Biocontrol of Soil Borne Pathogen of Potato Tuber Caused by *Rhizoctonia solani* through Biosurfactant based *Bacillus* strain

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ABSTRACT

Black scurf disease is a major problem for cultivation of potato worldwide that is managed by synthetic agrochemicals, that are increasing environmental pollution as well as residue in farm produce also. Therefore, now a day use of bio-agents for managing black scurf is attractive feature and need of the hour. A promising bio surfactant OD02 isolate, was isolated from oil contaminated soil, utilizing its secondary metabolite after preliminary biosurfactant screening tests. Furthermore, this strain exhibited positive test with haemolytic activity, emulsification index, oil spreading test, molecular characterization with 16S rRNA universal primers revealed that the strain belongs to *Bacillus* group, that was further chemically characterized using TLC, exhibiting reddish pink colour as lipopeptide and demonstrated antagonistic activity against *Rhizoctonia solani* under *in vitro* using dual culture (45±0.30%) and at 3% concentration under food poisoning method. *Bacillus subtilis HussainT-AMU* strain is very effective against phytopathogen *R. solani* and can be more explored in future diseases management strategies.

Keywords: Biological Control, Black scurf, *Bacillus spp.*, *Rhizoctonia solani*, Soil borne

INTRODUCTION

Black scurf is an important fungal pathogen of potato (*Solanum tuberosum* L.) in the category of soil and tuber borne. Infected seeds are the main sources of infection (Durak 2016). Black scurf was reported in Maine in 1913 by Morse and Shapovalo in the Maine Agricultural Station Bulletin #230, “The *Rhizoctonia Disease of Potatoes*” (https://extension.umaine.edu/publications/wp-
content/uploads/sites/52/2020/06/2273-reviewed-2020.pdf) and subsequently spread to all the potato growing areas, affects potato development from emergence to harvest. In addition to the development of unsightly sclerotia or ‘black scurf’ on tubers, this affects marketability of the crop (Bakali and Martin 2006). It is distributed in India in different regions at different levels of severity and is a major disease problem in fields where potato is cultivated yearly in the same field (Khurana et al 1998, Arora 2012). The black scurf disease cause yield loss up to 25% in the hill while in plain, it cause about 10% yield loss (Sharma, 2015). Out of various strategies available for the disease’s management, the chemical based strategies have been by far most dominating. Seed treatment with 3% boric acid has been identified as a safe and effective chemical treatment and has been recommended for the control of this disease (Arora 2005). Various methods of disease control, biological control agents, is the most preferred natural choice to manage plant diseases that can enhances crop yields by growth promoting attributes of environment friendly microorganisms (Kiewnick and Sikora 2006, Mohsin et al 2010, Hussain and Khan, 2020). Many researches showed that Bacillus strains can be used as biological control agents against R. solani infecting pepper (Ahmad et al 2003.), potato (Szczech et al 2006, Calvo et al 2010, Hussain and Khan, 2020), Tomato (Khedher et al 2015) plant, and so forth. Now a days, biosurfactant microbes are gaining very importance in plant protection. These biosurfactants are surface active compounds produced by a variety of microorganisms their mode of action, in biological control involves the formation of channels in the cell wall and perturbations of the cell surface of the pathogen (Raaijmakers et al 2006). The best-known biosurfactants in biological control are cyclic lipopeptides and rhamnolipids (Stanghellini and Miller 1997). The concept of biosurfactants involved in the biological control activity of plant pathogens by the bacterial strains was also proved by the work done by (Anjaiah et al 1998, Tambong and Hofte 2001). Surfactins and iturins are lipopeptides produced by Bacillus subtilis Cohn (Kavitha et al 2012). In previous studies (Pusey et al 1988 and Pusey and Wilson 1984) showed that Bacillus subtilis could be a potentially better biocontrol agent for the crop diseases produced by harmful fungi and bacteria. Gokte and Swarup (1988), Hussain et al 2020a also showed that cells and spores of B. subtilis were toxic to nematodes Meloidogyne incognita, Heteroderca cajani, Heteroderca avenae and Anguina tritici. The biocontrol methods are attracting more importance now a day, due to their safe environmental friendly in nature. In our study we focused on the oil contaminated soil near Paradwip port, isolated a new biosurfactant strain, preliminary characterization and identify its antagonistic activity against the pathogen Black scurf is reported here.

