

## Chitinolytic Activity of Native *Pseudomonas fluorescens* Strains

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### ABSTRACT

The chitinase producing ability of *Pseudomonas fluorescens* strains viz., PF1, PB2 and FP7 was evaluated in a culture medium with and without a chitin source. The addition of 1% (v/v) chitin in culture medium significantly increased the bacterial population and chitinase activity. Among three strains tested, FP7 responded well to the addition of chitin by producing 31.2% increased chitinase in culture. Western blot analysis with chitinase antibody detected six and five chitinase isoforms in culture inoculated with FP7 and PF1, respectively.

**Keywords:** Biological control, Chitin, Chitinase, *Pseudomonas fluorescens*.

### INTRODUCTION

The concept of biological control of soil borne disease by application of antagonistic fungi and bacteria has been showing great promise in recent years. Biocontrol of pathogenic fungi depends upon antibiosis, competition and lysis (Chet *et al.*, 1990). The lysis process mainly relies upon the production of lytic enzymes including chitinase and glucanase which cause degradation of the fungal cell wall (Chet *et al.*, 1987). Among the biocontrol agents, the chitinolytic *Trichoderma* spp. and *Pseudomonas* spp have been successfully used to control several pathogenic fungi (Sneh, 1981; Chet *et al.*, 1987; Nandakumar *et al.*, 2001; Viswanathan and Samiyappan, 2001). In general, the fungal chitinases have been investigated more extensively than the bacterial ones. However several bacterial species have been shown to produce chitinase in culture (Gooday, 1990; Frandberg and Schnurer, 1998; Ordentlich *et al.*, 1988; Viswanathan and Samiyappan, 2001).

Chitinases are a PR3 group of pathogenesis related proteins which degrade chitin, a major polysaccharide constituent of the cell wall of many higher fungi. Although chitinases are grouped mainly under PR3, the bacterial chitinase are grouped under PR8 which have exochitinolytic activity (Roberts and Selitrennikoff, 1988). Several bacterial species viz., *Pseudomonas*, *Serratia* and *Bacillus* are known to produce chitinase in culture and their production is further increased when the medium is supplemented with chitin source or fungal cell wall (Viswanathan and Samiyappan, 2001; Ordentlich *et al.*, 1988). The fluorescent pseudomonad strains PF1, FP7 and PB2 used in this study either individually or as a part of mixture strains had shown strong antifungal and antibacterial activity against *Rhizoctonia solani*, *Pyricularia oryzae*, *Xanthomonas oryzae* pv *oryzae*, *Fusarium oxysporum* f.sp *udam* under *in vitro* and field conditions (Vidhyasekaran *et al.*, 1997a,b; Vidhyasekaran *et al.*, 2001; Nandakumar *et al.*, 2001). Hence, in this study the chitinase producing ability of those *Pseudomonas*

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isolates in the presence and absence of chitin source was studied to correlate with its efficacy against plant pathogens.

## MATERIALS AND METHODS

### *Pseudomonas* Strains

Two *Pseudomonas* strains (PF1 and FP7 isolated from rhizosphere soil of blackgram and rice, respectively) used in this study were obtained from a culture collection at the Department of Plant Pathology, Tamil Nadu Agricultural University, India. The other strain, PB2, was isolated from rhizosphere soil of rice. The bacterial cultures were maintained on King's medium B (KMB) (King *et al.*, 1954) at 4 °C for routine use and in 30% glycerol at -70°C for long term storage.

### Preparation of Colloidal Chitin

Five grams of crab shell chitin (Sigma, USA) was slowly added into 100 ml of cold 0.25 N HCl with vigorous stirring and kept overnight at 4°C. The mixture was filtered through glasswool into 200 ml ice cold ethanol at 4°C with rapid stirring. The chitin suspension was centrifuged at 10,000 rpm for 20 minutes and the resultant chitin pellet was washed repeatedly with distilled water until the pH became neutral (Roberts and Selitrennikoff, 1988). The concentration was adjusted to 10 mg per ml.

