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IN VITRO CHARACTERIZATION OF *BACILLUS* SP. LBF-01 AS POTENT ANTIFUNGAL STRAIN AGAINST SOME CROP PATHOGENIC FUNGI

Microbiology

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ABSTRACT

The present work was conducted for characterization of antagonistic *Bacillus* sp. LBF-01 and its control mechanisms against *9 pathogenic strains* The antifungal strain LBF-01 formed the highest inhibition zone diameters of 12 mm - 21 mm against the fungal strains in PDA media and exhibited 78% inhibition in NA media followed by 89% PDA and MEA, 67% CMA and 56% OMA media against *A. alternata*, *C. gloeosporioides*, *F. moliniforme, F. oxysporum*, *Fusarium* sp., *A. parasiticus,* and *C. albicans* indicating broad spectrum antifungal activity. Microscopic evaluation of the strain against treated *Fusarium oxysporum* showed that the antifungal secondary metabolites affect on growing mycelia in terms of swelling, cytoplasm's coagulation and lysis of the mycelia. The strain is sensitive to common antibiotics used for human therapy indicating their non-virulent nature.

KEYWORDS

Antifungal; *Bacillus* sp. LBF-01; Fungal Pathogens.

1. INTRODUCTION:

Crops diseases need to be control for maintain the crop productivity and quality of food grains at regional, national and local scales. The grower management different types of fungal disease of crops through used of traditional chemical pesticides in agricultural field. The long used of chemical fertilizer in the crops field reduce the soil fertility and create the environmental pollution by excessive use and misuse these chemical pesticides, has led to considerable changes in people's attitude toward the use of pesticides in agriculture (Pal et al., 2006). Consequently, the farmer interest has been grown on alternatives to chemicals fungicides i.e. biopesticides to management fungal disease of crops. The use of biocontrol agents to control the diseases and pests in an ecofriendly manner is one of the best strategies and its commercial acceptability and applicability define by some experimental work which is being carried out all over world (Upadhyay et al., 2000).Microorganisms are used as broad spectrum antimicrobial biocontrol agents (BCA) due to their high capacity to suppressed pathogenic microbes. The rhizospheric and phyllospheric diseases controlled by *Bacillus* spp. which have highly potent bacterial BCAs (Rahman M., 2016). They have well developed secretary system producing structurally diverse secondary metabolites with a wide spectrum of antagonistic activity (Liu et al, 2007). Anthracnose disease caused by *Colletotrichum* sp. in various hosts including cereals, legumes, vegetables, perennial crops and tree fruits worldwide (Bailey and Jeger, 1992). The most destructive soil born disease spread by *Fusarium* species is vascular wilts, rots, and damping-off diseases of horticultural and food crops (Bodah, 2017). Chili and Tomato wilt is one of the chief diseases caused by *Fusarium* sp. (Chowdhury et al., 2019).

The main objective of this study was to *in vitro* evaluation of antifungal activity of *Bacillus* sp. LBF-01 against some pathogenic fungi of crop plants and its characterization through morphological, biochemical, physiological methods, and also to assess antibiotic susceptibility against some common antibiotics.

2. MATERIALS AND METHODS:

2.1. Experimental microorganisms and culture conditions

Bacillus sp. LBF-1 (GenBank accession number KX656669) was isolated from the inflorescence of mango plant and sub-cultured and maintained on nutrient agar (NA) slant medium at 4°C as stock cultures for performing different experiments. The referred strains of fungi were obtained from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. Reference strains include *Colletotrichum gloeosporiodes* MTCC-4618, *Colletotrichum lidemuthianum* MTCC-8474, *Fusaruim moniliforme* MTCC-2015, *Fusarium oxysporum* MTCC-2480, *Fusarium* sp., *Aspergillus parasiticus* MTCC-2796, *Aspergillus fumigatus* MTCC-1046, *Alternaria alternata* MTCC-8459 ,*Candida albicans* MTTCC-183. The above fungal strains were maintained on Potato Dextrose Agar (PDA)

2.2. Antifungal assessment of *Bacillus* sp. LBF-01 strain by dual culture overlay method.

In vitro antifungal activity of *Bacillus* sp. LBF-01was tested against all referred fungal strain using the dual culture overlay method as described by Mandal et al., 2007. Initially, 10µl of spore's suspension was mixed with 5ml of semi-solid malt extract agar (0.06%) and vortex gently for spores' homogenization. Five types of base media were used for antifungal test viz. PDA, NA, CMA, OMA and MEA for all experimental fungal strains. After that malt soft agar media with spores were then overlaid on each type of plates. The plates were then left for 30 min for solidification *Bacillus* sp. LBF-01 was spotted on the plates and incubated at 28°C for 4-7 days aerobically in incubator. Antagonist activity was observed after incubation at 28ºC up to 7 days and the zone of inhibition, if present, was determined by the protocol of Riungu et al., 2008. % Inhibition of mycelial growth = [(X – Y) /X] × 100, Where, X = Mycelial growth of the pathogen in absence of antagonists; Y= Mycelial growth of the pathogen in presence of antagonists. Antagonistic activity was carried out with three replications and repeated twice.

