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Screening of novel microorganisms for biosurfactant and biocontrol activity against *Phytophthora infestans*

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Abstract

In the present study, 95 isolates of bacteria were tested for their biosurfactant as well as biocontrol activity against *Phytophthora infestans*. The results revealed that only 15.8% isolates showed biosurfactant activity. The emulsification index ranged from 0-68% and 24.2% isolates showed positive reaction for biosurfactant properties. In emulsification assay and oil spreading test, 18.95% and 5.26% isolates, respectively scored positive for biosurfactant production. Among all, only five isolates were found effective against *P. infestans,* for biocontrol properties. *Pseudomonas aeruginosa*-1 showed 62.22% inhibition zone after 72 hrs while *P. aeruginosa-3* showed 46.42%. Forty-eight hrs old culture supernatants were highly effective in food-poisoning test, tuber slice test and detached leaf method against *P. infestans*. In whole potato plant test, bacterial cell based formulation, culture supernatant and bacterial cell suspension of *P. aeruginosa*-1 showed 10.42%, 9.94% and 17.96% diseases severity respectively, as against 53.96% in control. This isolate holds promise as biological control agent against *P. infestans* in field.

Key words

Bacteria, Biocontrol, Biosurfactant, *Phytophthora infestans*, Potato

Introduction

Potato is the third most important food crop in the world and late blight caused by oomycete *Phytophthora infestans* (Mont.) de Bary is the most serious disease of potato world over. Management of late blight has become more challenging during the last few decades because of the emergence of new virulent races of pathogen, especially A2 mating type (Daayf *et al*., 2001; Kato *et al*., 1997; Lambert and Currier, 1997) rendering newly released resistant potato cultivars susceptible in 5-7 years time the world over, including India. Hence, the only option left to manage late blight in these cultivars was the application of fungicides which have further complicated the situation with the appearance of fungicide resistant races. Therefore, quest for new alternative sources will always remain a priority so that the low to moderate level of resistance in such cultivars can be supplemented minimizing the use of fungicides that have a range of negative effects on humans and environment.

Several microorganisms have been used against plant disease for decades. A well known group of microorganism used is the fluorescent Pseudomonas which excretes secondary metabolites including antibiotics and biosurfactants that are inhibitory to plant pathogens (Haas and Defago, 2005). Biosurfactants are chemically a heterogenic group that includes glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds (Mulligan, 2005). However, biosurfactants could attract increased attention in the area of biological control of zoospore-producing plant pathogens only recently (De Jonghe *et al.,* 2005; Nielsen *et al*., 2006; Perneel *et al*., 2008). The biosurfactant produced by *P. putida* 267, were identified as putisolvin-like CLPs, provides excellent biocontrol activity against *Phytophthora* damping-off cucumber (Raaijmaker *et al*., 2006). The efficacy of biosurfactant produced by *Pseudomonas koreensis* used as a crude extract against the oomycetes *Pythium ultimum* in hydroponic tomato cultivation (Hultberg *et al.,* 2009). The ability of biosurfactant to bring about a

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reduction in plant disease caused by *P. infestans* has previously been reported by Tran *et al.* (2007), in a study focusing on tomato late blight. Reduction in potato late blight using biosurfactant, have also been reported by Martinez and Osorio (2007). The possibility of reducing potato late blight by biocontrol with the biosurfactant producing strains *Pseudomonas koreensis* 2.74 and its biosurfactant was observed in green-house trial and a clear direct effect of the biosurfactant on zoospores of *P. infestans*, whereas it caused only minor reduction in mycelia growth rate and had no effect on the rate of sporangia production in pure culture (Hultberg *et al.*, 2010).

In the potato–late blight disease, bio-control agents have been used sparingly to manage the disease. There is an urgent need to screen and find new bioagents to manage *P. infestans* in popular potato cultivars having little or no resistance to late blight. Therefore, an attempt was made in the present study to isolate novel microbial population for screening of their biosurfactant and biocontrol activity against *P. infestans*.

