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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

सारांश

आलुको कालो खोस्टे रोग विश्वमा आलु खेतीको एउटा प्रमुख समस्या हो र यो रोग बीउ आलुबाट सर्ने प्रवृत्तिको भएकोले यसलाई कृत्रिम कृषि रसायनको प्रयोगबाट व्यवस्थापन हुँदै आएको छ जसले गर्दा वातावरणीय प्रदुषण बढ्ने तथा फार्म उत्पादहरुमा विषादी अवशेष रहने समस्या रहेकोछ। त्यसैले हालका दिनहरुमा जैविक पदार्थहरुको प्रयोगद्वारा कालो खोस्टे रोगको व्यवस्थापन गर्ने तरिकाहरु बढी रुचाइएका छन् । तेलले बिगारेको माटोबाट आइसोलेट गरी उक्त आइसोलेटको द्वितीय जैविक मेटाबोलाइटलाई निकाली पूर्व स्क्रीनिङ गरी एक प्रचलित जैविक सर्फेक्टेन्ट ODO2 आइसोलेट गरिएको थियो । यस सर्फेक्टेन्टलाई हेमोलाइटिक गतिविधि, इमल्सिफिकेसन इन्डेक्स तथा तेलको वहाव परीक्षण साथै प्राइमर 16SrRNA सँगको यौगिक चारित्रिकरण गर्दा सकारात्मक नतिजा प्राप्त भएकोले यो सर्फेक्टेन्ट व्यासिलस समूहको हो भन्ने पुष्टि हुन्छ। साथै यसलाई थप TLC को प्रयोग गरी रासयनिक चारित्रीकरण गर्दा यसले लाइपापेप्टाइटको रातो गुलाबी रंग दियो र टेष्टट्युबमा गरिएको डुअल कल्चरमा *Rhizoctonia solani* को गतिविधिहरुसँग विपरित गतिविधिहरु (४५±०.३०%) र खाद्य विषादीकरण तरिकामा ३% गाठा गतिविधि देखायो । कालो खोष्टे लगाउने *R. solani* जीवाणुको विरुद्धमा प्रयोग गर्न *Bacillus subtilis HussainT-AMU* प्रजाति बढी प्रभावकारी पाइयो जसलाई भविष्यमा यस्ता रोगहरु व्यवस्थापन गर्ने रणनीतिहरुमा उपयोग ल्याउन सकिन्छ ।

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*, *Heterodera cajani*, *Heterodera 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 37ºC, 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 4ºC.

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

2010). 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 *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 37ºC. The mixture was supplemented with 100µl of 5M NaCl and mixed well. Then the addition of 100µl CTAB/NaCl solution which was preheated at 65ºC was done. Final mixture was incubated for reaction at 65ºC 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 –20ºC and incubated at room temperature for 30 min. followed by centrifugation at maximum speed for 15min., 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 GTT 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 94ºC, denaturation of 1min. at 94ºC, primer annealing for 1 min. at 54ºC, extension of 30sec at 72ºC, final extension for 5 min. at 72ºC; 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±2ºC, 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 mm^2 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±2ºC, 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 4ºC.

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.

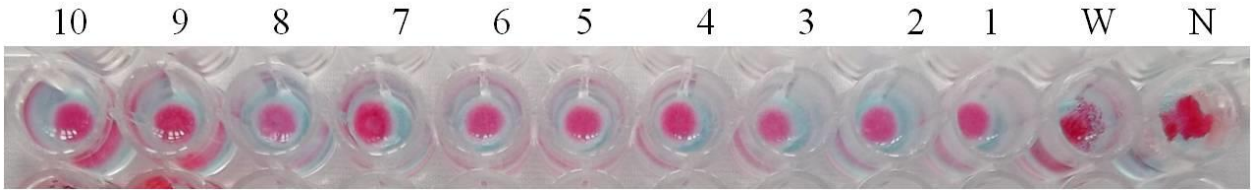

Figure 1. Penetration assay test for the screening of potential biosurfactant bacteria. Lane 1-10 randomly isolated suspected biosurfactant bacteria, W- Water, N-Negative control.

