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Biocontrol Activity of *Bacillus cereus* Against Tomato Wilting Pathogen, *Pseudomonas solanacearum*

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ABSTRACT: The application of chemical pesticides to control plant disease has harmful effects to both plant and animals. Here, *Bacillus cereus* wasisolatedfrom rhizospheric soil of Similipal Biosphere Reserve, Odisha, India. The isolate showed highly antagonistic activity against plant pathogen *Pseudomonas solanacearum* NCIM 5103.The antagonistic molecule observed from the isolate to be extracellular and water soluble. During study, the isolate synthesized optimum antagonistic molecules at pH 7, 37^0C and 1% xylose, 0.5% yeast extract in the medium. During in vivo study, the isolate showed biocontrol activity against the infection of tomato plant caused by *P. solanacearum.*

KEYWORDS: pot experiment/ antagonistic/optimization/ sustainable agriculture/ wilt.

I. INTRODUCTION

Soil born plant pathogens are the most important factors that cause major loss to the agricultural product throughout the world. Farmers significantly used different chemicals to protect crops from different diseases. The use of heavy amount of chemicals in the crops affects soil fertility and ecosystem. Other hand, most of chemicals are no degradable, which accumulated in plant cell and causes biomagnifications. Hence, sustainable agriculture may be achieved using different biological agents [1]. The antagonistic activities of microorganisms towards the protection of plants from different diseases are important characteristics in rhizospheric regions. These microorganisms are producing different secondary metabolites such as antibiotics or toxins, organic acids which lead to control different infection [2,3,4]. When testing biocontrol activity of microorganisms (bacteria and fungi), about 1% to 10 % showed at least some capacity to inhibit the growth of pathogens under *in-vitro* condition [5]. However, some microorganisms have retained their antagonistic activity in diverse environmental conditions. Since, last decades, research has repeatedly revealed that genetically diverse microorganisms can act as natural antagonists against various plant pathogens. Bacteria of different genera were isolated and proved their use as biocontrol agents include *Agrobacterium, Bacillus, Pseudomonas, Streptomyces* and fungi belonging to the genera *Ampelomyces, Candida, Coniothyrium and Trichoderma* [6]. In this view, the present study was undertaken, to study the biocontrol potential of a soil bacterium *Bacillus cereus* isolated from the rhizospheric soil collected from Similipal Biosphere Reserve against the tomato wilting bacterial pathogen *Pseudomonas solanacearum.*

II. MATERIALS AND METHODS

A. ISOLATION OF PHYTOPATHOGEN

During the investigation, tomato plant (*Solanum lycopersicum*) was selected for this study. The diseased plants were collected in sterilized poly bags and brought to the laboratory for further studies fromKuliana Block of Mayurbhanj District, Odisha. The infected stem (about 5 -10 cm length) was taken and washed with 2-3 times with sterilized distilledwater. Release of juicy substance from the infected stem was streaked onto nutrient agar medium and incubated at 37^0C for 24hr. After the incubation period the plates were checked for the development of pathogen colonies along the line of streaking.

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B. STUDY OF BIOCONTROL POTENTIAL OF THE ISOLATES

During field study, it was observed that the tomato plant from these areas was damaged by bacterium wilt. Therefore, the Bio-control potential of the isolate (*B. cereus*) was assessed against phytopathogen (*Pseudomonas solanacearum* NCIM 5103) by Disc Diffusion Method. *Bacillus cereus* was isolated from rhizospheric soil of Similiap Biosphere Reserve, Odisha, India. The isolates were inoculated into 150ml nutrient broth in 250 ml conical flasks for 48hr at 37^0C. After, the incubation, cell mass was separated by filtration and the liquid broth was taken for extraction of antagonistic metabolites with equal amount of ethyl acetate. The ethyl acetate was evaporated to yield the crude metabolites. The extract was dissolved with 2ml of DMSO (DimethyleSulphoxide) and taken for the study of antagonistic activity. Freshly grown culture pathogen (*Pseudomonas solanacearum* NCIM 5103) was swept on NA plate. Then, Sterilized filter paper discs (5mm) were placed on NA surface at equidistance and 10µl of extract was transferred onto the paper disc aseptically. The plates were incubated at 37^0C for 24hrs. After, the incubation, the plates were observed for zone of inhibition around the disc. The zone sizes were measured and compared. All the experiments were carried out in triplicates.

