



Characterization of Biosurfactant from *Bacillus* Isolates as Antifungal Agent

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ABSTRACT: Use of green compounds to achieve the sustainable agriculture is the present necessity. Biosurfactants are one of the green compounds reported to be produced by bacteria, yeasts, and fungi which are considered to be less toxic and eco-friendly with extensive applications in pharmaceutical, cosmetics, and food industries. *Bacillus* group is one of the examples of biosurfactant producers. In the present study soil isolates of *Bacillus* species were screened for the production of biosurfactant by oil spread assay. The activity was found positive in three isolates which were assayed for antifungal activity against the phytopathogens *R. oryzae-sativae* and *F. solani*. The biocontrol activity was observed in both extracellular and the homogenized biomass fractions of *B. subtilis* BP-9, *B. subtilis* BP-13 and *Bacillus* isolate PRIS-1. Different methods as acid precipitation, organic solvent extraction and salting out by ammonium sulfate were used for isolation of biosurfactant lipopeptides from the extracellular fractions of *B. subtilis* BP-9, *B. subtilis* BP-13 and *Bacillus* species PRIS-1. The antifungal as well as biosurfactant property was confirmed in the acid precipitated fraction of *Bacillus* isolate PRIS-1. The compound was identified as a high molecular weight protein by SDS-PAGE with approximate size of 100kD. © 2015 iGlobal Research and Publishing Foundation. All rights reserved.

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INTRODUCTION

Biosurfactants are surface-active compounds produced by living organisms. Biosurfactants derived mainly from microorganisms have attracted much attention because of advantageous characteristics such as structural diversity, low toxicity, higher biodegradability, better environmental compatibility, higher substrate selectivity, and lower critical micelle concentration (CMC)[1,2,3,4]. There are different types of biosurfactant stated based on their physico-chemical properties as glycolipids, lipopeptides, neutral lipids, phospholipids, fatty acids, and polymeric [5,6,7,8,9]. It has potential to be applied in pharmaceutical, cosmetics, petroleum, and food industries [6,10,11,12,13], used as bioremediation agent for treatment of soil and water [14] and used for removal of

hydrocarbons as well as heavy metals [15]. In addition, it has demonstrated antifungal, antiviral, antitumor, insecticidal, and anti-mycoplasma activities. Surfactants are also reported to inhibit the aflatoxin production by *Aspergillus* sp. which infects crops [16]. Thus the surfactants (synthetic and biological) play diverse role in plant pathogen elimination directly or indirectly and at different processes related to agriculture. In view of sustainable development biosurfactant would provide a new hope to overcome the problem of loss of productivity in agricultural sector due to fungal diseases. Approx. ±0.2 million tons of surfactants are used in crop protection and agrochemical formulations[17]. Biosurfactants from several microbes (i.e. *Pseudomonas* spp., *Acinetobacter*

spp., *Bacillus* spp., and *Candida* spp.) have antimicrobial and antifungal activity against plant pathogens and therefore they are considered to a promising biocontrol agents for achieving sustainable agriculture [18,19,20]. Considering the antagonistic and antifungal properties of *Bacillus* spp present study is performed for screening of *Bacillus* isolates from soil for production of biosurfactant and its characterization for antifungal property.

MATERIALS & METHODS

Bacterial and fungal cultures

In the present study six soil isolates of *Bacillus* species viz. *B. subtilis* BP-1, *B. subtilis* BP-2, *B. subtilis* BP-5, *B. subtilis* BP-9, *B. subtilis* BP-13 and *Bacillus* isolate PRIS-1 were used. These cultures were obtained from Department of Biotechnology, Sir Padampat Singhanian University, Udaipur. The bacterial cultures were maintained on nutrient agar slants. The fungal cultures of *Rhizoctonia oryzae-sativae* (MTCC code-2162) and *Fusarium solani* (MTCC code 2671) were obtained from MTCC, Chandigarh (India) and maintained on Potato Dextrose Agar (PDA) slants. Both the cultures were stored in refrigerator at temperature $7^{\circ}\text{C}\pm 2$ with regular sub-culturing at an interval of 1 month.

Biosurfactant activity

Biosurfactant assay was performed with the culture broths of the *Bacillus* isolates. To determine the presence of a lipoprotein by oil displacement method 10 μl of crude oil was dropped over the surface 40 ml of distilled water in a petri dish to form a thin oil layer and onto it 10 μl of extract was gently dropped and checked for the formation of a clear zone [21]. The positive cultures were screened and further used for detection of antifungal property.

