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In vitro Biofilm Formation of *Pseudomonas fluorescens*, A Promising Technique for Waste Water Treatment

Ravishankar Naik[1], Vaishali Pawar[2], Deepika Suryawanshi[3]

Department of Biotechnology, Padmashri Vikhe Patil College, Loni, Pravaranagar.

Abstract: ***In recent, the search of plant growth promoting rhizobacteria (PGPR) for sustainable crop production is increasing drastically. The Pseudomonas fluorescens, isolated from rhizosphere of wheat fields analyzed for bio-control agents, PGPR, biofilm formation for waste water treatment. The isolates showing fluorescence under UV light ,characterized using morphological and biochemical characteristics. Pot assay proved its growth promoting ability by enhancing growth of Vigna radiata plants(treated) compared to control (untreated) and antibacterial and antifungal activity was evaluated against rhizospheric bacteria (Rhizobium leguminosarum and Azotobacter agilis) and fungal pathogens (Aspergillus niger and Fusarium oxysporum) respectively. No inhibition seen against rhizospheric bacteria but inhibit fungal pathogens indicating quorum sensing mechanism involved in biocontrol activity. The efficiency in vitro biofilm formation and effect of different concentrations of antibiotics on biofilm formation was studied. This biofilm used to treat municipal waste water by passing waste water through it by maintaining constant flow rate of 1ml/min. Effective use of biofilm formed for enhanced waste removal in wastewater treatment application was studied with pH, temperature, color, determination of solid waste and MPN test. Results from this study provide application of this bacterium as PGPR, control fungal root diseases and for purification of waste water increasing its commercial use.***

Key words: *Pseudomonas fluorescens*, Biocontrol agents, Pot assay, Biofilm formation, Waste water treatment

1. Introduction

Plant Growth Promoting Rhizobacteria (PGPR) are soil bacteria which rapidly colonize the root zone and suppress soil borne pathogens at the root surface (Meera and Balabaskar, 2012; Maleki *et al*., 2010). PGPR strains also increase plant growth by secreting some plant growth promoting substances, siderophore, antimicrobial substances as HCN, antibiotics, and enzymes directly or indirectly, which inhibit the growth of plant pathogenic micro-organisms (Deshwal VK *et al* ., 2013). Amongst these organisms, *Pseudomonas fluorescens* and *Pseudomonas putida* (Koche *et al.*, 2013) are the most efficient group of rhizobacteria involve in biocontrol of plant diseases (Maleki *et al* ., 2010). The biocontrol and plant growth promoting ability of *Pseudomonas fluorescens* is mainly due to production of secondary metabolites such as antibiotics namely phenazine-1-carboxylic acid (PCA), pyocyanin, 2-acetamidophenol, pyrrolnitrin, pyoluteorin, 2, 4-diacetylphloroglucinol (DAPG), viscosinamide, tensin and biosurfactant (Koche *et al* ., 2013), phytohormones as indole-3- acetic acid(IAA) (Bholay *et al* .,2012) volatile compound hydrogen cyanide(Rekha *et al* ., 2010) and siderophores (Meera and Balabaskar, 2012). These characteristics make *Pseudomonas species* a good candidate to be used as seed inoculants, root dips for biological control of soil-borne pathogen, antibacterial agents (Rekha *et al.*, 2010), antifungal agents (Koche *et al* ., 2013) and an effective substitute for chemical pesticides to suppress plant diseases (Compant *et al*.,2005).The biocontrol activity in *Pseudomonas fluorescens* is thought to be regulated by Quorum sensing mechanism (Hai-Lei Wei and Li-Qun Zhang, 2006).