C. MOLECULAR IDENTIFICATION OF THE ACTIVE ISOLATE

The isolate S5-1 showed better antagonistic activity against the tested plant pathogen *Pseudomonas solanacearum* NCIM 5103 and it was selected for further study. The molecular characterization was carried out by partial sequencing of16S rRNA using primer 518F and 800R. The rDNA sequence of 16S ribosomal RNA gene was amplified by PCR and was carried out by Macrogen Inc. South Korea. A comparative study of rDNA sequences of bacterial isolate (S5-1) with other bacteria was done using BLAST algorithm at the website www.ncbi.nlm.nih.gov.

D. CONDITION OPTIMIZATION FOR METABOLITE PRODUCTION
E. INCUBATION PERIOD, TEMPERATURE AND PH

The isolate S5-1 was incubated in different flask containing150ml Nutrient broth, for 18, 24, 30, 36 and 42 hrs and subsequently at different pH (4-10) and temperature (25^0C , 31^0C , 37^0C,43 ^{0}C & 49^0C). The metabolites was extracted from cultured broth with ethyl acetate and screened for antagonistic activity against *P. solanacearum* by Disc Diffusion Method (DDM) as described earlier.

F. CARBON AND NITROGEN SOURCES

This experiment was designed to study the effect of different carbon and nitrogen sources on biocontrol activity of the isolate. The carbon sources like fructose, galactose, dextrose, xylose, raffinose, arabinose, starch, maltose, trehalose, and sucrose were used at different concentration of 0.5%, 1%, 1.5%. The isolate (S5-1**)** was grown separately in the carbon supplemented medium at optimum pH, temperature and incubation period. Extraction of metabolites and biocontrol activity studies were carried out as per the method described earlier. Similarly, The nutrient medium was supplemented with different nitrogen sources (Ammonium Nitrate, Ammonium Sulphate, Sodium Nitrate, Calcium Nitrate, Asparagine, Potassium Nitrate and Glycine) in varied concentrations (0.1%, 0.5%, 0.7%). and the biocontrol activity was studied.

G. STUDY OF BIOCONTROL ACTIVITY (POT EXPERIMENT)

Three-week-old tomato plants were taken. The roots of tomato plant were washed by sterile distilled water three times. Then, it was rinsed by 60 % alcohol and immediately washed by sterile distilled water. Further, the roots were treated with tween 80 for removal of adhering microorganisms and spores. The pots were prepared by sterilized soil with requisite amount of humus. The experiment was conducted in five sets (A, B, C, D, E). Set A: Pot contains sterilized soil and root treated with *P. solanacearum.* Set B: Pot contains sterilized soil and root treated with S5-1(biological control agent). Set C: Pot contains soil inoculated with *P. solanacearum* and root treated with S5-1. Set D: Pot contains soil inoculated withS5-1 and plant root treated with *P. solanacearum.* Set E: Pot contains soil inoculated withS5-1and *P. solanacearum* and plant root was without treatment.Thedensity of the inoculums in the potted soil for the both the organisms (*B. cereus* and *P. solanacearum*) were 4.1 $X10^7$ and 5X 10^7 respectively. Similarly, root treatment of plant was carried out by dip with overnight culture of the isolate for 2 two hrs. All the experimental sets were grown in green house.

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H. CHARACTERIZATION OF BIOACTIVE COMPOUND BY TLC ANALYSIS

The ethyl acetate dissolved compound was taken for TLC analysis. In the analysis, different solvents were taken i.e. Ethyl acetate-hexane-water (3:6:1), and ethyl acetate-hexane-water (3:5.5:1.5). The silica gel- 254 GH (Hi-media) and thickness of 0.25 mm was taken during TLC.