MATERIALS AND METHODS

Survey and Sample Collection
Soil samples mixed with crude oil/coal powder were collected (beneath 5cm of surface) from near Paradwip port site, Paradwip, Odisha (20°15′55.44″N) during 2017. Soil samples were collected in clean plastic bags. These samples were used immediately to prevent any deterioration.

Isolation of biosurfactant bacteria
At plant pathology lab., Dept. Of Botany, AMU, Aligarh in 2017, five gram of oil contaminated soil sample was inoculated in 100 ml minimal medium (Miller, 1972) and incubated at 37°C, at 200rpm for 48hrs. From that 100ml stock serial dilution technique was used for isolation of biosurfactant bacteria, 1 ml from the soil suspension at (10^2 to 10^7) dilution was spread on each petri plate of RA2 agar medium (Himedia, Mumbai) and these plates were incubated at 37°C for 48 hrs dark bacterial incubator. Morphologically different colonies were randomly picked/selected and purified on Nutrient agar medium plates (with 0.1% petrol mixed) (Shoeb 2006). The selected bacterial isolates were stored on Nutrient agar (Himedia, Mumbai) petri plates as well as on Nutrient agar slants (with 0.1% petrol mixed) slants and kept under 4°C.

Screening methods for biosurfactant activity
The isolated bacteria were tested further for biosurfactant production through following methods:

Penetration Assay (PA)
This method relies on the phenomenon of silica gel entering the hydrophilic phase from hydrophobic paste much more quickly in the presence of biosurfactant which leads to a colour change (Walter et al
In this assay, the cavities of 96 well microtiter plate (Corning, USA) were filled with 150μl of a hydrophobic paste made up of oil (petrol) and silica gel. The paste was covered with 20μl of oil. The 10μl of 1% safranin was added to 90μl of the culture supernatant. In the control, uninoculated medium was added instead of culture supernatant. This coloured supernatant was then placed on the surface of the paste. The upper phase changes from clear red to cloudy pink red within 15 minutes if biosurfactant is present. Biosurfactant free supernatant was turned cloudy but stayed red. Based on the results of qualitative screening tests, positive isolates showing good surfactant activity were selected for further studies.

Oil Spreading Method (OSM)
For this test, oil (Petrol) was layered over water in a petriplate (9cm). 10μl supernatant (from culture broth) was added to the surface of oil (petrol) as described by (Morikawa et al 2000). Occurrence of clear zone was an indication of biosurfactant production. Water drop was used as a negative control (Morikawa et al 1993).

Haemolytic Activity (HA)
The pure culture of bacterial isolates (24hrs) was streaked on the freshly Sheep blood agar plates (Potous Medium, New Delhi) and incubated at 37°C for 24-48 hrs to assay haemolytic activity. Haemolytic activity was detected with the presence of a clear zone (Haemolysis) around bacterial colonies. The plates were visually inspected for clear zones of clearing around colonies (Satpute et al 2008).

Emulsification Index (EI24%)
The emulsifying capacity was evaluated by an emulsification index. The EI24% of samples was determined by adding 2 ml of petrol and 2 ml of the cell-free broth in test tube, vortexed at high speed for 2 min and allowed to stand for 24hrs. The percentage of emulsification index was calculated by using the following equation (Youssef et al 2004).

\[ EI24\% = \frac{\text{Height of emulsion formed}}{\text{Total height of solution}} \times 100 \]

Isolation of Fungal pathogen
The field collected infected tubers with black scurf of potato were surface sterilized by 0.25% Sodium hypochlorite for two minutes and extensively washed with sterile distilled water. The black sclerotia presented on the surface of potato were peeled and placed on petri plates containing potato-dextrose-agar (PDA) and incubated at 26±2°C for 48-72 hr. Subsequently, grown fungal culture was purified by hyphal tip method and identified by compound microscopic. Pure culture of \textit{R. solani} was preserved at 4°C in culture tubes and petri plates containing PDA for further study at department Plant pathology lab.