### Spot Bioassay

The ability of bacterial strains to degrade chitin was proved by the spot bioassay method on KMB containing 1.0% chitin (v/v) (Viswanathan and Samiyappan, 2001). After pouring the chitin containing medium into the Petri plates, four sterile paper discs were placed over the medium and moistened with 48 hours-old fresh cultures of PF1, FP7, PB2 and sterile KB broth separately,

incubated for 48 hours and observed for a zone of clearing. Three replications were employed for each strain.

### Bacterial Growth

From the stock culture, fresh cultures were prepared on plates of KMB. One loopful of 48 hour-grown fresh culture was inoculated into 10 ml of the following medium *viz.*, King's broth B, King's broth B + Chitin (1%), Water + Chitin (1%) and Water alone. They were then incubated in a mechanical shaker under constant shaking at 150 rpm for 48 hours at room temperature ( $28 \pm 2$  °C). The bacterial cultures at their logarithmic phase of growth were centrifuged at 6,000 rpm for 10 minutes. The supernatant was freeze dried and used as a total protein source for electrophoresis (Viswanathan & Samiyappan, 2001). The total protein content was determined by the method of Bradford (1976) with bovine serum albumin (BSA) as the standard.

### Determination of Bacterial Population

After 48 hours, one ml of bacterial culture was taken from the liquid culture. The bacterial cells were collected by centrifugation at 6,000 rpm for 5 minutes and resuspended in one ml of 0.1 M phosphate buffer. The population of the bacteria was calculated spectrophotometrically at 595 nm (Thompson, 1996).

### Chitinase Assay Procedure

Chitinase activity in bacterial culture was assayed colorimetrically according to the procedure developed by Boller and Mauch (1988). The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml enzyme solution and 0.1 ml colloidal chitin (10 mg/ml). After incubation for 2 hours at 37°C, the reaction was stopped by centrifugation at 1,000 g for 3 minutes. An

aliquot of the supernatant (0.3 ml) was pipetted into a reagent tube containing 30  $\mu$ l of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20  $\mu$ l of 3% (w/v) snail gut enzyme (Helicase) for one hour. After one hour, the reaction mixture was brought to pH 8.9 by the addition of 70  $\mu$ l 1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 minutes and then rapidly cooled in an ice bath. After addition of 2 ml of dimethylamniobenzaldehyde (DMAB), the mixture was again incubated for 20 minutes at 37°C. Immediately thereafter, the absorbance was measured at 585 nm. N-acetylglucosamine (GlcNAc, Sigma, USA) was used as a standard. The enzyme activity was expressed as nmol of GlcNAc equivalents/min/ml of bacterial culture.

#### **Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Total Extra Cellular Proteins**

SDS-PAGE was carried out by the method described by Laemmli (1970). A stacking gel with 4% acrylamide was layered on top of a 12% separating gel in a Sigma slab gel apparatus. Samples with an equal amount of protein (50  $\mu$ g) were dissolved in a sample buffer and used for electrophoresis. Medium range molecular weight marker (Genei, India) was used as the standard. The protein bands were visualized by staining with Commassie brilliant blue overnight. The approximate molecular weight of desired proteins in kiloDaltons (kDa) was determined by comparing the distance between the gel and the protein bands to the logarithmic transformation of molecular weight plotted against the distance traveled by standards (Weber and Osborn, 1969).

#### **Western Blotting**

After SDS-PAGE, the proteins were electroblotted onto 0.45  $\mu$ m nitrocellulose membrane (Sigma, USA) according to the procedure given by Gallagher *et al.* (1995). The

