



Production of cell wall degrading enzymes and antibiotic by *Pseudomonads* for assessing their biocontrol potential

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Received: 6 October 2018; Accepted: 11 January 2019

ABSTRACT

A comprehensive study on bio-control efficacy of different *Pseudomonas* species was made in terms of cell wall degrading enzymes, viz. chitinase, lipase, protease and antibiotic 2, 4- diacetylphloroglucinol (2,4- DAPG) production abilities in respect to changing temperatures and pH. Observations revealed that all the isolates of *Pseudomonas* spp. under study produced all the three cell wall degrading enzymes and 2,4- DAPG significantly. However, the isolates PS-11 and PS-30 exhibited maximum enzyme producing abilities. Moreover, PS-11 could be considered to be the maximum heat tolerant among the isolates as it maintain remarkable sustainability even at 55° C in the ability to produce enzymes with minimum losses in activities 0.1026 to 0.718 in chitinase, 3.1167 to 2.1794 in lipase and 0.5827 to 0.4075 in protease activities due to rise of temperature from 45° C to 55° C. In pH variation studied at 35°C, it was observed that chitinase, lipase and protease production abilities of most of the isolates of *Pseudomonas* spp. were maximum at pH value of 6.5 and decreased on further lowering to 5.5 or rising to pH 8.5. PS-30 recorded the highest production of 2,4-DAPG (767.37 µg/ml) at 30° C and pH 6.5.

Key words: Biocontrol, Chitinase, 2,4-DAPG, Lipase, Protease, *Pseudomonas*

Plant pathogens are reported to be the major cause of crop yield losses (Prasad and Lallu 2006, Mallick *et al.* 2015). For prevention of such losses due to plant diseases, chemical compounds have been used but abuse in their applications has led to the development of resistance to plant pathogens besides the repercussions on environment and human health. Therefore, an alternative and better management strategy like use of biocontrol agents need to be introduced to prevent the crop damage and hence to improve the agricultural production (Nasseby and Pascual 2000). *Pseudomonas* genus belonging to family *Pseudomonaceae* is a soil borne gram- negative, aerobic bacteria (Euzaby 1997). They are reported to produce many secondary metabolites including extracellular excretion of hydrolysing enzymes having cellulolytic and chitinolytic activities, antibiotics, plant growth promoters and iron-chelating siderophores and hence the bacteria develop the activity to inhibit the plant pathogens directly or /and indirectly through induction of systemic resistance to phytopathogens (Naseby *et al.* 2000). The excretion of these cell wall hydrolysing enzymes and antibiotic production makes *Pseudomonas* species potent

biocontrol agents against plant pathogens. However, effectiveness of *Pseudomonas* as biocontrol agent depends upon the survival ability of bacteria and the production efficacy of these enzymes and antibiotic. Agro-climatic and soil conditions of various crops depend upon the season and growth stages of crop which are affected by temperature and soil pH that plays a very specific and key role in the plant growth and production. Therefore, for wide application of *Pseudomonas* as an efficient biocontrol agent, the bacterial survivability and sustainability in production of pathogen cell degradation enzymes/antibiotics under various crop conditions are pre requisite.

Keeping this in view, the present study was under taken to investigate the impact of high temperature and change in pH on novel *Pseudomonas* spp. (isolated from soil) in terms of production ability of chitinase, lipase, protease and antibiotic 2,4- diacetylphloroglucinol (2,4-DAPG) so as to sort out the most potential *Pseudomonas* isolate which could be exploited for wide range of application including biocontrol.

MATERIALS AND METHODS

Microbial cultures: Isolates of *Pseudomonas* spp. (PS-7, PS-10, PS-11, PS-15, PS-17, PS-18, PS-22, PS-26, PS-29 and PS-30) were procured from the Division of Biochemistry, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu. These bacteria were earlier isolated from soil of Jammu

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and identified as *Pseudomonas* species (Sharma 2014). The isolates were maintained in the King's B media, regularly subcultured and maintained in slants. In order to investigate the effect of temperature and pH on bio-control efficacy of *Pseudomonas* isolates in terms of cell degradation enzymes and antibiotic production, the cells were grown at wide range of acidic to alkaline pH (5.5, 6.5 and 8.5) and at temperatures ranging from 30 to 55°C. The bacterial growth was given constant shaking and after 24 hr, the cultural filtrate was collected after discarding the cells by centrifugation.