Materials and Methods

Isolation of bacteria: Rhizospheric/phyloplanic samples were collected from different regions (Northern, Southern and Eastern) of India for isolation of bacteria. Phylloplane (1g) / rhizospheric soil (1g) sample was placed in 9 ml of distilled water in a 50 ml test tube serially diluted up to 10^{-7} concentration. An aliquot (0.1 ml) from 10^{-4} to 10^{-7} was placed in the Petri plate, which were poured with 20 ml of sterilized Nutrient Agar Medium (NAM), for isolation of bacterial bio-agents (Aneja, 2003). The selected colonies were purified twice to obtained pure culture and maintained in double distilled water amended with 20% glycerol at -20°C for further study.

Screening for biosurfactant activity : For preparation of culture supernatant a loop of 24 hrs old bacterial colonies of bacteria grown on Nutrient Agar Medium was transferred to 250 ml sterile conical flask containing 100 ml sterile Nutrient Broth and kept for 48 hrs at 32ºC in a rotary shaker incubator at 150 rpm. After 48 hrs of shaking and incubation the culture broth was centrifuged at 12,000 rpm for 20 min at 4ºC (Cooper and Goldenberg, 1987). For screening of biosurfactant activity of isolates six methods were used. These methods were emulsification index per cent (Cooper and Goldenberg, 1987), emulsification assay EU ml^{-1} (Patil and Chopade, 2001), haemolytic activity (Bicca *et al*., 1999), drop collapse assay (Youssef *et al*., 2004), tilted glass slide method (Peerson and Molin, 1987) and oil spreading test (Morikawa *et al*., 1993).

Identification of bacteria: One of the representative bacterial isolate, which showed both bicontrol and biosurfactant activities out of best five, was identified from Microbial Type Culture Collection and Gene Bank, Chandigarh up to genus level using biochemical testes. However, species of all bacteria were identified by using PCR technique with 16s rRNA based primers (Spilker *et al*., 2004; Pavel *et al*., 2005).

Screening for biocontrol activities: All the isolates were tested for their biocontrol activity against *P. infestans* using dual culture method. Bacterial cell suspension was prepared and 5 µl aliquots of the bacterial suspension were placed around 5 mm plug of *P. infestans* at four places at equal distance in full grown plates of *P. infestans* on rye A media and kept at 20ºC. A petriplate of *P. infestans* culture without inoculation of bacterial cell suspension was kept as control. Five replications were maintained for each of the bacteria. Data were recorded at 24, 48 and 72 hrs after incubation and diameter of inhibition zone (% inhibition) was measured (Daayf *et al*., 2003).

Bacteria which showed promising results in above test (i.e. ≥ 40% inhibition) were further tested using post inoculation dual culture. Radial growth diameter of *P. infestans* was measured at different time intervals (5, 7, 9 and 11 d) and inhibition over control was calculated (Deswal, 2012).

Bacterial isolates which showed mean inhibition above 50% in post inoculation test were again tested using parallel inoculation dual culture to check the 100% efficacy of bioagents. Radial growth diameter of *P. infestans* was measured at different time intervals (5, 7, 9 and 11 d) and inhibition over control was calculated (Deswal, 2012). Among 95 bacterial isolates, two *Pseudomonas aeruginosa* isolates (PA-1 and PA-3) showed best results in dual culture and hence the bacterial cell suspension and culture supernatant of these two isolates were used in further tests *i.e.,* food-poisoning, detached leaf and tuber slice. For these tests, zoospore suspension of *P. infestans* was prepared as method described by Gupta *et al*. (2004).

For food poisoning test culture supernatants of bacteria (PA-1 and PA-3) were prepared and used at a concentration of 0.25 % against *P. infestans* as suggested by Grover and Moore (1962). Radial growth of *P. infestans* was measured at different interval and inhibition percentage was calculated (Rani *et al.*, 2007).