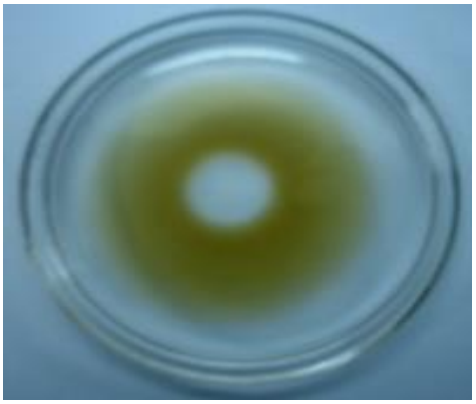

Figure 2. Oil displacement test with OD02 culture filtrate

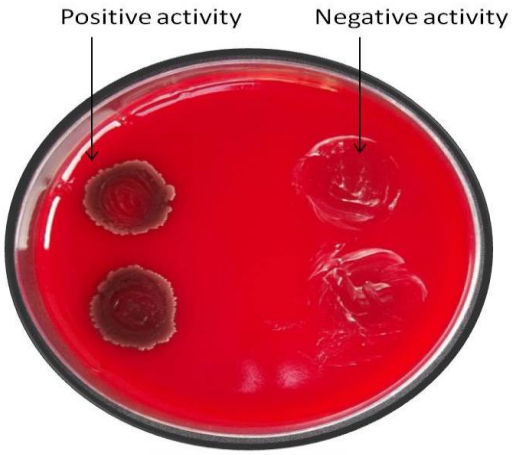

Figure *3.* Haemolytic activity test, with OD02 bacteria.

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 (Guttierez 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.

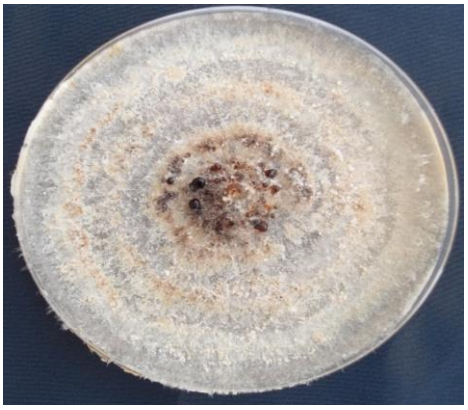

Figure *5. R. solani* isolated from potato tuber skin.

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**).

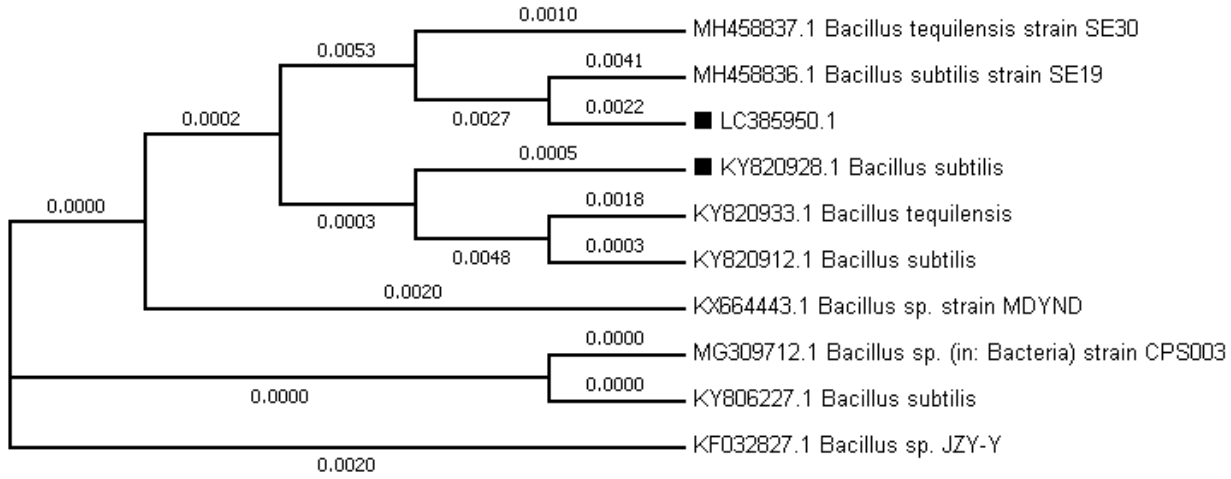

Figure 6. Phylogenetic tree relationship between *B. subtilis HussainT-AMU* and other *Bacillus* species.

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.

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*.