III. RESULTS

In the field survey and interaction with the farmers, it was observed that 25-35% of tomato crops were lost by wilting. The infected plants were collected and

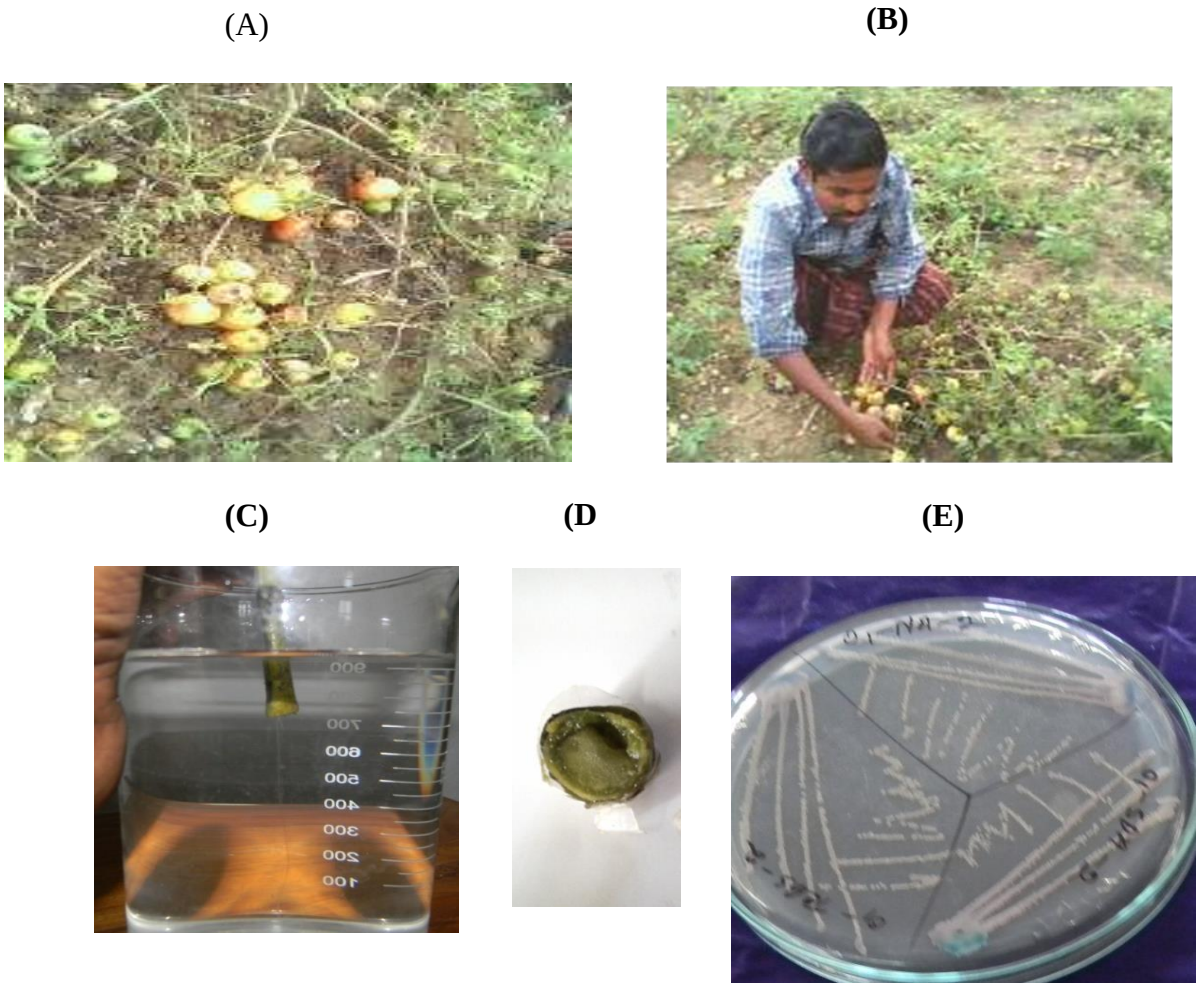

Figure 1: Study of the pathogenic characters of infected plants: (A & B) Field observation; (C) an infected stem dipped in sterile distilled water (release of juice into the medium); (D) section of an infected stem; (E) growth of the pathogen from the infected juice on NA plate.