Antifungal activity of Bacillus isolates

The bacterial cultures grown in nutrient broth medium at 37°C for 48h were used to detect the antagonistic activity against the phytopathogens *R. oryzae-sativae* and *F. solani*. A 5 mm diameter mycelial disc of fungi was placed in the center and bacterial isolate were spot inoculated at 3 cm distance from disc on a potato dextrose agar (PDA) [22]. The zone of inhibition was observed after every 24h of incubation at 28°C .

Detection of antifungal property in extracellular fraction

The selected positive cultures were further used for detection of the extracellular production of the antifungal active compound. The supernatant was separated from the biomass by centrifugation at 10000rpm for 15min. The culture biomass was suspended in sterile distilled water and homogenized to release intracellular components [23]. The antifungal assay with intracellular (homogenized biomass) and extracellular (the supernatant) fractions was carried out on 1% starch agar. The mycelia was inoculated in the center of the petri plate and allowed to grow and after 48 h, wells (0.65 cm in diameter) were created at a distance of 0.5 cm away from the rim of the mycelial colony. Each well was filled with 30 μl of the sample and the plates were incubated at 28°C until mycelia growth had developed in the control plate without any sample [24].

Extraction of the active component

Overnight grown cultures of *B. subtilis* BP-9, *B. subtilis* BP-13 and *Bacillus* isolate PRIS-1 in nutrient broth at 37°C were used for extraction of active compound. To extract the extracellular products the biomass was separated by centrifugation at 10000rpm for 15 min at 4°C . The extraction was performed by three methods viz. organic solvent extraction, ammonium sulphate precipitation and acid precipitation.

Extraction with organic solvent

Culture supernatant was stirred vigorously for 20 min with organic solvents, chloroform– methanol (2:1, v/v) in 1:1 (v/v) ratio [25]. The solvent phase was evaporated and the sediments were dissolved in buffer (0.1mol l^{-1} Tris, pH7.0).

Ammonium sulphate precipitation method

Proteins were precipitated by the slow addition of ammonium sulphate to the supernatant fluid up to 40% saturation, and stirred at 7°C for 24h [26]. The precipitate was separated by centrifugation at 4°C and 10000 rpm for 20min. The pellet was re-suspended in buffer (0.1 mol l^{-1} Tris buffer, pH7).

Acid precipitation method

The culture supernatant was acidified by adding 2 N HCl to obtain a final pH of 2.0 and stored overnight at 4°C for 24h [27]. The acid precipitate was collected by

centrifugation at 12,000rpm for 20 min at 4°C and dissolved in a methanol.

Analysis of extracts

All these extracts were tested for the presence of the active component by the antifungal assay method as mentioned above [24]. The plates were incubated for 72 h at 27°C and the inhibitory activity of each extract was expressed as the percent growth inhibition, compared to the control. The extract was also analyzed for biosurfactant activity by oil spread assay using 10µl of extract.

Analysis of extracts by SDS PAGE

The extracts were analyzed for presence of protein by performing discontinuous SDS-PAGE using 4% stacking gel and 12% resolving gel. Electrophoresis was performed at 100V using tris-glycine buffer.

RESULTS & DISCUSSION

All six *Bacillus* cultures showed a clear zone formation in the oil spread assay and were considered positive for biosurfactant activity. The maximum zone of clearance in oil spread assay was observed in *Bacillus* isolate PRIS-1 (Table 1). These cultures were further used for detection of the antifungal activity. Fungal growth inhibition was observed in five *Bacillus* isolates out of six cultures namely *B. subtilis*BP-2, *B. subtilis* BP-5, *B. subtilis*BP-9, *B. subtilis*BP-13 and *Bacillus* species PRIS-1. Amongst all the isolates *B. subtilis* BP-13 have shown the maximum inhibition of 14 mm against *F. solani* (Fig 1, Table 1) and least in *B. subtilis* BP-2, *B. subtilis* BP-5.

Furthermore for determining the intracellular and extracellular production of antifungal activities in the selected isolates; *B. subtilis* BP-9, *B. subtilis* BP-13 and *Bacillus* isolate PRIS-1, the antifungal assay was performed with the homogenized biomass pellet suspended in buffer (intracellular fraction) and the culture supernatant (extracellular fraction). The antifungal activity against both the phytopathogenic fungi was observed positive in both the fractions of the three *Bacillus* isolates. The maximum positive inhibitory activity was showed by *Bacillus* isolate PRIS-1 (Fig 2). These results confirmed a strong antifungal activity in *Bacillus* isolate PRIS-1 and presence of active component both as intracellular as well as extracellular.