electrophoretic transfer of proteins from gel to membrane was carried out in a BioRad semi-dry blot transfer apparatus using transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) at 0.14 mA for 30 minutes. The membrane was stained with Ponceau S stain (Sigma, USA) for 5 minutes to check the resolution and transfer quality. The blot was destained using 1x PBS (0.2 g  $\text{KH}_2\text{P}_04$ , 1.15 g  $\text{Na}_2\text{HP}_04$ , 0.2 g KCl and 8 g NaCl per L, pH 7.4) for two minutes. After destaining, the membrane was blocked for 2 hours at room temperature ( $28 \pm 2^\circ\text{C}$ ) in a phosphate buffer saline (PBS) containing 1% Tween20 and 5% fat-free milk powder at room temperature and probed with diluted (1:3000) primary antibodies (barley 28 kDa chitinase anti-rabbit antibody, a gift from Dr. S. Muthukrishnan, Kansas State University, USA) for 3 hours in PBS containing 0.1% Tween20 and 5% fat-free milk powder. The membrane was washed with PBST three times for 10 to 15 minutes each time to remove the unbound antibodies and incubated in secondary antibodies (affinity purified goat antirabbit immunoglobulin (IgG) conjugated with alkaline phosphatase, from Genei, Bangalore, India) at a dilution of 1:5000 for 3 hours. Immunological reactions were visualized by soaking the membranes in alkaline phosphatase colour development reagents (Gallagher *et al.*, 1995).

## **RESULTS**

#### **Spot Bioassay and Bacterial Population in Chitin Containing Medium**

The three bacterial strains showed a visible zone of clearing around the paper disc on chitin containing medium (data not shown). Further, the presence of chitin in liquid medium increased the population of bacterial antagonists. A population increase of 25% was observed in chitin containing medium inoculated with FP7 strain, whereas PF1 and PB2 recorded 12.8 and 11.8% increases in population, respectively (Table 1). The bacteria grown in the medium containing chitin

**Table 1.** Effect of chitin amendment on bacterial population.

Medium	<i>Pseudomonas</i> strains		
	Bacterial population (x 10 <sup>8</sup> cfu/ml of culture )		
	PF1	FP7	PB2
King's broth B	136.5 <sup>b</sup>	159.0 <sup>b</sup>	131.5 <sup>b</sup>
King's broth B + Chitin	154.0 <sup>a</sup>	199.0 <sup>a</sup>	147.0 <sup>a</sup>
Water alone	33.5 <sup>d</sup>	38.5 <sup>d</sup>	30.5 <sup>c</sup>
Water + Chitin	45.5 <sup>c</sup>	62.5 <sup>c</sup>	44.5 <sup>d</sup>

Values are the mean of three replications. In a column, the values followed by common letters are not significantly different from DMA T at 5% level.

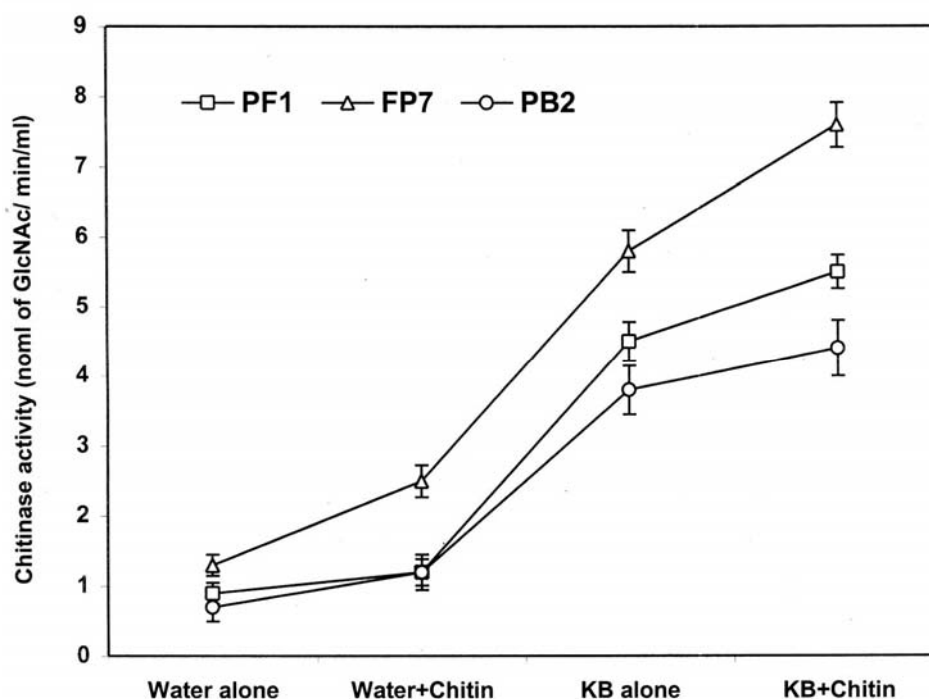
alone also reached an appreciable amount of population (Table 1).