Quorum sensing (QS) is a communication mechanism of bacteria based on production of low-mass signaling molecules called auto inducers such as peptide based signals in various Gram-positive organisms and N-acyl homoserine lactone (AHL) signals found in many Gram-negative bacteria (Waters and Bassler, 2005). These auto inducers diffuse from the bacterium membrane into the environment, whose concentration is related to the population density of producing organisms. These signalling molecules can be sensed by the bacterial cells and allows the population to initiate a coordinated action once a critical concentration has been reached (K. Braeken *et al.*, 2008). Quorum sensing thus helps bacteria to coordinate, respond quickly to environmental changes, biofilm formation etc. (Avantika Lal, 2009; Cvitkovitch *et al.*, 2003). Biofilm are communities of microorganisms attached to a solid surface and enclosed in an exopolysaccharide matrix (Thien-Fah C.Mah and George A.O'Toole, 2001). It includes attachment of bacteria to the substratum (reversible attachment), bacterial growth and division leading to colonization of surrounding area and formation of biofilm (irreversible attachment) (S. M. Hinsa and G. A. O'Toole, 2006).Biofilm can form on variety of surfaces as living tissues, indwelling medical devices, industrial or potable water system piping or natural aquatic systems (Donlan, 2002). These are effectively applied in waste water treatment, industrial water, air pollution etc. (L. Yotova and D. Marinkova, 2012; Bryers, 2000). *P.fluorescens* is also known in biofilm formation. Biofilm systems has many advantages in waste water treatment such as low space requirements, reduced hydraulic retention time, resilience to changes in the environment, increased biomass residence time, high active biomass concentration, enhanced ability to degrade recalcitrant compounds as well as a slower microbial growth rate resulting in lower sludge production compared to suspended growth systems (Chen.C.Y and Chen. S.D, 2000; Verma *et al.*, 2006).Water is life but due to globalisation and industrialization this water becomes hazardous for life (Sofia

Andersson, 2009), hence there is need to purify the water by biological method. So here an attempt has been done to use *P.fluorescens* biofilm for waste water treatment.

The aim of the study was to analyse this bacteria as a biocontrol agent and PGPR, formation of biofilm and its use in waste water treatment. So in the present study, *Pseudomonas fluorescens* was isolated and characterized from rhizospheric soil of wheat fields and its antagonistic activity was studied against rhizospheric bacteria (*Rhizobium leguminosarum* and *Azotobacter agilis)* and soil borne fungal pathogens (*Aspergillus niger* and *Fusarium oxysporum)*. Pot assays were carried out to study its potential as a PGPR. Also, in vitro biofilm was formed and used for waste water treatment.

2. Material and Method

2.1 Isolation of *Pseudomonas fluorescens* from rhizospheric soil of wheat fields

Isolation of *Pseudomonas fluorescens* was made from rhizosphere of wheat fields. The rhizosphere soil particles loosely adhering to the roots were gently collected and dissolved in sterile distilled water. The mixture was shaken for 10-20 min to obtain standard soil suspension. *P. fluorescens* was isolated by following the serial dilutions and spread plate method using the Cetrimide and specific King's B medium (Meera and Balabaskar, 2012). 1ml of soil suspension from aliquot dilutions (10^{-5} and 10^{-6}) was aseptically spread on sterile medium and incubated at 28 ± 2^0C for 24 to 48 hrs. Colonies showing fluorescence on King's B medium under UV light was used further for morphological and biochemical characterization (S.Reetha *et al*., 2014).

2.2 Identification of the isolates by studying morphological and biochemical characterization

Morphological characters such as size, shape, color, elevation, margin, opacity, bacterium shape, Gram staining and motility were performed for identification of the isolates. For biochemical characterization, various tests such as catalase test, oxidase test, starch hydrolysis, gelatin liquefaction, Citrate Utilization test, H_2S, Urea hydrolysis test were performed (Deshwal *et al* ., 2013; Meera and Balabaskar , 2012).

2.3 Antibacterial and Antifungal Activity

The antibacterial and antifungal effect of *Pseudomonas fluorescens* against rhizospheric bacteria (*Rhizobium leguminosarum* and *Azotobacter agilis)* and soil borne fungal pathogens (*Aspergillus niger* and *Fusarium oxysporum)* was evaluated by well diffusion method respectively. Antibiotics such as Ampicillin (antibacterial) and gentamycin(antifungal) were used as a positive control and saline as negative control. The plates were incubated for16-18 h at 37°C for antibacterial and 3-4 d at 28°C for antifungal activity and the zone of inhibition were recorded (V.Rekha *et al* ., 2010)

2.4 Effect of *Pseudomonas fluorescens* on growth of plants by Pot assay:

In pot assay, three different soils such as autoclaved, unautoclaved (normal soil) and unautoclaved soil inoculated with *Pseudomonas fluorescens* were used as a potting mixture for sowing. Autoclaved and Unautoclaved soil were used as a control. Twenty five seeds of Green gram (*Vigna radiata*) were used for sowing in pots containing 45g soil. The plants were given water as per daily requirement and observed for height of the plants and seed germination (Bholay *et al*., 2012).