The cultures grown in nutrient broth were used for the extraction of biosurfactant. The extract was obtained by three different methods viz. organic solvent extraction, salting out (ammonium sulfate precipitation) and acid precipitation methods. In the analysis for the presence of active antifungal component in the extracts, the positive activity was observed only in the extract obtained by acid precipitation method (Fig 2, Table2). No significant activity was observed in the other two extracts. The biosurfactant property of the active component in the purified extract was confirmed by oil displacement assay. The molecular characterization of the active components in the extracts was performed by SDS PAGE. The results showed a single band of protein approximately of 100kDa molecular weight in the *Bacillus* isolate PRIS-1 extract, which confirmed the active component as high molecular weight protein (Fig. 3).

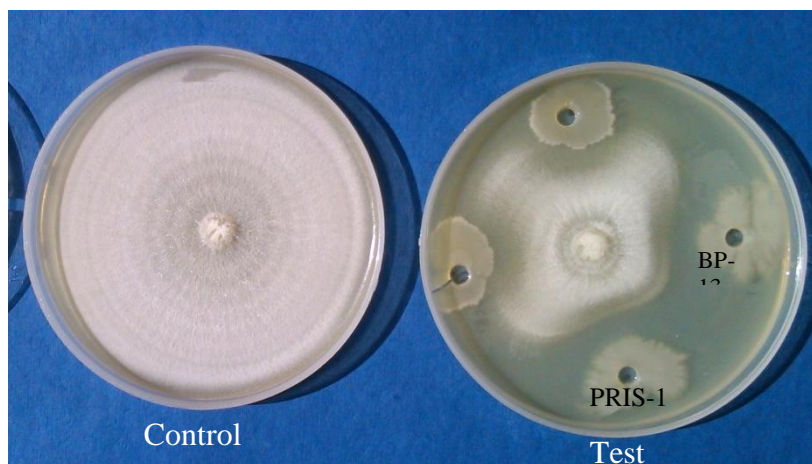


Fig. 1: Antagonism behavior shown by *B. subtilis* BP-13 and *Bacillus* isolate PRIS-1 in dual culture assay against *F. solani* compared to the radial growth of *F. solani* in control plate.

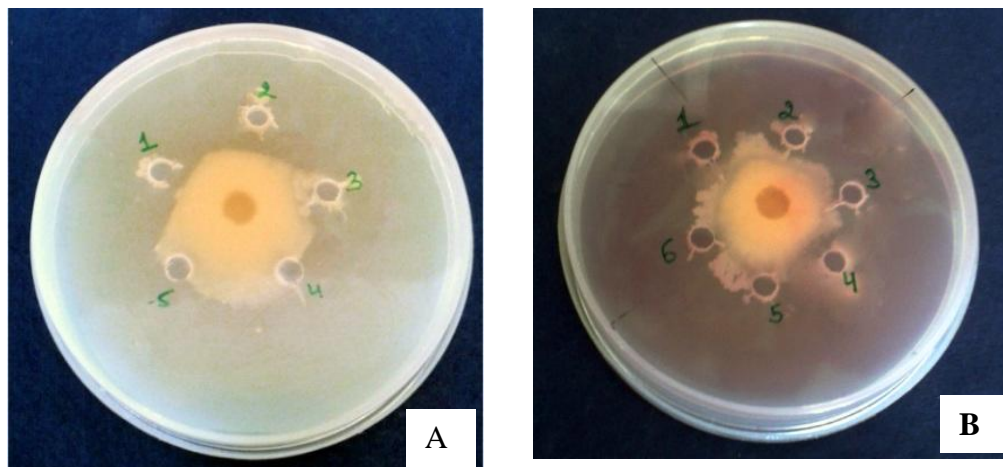


Fig. 2: Fungal growth inhibition of pattern.

A: *Bacillus* isolate PRIS-1 against *F. solani*; 1 & 2: Biosurfactant extract (acid precipitation); 3: Cell Biomass homogenate, 4: Organic solvent extract; 5: Control.

B: *Bacillus* isolate PRIS-1 against *R. oryzae*; 1: Culture supernatant; 2: Homogenized Cell biomass; 3 & 4: Biosurfactant extract (acid precipitation); 5: Organic solvent extract; 6: Control.

It is very well known fact that *Bacillus* spp. are among the important soil inhabitant and are safe bacteria. This group of bacteria were instantly studied with regard to the production of bioactive compounds and have been extensively studied for their abilities for synthesizing a vast array of beneficial substances [28] and used as potential antimicrobial agents [29,30,31]. There were several reports on *Bacillus* potential as biological control agent against many common phytopathogens [32,33]. In the present study *Bacillus* Isolates have shown the in vitro antifungal activity in *Bacillus* isolates against *Fusarium solani*. Similar results were obtained by Sadfi et al. [34].