Bacterial cell suspensions and culture supernatants of bacteria (PA-1 and PA-3) were tested separately by detached leaf method using susceptible popular potato cv. Kufri Bahar by the method suggested by Umaerus and Lihnell, (1976). Bacterial cell suspension (48 hrs old culture) was prepared by transferring two loops from a culture growing on a NAM into 15 ml of sterile distilled water and adjusting to an absorbance of 1.00 at 620 nm (10^9 cfu ml^{-1}). This bacterial suspension was applied to a leaflet by dipping for 20 min. After 15-20 min drying, the underside of each leaf was inoculated using filter paper discs (0.3 x 0.3 cm) dipped in zoospore suspension ($6x10^4$zoospores ml^{-1}) of *P. infestans*. The tray containing treated and untreated leaves (control) were incubated in a growth chamber at 18±1^{0}C and ≥ 95% RH. For bacterial culture supernatant test, culture supernatant was prepared from 24, 48, 72 and 96 hrs old culture broth of bacteria

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and treated as above. The lesion area was measured as suggested by Rani *et al*. (2007) and percentage control was also calculated according to Daayf *et al*. (2003).

For tuber slices test, culture supernatants of bacteria (PA-1 and PA-3) were prepared. The tuber slices were dipped in 100% culture supernatant for 15-20 min and then dried by using sterile filter paper. The treated tuber slices were placed in 90 cm Petri dishes and inoculated using filter paper discs (0.3 x 0.3 cm) dipped in zoospore suspension ($6x10^4$ zoospores ml^{-1}). Tuber slices were also dipped in nutrient broth devoid of bacteria and used as control. Five replications were maintained for each treatment and lesion area was measured (Singh and Bhattacharyya, 1995).

Preparation of bio-formulation : The bio-formulation of *P. aeruginosa* (PA-1), which showed best results in detached leaves and tuber slice experiments, was developed using a mixture of 10 g of carboxymethylcellulose (CMC) and 1 kg of talc and 5 g of calcium carbonate to adjust pH to 7.0 (Vidhyasekaran and Muthamilan, 1995). The mixture was autoclaved for 20 min at 15 psi and 121 ºC for three consecutive d. *P. aeruginosa*-1 was grown on *Pseudomona*s specific media (Hi-media) for 48 hrs at 32 ºC and 3 plates of bacterial cell were mixed into the autoclaved talc material by using a sterile glass rod under laminar flow and sealed in sterile polythene and kept for 2 d at 32ºC. After 2 d, 1 g of bio-formulation prepared was added to 9 ml distilled water and diluted up to 10^{-10} time for counting cfu ml^{-1}.

For whole plant experiment, different forms of *P. aeruginosa*-1 was used as different treatments. The treatments *i.e.,* culture supernatant (100%), bacterial cell suspension (10^9cfu ml^{-1}) and bacterial cell based formulation (10^9cfu ml^{-1}) were tested separately against late blight on potato cv. K. Bahar. Plants of cv. K. Bahar were raised in 24" earthen pots under polyhouse. Forty days after planting, the plants were divided into 6 sets, each containing 4 replicates. All the sets were sprayed with different treatments. One of the six sets is sprayed with 100 ml per plant bacterial cell suspension of *P. aeruginosa*-1 (10^9cfu ml^{-1}), another with culture supernatant of *P. aeruginosa*-1 (100% conc.). Third set was sprayed with bacterial cell based formulation of *P. aeruginosa*-1 (10^9cfu ml^{-1}) and fourth with mixture of china clay and CMC powder only (control). Fifth set was sprayed with mancozeb (0.2%) and the last was sprayed with distilled water which served as control. All the sets were kept in growth chamber at 18±1°C and >95% RH. After 24 hrs, all the sets were inoculated with *P. infestans* zoospore suspension ($6x10^4$zoospores ml^{-1}). Data on disease severity (%) were recorded after 3, 5, 7, 9, and 11 d after inoculation of pathogen (Gupta *et al*., 2004). The test to ascertain biosurfactant activities as well as biological control activity were repeated thrice to confirm the results. Experiments were conducted using Completely Randomized Design (CRD) and the data were subjected to statistical analysis using computer software IRRISTAT (version 4.4.20030719).