Enzyme assays: Chitinase activity was determined by following the method of Miller (1959). The values were calculated comparing with the standard curve of N- acetyl glucosamine. Enzyme specific activity was expressed as μmole of N- acetyl glucosamine/min/mg protein.

Lipase activity was determined by following the method of Gupta *et al.* (2002). The absorbance of produced p-nitro phenol was taken at 410 nm and then quantified comparing with the standard curve of p-nitro-phenol. The enzyme lipase activity was expressed as μmole of p-nitro phenol/min/mg protein.

Protease activity was determined by following the method of Sacherer *et al.* (1994). The values were calculated using standard curve of tyrosine and protease activity was expressed in μmol of tyrosine/min/mg protein.

2,4-diacetylphloroglucinol (2,4-DAPG) was extracted from the isolates of *Pseudomonas* spp. by following the method of Rosales *et al.* (1995). 2,4-DAPG extracted from isolates of *Pseudomonas* spp. was quantified using standard curve equation of the standard phloroglucinol (Rosales *et al.* 1995).

Separation and identification: Separation and identification of 2, 4-DAPG was done using Thin Layer Chromatography (TLC). A uniformly spread slurry of silica gel G (25g of silica gel G powder with 50 ml of water) to a thickness of 250 μm on glass plate (20 $\times$ 20 cm

and 0.5 cm thickness) was made by using applicator. The plates were air dried to allow the binder to set. The plates were then activated at 120°C for 1 h keeping in an oven just before use. 20 μl methanol dissolved samples were applied to 250 μm thick silica gel chromatography plates and developed in a acetonitrile/ methanol/ water (1:1:1) solvent system. Spots were visualized by spraying with diazotized sulphanic acid. Rf values of the spots were compared with synthetic phloroglucinol (50 mg/ml methanol).

Statistical analysis: The data of the study was statistically analysed by analysis of variance (ANOVA) using Tukey's test.

RESULTS AND DISCUSSION

Pseudomonas species are observed to excrete cell wall degrading enzymes, viz. glucanases, proteases, lipase, and antibiotic, viz. 2,4- diacetylphloroglucinol (2,4- DAPG) and hence these bacteria develop the activity to manage various plant pathogens (Dunne *et al.* 1997). The present study was under taken with the objective to screen out potential biocontrol *Pseudomonas* species have the highest capacity to produce cell wall degrading enzymes and antibiotic (2,4-DAPG) possessing heat tolerance and wide pH range sustainability. The results of enzymes and 2,4-diacetylphloroglucinol assay were found to vary significantly among the isolates of *Pseudomonas* spp. as well as with temperature and pH combinations.

The data in Table 1 revealed that all the *Pseudomonas* isolates were observed to produce the significant amount of enzymes under study, viz. chitinase, lipase and protease and their activities varied with the rise of temperature from 30 to 55°C showing the maximum values at 35°C in all the isolates and a decreasing trend was recorded afterwards with a rise in temperature. Among the isolates, PS-18 and PS-30 were observed more efficient in production abilities of enzymes with the exception of PS-10, PS-11 for chitinase production.

Table 1 Effect of temperature on chitinase, lipase and protease production potential of *Pseudomonas* isolates at pH 6.5