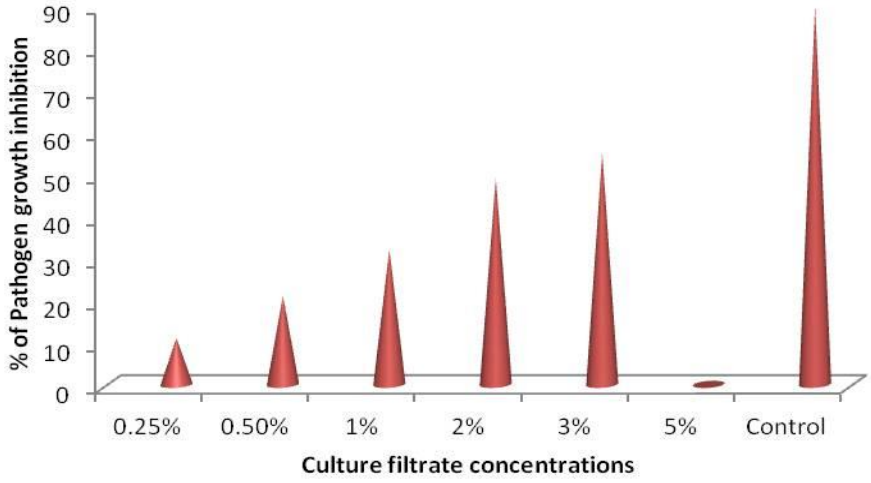

Figure 9. Pathogen (*R.solani*) growth inhibition by *B. subtilis HussainT-AMU* under food poisoning method

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 >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 trancatum*, *Drechslera graminea*, *Fusarium oxysporum*, *F. solani*, *M. phaseolina*, *Rhizopus arrhizus* and *Sclerotium rolfsi* with *P.aeruginoas 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 *Dreschlera graminaeat* 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 *Pythium* and *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-zoospore 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 bioproducts 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 HussainT-AMU* and its metabolite can be very effective for the integrated diseases management of the plant pathogens in near future.

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REFERENCES

Ahmed AS, M Ezziyyani, C Pérez Sánchez, and MzzE Candela. 2013. Effect of chitin on biological control activity of *Bacillus* spp. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. European Journal of Plant Pathology **109**:6:633–637.

Arora RK.2012**.** Eco-friendly management of soil and tuber borne diseases of potato. Indian Phytopathology **65**:116-21.

Arora RK.2005. Efficacy of boric acid spray for control of black scurf in unwashed and washed potato tubers. Potato J **32**:183-184.

Anjaiah V, N Koedam, B Nowak-Thompson, JE Loper, M Hofte, JT Tambong and P Cornelis. 1998. Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. Mol Plant Microbe Interact **11**:847–854.

Anandaraj B, P Thivakaran.2010. Isolation and production of biosurfactant producing organism from oil spilled soil. J. Biosci. Technol. **1**(3): 120-126.

Ahmadzadeh M, AS Tehrani and KT Jahromi. 2004. Study on production of some antimicrobial metabolites by *fluorescent pseudomonads*. Iranian J. Agric. Sci., **35**(3): 731-739.

Bakali, AM EL, MP Martin.2006.Black scurf of potato. M y c o l o g i s t. **2 0**:130 – 132

Bernheimer AW, LS Avigad.1970.Nature and properties of a cytological agent produced by *Bacillus subtilis*. J Gen Microbiol **61**:361–369

Baker K, R Cook.1974. Biological control of plant pathogens. W.H freeman Company, San Francisco, USA, **p.**433.

Banat IM, Makkar RS, Cameotra SS.2000. Potential commercial Application of Microbial Surfactants. Applied Microbiol. Biotechnol **53**:495-508.

Bonilla M, C Olivaro, M Caronal, A Vazquez, M Soubes.2005. Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. J Appl Microbiol **98**:456–463.

Carter G.1984. Diagnostic Procedures in Veterinary Bacteriology and Mycology,4th **ed**. Springfield, IL, USA: Charles C Thomas.

Carrillo P, C Mardaraz, IS Pitta-Alvarez, AM Giulietti.1996. Isolation and selection of biosurfactant-producing bacteria. World Journal of Microbiology and Biotechnology **12**:82-84.

Calvo E. Ormeño-Orrillo, E. Martínez-Romero, and D. Zúñiga.2010. Characterization of *Bacillus* isolates of potato rhizosphere from Andean soils of Peru and their potential PGPR characteristics, Brazilian Journal of Microbiology **41**: 4 899–906.