Results and Discussion

In total, 95 bacteria were isolated (rhizosphere and phylloplane) from the samples collected from different parts of the country. In the present study six methods viz., emulsification index (%), emulsification assay (EU ml^{-1}), haemolytic activity, drop collapse, tilted glass slide and oil spreading test were used for evaluating the biosurfactant activity of different bacterial isolates isolated from different niche. Out of these methods emulsification index (%) was found to be the best (24.21%) followed by emulsification assay (EU ml^{-1}; 18.85%), haemolytic activity (16.85%), tilted glass slide (16.84%) and drop collapse (12.63%) respectively. Oil spreading test proved to be the least effective (5.26%) method. Emulsification index has also been found to be the best method for identifying bacterial isolates, having biosurfactant activity, by several workers in the past (Banat *et al*., 2000; Bento *et al.,* 2005; Techaoei *et al*., 2007; Karthik and Rao 2010). However, Satpute *et al.* (2008) suggested emulsification index (%) is not reliable for isolation of biosurfactant producing bacteria and it was observed that emulsification assay can be effectively used for assaying biosurfactant activity. However, emulsification assay was reported by Patil and Chopade (2001) yet this method has not been used extensively so far.

Haemolytic assay has been used by many workers in the past for assaying biosurfactant activity of microorganisms (Carrillo *et al*., 1996; Rashedi *et al*., 2005; Maneerat and Pheetrong, 2007; Samadi *et al.,* 2007). However, Rismani *et al*. (2006) and Moran *et al*. (2002) reported that haemolytic assay allows a rapid assessment of biosurfactant in culture and no special equipment or expensive reagents are needed. In the present study, drop collapse test and oil spreading test were also efficient in identifying the isolates having biosurfactant property. Rismani *et al.* (2006) also reported that oil spreading test was very effective for biosurfactant screening, in their experiments, with *Bacillus* spp. Although, the mechanism of oil spreading by surfactant have not yet been clarified on the molecular level, but this method provides a sensitive and easy system of biosurfactant identification. These results were also supported by the findings of Karthik and Rao (2010) and Satpute *et al*. (2008).

Results obtained from MTCC, Chandigarh confirms that the isolate belongs to genus *Pseudomonas* strain X (suspected species *aeruginosa*). However, confirmation of species was done by using molecular technique which showed the isolate belong to the species *aeruginosa* (data not shown).

Out of 95 bacterial isolates, only five isolates namely *P. aeruginosa*-1*, P. aeruginosa*-2, *P. aeruginosa* -3, *P. aeruginosa*-4*,* and *P. aeruginosa*-5 exhibited best biocontrol activities when tested in dual culture method. These isolates showed 62.22%, 38.33%, 46.22%, 32.66%, and 35.33% inhibition, respectively (Fig.1**)**. The promising isolates which exhibited excellent biosurfactant activity also performed better in dual culture test.

The ability of biosurfactant to bring about a reduction in plant disease caused by *P. infestans* has previously been reported (Tran *et al*., 2007; Martinez and Osorio, 2007). Hultberg *et al.* (2010) also reported significant reduction in late blight disease due to a biosurfactant producing strain and its biosurfactant was observed in green-house trial and a clear direct effect of the biosurfactant on zoospores of *P. infestans* was recorded, whereas it caused only minor reduction in mycelial growth rate and had no effect on the rate of sporangia production in pure culture.

The isolates which showed best biosurfactant activity as well as maximum inhibition in full grown plate were further tested for their biocontrol activity in post inoculation dual culture method. It is evident from the data (Table 1) that after 11 d of inoculation, percent inhibition over control was 55.83% and 53.91% by *P. aeruginosa*-1 and 3, respectively. The isolates which showed more than 50% inhibition were further tested in parallel inoculation and both the isolates checked the growth up to 100%. The dual culture test was performed in three ways to ascertain the efficacy of the bio-agents. These results were supported by the findings of Deswal (2012) who reported that the strains of *Pseudomonas aeruginosa* inhibited the growth of *Sclerotinia sclerotiorum*. Similar results were obtained in a study conducted by Mansoor *et al*. (2007) where the strain of *Pseudomonas aeruginosa* inhibited the growth of *Macrophomina phaseolina, Fusarium solani* and *Fusarium oxysporum* in dual culture plate assay. For further confirmation of their biological properties and mode of action, these isolates were screened in food poisoning test at different concentration of culture supernatant. The isolates exhibited cent per cent inhibition of *P. infestans* at 0.25% concentration (data not shown). The results of effect of age of culture supernatant of bioagent on the lesion size revealed that 48 hrs old cultur supernatant was most effective as no lesions were observed on detached leaves inoculated with *P. aeruginosa*-1 and 3 as against 4.31 cm^2 in control after 5 d of inoculation (Table 2). This was followed by 72 hrs old culture supernatant while 24 and 96 hrs old culture supernatants were in effective. When tested on tuber slices, there was no growth of *P. infestans* after 4 d of inoculation as against 1.50 cm^2 in control and even after 9 d post inoculation there was little growth of the pathogen (0.33 & 0.44 cm^2) as against 2.60 cm^2 in control (Table 3).