Isolate	Specific activity of chitinase (μmole glucosamine/min/mg protein)				Specific activity of lipase (μmol p-nitro phenol/min/mg protein)				Specific activity of protease (μmole tyrosine /min /mg protein)			
	Temperature				Temperature				Temperature			
	30°C	35°C	45°C	55°C	30°C	35°C	45°C	55°C	30°C	35°C	45°C	55°C
PS-7	0.0564 ^c	0.0569 ^c	0.0219 ^b	0.0051 ^a	1.8255 ^g	1.8259 ^g	0.7019 ^e	0.1627 ^a	0.2785	0.2787 ^c	0.1071 ^e	0.0248 ^a
PS -10	0.1050 ^e	0.1050 ^e	0.0413 ^e	0.0216 ^c	2.7850 ⁱ	2.7900 ⁱ	1.0979 ^h	0.5741 ^f	0.2800 ^c	0.2800 ^c	0.1102 ^f	0.0576 ^d
PS -11	0.0950 ^d	0.0953 ^d	0.1026 ⁱ	0.0718 ^h	2.8482 ^j	2.8491 ^j	3.1167 ^j	2.1794 ^j	0.5408 ^e	0.5411 ^e	0.5827 ^j	0.4075 ^j
PS -15	0.0425 ^b	0.0427 ^b	0.0199 ^a	0.0109 ^b	1.2617 ^e	1.2621 ^e	0.5891 ^d	0.3233 ^d	0.2000 ^b	0.2000 ^b	0.0933 ^c	0.0512 ^c
PS -17	0.1100 ^f	0.1100 ^f	0.0457 ^f	0.0363 ^f	1.0350 ^d	1.0350 ^d	0.4303 ^c	0.3420 ^e	0.3450 ^d	0.3450 ^d	0.1434 ^g	0.1140 ^h
PS -18	0.1839 ^j	0.1842 ⁱ	0.0672 ^h	0.0489 ^g	2.7130 ^h	2.7135 ^h	0.9913 ^g	0.7209 ^h	0.2754 ^c	0.2760 ^c	0.1008 ^d	0.0733 ^f
PS -22	0.0383 ^a	0.0383 ^a	0.0334 ^c	0.0318 ^c	1.0180 ^c	1.0182 ^c	0.8874 ^f	0.8454 ⁱ	0.2042 ^b	0.2045 ^b	0.1782 ^h	0.1670 ⁱ
PS -26	0.1312 ^h	0.1315 ^h	0.0563 ^g	0.0490 ^g	0.7725 ^a	0.7729 ^a	0.3312 ^b	0.2879 ^c	0.1910 ^a	0.1912 ^a	0.0819 ^b	0.0712 ^e
PS -29	0.1254 ^g	0.1258 ^g	0.0372 ^d	0.0243 ^d	0.7807 ^b	0.7809 ^b	0.2309 ^a	0.2127 ^b	0.1687 ^a	0.1691 ^a	0.0500 ^a	0.0461 ^b
PS -30	1.0180 ^j	1.0182 ^j	1.3175 ^j	0.4686 ⁱ	1.3344 ^f	1.3347 ^f	1.7271 ⁱ	0.6143 ^g	0.2200 ^b	0.2203 ^b	0.2851 ⁱ	0.1010 ^g

Values are the means of three replications for each treatment and the same letter within the column are not significantly different at $\alpha=0.05$ by Tukey's test.

However, the PS-11 and PS-30 recorded highest values at 45°C possessing 0.1026 and 1.3575 μ mole of N-acetyl glucosamine, 3.1167 and 1.7271 μ mole of p-nitro phenol and 0.5827 and 0.2851 μ mole of tyrosine per minute per mg of protein in chitinase, lipase and protease respectively. It was observed that even at 35° C, these two isolates were found superior; chitinase activity was observed highest in PS-30 (1.0182 μ mole of N-acetyl glucosamine/min/mg protein) whereas lipase and protease activities were recorded maximum in PS-11 (2.849 μ mole of p-nitro phenol/min/mg protein and 0.5411 μ mole of tyrosine/min/mg protein). PS-10, PS-18, PS-15, PS-26, and PS-17 were also found to have fairly good potential in enzyme producing ability at 35°C. Overall observations revealed that *Pseudomonas* isolates (PS-11 and PS-30) were heat tolerant along with highest enzyme producing abilities. However, among them, PS-11 showed higher sustainability at 55° C in the production of all the three enzyme with minimum loss (0.1026 to 0.718 in chitinase, 3.1167 to 2.1794 in lipase and 0.5827 to 0.4075 in protease activity) due to rise of temperature from 45 to at 55°C. Therefore, PS-11 and PS-30 were found as high temperature/heat tolerant cell wall hydrolysing isolates and PS-11 was the best among them. Al-Saleh and Zahran (1999) studied the effect of temperature in the range of 7 to 37 °C on growth and lipase production of psychrotrophic strains of *Pseudomonas fluorescens* (RM4) and observed that optimum temperature for lipase production was 37 °C. Similar results were obtained by Vasantha *et al.* (2012) who reported protease production at temperature range of 30 - 50°C. However, increase in temperature beyond 50° C led to decline in production of enzyme proving that temperature plays a major role in protease production.