PCR based 16S rRNA gene amplification and Sequencing
The freshly inoculated 24hrs bacterial broth culture of OD02 was inoculated in sterilized nutrient broth (0.1% petrol mixed) used for DNA extraction. After 24 hrs bacterial DNA was extracted by CTAB method as follows: About 1.5 ml culture cultivated on nutrient broth was centrifuged to obtain pellet. The pellet was suspended in 1.5ml Tris EDTA by vigorous pipetting to ensure proper distribution of the culture in TE buffer followed by centrifugation at 10,000 rpm for 5min. This step was repeated by decanting and further addition of TE buffer followed by centrifugation. By discarding supernatant fresh TE buffer (740μl) was added along with 20μl Lysozyme (100mg/ml) and mixed 6 well by incubating for 5min. at room temperature. After incubation, 40μl of 10% SDS was carefully added, followed by 8μl of proteinase K (10mg/ml) while mixed well. The total mixture was incubated for 1hr at 37°C. The mixture was supplemented with 100μl of 5M NaCl and mixed well. Then the addition of 100μl CTAB/NaCl solution which was preheated at 65°C was done. Final mixture was incubated for reaction at 65°C in pre-adjusted water bath for 10min. After incubation, the mixture was supplemented with 0.5ml chloroform: isoamyl alcohol (24:1) and mixed well. Followed by mixing, tubes were spun down at maximum speed for 10min. at room temperature. Developed upper aqueous layer was transferred to a fresh tube. To aqueous layer 0.5 ml phenol: chloroform: isoamyl alcohol (25:24:1) was added and...
mixed well. Liquid was then centrifuged at maximum speed for 10 min. at room temperature, while obtaining an upper aqueous layer was transferred to a fresh tube. It was added with 0.6 vol. of isopropanol kept initially in −20°C and incubated at room temperature for 30 min. followed by centrifugation at maximum speed for 15 min., supernatant was decanted and pellet washed with 70% ethanol. It was centrifuged at maximum speed for 5 min. Supernatant was removed and pellet air dried by keeping it at room temperature for 5-10 min. On a dry pellet 20μl TE was used for resuspension and stored at −20°C until further use. The successful isolation of genomes was analyzed on 0.8% agarose gel.

About 20ng of bacterial gDNA was used to amplify 16S rRNA gene applying following 16S Universal primers 16S (F) AGA GGT TGA TCC TGG CTC AG, (R) AAG GAG GTG ATC CAG CCG CA. The PCR amplifications of 16S rRNA gene of bacterial isolates were performed for the total of the 50μl reaction mixture. The amplification mixture comprised of 32.0 μl nuclease freewater, 5.0 μl PCR buffer 10x, 2.0 μl dNTP (10 mM), 4.0 μl forward primer (10 μM), 4.0 μl reverse primer (10 μM), 1.0μl Taq DNA polymerase enzyme (1U/ μl) and 200ng DNA template. The temperature range for 50 μl each PCR reaction was programmed at the following conditions: initial denaturation of 3 min. at 94°C, denaturation of 1min. at 94°C, primer annealing for 1 min. at 54°C, extension of 30sec at 72°C, final extension for 5 min. at 72°C; total 30 cycles. The amplified PCR products were sent to the private firm for sequencing that was performed in ABI prism 3100 Genetic Analyzer (Applied Biosystems). The sequences were checked against the microbial nucleotide databases using BLASTN search algorithms (Mendez 2014) (http://www.ncbi.nlm.nih.gov/ BLAST).

**Phylogenetic analysis**

For the phylogenetics analysis, DND file obtained from CLUSTAL alignment was used for the phylogram built up by using the MEGA 6.06 software. In an output, built phylogram was documented close homology of the bacteria isolated (showcased with the accession number) with the best matched bacterial sequence and highlighted by marking in a Phylogram.

**Chemical characterization of biosurfactant**

**Thin layer chromatography (TLC) analysis:** The partially purified biosurfactant obtained from *B. subtilis HussainT-AMU* strain was characterized by TLC plate made up of aluminum sheet silica gel 60F24 plate (Merck) with solvent system using chloroform: methanol: acetic acid (75:15:2). Ninhydrin reagent was sprayed to confirm the type of biosurfactant (Anandraj et al 2010).

**Evaluation of *B. subtilis HussainT-AMU* against *R. solani:** This novel bacteria *B. subtilis HussainT-AMU* is maintained at Dept. of Botany, Aligarh Muslim University, Aligarh was used for present study.