their symptoms and type of diseases were studied. When the infected plant parts were put in the water, a slime and juice substances was obtained which provide the preliminary idea that plant might have been infected by bacterial pathogens (Fig. 1). The infected juice was streaked on nutrient agar plate and incubated. The bacterial colonies were developed along the streaking lines suggesting that infection made by bacterium. Again, it was confirmed by microscopic observation. Therefore, biocontrol activities of rhizopheric bacteria were studied against plant pathogens

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i.e.*Pseudomonas solanacearum* NCIM 5103. In the plate assay method, the bacterialisolate S5-1 was selected for studying as biocontrol agent against the plant pathogens *Pseudomonas solanacearum* NCIM 5103.

A. IDENTIFICATION OF THE SOURCE ORGANISM

The bacterial isolate (S5-1) was identified as *Bacillus* sp. by Gram's staining and series of biochemical reactions. The species confirmation was carried out by 16S rDNA. sequence analysis. The sequence was annotated and submitted to NCBI data base with accession number KM 273138. BLAST analysis of the sequence revealed that the species showed 99.9% closest homology with *Bacillus cereus*. Molecular phylogeny of the isolate with other bacterial isolates (38 taxas) revealed that the isolate clustered within the *Bacillus thuringeinsis* group with supported bootstrap of 98%. (Fig.2). The evolutionary history was inferred using the Maximum Parsimony method. The consensus tree inferred from 14 most parsimonious trees is shown. Branches corresponding to partitions reproduced in less than 50% trees are collapsed. The consistency index was (0. 533071), the retention index was (0.876792), and the composite index was 0.484090 (0.467392) for all sites and parsimony-informative sites. The percentage of parsimonious trees in which the associated taxa clustered together is shown next to the branches. The MP tree was obtained using the Close-Neighbor-Interchange algorithm with search level 1 in which the initial trees were obtained with the random addition of sequences (10 replicates). The analysis involved 39 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 1482 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [7].

B. EFFECT OF ENVIRONMENTAL FACTORS ON BIOCONTROL ACTIVITY

The effect of different environmental factors i.e. pH, incubation period, temperature on the biocontrol activity of the isolate was studied against *Pseudomonas solanacearum* NCIM 5103. The production of secondary metabolites by bacterium (S5-1) in term of zones of inhibition was considerably influenced by incubation period. The antagonistic activity of the isolate was observed at 24hrs of incubation but the maximum activity was recorded at 30 hrs (i.e. 25±2 mm zone of inhibition). Thereafter, activity was
decreased significantly (Table 1). During this study it was observed that, pH and temperature affected production of secondary metabolites of the isolate. The highest inhibition was observed to be 24±3 mm of zone inhibition at pH 7 and 37^0C. However, the activity drastically decreased at low and high pH (Table 1). The activity of the isolate was retained at varied temperature of 25^0C to 49^0C.