### Chitinase Activity

Chitinase produced by *Pseudomonas* was assayed in culture filtrates of PF1, FP7 and PB2 strains. Chitin amendment significantly increased the chitinase activity of *Pseudomonas* strains. Among the three strains, FP7 responded well to the addition of chitin and

produced 31.2% increased chitinase in chitin-amended medium. However, only a moderate response to the addition of chitin was noticed in the PF1 and PB2 strains. (Figure 1)

### Extra-cellular Protein and Chitinase Patterns of Bacterial Strains



**Figure 1.** Chitinase activity of *Pseudomonas fluorescens* strains grown in chitin amended medium; Error bars indicate  $\pm$  SE.



**Figure 2.** Extra cellular protein profile of bacterial strains.

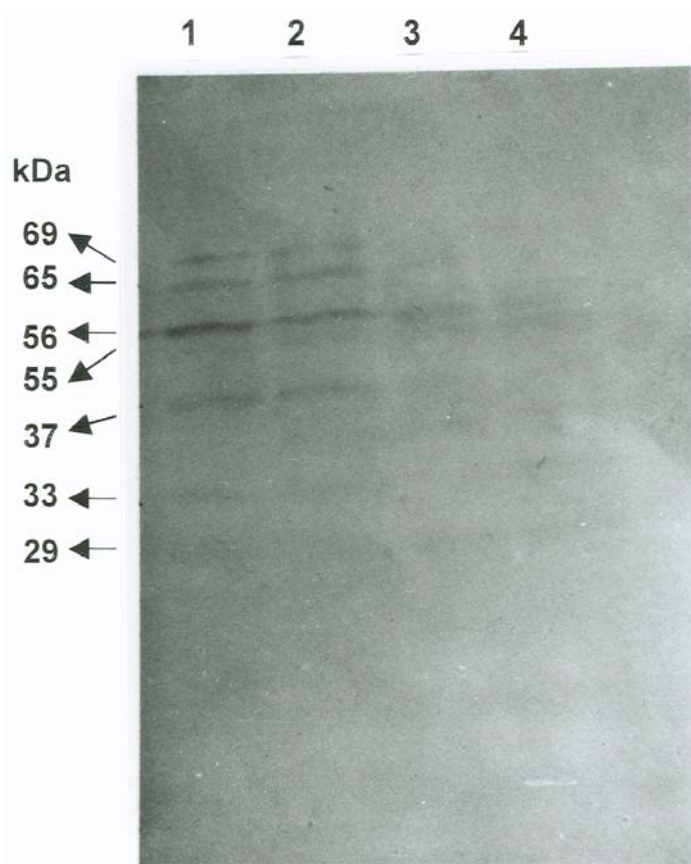
Lane 1: Protein marker; 2: FP7 in King's medium B; 3: FP7 in King's medium B + 1% chitin; 4: FP7 in chitin alone; 5: FP7 in water alone; 6: PF1 in King's medium B; 7: PF1 in King's medium B + 1% chitin; 8: PF1 in chitin alone; 9: PF1 in water alone.

SDS-PAGE analysis of a culture filtrate of bacterial strains grown in KMB with 1.0% chitin showed the induction of new proteins (Figure 2). Western blot analysis with polyclonal barley chitinase antibody detected six chitinase isoforms with molecular weights of 69, 65, 56, 55, 37 and 33 kDa and five isoforms with molecular weights of 65, 56, 55, 33 and 29 kDa in FP7 and PF1 culture filtrate, respectively. The addition of chitin induced the production of 55 kDa isoform in the FP7 strain and 65 kDa in the PF1 strains (Figure 3).