**Dual culture assay**

Bio-efficacy of *B. subtilis HussainT-AMU* was evaluated against *R. solani* in dual culture test. Mycelial discs (5 mm) were cut from the 2 to 3 days old cultures of *R. solani* with the help of a cork borer and placed in the petri plates containing sterilized potato dextrose agar at the periphery. The bioagents were streaked on the other side of petri plate at equal distance. All the petri plates were incubated at 28±2°C, till the radial growth of control petri plates were full. Radial growth and percent inhibition of test pathogens was measured as per Vincet 1927, at different time intervals (24, 48, 72 and 96 hrs).

**Food-poisoning method**

The culture filtrate of *B. subtilis HussainT-AMU* was prepared as described by Tomar et al 2013, and were sterilized with 0.45μm filters and tested against *R. solani*. Different concentrations of culture filtrates (0.25, 0.5, 1.0, 2.0, 3.0, and 5.0%) were used in food-poisoning method (Grover and Moore 1962). Calculated volume of the culture filtrate was added to the molten PDA medium and poured aseptically to the petri plates and allowed to solidify. Five mm² of freshly growing (3-4 days) *R. solani* culture was placed at the centre of the petri dishes. Unamended medium served as the control. Three replications were maintained for each treatment. All the petri plates were incubated at 28±2°C, till the radial growth of control petri plates were full. Radial growth of *R. solani* was measured at different intervals and inhibition percentage was calculated (Mohsin et al 2010).
RESULTS
Isolation of bacteria from soil
Total, 12 unique different aerobic bacterial populations were isolated from crude oil/coal powder soil samples collected from Paradwip port, Odisha. Repeated streaking and sub-culturing of these bacterial isolates were purified and screened for the initial biosurfactant production bacteria test on specific growth media and after that for the biosurfactant production conformation tests was carried out. Those isolates which exhibited best biosurfactant activity were carried for further screening and were maintained at 4°C.

Screening for Biosurfactant production activity
Our study showed that all the bacterial isolates producing biosurfactant activity display good emulsification with oil. The culture supernatant placed on the surface of the hydrophobic paste, resulted in colour change in upper phase from red to cloudy white with OD02 isolate only which was noted as positive test. Partial change in colour or appearance of red colour wells was observed in rest OD01,03-10 isolates as shown in Figure 1. Overnight culture of OD02 isolated bacteria was centrifuged and added to oil (petrol) containing petri plate. OD02 isolate showed the clear zone (2.3cm) by being able to displace the oil (petrol) around the colony indicating biosurfactant production Figure 2. Rest other OD(01,03-10) isolates showed partial activity (between 0.3-0.5cm). No clear zone was observed with control (water). The haemolytic activity was observed in only OD02 isolate, results showed positive haemolytic activity (complete clear zones of more than 2cm) in Figure 3 while other rest of isolates OD(01,03-10) showed partial activity which were not considered in our further study. Emulsification index of >50% or more was considered as significantly positive emulsification activity. In our study, revealed that among twelve bacterial isolates, only OD02 exhibited >70% positive emulsification activity with hexane, showed 57% positive emulsification activity with xylene, and 95% were miscible with oil (petrol) Figure 4. Meanwhile, OD(01,03-10) of the isolates exhibited partial emulsification with 3 hydrocarbons tested.

![Figure 1](image1.png)

**Figure 1.** Penetration assay test for the screening of potential biosurfactant bacteria. Lane 1-10 randomly isolated suspected biosurfactant bacteria, W- Water, N-Negative control.

![Figure 2](image2.png)

**Figure 2.** Oil displacement test with OD02 culture filtrate.
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**Figure 3.** Haemolytic activity test, with OD02 bacteria.