Chun J, KS Bae. 2000. Phylogenetic analysis of *Bacillus subtilis* and related taxa based on partial gyrA gene sequences. *Anton Van Leeuw J Microbiol*.**78**:123–127.

Dodman, R. and Flentje, M. T. 1970. The mechanisms and physiology of plant penetration by *Rhizoctonia solani.* In : Parmeter, J. R. Jr. (**ed**.). *Rhizoctonia solani :* Biology and Pathology. University of California Press, Berkeley, USA.149-160.

Demirci E, E Dane, C Eken.2011.*In vitro* antagonistic activity of fungi isolated from sclerotia on potato tubers against *Rhizoctonia solani.* Turk J Biol **35**:457-462.

D'aes J, GKH Hua, K De Mayer, J Pannecoucque, I Forrez, M Ongena, LEP Dietrich, LS Thomashow, DV Mavrodi and M Hofte.2011. Biological control of *Rhizoctonia* root rot on bean by phenazine and cyclic lipopeptide-producing *Pseudomonas* CMR12a. Phytopathology **101:**996-1004.

Durak E.2016. Biological Control of *Rhizoctonia solani* on potato by using indigenous *Trichoderma* spp. AIP Conference Proceedings 1726, 020020.

Errampalli D and HW Johnston.2001. Control of tuber-borne black scurf (*Rhizoctonia solani*) and common scab (*Streptomyces scabies*) of potatoes with a combination of sodium hypochlorite and thiophanatemethyl preplanting seed tuber treatment. Can. J. Plant Pathol. **23**:68–77.

Frank JA, Leach SS.1980.Comparison of tuberborne and soilborne inoculum in the *Rhizoctonia* disease of potato. Phytopathology **70**: 51-53

Geisslera M, C Oelligb, K Mossa, W Schwackb, M Henkela, R Hausmann.2017. High-performance thin-layer chromatography (HPTLC) for thesimultaneous quantification of the cyclic lipopeptides Surfactin,Iturin A and Fengycin in culture samples of Bacillus species. Journal of Chromatography B, **1044**–1045:214–224.

Guttierez, W. A., Shew, H. D., and Melton, T. A. 1997. Sources of inoculum and management of *Rhizoctonia solani* damping-off on tobacco transplants under greenhouse conditions. *Plant Dis*., **81**:604-606.

Gokte N, Swarup G (1988) On the potential of some bacterial biocides against root knot nematode 666 and cyst nematodes. Indian J Nematol 18:152–153 Gokte N, Swarup G (1988) On the potential of some bacterial biocides against root knot nematode 666 and cyst nematodes. Indian J Nematol 18:152–153

Gokte N, G Swarup.1988. On the potential of some bacterial biocides against root-knot and cyst nematodes. Indian Journal of Nematology. **18**:152-153.

Grover RK and JD Moore.1962. Toximetric studies of fungicides of brown rot organism *Sclerotinia fruticola* and *S. laxa*. Phytopathology **52**:876-880. https://extension.umaine.edu/publications/wp-content/uploads/sites/52/2020/06/2273-reviewed-2020.pdf

Hussain A, MS Awan, SW Khan, MAS Ali, QAA AlI .2014. Bioefficacy of botanical extracts and bioagents against sclerotial isolates of *Rhizoctonia solani*. J Biodiv Envir Sciences. **4**: 370-380.

Hussain T, M Haris, A Shakeel, AA Khan, MA Khan.2020a.Bio-nematicidal activities by culture filtrate of *Bacillus subtilis HussainT-AMU*: new promising biosurfactantbioagent for the management of Root Galling caused by *Meloidogyne incognita*. Vegetos **33**:229-238

Hussain T and AA Khan.2020.*Bacillus subtilis HussainT-AMU* and its antifungal activity against Potato black scurf caused by *Rhizoctonia solani*. Biocatalysis and Agricultural Biotechnology.**23**:101433.

Hussain T and AA Khan.2018.A combination of rapid and easy assays of biosurfactant producing bacteria strain isolated from automobiles repairing workshop in Aligarh. Proceedings of the Voronezh State University of Engineering Technologies **80**(3):153-163.