2.3. Morphological characterization by SEM Study.

Morphological studies of *Bacillus* sp. LBF-01 was evaluated on the basis of different morphological growth parameters viz. colour, shape and size, appearance, colony diameter and margin and Gram nature. The strain was inoculated on Nutrient Agar (NA) plates and incubated at 37°C for 24 hrs. The fresh growing colonies were observed under microscope for morphological characterizations**.** To analyse the bacterial cell morphology by SEM, samples were prepared by standard protocol.

2.4. Physiological Characterization of *Bacillus* LBF-01

2.4. 1. Salinity tolerate stability

To evaluate salt tolerance ability of the isolates, fresh active culture bacteria transfer into liquid medium at different salt concentrations. The salt concentrations (NaCl) tested were 0, 0.5, 5.0, 10.0, 15.0, and 20.0% (w/v). All of the tubes with distinct growth were measured after 12 hrs in terms of optical density at 610 nm in triplicate trials.

2.4.2. Temperature tolerates stability

The ability to tolerate high temperature of the isolates was tested for their growth in different temperatures variation. The temperatures were at 20 °C, 28 °C, 37 °C, 47 °C, 57 °C and 67 °C. The growth was measured after 12 hrs in terms of optical density at 610 nm in triplicate trials.

2.4.3. Initial media pH tolerates stability:
The initial high and low pH variations of the media were assayed for ability to tolerate for their growth parameters. The pH of the media was taken in the range from 4.0 to 11.0. All of the culture tubes with distinct growth were measured after 12 hrs in terms of optical density at 610 nm in triplicate trials.

2.5. Biochemical characterization
Biochemical characterization of antifungal phyllobacterial strain LBF-1 was carried out for preliminary identification of the potent isolated strains.

2.5. 1. Carbohydrate fermentation
Carbohydrate fermentation analysis of 35 sugars was performed using the kit (Hi-Media, Mumbai, India). Kit contains Part A, Part B each having 12 carbohydrates utilization tests and Part C containing 11 sugars and 1 control. *Bacillus* sp. LBF-01 was grown actively in NB media at 37°C for 24 hrs in Biological incubator. Then open the kit aseptically, peel off the sealing foil and inoculate each well with 20 µl of the above inoculums by surface inoculation method. Alternatively, the kit can also be inoculated by stabbing each individual well with a loopful of inoculums and incubated at 37°C for 24 hrs.

2.5. 2. Biochemical analysis of *Bacillus* sp. LBF-01 using Gram-negative and Gram-positive test kit
The isolated strain was grown actively in NB media at 37°C for 24 hrs in biological incubator. Then open the KB013 kit aseptically, peel off the sealing foil and inoculate each well with 20 µl of the above inoculum by surface inoculation method. Alternatively, the kit can also be inoculated by stabbing each individual well with a loopful of inoculum and incubated at 37 ˚C for 24 hrs.

2.5.4. Characterization for Antibiotics Susceptibility of *Bacillus* sp. LBF-01
Antibiotic sensitivity assay of bacterial isolate was determined using antibiotic kit (Hi-Media, Mumbai, India). The 24 hrs actively growing fresh bacterial culture was spread on sterile NA plates and antibiotic kit placed on the centre of each plates. Antibiotic sensitivity of the bacterial isolates was studied by the Dodeca antibiotic assay kit manufactured by Hi-Media, India. The diameter of the inhibitory zone was measured and compared with the standard chart supplied by the manufacturer of the antibiotic disk.

