects (Currently NBAIR- National Bureau of Agricultural Insect Resources)

LB- Luria Bertani

NaCl- Sodium chloride

M- Molar

SDS-PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

**Conflict of interest:** None declared

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