## DISCUSSION

Biological control with fluorescent pseudomonad offers an effective method of managing plant pathogens (Chet *et al.*,

1990; Ramamoorthy *et al.*, 2001). These bacteria inhibit the fungal pathogens by producing antibiotics, lytic enzymes and by inducing resistance systemically in the plant by activating defensive genes such as chitinase,  $\beta$ -1,3-glucanase, peroxidase and phenylalanine ammonia lyase (Friendlender *et al.*, 1991; Punja and Zhang, 1993; Ramamoorthy *et al.*, 2001; Viswanathan and Samiyappan, 2001). Chitinases are well known to lyse the fungal cell wall (Chet *et al.*, 1987). Addition of chitin or cell wall material to the fungus culture medium has been used in many studies to prove the chitinolytic activity (Ordentlich *et al.*, 1988; Frandberg and Schuurer, 1998; Viswanathan and Samiyappan, 2001). In the present study, the appearance of a visible zone of clearance indicates that the *Pseudomonas* strains are able to degrade and utilize the



**Figure 3.** Western analysis of bacterial chitinase.

- 1: EP7 in King's medium B.
- 2: EP7 in King's medium B+1% chitin.
- 3: PF1 in King's medium B+ 1%chitin.
- 4: PF1 in King's medium B.

complex chitin polymer for their growth. This was reflected in an increased population of bacteria in culture medium containing chitin or even on chitin alone. The increased population and the addition of chitin might have resulted in enhanced chitinase activity. In the present study, polyclonal barley chitinase antibody detected six and five isoforms of chitinases in culture filtrates of FP7 and PF1 grown in King's broth B (with and without 1.0 percent chitin), respectively. Addition of chitin induced one isoform in each strain. Induction and enhanced activity of chitinase in the chitin containing medium have been reported previously (Ordentlich *et*

*al.*, 1988; Viswanathan and Samiyappan 2001). Watanabe *et al.* (1990) reported that six isoforms (74, 69, 52, 39, 38 (pI 6.6) and 38 kDa (pI 5.9)) of chitinases are produced by *Bacillus circulans* grown in YNB medium containing 0.2% chitin. Among them, two isoforms (79 and 69 kDa) had the highest colloidal chitin degrading activities. The insoluble polymer chitin could selectively enhance the growth and development of chitinolytic bacterium (Kokalis-Burella *et al.*, 1991). More recently, Viswanathan and Samiyappan (2001) reported that the replacement of glycerol in King's medium with colloidal chitin enhanced the bacterial

population in addition to having enhanced antifungal activity against *C. falcatum*.

The addition of chitin to soil leads to an increase in the population of chitinolytic microbes which, in turn, reduce the plant diseases caused by fungal pathogens (Sneh *et al.*, 1981; Benhamou *et al.*, 1996; Lafontaine and Benhamou, 1996). Hallmann *et al.* (1996) reported the chitin-mediated changes in bacterial population in soil and plant systems associated with nematode control. Furthermore, the bacterial formulation containing chitin effectively controlled some of the soil borne disease. The chitin amended talc-based formulation of bacterial strains used in this study effectively reduced the sheath blight incidence under field conditions (Radjacommarie *et al.*, 2001). Hence it is worth considering the utilization of chitinolytic bacteria, particularly *Pseudomonas fluorescens* in the control of plant pathogens.

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### فعالیت کیتینولیتیک استرینهای بومی *Pseudomonas fluorescens*

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#### چکیده

توانایی استرینهای PB2، PF1 و FP7 باکتری *Pseudomonas fluorescens* در تولید آنزیم کیتیناز در محیط کشت همراه و یا بدون کیتین بررسی شد. اضافه کردن ۱٪ (V/V) کیتین به محیط کشت به صورت معنی دار باعث افزایش جمعیت باکتری و فعالیت کیتیناز گردید. در میان ۳ استرین آزمایش شده، استرین FP7 نسبت به اضافه کردن کیتین عکس العمل خوبی نشان داد و موجب ۳۱/۲ درصد افزایش کیتیناز در محیط کشت گردید تجزیه Western blot با پادتن کیتیناز به ترتیب باعث شناسایی ۵ و ۱۵ ژنوم کیتیناز در محیط کشت تلقیح شده با استرینهای FP7 و PF1 شد.