**Figure 4.** Emulsification assay test

**Isolation, purification and identification of pathogen**

Naturally infected potato tubers were collected from the agricultural field at Aligarh-Hathras region in 2017 for isolation and purification of the causal fungus in the laboratory. Cultures were purified by single hyphal tip method and were maintained on PDA at 28±2°C (Figure 5). The isolated fungi were identified on the basis of following morphological characteristics. The genus *R. solani* belongs to form Class Deuteromycetes that does not make vegetative spores and present as mycelium and sclerotia. It produces shade of brown hypha, constriction at the point of branching and right angle branching in matured hyphae. Sclerotia were undifferentiated aggregations of thick-walled cells, small (1-3-mm diameter) irregular-shaped, brown to black structures (Gutierrez et al 1997). Similar result on isolation, purification and identification were reported by Parmeter and Whitney (1970), Domand and Flentje (1970). White cottony raised mycelia growth observed in Potato Dextrose Agar medium.

**Figure 5.** *R. solani* isolated from potato tuber skin.
PCR based method identification
Results obtained from sequencing showed that atypical better bacterial OD02 belongs to genus Bacillus later named as Bacillus subtilis HussainT-AMU (NCBI Accession No. LC385950) (Figure 6).

![Figure 6. Phylogenetic tree relationship between B. subtilis HussainT-AMU and other Bacillus species.](image)

Thin Layer Chromatography
The sediment obtained was placed in the TLC plate and the plates when sprayed with Ninhydrin reagent (95Ethanol+5H_2SO_4+1gm Ninhydrin reagent) and dried for 10min in hot air oven at 100ºC. The plate showed red-pink spots indicating the presence of lipopeptides class of biosurfactants in the extract (Figure 7).

![Figure 7. Chemical characterization of metabolite produced by B. subtilis HussainT-AMU through Thin Layer Chromatography.](image)
Inhibitory effect of *B. subtilis HussainT-AMU* on the growth of *R. solani* in dual culture and Food poisoning method

*B. subtilis HussainT-AMU* was tested for its bio-efficacy against *R. solani* using dual culture method. The new *Bacillus* strain significantly reduced the growth of pathogen (45±0.30%), in comparison to control (Figure 8). Pathogen could not grow well in the presence of bacterial secondary metabolites. There was a significant difference between the bacterial treatments to % inhibition of fungal radii. Growth of *R. solani* was significantly inhibited by the by antagonistic bacteria tested. 59.60% inhibition of fungal radii was exhibited by *B. subtilis HussainT-AMU* strain most efficiently. Our result revealed that the culture filtrate was effective and significantly inhibiting the growth of *R. solani* at concentration of 3% and above (Figure 9).

![Figure 8. Dual culture assay with *R. solani* with *B. subtilis HussainT-AMU*.](image)

![Figure 9. Pathogen (*R.solani*) growth inhibition by *B. subtilis HussainT-AMU* under food poisoning method](image)