Cell wall of pathogenic fungi and insect/pest are composed of chitin and lipids in fungi. So chitinase, lipases and proteases cause the degradation of the cell wall of the fungi and protect the plant by inhibiting pathogen growth

or checking further propagation (Mondal *et al.* 2016). Therefore, *Pseudomonas* species which excrete more hydrolysing enzymes can be considered to be potent bio control agents. *Pseudomonas* species have been reported as potential biocontrol agents against various plant diseases (Weller 2007) and the biocontrol efficiency was found proportional to production ability/specific activities of the hydrolysing enzymes chitinase, lipase and protease and production of 2, 4-DAPG antibiotic (Naseby *et al.* 2000).

Similarly, the enzyme production ability of *Pseudomonas* isolates at various pH were studied with the variation of growth medium pH from 5.5 to 8.5 at the temperature of 35°C. The data in the Table 2 revealed that chitinase activity of most *Pseudomonas* isolates were found maximum at pH of 6.5 and decreased on further lowering of pH to 5.5 or rising to 8.5. However, some isolates, viz. PS-18, PS-22, PS-26, PS-29 and PS-30 recorded highest chitinase activity values at alkaline pH, i.e. 8.5 showing specific activity of 0.2330, 0.0475, 0.2415, 0.2441 and 2.3837 μ mole of N-acetyl glucosamine produced/minute/mg of protein respectively, while the rest of isolates studied showed reduction in chitinase activity at pH value of 8.5. Similar to chitinase, the lipase and protease production abilities of most of the isolates were observed to be maximum at 6.5 pH and declined with changes of pH (acidic as well as alkaline). Isolates PS-15 and PS-11 showed highest values at pH 5.5 and pH 6.5 respectively. However, isolates PS-18, PS-22, PS-26, PS-29 and PS-30 possessed maximum lipase and protease activities at pH 8.5 recording values of 3.4330, 1.2633, 1.4198, 1.5155 and 3.1247 μ mole of p-nitrophenol and 0.3492, 0.2537, 0.3512, 0.3281 and 0.5157 μ mole of tyrosine/min/mg of protein respectively. In assay of all the three enzymes, isolate PS-30 was found most potential having highest production capacities of 2.3837 μ mole of N-acetyl glucosamine, 3.1247 μ mole of p-nitrophenol and 0.5157 μ mole of tyrosine produced/min/mg of protein

Table 2 Effect of pH on chitinase, lipase and protease production of *Pseudomonas* isolates at 35°C