Table 1: Effect of environmental factors (Incubation period, pH and Incubation temperature) on antagonistic activity of the isolate *B. cereus* against pathogens *P. solanacearum*

Zone of Inhibition (mm)					
Incubation Period		**pH**		**Incubation Temperature**	
hrs	**Mm**	**pH**	**mm**	**^{0}C**	**Mm**
18		**4**	-	**25**	18±1
24	18±2	**5**	-	**31**	20±2
30	25±2	**6**	14±1	**37**	25±3.5
36	21±3	**7**	24±3	**43**	19±2
42	20±1.5	**8**	18±2	**49**	12±1
-	-	**9**	12±1	-	-
-	-	**10**	-	-	-

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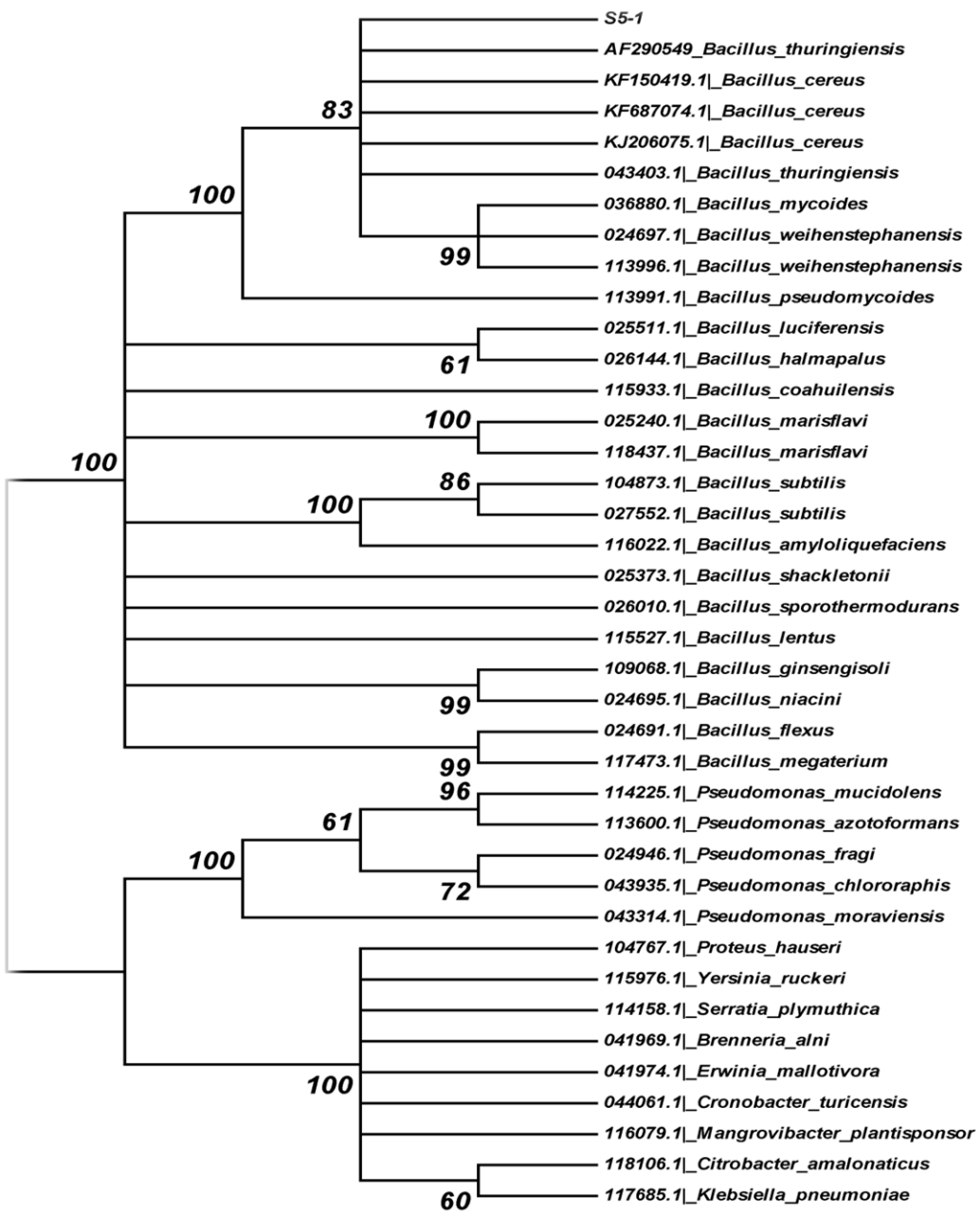

Figure 2: Phylogenetic tree generated by maximum Parsimony showing evolutionary relationship of *Bacillus cereus* (S5-1) along with 38 closely related bacterial sp.