3. RESULTS

3.1. Antifungal activity of *Bacillus* sp. LBF-01
The antifungal spectrum and % of mycelia inhibition by *Bacillus* sp. LBF-01 was tested against *A. alternata, C.gloeosporioides*, *C.lindemuthianum*, *F. moliniforme, F. oxysporum*, *Fusarium* sp, *A. parasiticus, A. fumigatus* and *C. albicans* fungal strains in PDA base media with ME overlaid plates as shown in Figure-1,2 and 3. The antifungal strains LBF-01 formed the highest inhibition zone diameters 18mm, 16mm, 18mm, 21mm, 20 mm, 21mm and 12mm in PDA media against *A. alternata*, *C. gloeosporioides*, *F. moliniforme, F. oxysporum*, *Fusarium sp, A. parasiticus,* and *C. albicans* respectively. The strain exhibited 78% inhibition in NA media followed by 89% PDA, MEA,67% CMA, and 56% OMA, respectively. Effect of *Bacillus* sp. LBF-o1 on mycelia growth of *Fusarium oxysporum* shown in figure-4 and Microscopic observation showed the different types of mycelial abnormalities .

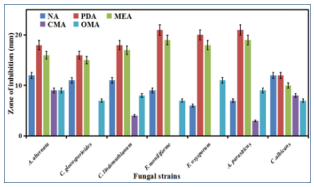

Figure-1:-Antifungal activity of *Bacillus* sp. LBF-01 against 9 fungal pathogens.

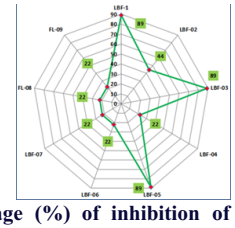

Figure-2:-Percentage (%) of inhibition of mycelia growth of *Bacillus* sp. LBF-01 against 9 fungal pathogens in PDA media.

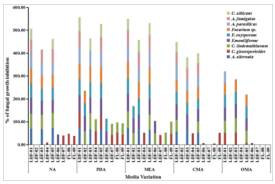

Figure-3:-Media optimization for better antifungal activity of *Bacillus* sp. LBF-01 against 9 fungal pathogens from five different base media.

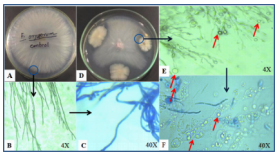

Figure-4: Antifungal activity of *Bacillus* sp. LBF-01 against *Fusarium oxysporum.*
(A) Control plate; (B) normal mycelia under 10X microscope; (C) Normal stained mycelia under 40X microscope; (D) Treated plate; (E) Red arrow indicates the treated mycelia showing different types of swelling or gall; (F) Red arrow indicates the coagulation of cytoplasm's to form granules.

3.2. Morphological characterization of *Bacillus* sp. LBF-01
Plate shows an electron micrograph of ultra-structure of *Bacillus* sp. LBF-01 revealed the rod shaped bacteria morphology.

Parameters	LBF-01
Colony Shape	Flat
Colour	White
Gram- staining	Gram-Positive
Colony margin	Undulate
Cell shape	Rod
Consistency/Texture	Smooth shiny Moist, dry

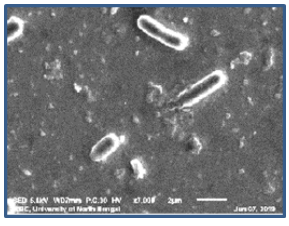

Figure-5:- SEM microscopic study of *Bacillus* sp. LBF-01.

3.3. Physiological characterization of *Bacillus* sp.LBF-01.
The maximum salt tolerance ability by the strain LBF-01 was 15 % (w/v) and tolerates temperature 15- 57°C and pH 11.

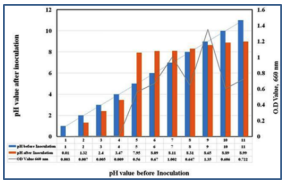

Figure-6:-Growth behavior of *Bacillus* sp. LBF-01 in different initial pH media.

3.4. Biochemical characterization of *Bacillus* sp. LBF-01
The media colour of maltose, fructose, dextrose, trehalose, sucrose, mannose, inulin, glycerol, sorbitol, mannitol and cellobiose carbohydrates were changes from red to yellow indicates the positive result by LBF-01 shown in Table-1 and Table 2. *Bacillus* sp. LBF-01 not able to changes media colour in test kit due to negative biochemical result except arabinose. *Bacillus* sp. Gram-positive test kit indicated that isolated LBF-01was Gram positive *Bacillus* sp. bacteria. The used of *Bacillus* genus identification kit for the strain LBF-01 was showed positive result against arginine, sucrose, mannitol, glucose, arabinose, Voges-Proskaurs, and catalase.