DISCUSSION

Both soil-borne and tuber-borne inoculum of *R. solani* is important in disease on potato (Frank and Leach 1980, Demirci 2011). Present chemical and cultural control methods have reduced the soil-borne and tuberborne inoculum, although research has been directed toward the use of antagonists for biocontrol of black scurf on potato. In the present study, rhizosphere associated bacteria was evaluated for its antagonism against *R. solani*, causative agent of potato black scurf. Biosurfactants are amphipathic compounds which contain hydrophobic and hydrophilic moieties that reduce surface tension and interfacial tension between individual molecules at the surface and the interface.
Biosurfactant are known to have good penetration ability (Youssef et al 2004). Ability of some strains to show positive biosurfactant production following one method and negative following other methods makes it very difficult to confirm biosurfactant production using two or three methods. In view of this, several screening methods were considered in order to identify the potential organism that could produce biosurfactant. Compared to other isolates OD-02 exhibited good potential activity in the entire screening test for biosurfactant production activity. Hence, it was chosen as promising bacteria for further experimental study. Penetration method relies on the phenomenon of silica gel entering the hydrophilic phase from hydrophobic paste much more quickly in the presence of biosurfactants which leads to a colour change (Walter et al 2010). Biosurfactant free supernatant was turned cloudy but stayed red in comparison to OD02 isolate. The oil spreading method is rapid and easy to carry out, requires no specialized equipment, and only requires a small volume of sample (Plaza et al 2006). In our study, only OD02 was found to be positive by oil-spreading method indication production of biosurfactant. Displacement of oil clearly is a sign of extracellular surfactants present in the supernatant of cultures that is in compliance with our study also. Haemolytic activity appears to be a good screening criterion in the search for biosurfactant-producing bacteria (Carter 1984; Hussain and Khan, 2018). The haemolytic activity of biosurfactants was first discovered when Bernheimer and Avigad (1970) reported that the biosurfactant produced by B. subtilis, surfactin, lysed red blood cells. Blood agar lysis has been used to quantify surfactin (Moran et al 2002). This test is generally carried out as a primary method for screening of biosurfactant-producing bacteria. Isolates were tested for haemolytic activity, which is regarded by some authors as indicative for biosurfactant production and used as a preliminary method for bacterial screening that was in agreement with previous findings (Mulligan et al 1984, Carrillo et al 1996, Banat et al 2000, Zhang et al 2012, 2016). To determine the potential of a good biosurfactant emulsification activity is one of the good criteria (Bonilla et al 2005). In the present investigation OD01, 03-10 isolates showed partial emulsification potential, and only OD02 isolate gave a good emulsification index (over 50%) with all 3 hydrocarbons tested, which included hexane, xylene, and oil (petrol). Sequencing of 16S rRNA allows the accurate identification of bacterial genera from various microbes species. Bacillus is an important bacteria genus because of the synthesis of a wide range of metabolites with diverse properties (Jacobsen et al 2004, Velmurugan et al 2009). The 16S rRNA sequence analysis, and the high similarity 99% values confirmed that new Bacillus belong to the same species (Liu et al 2014) respectively, of which the phylogenetic tree is given in Figure 6. The precise definition of species and subspecies analysis of other genes is needed (Chun and Bae 2000). Minaxi and Saxena, (2010) observed that the inhibition of various pathogens viz., Colletotrichum truncatum, Drechslera graminea, Fusarium oxysporum, F. solani, M. phaseolina, Rhizopus arrhizus and Sclerotium rolfsii with P.aeruginosa RM-3 under dual culture method. The antagonistic activities of Pseudomonas sp. Bacillus sp. were showed positive inhibition of mycelial growth of P. infestans, Fusarium spp and R. solani under in vitro that is in agreement with previous findings of Tomar et al 2014, Hussain and Khan 2018a, Hussain et al 2019. Ahmadzadeh et al 2004 reported that antagonistic rhizobacteria, more specifically fluorescent pseudomonads and certain Bacillus species possessed the ability to inhibit fungal and bacterial root diseases of agricultural crops. Mycelial growth was completely inhibited, when the volume of the culture filtrate increased. The level of reduction was being of very low (below 3%). However, Minaxi and Saxena, (2010) reported that P. aeruginosa strain RM-3 was effective for inhibiting the growth of M. phaseolina and Drechslera gramineae at 3%. Some researchers have found that B. subtilis showed significant inhibitory effects on the growth of R. solani by reduced disease incidence and promoted plant growth (Calvo et al 2010, Khedher et al 2015, Hussain and Khan 2020, Khan et al 2020).D’aes et al (2011) reported that biocontrol research on biosurfactant has predominantly focused on the control of zoospore-producing plant pathogen, such as Phytophthora spp. where the mode of action lies in zoospore lysis. Only a few studies have demonstrated the involvement of biosurfactant in biological control of non-zooospor producing plant pathogen which did not produced any positive response. They also reported that among the great variety of beneficial bacteria and metabolites already discovered, their attention has been focused on lipopeptides and the biosurfactant produced by Bacillus spp. The ability of different Bacillus species to prevent several plant diseases has resulted in the commercial development and registration of several Bacillus pest management bioproduts that can be integrated into pest management regimens to efficiently control plant diseases. Some potato varieties differ in their susceptibility to Rhizoctonia; however, no resistant varieties are currently available. To date no variety has been found with immunity
to the sprout nipping and stem lesion phase, although some varieties show varying degrees of resistance to formation of sclerotia on tubers. Tuber inoculum is more important than the soil inoculum as the primary cause of disease. It is very important to develop fast and efficient methods to select biocontrol microorganisms, especially when evaluating a large number of candidates. In vitro evaluations allow for a reduction in the number of isolates, which makes further in vivo tests viable. Hence, it was reconfirmed in our research work also that only a very good control of R. solani fungus was observed by particular strain of B. subtilis Hussain-T-AMU and its metabolite can be very effective for the integrated diseases management of the plant pathogens in near future.

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