C. EFFECT OF CARBON AND NITROGEN SOURCES ON BIOCONTROL ACTIVITY

During investigation, it was observed that production of secondary metabolite was greatly influenced by different carbon and nitrogen sources. The highest activity in term of zone of inhibition was found to be 26±2.3 mm when medium was supplemented with carbon sources of 1% xylose followed by 0.5% and 1% of fructose (20±1.5 mm), 1% of arabinose

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(19±2 mm), 1% of raffinose (18±1 mm) and 1% of starch (18±1.5mm) (Table 2). All the 10 types of carbon sources enhanced the biocontrol activity in terms of zone of inhibition at 1% level in the medium. Similarly, the effect of different nitrogen sources was also studied (Table 3). It was observed that nitrogen sources like yeast extract, peptic digest, ammonium sulphate and ammonium nitrate in the medium enhanced the biocontrol activity against the phytopathogen studied. Surprisingly, the isolate did not show any zone of inhibition or activity against the pathogen when the medium was supplemented with calcium nitrate, sodium nitrate and potassium nitrate. Among nitrogen sources yeast extract at 0.5% in the medium exhibited better activities (30±2 mm zone of inhibition).

Table 2: Effect of carbon sources on antagonistic activity against *Pseudomonas solanacearum*

	Zone of inhibition (mm)		
	0.5%	1%	1.5%
Fructose	20±1.5	20±1.5	18±2
Galactose	12±0.7	12±1	12±1
Dextrose	17±1.9	17±1.7	15±2.1
Xylose	22±2	26±2.3	24±2
Raffinose	14±1.6	18±1	16±2
Arabinose	15±1	19±2	18±2.2
Starch	18±2	18±1.2	13±1.5
Maltose	-	-	-
Trehalose	17±1	17±1.7	14±1.1
Sucrose	12±1.3	14±1.3	14±1
Control		12±1	

Table 3: Effect of nitrogen sources on antagonistic activity against *Pseudomonas solanacearum*

	Zone of inhibition (mm)		
	0.1%	**0.5%**	**0.7%**
Calcium nitrate	-	-	-
Yeast extract	25±2	30±1.8	27±2.4
Peptic digest	25±2.2	24±2	20±2
Sodium nitrate	-	-	-
Potassium nitrate		-	-
Ammonium sulphate	-	14±1	14±1.1
Ammonium nitrate	12±1	15±1.2	13±1
Control		**12±1.2**	

(A) **(B)**

Figure 4: Depicting biocontrol activity of the isolate on pot experiments under green house condition: (A) Wilting of tomato plant (treated with *P. solanacearum)*; (B) Indicating Biocontrol properties of the isolate *B. cereus*, the plant root was treated with our isolate *B. cereus* and challenged by *P. solanacearum*

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D. STUDY OF BIOCONTROL ACTIVITY OF THE ISOLATE (POT EXPERIMENT)

The biocontrol activity of the isolate against *P. solanacearum* was evaluated by field trial. Pot experiments were conducted taking 3 weeks old tomato plant. It was observed that, 10% of the plants were infected in the pot containing Soil with *P. solanacearum* and plant root treated with S5-1. While, 80% of the plants were infected in the pots where, the plant root was treated withP. *solanacearum* (Table 4). However, the plant only treated with isolate S5-1 did not show any sign of infection, indicating, non-pathogenic in nature. This indicates retention of biocontrol properties of our isolate under field conditions (Fig.3).