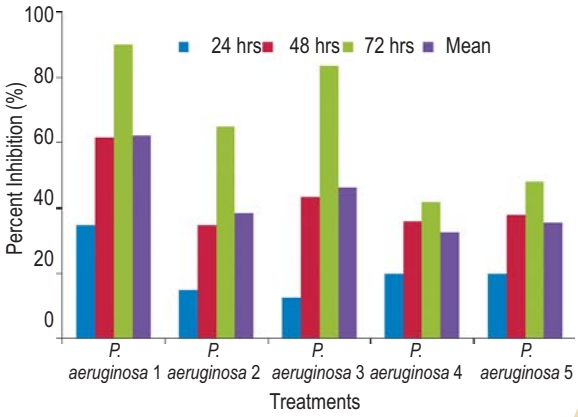

Fig. 1 : Preliminary evaluation of bacterial isolates for their biocontrol activity against *P. infestans* using dual culture

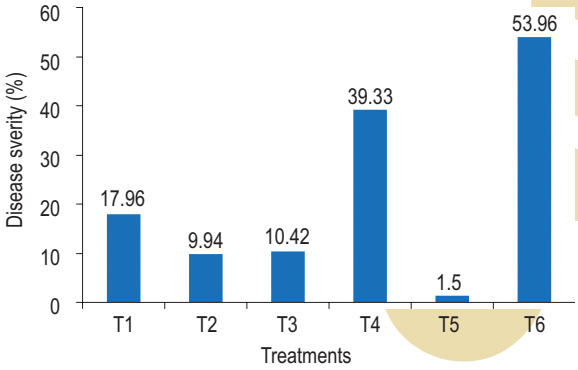

Fig. 2 : Effect of different forms of bioagent on late blight development using whole plant method T1-Bacterial suspension *Pseudomonas aeruginosa* 1, T2-Culture filtrate *P. aeruginosa* 1, T3-Bioformulation of *P. aeruginosa* 1, T4- Talc powder, T5-Mancozeb (0.2%), T6-Distilled water spray (control)

When these isolates were tested on detached leaves with bacterial cell suspension of absorbance 1.0 at O.D 620 nm (10^9cfu ml^{-1}), it was found that after five d of inoculation, lesion sizes were 0.06 and 0.21 cm^2 against 4.56 cm^2 in control (Table 4). Based on the results of food poisoning, tuber slice and detached leaves it is concluded that *P. aeruginosa*-1 was better than *P. aeruginosa*-3 against *P. infestans*. Consequently, it was used to prepare bacterial cell based formulation. *P. aeruginosa*-1 was finally tested in different forms using whole plant method and disease severity was recorded as 17.96%, 9.94% and 10.42% (Fig.2) in bacterial suspension, culture supernatant and bacterial cell based formulation respectively after 11 d of inoculation. A significant reduction in the severity of late blight infection was achieved when spores of bio-antagonist were applied either simultaneously with *P. infestans* or 12 hrs before planting of tubers in green house experiment (Cohen *et al.,* 1993). Daayf *et al.* (2003) reported that bacterial cell suspensions of few bacteria were not effective against *P. infestans* on either plates or potato leaves, whereas their culture supernatants were effective which may be due to secondary metabolites or staling products present in their culture supernatants. Our results revealed that 48 hrs old bacterial cell suspension and culture supernatant were effective for inhibiting growth of *P. infestans,* which may be due to secretion of metabolites, after 48 hrs and antibiosis may be the mode of action of bacterial strain that have biosurfactant producing capacity.