Hussain T and AA Khan.2018a.Biological control of black scurf of potato caused by *Rhizoctonia solani* using lipopeptides produced by novel biosurfactant bacteria: *Bacillus subtilisHussainT-AMU* isolated from Paradwip port, Odisha, India. National Conference on Biointensive Approaches in Plant Protection Strategies and their Socio-economic impacts held on 29th-30th Oct. 2018 at Dept. of Plant Protection, Aligarh Muslim University, Aligarh, India under Indian Phytopathological society, Mid-Eastern Zone region Annual meeting. OP, **pp** 31.

Hussain T, AA Khan and S Tomar. 2019.Biocontrol of dry rot of potato seed tubers caused by *Fusarium* sp. by using novel biosurfactant bacteria *Bacillus firmus HussainT-AMU: Lab.66* producing cyclic lipopeptides. Indian Plant science Congress 2019, from 23rd to 25th Jan.2019 at SRM Institute of Science and Technology, Kattankulathur, TN.**pp**.101.

Jacobsen BJ, NK Zidack, BJ Larson.2004. The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. *Phytopathology* **94**: 1272–1275.

Juan-Abgona RV, N Katsuno, K Kageyama, M Hyakumachi.1996. Isolation and identification of hypovirulent *Rhizoctonia* spp. from soil. Plant Pathol, **45**: 896-904.

Khan MA, S Tomar and T Hussain.2020.Efiicacy of biosurfactant producing bacteria against fungal disease of potato.4th IUPAC International Conference on "Agrochemicals Protecting Crops, Health and Natural Environment –Discovery and development of synthetic and natural products for health and pests management" (APCHNE-2020) being organized by Department of Chemicals & Petrochemicals, Society for Promotion of Sustainable Agriculture (SPSA) and Institute of Pesticide Formulation Technology, Gurugram, during 7-10 January 2020, in New Delhi, India. **pp**.16.

Khurana SMP, SK Pandey, RL Bhale, BK Patel and BS Lakra.1998**.** Surveillance for potato diseases in India over last five years. Indian Journal of Potato Association **25** : 16-20.

Khedher SB, O Kilani-Feki, M Dammak, H Jabnoun-Khiareddine, M Daami-Remadi, and S Tounsi.2015. Efficacy of *Bacillus subtilis* V26 as a biological control agent against *Rhizoctonia solani* on potato, Comptes Rendus Biologies **338**: 12:784–792.

Kiewnick S and RA Sikora.2006. Biological control of the root-knot nematode *Meloidogyne incognita* by *Paecilomyces lilacinus* strain 251. Biol. Control **38**:179–187.

Kavitha PG, EI Jonathan and S NakkeeraN.2012. Effects of crude antibiotic of *Bacillus subtilis* on hatching of eggs and mortality of juveniles of *Meloidogyne incognita*. Nematol. Medit **40**:203-206.

Liu HL, YY Li, RC Ju, and HT Zhao.2014. Isolation, identification and fermentation optimization of antagonistic *Bacillus subtilis* KC-5, China Biotechnology, **34**:96–102.

Miller JH. 1972. Experiments in Molecular Genetics.Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., **p.** 433.

Moran A, Alejandra M, Martinez F et al.2002.Quantification of surfactin in culture supernatant by hemolytic activity. Biotechnol Lett **24**:177–180.

Morikawa, M, H Diado, T Takao, S Murata, Y Shimonishi, T Imanaka.1993. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. J Bacterio. **175**: 6459–6466.

Morikawa, M, Y Hirata, T Imanaka.2000. A study on the structure function relationship of lipopeptide biosurfactants. Biochim Biophys Acta **1488**:211–218.

Minaxi and Saxena, J.2010. Characterization of *Pseudomonas aeruginosa* RM-3 as a potential biocontrol agent. Mycopathologia, **170** (3):181-193.

Mohsin T, Y Sumera and HY Fauzia. 2010. Biological control of Potato Black scurf by Rhizosphere associated Bacteria. Brazalian Journal of Microbiology. **41**:439-451.

Mendez J. 2014. Characterization of phosphate-solubilizing bacteria isolated from the arid soils 523 of a semi-desert region of north-east Mexico. Biol Agric Hortic **30**:211-217.

Mulligan CN, DG Cooper, RJ Neufeld.1984. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol. **62**:311–314.

Parmeter J R Jr and HA Whitney. 1970. Taxonomy and nomenclature of the imperfect state. In Parameter, I. R. Jr. (**ed**.) *Rhizoctonia solani,* Biology and Pathology, London, Berkeley CA: University of California Press. 7-19.