Isolate	Specific activity of chitinase (μ mole glucosamine/min/mg protein)			Specific activity of lipase (μ mol p-nitro phenol/min/mg protein)			Specific activity of protease (μ mole tyrosine /min /mg protein)		
	pH			pH			pH		
	5.5	6.5	8.5	5.5	6.5	8.5	5.5	6.5	8.5
PS-7	0.00097 ^a	0.0569 ^c	0.0549 ^c	0.0311 ^a	1.8259 ^g	1.7637 ^f	0.0048 ^a	0.2787 ^c	0.2692 ^d
PS-10	0.00270 ^c	0.1050 ^e	0.0940 ^e	0.0719 ^d	2.7900 ⁱ	2.4995 ^g	0.0072 ^b	0.2800 ^c	0.2508 ^b
PS-11	0.00912 ^e	0.0953 ^d	0.0916 ^d	0.2771 ⁱ	2.8491 ^j	2.7817 ^h	0.0518 ⁱ	0.5411 ^e	0.5201 ^j
PS-15	0.04248 ^h	0.0427 ^b	0.0380 ^a	1.2557 ^j	1.2621 ^e	1.1238 ^b	0.1330 ^j	0.2000 ^b	0.1781 ^a
PS-17	0.00870 ^c	0.1100 ^f	0.1043 ^f	0.0820 ^c	1.0350 ^d	0.9818 ^a	0.0273 ^h	0.3450 ^d	0.3273 ^c
PS-18	0.00800 ^d	0.1842 ⁱ	0.2330 ^g	0.1187 ^h	2.7135 ^h	3.4330 ^j	0.0121 ^d	0.2760 ^c	0.3492 ^g
PS-22	0.00190 ^b	0.0383 ^a	0.0475 ^b	0.0552 ^b	1.0182 ^c	1.2633 ^c	0.0111 ^c	0.2045 ^b	0.2537 ^c
PS-26	0.01030 ^f	0.1315 ^h	0.2415 ^h	0.0606 ^c	0.7729 ^a	1.4198 ^d	0.0150 ^e	0.1912 ^a	0.3512 ^h
PS-29	0.01450 ^g	0.1258 ^g	0.2441 ⁱ	0.0931 ^f	0.7809 ^b	1.5155 ^e	0.0193 ^g	0.1691 ^a	0.3281 ^f
PS-30	0.07560 ^j	1.0182 ^j	2.3837 ^j	0.1057 ^g	1.3347 ^f	3.1247 ⁱ	0.0174 ^f	0.2203 ^b	0.5157 ⁱ

Values are the means of three replications for each treatment and the same letter within the column are not significantly different at $\alpha=0.05$ by Tukey's test.

Table 3 Production of 2,4-DAPG by different *Pseudomonas* isolates

Isolate	2,4- DAPG (µg/ml)
PS-7	52.14 ^b
PS-10	36.40 ^a
PS-11	54.11 ^c
PS-15	586.34 ⁱ
PS-17	161.34 ^e
PS-18	177.08 ^f
PS-22	240.05 ^g
PS-26	62.96 ^d
PS-29	354.17 ^h
PS-30	767.37 ^j

respectively at pH 8.5. However, highest heat resistant isolate, PS-11 was observed to possess maximum production potential of all the three enzymes at pH 6.5. An earlier study on the activities of these enzymes at different temperature (20°C, 30°C, 40°C and 50°C) and pH reported to show optimal product at 40° C and at pH level of 7-8 (Kathiravan *et al.* 2012). However, Senol *et al.* (2014) reported that the optimum activity of the purified chitinase enzyme was at pH 4.0 and at 50° C of temperature. In this study PS-11 and PS-30 were observed to be more heat tolerant showing highest activity values at 45° C but at different pH (8.5 for PS-30 and 6.5 for PS-11).

Extraction and quantification of 2, 4-DAPG: The values of extracted 2,4-DAPG from isolates of *Pseudomonas* spp. ranged from 0.89 to 0.94 which were close to the R_f of synthetic phloroglucinol (0.88) (Fig 1). Rosales *et al.* (1995) also obtained similar types of results. The results in Table 3 revealed that the isolate PS-30 showed the highest production of 2, 4-DAPG, i.e. 767.37 µg/ml and PS-10 showed the lowest production of 2, 4-DAPG, i.e. 36.40 µg/ml at 35°C and pH 6.5. The amount of 2, 4-DAPG produced by *Pseudomonas* isolates ranged from 36.40 to 767.37 µg/ml with a mean production of 2.4920 µg/ml. Antibiotics which have role of inhibition/stop the growth of bacteria and fungi, also check the growth of pathogens (Rousk and Baath 2011). Similar results were obtained by Sharma *et al.* (2018) who reported the production of antibiotic, i.e 2,4-DAPG from various *Pseudomonas* isolates having R_f values in the range of 0.70-0.89.

On the basis of overall observations, it is concluded that all ten *Pseudomonas* spp. isolated from soil of agricultural fields of Jammu region possess potential biocontrol properties as all of them produced chitinase, lipase and protease cell wall/coat protein hydrolysing enzymes and considerable amount of 2,4-DAPG antibiotic. These isolates could be further exploited for developing management of various soil borne plant diseases.

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