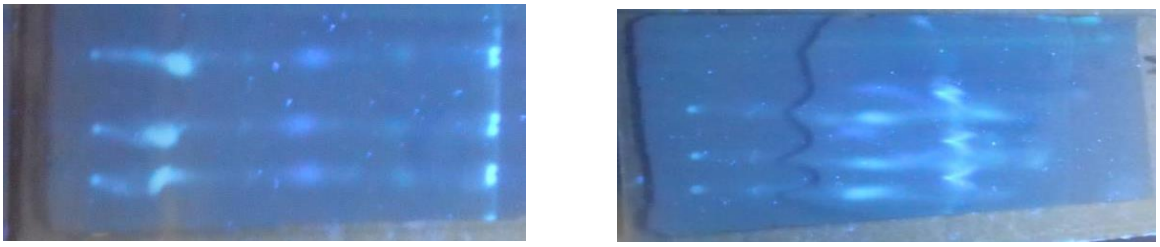
Table 4: Effect of biocontrol activity (pot experiment)

No of Observation	Plant root with*P. solanacearum*	Plant root with S5-1	Soil with *P. solanacearum* + root S5-1	Soil with S5-1+ plant root with *P. solanacearum*	Soil with *P. solanacearum* and S5-1
1	Infected	No infection	No infection	No infection	No infection
2	Infected	No infection	No infection	Infection	No infection
3	Infected	No infection	No infection	Infection	No infection
4	Infected	No infection	Infection	No infection	Infection
5	No infection	No infection	No infection	No infection	No infection
% of infection	80%	00%	10%	40%	10%

*Each observation contains 10 plants

E. TLC ANALYSIS OF THE COMPOUNDS

The separation of crude metabolites was carried out by TLC using different solvents. The extract showed different spots on TLC plate when used different solvents like : Ethyl acetate-hexane-water (3:6:1), solvent B: solvent C: ethyl acetate-hexane-water (3:5.5:1.5). under UV light at 254 nm. The R_f values for solvent A was calculated to be 0.15, 0.31, 0.83 (Fig.4A). But, the crude was separated in to four different bands when solvent B used as mobile phase and R_f value was calculated to be 0.13, 0.2, 0.45 and 0.84 respectively (Fig. 4B).



A **B**

Figure 5: TLC analysis of crude extract

IV. DISCUSSION

The isolate was isolated from rhizospheric soil of Similipal Biosphere. The isolate showed biocontrol activities against tomato wilting bacterium pathogen *P. solanacearum* during the investigation. The isolate was identified as *Bacilluscereus* by both morphological and molecular characterization. The BLAST analysis showed that the sequence of the isolate have closest homology with 99.9% of *Bacillus cereus.Bacillus cereus* is a large, gram-positive, endospore forming bacterium that is very common in rhizospheric soil [8,9]. Different researchers have isolated *Bacillus cereus*

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from different sources having antimicrobial activity [10,11,12,13].*Bacillus* as soil bacteria that biocontrol agents are quite important in the management of pests and different plant diseases [14]. Due to plant growth activity of *Bacillus* spp. play important role in maintaining soil quality and serve macro and micro nutrient to plant for growth. *Gardener* [15]reported that varieties of *Bacillus* and *Paenibacillus*help to promote the health of crops and control diseases by producing secondary metabolites. The need of biofertilizer for improve soil health, productivity and maintaining the ecosystem as explicate the *Bacillus* sp. Like, *B. cereus* RS87 significantly promoted growth of root length, plant height and seedling emergence over control and produced IAA [16]. *B. cereus* was effective in suppressing alfalfa diseases, enhancing the emergence of seedling and increasing nodulation in common beans [17].