Plaza G, I Zjawiony, I Banat.2006. Use of different methods for detection of thermophilic biosurfactant-producing bacteria from hydrocarbon-contaminated bioremediated soils. J Petro Science Eng. **50**:71–77.

Prasad CS, V Gupta.2002. Studies on bio efficacy of *Trichoderma harzianum* in management of stem rot of potato (*Rhizoctonia solani*). Natl Acad Sci Lett **25**:357-359.

Pusey PL, MW Hotchkiss, HT Dulmage, RA Banumgardner, EI Zehr.1988. Pilot tests for commercial production and application of *Bacillus subtilis* (B-3) for postharvest control of peach brown rot. Plant Disease **72**:622–626.

Pusey PL, CL Wilson.1984.Postharvest biological control of stone fruit brown rot by *Bacillus subtilis.* Plant Disease **68**:753–756.

Raaijmakers JM, I de Bruijn and MJD de Kock.2006. Cyclic lipopeptide production by plant-associated *Pseudomonas* species: diversity, activity, biosynthesis and regulation. Mol Plant Microbe Interact **19**:699–710.

Sharma S.2015.Black Scurf and stem canker. In A manual on diseases and pest of potato-Technical Bulletin No. 101 (Ed. BP singh, M Nagesh, Sanjeev Sharma, Vinay Sagar, A Jeevvlatha and J Sridhar) ICAR-central potato research institute, Shimla, HP, India. 2015. **pp**. 11-13.

Sharma BL and HS Sohi.1965. Control of black scurf diseases of potato in Himachal Pradesh. Indian Potato J **7**:12-17.

Shoeb E. 2006. Genetic Basis of Heavy Metal Tolerance in Bacteria. Ph.D. thesis. University of Karachi, Karachi, Pakistan.

Satpute SK, BD Bhawsar, PK Dhakephalkar, BA Chopade. 2008. Assessment of different screening methods for selecting biosurfactant producing marine bacteria. Indian J Marine Sci. **37**:243-250.

Stanghellini ME and RM Miller.1997. Biosurfactants: their identity and potential efficacy in the biological control of zoosporic plant pathogens. Plant Diseases **81**:4–12.

Somani AK.1986. Non-hazardous chemical control of black scurf of potato. Indian J.Agric. Sci, **56**:366-369.

Szczech and M Shoda.2006. The effect of mode of application of *Bacillus subtilis* RB14-C on its efficacy as a biocontrol agent against *Rhizoctonia solani*, Journal of Phytopathology, **154**(6):370–377.

Tambong JT and Ho¨fte, M.2001. Phenazines are involved in biocontrol of *Pythium myriotylum* on cocoyam by *Pseudomonas aeruginosa* PNA1. Eur J Plant Pathol **107**:511–521.

Tomar S, BP Singh, M Lal, MA Khan, T Hussain, S Sharma, SK Kaushik and S Kumar.2014. Screening of novel microorganism for biosurfactant and biocontrol activity against *Phytophthora infestans.* Journal of Environmental Biology, **35**:893-899.

Vincet JM.1927.Distortion of fungal hyphae in the presence of certain inhibitors. Nature, **159**:850.

Velmurugan N, MS Choi, SS Han, YS Lee. 2009. Evaluation of antagonistic activities of *Bacillus subtilis* and *Bacillus licheniformis* against wood-staining fungi: *in vitro* and *in vivo* experiments. *J Microbiol* **47**:385–392.

Walter V, Syldatk, C, R Hausmann.2010. Screening concepts for the isolation of biosurfactant producing microorganisms. Adv Exp Med Biol **672**:1-13.

Youssef NH, KE Duncan, DP Nagle, KN Savage, RM Knapp, MJJ McInerney .2004.Comparison of methods to detect biosurfactant production by diverse microorganisms. Microbiol Methods **56** (3):339–347.

Zhang J, Q Xue, H Gao, H Lai, P Wang.2016. Bacterial degradation of crude oil using solid formulations of *Bacillus* strains isolated from oil-contaminated soil towards microbial enhanced oil recovery application. RSC Advances **6** (7):5566-5574.

Zhang X, D Xu, C Zhu, T Lundaa, KE Scherr.2012. Isolation and identification of biosurfactant producing and crude oil degrading *Pseudomonas aeruginosa* strains. Chemical Engineering Journal **209**:138-146.

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