The antimicrobial activity of *Bacillus* spp is attributed to production of HCN, siderophore, hydrolytic enzymes and antibiotics [35]. It is also likely that the most effective biological control strains act *via* multiple mechanisms. Antimicrobial activity of several biosurfactants has been reported in the literature for many different applications [36]. The most commonly isolated biosurfactants are glycolipids and lipopeptides. They include rhamnolipids released by *Pseudomonas aeruginosa* [37], sophorolipids from *Candida* sp. [38], as well as surfactin and iturin

produced by *Bacillus subtilis* strains [39]. In the present study the bioactive compound produced by PRIS-1 having a biosurfactant activity has been identified with the ability to inhibit the mycelial growth. The compound was identified as protein by SDS PAGE -having a molecular weight of approximately 100 kDa. Two categories of biosurfactants were identified to be produced by *Bacillus* species, low molecular weight and high molecular weight [40]. In the present study the antifungal biosurfactant was found to be of high molecular weight of around 100KD and may be considered to be a lipopeptide.

It can be concluded that strains, *Bacillus* isolate PRIS-1, produced antagonistic fungal biosurfactant compound which might be lipopeptide in nature. In addition, it might be used in various industrial fields, including the food industry, clinics, cosmetics and specialty chemical industries, and for cleaning oil spills by bioremediation. Therefore, we expect that these lipopeptides may be useful in agriculture and various industries as biocontrol agents and biosurfactants, respectively, although further study is needed.

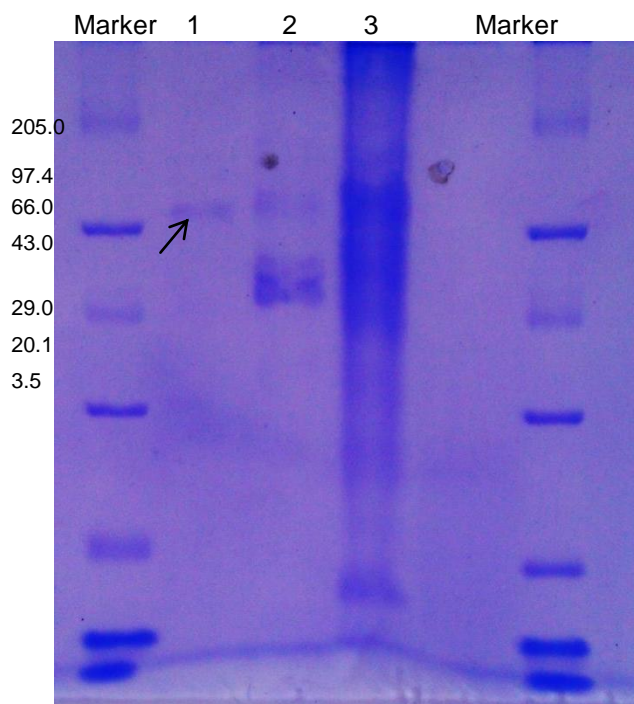


Fig. 3: SDS PAGE Pattern

Table 1: Comparative study of biosurfactant activity by oil displacement method and the antagonism pattern against the phytopathogenic fungi by *Bacillus* isolates in dual culture assay.

Sr. No.	Isolates	Biosurfactant activity by Oil Displacement (clear zone in mm)	Antifungal Activity Against	
			<i>Rhizoctonia oryzae</i>	<i>Fusarium solani</i>
1.	<i>Bacillus subtilis</i> BP 1	25	-	-
2.	<i>Bacillus subtilis</i> BP 2	35	+	+
3.	<i>Bacillus subtilis</i> BP 5	60	+	+
4.	<i>Bacillus subtilis</i> BP 9	40	++	++
5.	<i>Bacillus subtilis</i> BP 13	70	++	++
6.	<i>Bacillus</i> isolate PRIS 1	80	++	++

Note: (-) : No effect ; (+) : inhibition range 2mm to 10mm; (++) : inhibition range 10mm to 20mm

Table 2: Antifungal characteristic of extracts from different *Bacillus* isolates obtained by acid precipitation method.

Sr. No.	<i>Bacillus</i> Isolates	Against <i>F. solani</i>		Against <i>R. oryzae-sativae</i>	
		*Fungal Growth diameter in test (T) mm	% Inhibition $\frac{(C-T) \times 100}{C}$	*Fungal Growth diameter in test (T) mm	% Inhibition $\frac{(C-T) \times 100}{C}$
1	<i>B. subtilis</i> BP-9	35	44.44	58	3.3
2	<i>B. subtilis</i> BP-13	31	50.79	50	16.6
3	<i>Bacillus</i> isolate PRIS -1	25	60.32	20	66.66

*Growth diameter of mycelium in direction of spot inoculated *Bacillus* culture.

Growth diameter of *F. solani* in control plate (C) = 63mm

Growth diameter of *R. oryzae-sativae* in control plate (C) = 60mm

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