2.5 Biofilm formation:

Overnight grown *P. fluorescens* colonies in nutrient broth were used for the experiment and its O.D. (495 nm) was adjusted to 0.65.The resulting bacterial suspension was diluted to 1:6 (1ml bacterial culture + 5ml pre-warmed medium) & incubated at 37℃ in CO_2 incubator for approximately 3 hrs in order to reach mid log phase. Dilute mid log growth suspension with pre-warmed media and place 1ml of dilution into each well of a 6 well microtiter chamber plate. Incubate at 37℃ in CO_2 incubator. Approximately after 16 hrs, aspirate medium from corner of each chamber. Then add 1 ml fresh pre-warmed medium along well of chamber so as not to create shear forces within the chamber which could disturb the biofilm. For incubation of longer time (more than 24 hrs) the medium need to be changed every 12 hrs to maintain bacterial viability (Modified from L. Rinaudi *et al*., 2006; Jurcisek *et al*., 2011). Biofilm formation was evaluated by the graph plotted based on Image J software.

2.6 Effect of antibiotics on biofilm formation

Biofilm was formed in polystyrene 6 well plates. Different antibiotics (ampicillin, gentamycin) and antimycotics were used. Different concentrations of each antibiotic (50 mg/ml) i.e., (50ul, 100ul, 150ul and 200ul) were added into the well containing biofilm. All the experiments were done in three replicates. Observations were taken up to 3 days daily under microscope and graphs were plotted for the same using Image J software to check for biofilm disruption.

2.7 Effect of Biofilm on waste water treatment

Treatment of waste water was done at lab level. Water sample used for the experiment was Municipal waste water (500 ml) collected from the drainage. Temperature and pH of the sample was recorded at the time of collection. Waste water was passed on the biofilm formed in polystyrene plate (radius-5.5cm) at a flow rate of 1 ml/min. Treated water was collected. Both the water samples, before and after treatment was analyzed for parameters as pH, temperature, color, solid waste, and MPN test (V. Girdoniya, 2011).

3. Results and Discussion

3.1 Cultural, Morphological and biochemical characteristics:

The colonies of *P. fluorescens* were obtained on Cetrimide and Kings Rany B medium after incubation for 48 hrs at 28°C. The colonies were round in shape, yellowish white, opaque with regular margin, sticky consistency, flat, 2-3 mm in dia., motile and Gram negative rods (Table.1), which are summarized based on Bergey's Manual of Determinative Bacteriology. The colonies also showed fluorescence under UV light on Kings B medium. The isolate showed positive results for Catalase test, Oxidase test, Gelatin hydrolysis test, Citrate utilization test and Urea hydrolysis test, whereas negative for Starch hydrolysis and H_2S test(Table.2). Similar results were also observed by Deshwal *et al*., 2013 and Meera and Balabaskar, 2012, which indicates that the isolated bacterium is *P. fluorescens.*

Table 1: Colony Characters

Characters	*Pseudomonas*
Shape	Round
Size	2 mm
Colour	Yellowish white
Elevation	Flat
Margin	Regular
Consistency	Sticky
Opacity	Opaque
Motility	Motile
Gram staining	Gram negative rods

Table 2: Biochemical characters:

Test	Result
Catalase test	Positive
Oxidase test	Positive
Starch hydrolysis test	Negative
Gelatin hydrolysis test	Positive
Citrate utilization test	Positive
Urea hydrolysis test	Positive
H_2S	Negative

3.2 Antibacterial and Antifungal activity:

Isolated *P.fluorescens* inhibits the growth of soil-borne fungal pathogens (*Aspergillus niger* and *Fusarium oxyporum*)(Fig.1), which affects the yield of plants whereas does not inhibit the growth of rhizospheric bacteria (*Azotobacter agilis* and *Rhizobium leguminosarum)*,which are beneficial for the plant yield. The bacterium produces secondary metabolites with antibiotic/ antifungal activities which suppress soil borne diseases (Koche *et al* ., 2013; Ardebili *et al*., 2011).This may be due to the quorum sensing mechanism involved in regulation of biocontrol activity in *P.fluorescens* (Hai-Lei Wei and Li-Qun Zhang ,2006).