Based on the present study, comprising of dual culture, food poisoning, detached leaves, tuber slices and whole plant

Table 1 : Effect of selected *Pseudomonas* isolates on the radial growth of *Phytophthora infestans* on post inoculation at 20°C

Bio-agent	Radial growth (mm) and inhibition (%)									
	5th day		7th day		9th day		11th day		Mean	
	Growth	Inhibition %	Growth	Inhibition %	Growth	Inhibition %	Growth	Inhibition %	Growth	Inhibition %
P. aeruginosa 1	22.50	43.75	27.50	54.10	33.00	61.10	32.00	64.44	28.75	55.83
P. aeruginosa 2	23.00	42.50	28.70	52.16	34.10	59.90	35.00	61.11	32.25	48.60
P. aeruginosa 3	30.00	25.00	33.00	45.00	33.00	61.10	33.00	63.33	30.20	53.91
P. aeruginosa 4	40.00	0.00	40.00	33.30	45.00	47.00	50.00	44.44	43.75	31.17
P. aeruginosa 5	40.00	0.00	39.00	33.30	41.00	51.70	43.70	51.44	40.92	34.11
Control	40.00	-	60.00	-	85.00	-	90.00	-	68.75	-
LSD (P = 0.05)	0.91		0.12		0.23		0.28		0.26	

Table 2 : Effect of age of culture filtrates of *Pseudomonas* isolates on development of *P. infestans* on detached leaf

Bio-agent	Different hours old culture filtrate and lesion area (cm^2) after days											
	24 hr			48 hr			72 hr			96 hr		
	4th day	5th day	Inhibition over control (%)	4th day	5th day	Inhibition over control (%)	4th day	5th day	Inhibition over control (%)	4th day	5th day	Inhibition over control (%)
P. aeruginosa 1	4.00	4.17	25.14	0.00	0.00	100.00	1.50	1.72	60.00	5.00	5.00	0.00
P. aeruginosa 3	4.40	4.45	21.00	0.00	0.00	100.00	2.20	2.39	44.72	3.29	5.00	0.00
Control	5.50	5.57	-	4.00	4.31	-	4.20	4.30	-	5.00	5.00	-
LSD (P= 0.05)	1.16	2.09		1.31	1.33		2.28	2.48		2.24	3.62	

Table 3 : Effect of 48 hrs old culture filtrate of *Pseudomonas* isolates on the growth of *P. infestans* on tuber slice

Bio-agent	4th day		9th day		Mean	
	Lesion area (cm^2)	Reduction over control (%)	Lesion area (cm^2)	Reduction over control (%)	Lesion area (cm^2)	Reduction over control (%)
P. aeruginosa 1	0.00	100	0.33	87.31	0.16	93.65
P. aeruginosa 3	0.00	100	0.44	83.08	0.22	91.54
Control	1.50	-	2.60	-	2.05	-
LSD (P= 0.05)	0.7		2.0		1.0	

Table 4 : Effect of bacterial spore suspension of *Pseudomonas* isolates against *P. infestans* with detached leaf method

Bio-agent	4th day		5th day		Mean	
	Lesion area (cm^2)	Reduction over control (%)	Lesion area (cm^2)	Reduction over control (%)	Lesion area (cm^2)	Reduction over control (%)
P. aeruginosa 1	0.02	99.15	0.06	98.87	0.04	99.01
P. aeruginosa 3	0.06	97.46	0.21	95.4	0.13	96.43
Control	2.36	-	4.56	-	3.46	-
LSD (P= 0.05)	0.2		0.4		0.3	

inoculation tests it can be safely concluded that *P. aerugionsa*-1 isolate possessed both biosurfactant and biocontrol properties. Use of such biocontrol agent should become an important component of Integrated Disease Management in the entire potato growing regions affected by late blight. Besides, it will also help to manage late blight on old, susceptible but popular potato cultivars, thereby reducing the dependence on costly, environmentally hazardous fungicides.

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