Bacillus cereus 28-9 is a chitinolytic bacterium isolated from lily plant in Taiwan. This bacterium exhibited biocontrol potential on Botrytis leaf blight of lily. For plant disease control, *B. cereus* UW85, which is capable of producing two antibiotics responsible for disease suppression[18], has been proven to be a reliable biocontrol agent of *Phytophthora* damping off and root rot of soybean [19]. *Bacillus cereus* strain UW85 is known to produce both zwittermycin and kanosamine. The production of multiple antibiotics probably helps to suppress diverse pathogenic microbial competitors in rhizospheric soil. *Bacillus cereus* U92 was determined as the most efficient strain that reduce the pathogenic infection

During our investigation, we made an attempt to study the effect of different environmental (temperature, pH, incubation period) and nutrients (carbon, nitrogen) factors on production of secondary antagonistic metabolites and evaluated in term of zones of inhibition. It was observed that incubation period, pH and temperature greatly affected the biocontrol activity of the isolate(Table 1). *Gupta* et al.[20] reported that antimicrobial activity of *Bacillus* sp. against bacterial pathogens was found to be maximum at 24 hrs of incubation period. *Kumar* et al. [21] reported that maximum biocontrol activity of *B. subtilis* against fungal plant pathogens *Microsporumfulvum* and *Trichophyton* sp. at 48hrs. Sadfi et al. [22] also reported that *B. cereus* X16 showed antimicrobial activity against plant pathogen*Fusarium roseumvar. sambucinum*and it was maximum at 48hrs incubation period. The isolate showed optimal activity when cultured in a medium with pH 7.0 and incubated at 37^0C (Table 1). Our observations are in agreement with report of *Wilson* [23]. Huang *et al*. [24] who reported that *Bacillus cereus* showed maximum biocontrol activity at pH 7-7.5. Similar result was also reported by Kumar *et al*., [21] (2009), that *B. subtilis* showed the maximum biocontrol activity at pH 7. *Karunya* et al. [25] also reported that *Bacillus* sp. showed maximum antimicrobial activity against plant pathogens *Aspergillus niger*, *Aspergillus flavus* and *Penicillium chrysogenum*at 35^0C.

Similarly, while studying the effect of different C & N sources it was observed that selective C and N sources affected the antagonistic properties of the isolate against the test pathogen *P. solanacearum*, maximum activity in terms of zone of inhibition was observed when the isolate was grown in a batch fermentation, with medium containing 1% xylose, followed by arabinose, fructose, raffinose, starch trehalose etc. Interestingly maltose had no effect on the antagonistic activity of the isolate. Different carbon sources, like dextrose [26], lactose [27], [28]sucrose, fructose [29] effect significantly the production of antimicrobial activity were recorded.

Nitrogen sources showed a notable effect on the production of the antimicrobial metabolite by the bacterium. In study yeast extract was recorded as a significant effect on the antimicrobial production followed by meat peptone and beef extract. In corroboration to our findings, nitrogen sources significantly affect antibiotic formation [30], based on the biosynthesis pathways. The results of the present study indicated that nutrient in the fermentation media play an important role in the onset and intensity of secondary metabolites. In the investigation, it was found that yeast extract and peptic digest are suitable nitrogen sources for augmentation of the antimicrobial activity[31]. *Singh* and *Rai [32]* also reported that yeast extract and peptone are suitable nitrogen source in the medium for production of antimicrobial compound. Since, the crude extract was prepared from the culture of the isolate, and showed antimicrobial activities, thus it can be told that the antimicrobial secondary metabolites of *B. cereus* is extracellular and water soluble. On increase of the quantity of culture supernatant, there is increase in activity indicates the production of the metabolites (S) is nutrient dependent and also environmental conditions regulate production of these metabolites. Also pot experiments revealed that isolate having efficacy to control plant pathogenic bacterium in field condition.

V. CONCLUSION

The *Bacillus* is the gram-positive soil bacteria found in every ecosystem. This research showed that *Bacillus* species are quite important and effective as biocontrol agents. By viewed of different researcher, the efficacy of *Bacillus cearus* is

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observed to promote growth in plants through different mechanisms like, mineralization of macro and micro elements, production of plant growth hormones. Therefore, the isolate could be exploited as plant growth promoters in sustainable agriculture with further laboratory tests like its toxicity and effectiveness.

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