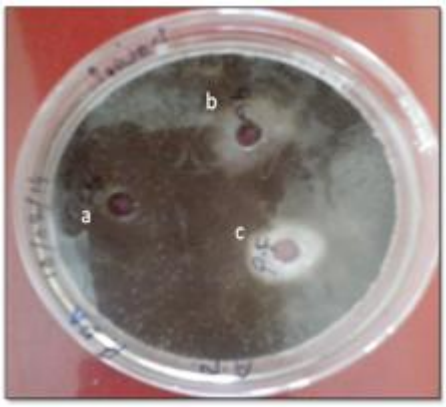

Figure 1: Effect of *P.fluorescens* on *Aspergillus niger*
a- saline solution, b- gentamycin, c- *P.fluorescens*
Zone of clearance seen around gentamycin and *P.fluorescens*

3.3 Effect of *Pseudomonas fluorescens* on growth of plants by Pot assay:

Three plants from each group were randomly selected for recording the height of the plants which was measured using the centimeter scale.In the pot assay, controli.e autoclaved and unautoclaved soil showed less growth of plants as compared to the treated soil.Of the three soils used unautoclaved soil inoculated with *P.fluorescens* showed best results compared to unautoclaved and then autoclaved (Table.3).

Table 3: Effect of *P.fluorescens* on growth of plants

Treatment	Autoclaved	Unautoclaved (normal)	Unautoclaved +*P.fluorescens*
Height of the plants(cm)Day13	3.73±0.25	4±0.5	5.8±0.26

The values are mean ±SD for three samples in each group

3.4 Biofilm formation and effect of antibiotics on it:

P.fluorescens suspension was inoculated in polystyrene plates containing medium using above procedure and monitored daily to check the standard time for biofilm formation. The graph was plotted using Image J software. There was continuous growth of biofilm seen from day1 onwards by replacement of medium and on 10th day biofilm was found to properly attach to the substratum (Fig.2). Biofilm showed resistance against various antibiotics upto certain extent (200 ul) and it was not degraded(Fig. 3) based on the graphs produced.

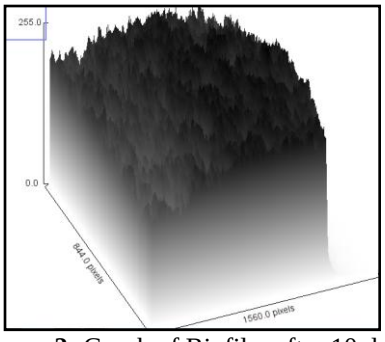

Figure 2: Graph of Biofilm after 10 days

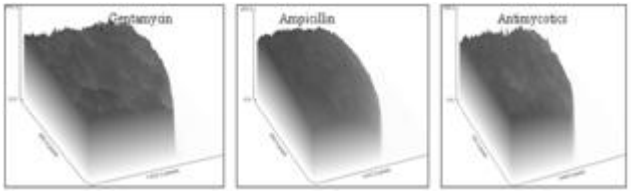

Figure 3: Effect of antibiotics on biofilm formation

No distinguishing change in the graph of biofilm seen after addition of antibiotics.

3.5 Effect of Biofilm on Waste Water Treatment

The untreated and treated water samples were assessed for parameters as pH, temperature, colour, solid waste and MPN test. These parameters were measured using standard methods. The physic-chemical analysis of Municipal waste water before and after treatment by passing through biofilm is given in table 4:

Table 4: Physico-chemical properties of waste water before and after treatment

Parameters	Before treatment	After treatment
Color	Brown	Almost colourless
Ph	5.3	6.8
Temperature	33°C	30°C
Solid waste	13gm in 500ml	10gm in 500ml
MPN test	Positive test (Production of gas and acid), Indicates presence of coliforms which can cause various disease	Negative test (No gas produced and acid production was reduced)

Figure 4: Waste water before and after treatment

4. Conclusion

The isolated bacterium was proved to be *Pseudomonas fluorescens* based on morphological, cultural and biochemical characteristics. No antibacterial activity was seen against rhizospheric bacteria (*Rhizobium leguminosarum* and *Azotobacter agilis)* but inhibited the growth of soil borne fungal pathogens (*Aspergillus niger* and *Fusarium oxysporum)*. Also, *Pseudomonas fluorescens* increased the growth of plants as compared to autoclaved soil and unautoclaved soil. Biofilm was successfully formed on polystyrene plates and used for waste water treatment, which showed positive results in treating waste water.These results summarized that *Pseudomonas fluorescens* can be effectively used as a biocontrol agent, plant growth promoter and in waste water treatment by biofilm formation which also shows resistance to antibiotics.Combined use of *P.fluorescens in* waste water treatment and as a plant growth promoter can be more efficient in agriculture.

5. Future Scope

Treated water from biofilm will be used for agriculture purpose, molecular study of *P. fluorescens*, isolate and purify PF1 protein, waste water treatment of plastic and pharmaceuticals industries.

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