Table-1:- Carbohydrate fermentation test of *Bacillus* sp.LBF-01

SL.No.	Names of Carbohydrates	LBF-01	SL.No.	Names of Carbohydrates	LBF-01
1	Lactose	+++	19	Sorbitol	++
2	Xylose	+++	20	Mannitol	+++
3	Maltose	+++	21	Adonitol	-
4	Fructose	+++	22	Arabitol	-
5	Dextrose	+++	23	Erytritol	-
6	Galactose	-	24	α-Methyly-D-glucoside	-
7	Raffinose	-	25	Ramnose	-
8	Trehalose	+++	26	Cellobiose	+++
9	Melibiose	-	27	Melezitose	-
10	Sucrose	+++	28	α-Methyly-D-mannoside	-
11	L-Arabinose	++	29	Xylitol	-
12	Mannose	+++	30	ONPG	-
13	Inulin	+++	31	Esculin hydrolysis	-
14	Sodium gluconate	-	32	D-arabinose	+
15	Glycerol	+++	33	Citrate utilization	-
16	Salicin	+++	34	Malonate utilization	-
17	Dulcitol	-	35	Sorbose	-
18	Inositol	+++			

Here, the symbols '+++' indicates good fermentation; '++' indicates moderate fermentation; '+' indicates poor fermentation, and '-' indicates no fermentation, as evidenced by the color change of the indicator of the well.

Table-2:-Biochemical analysis of the potent strains through Gram negative *Bacillus* sp. test kit and Gram positive *Bacillus* sp. test kit

SL. No.	Name of the test	LBF-01	SL. No	Name of the test	LBF-01
1	Citrate utilization	-	1	Malonate	-
2	Lysine utilization	-	2	Voges Proskaurs	++
3	Ornithine utilization	-	3	Citrate	++
4	Urease	-	4	ONPG	-
5	Phynaylalanine	-	5	Nitrate Reduction	-
6	Nitrate	-	6	Catalase	+++
7	H2S production	-	7	Arginine	+++
8	Glucose	-	8	Sucrose	++
9	Adonitol	-	9	Mannitol	+
10	Lactose	-	10	Glucose	+
11	Arabinose	++	11	Arabinose	++
12	Sorbitol	-	12	Trehalose	+++

3.5. Characterization for Antibiotics Susceptibility

The antibiotic susceptible assay of antifungal strain LBF-01 against 12 antibiotics which is shown in figure-7. All the antibiotic exhibited clear zone against strains LBF-01.

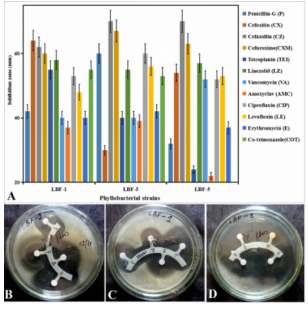

Figure-7: Antibiotics susceptibility assay of Bacillus sp. LBF-01. (A) Zone of inhibition against the antibiotics; (B-D) The antibiotic assay plate showing the inhibition zones.

4. DISCUSSION

The global food security has been challenged by plant pathogens which have the most devastating threats to the human life by increase the malnutrition, and poverty (Strange and Scott, 2005). The effect of accumulation of toxic microbial metabolites within plant tissues through food chain promoting hazardous diseases to human and animals. Chemical fungicides had been mainly used as the common strategy for controlling plant diseases, however, these compounds resulted in emerging of sever fungicides resistance plant pathogens, fastidious human disease. Thus, searching for an ecofriendly biocontrol microbes is a challenge for providing a pesticide-free food. Herein, the potency of *Bacillus* sp. LBF-01 for controlling variou pathogenic fungi of crops and enhance their productivity along with food safety proved as strong biocontrol bacteria.

The antagonistic strains LBF-01was exhibited broad spectrum antifungal activity against all referred fungal strains except *C. gloeosporioides* in NA media. The potentiality of said strains was 87.5%, 87.5% and 100% against 8 referred fungal strains shown in figure-1. The antifungal activity of *Bacillus vallismortis* against plant pathogenic fungi like *A. alternata, R. oryzae, F. oxysporum, F. moniliforme, Colletotrichum* sp., *Helminthosporium* sp.and *Magnaporthe grisea* in dual culture plate assay showed 50% inhibition (Kaur et al., 2015). *Investigators also reported that Bacillus amyloliquefaciens* NJN-6 inhibited the mycelial growth and spore germination of *Fusarium oxysporum* f. sp. *cubense through the production of*volatile compounds (VOCs) (Yuanet al., 2012). *Bacillus subtilis*, strain ALICA was reported to have its antifungal activity in dual plate assay and cell-free culture filtrate (25%) against five different phytopathogenic fungi *Alternaria alternata, Macrophomina* sp., *Colletotrichum gloeosporioides, Botrytis cinerea,* and *Sclerotium rolfesii* through three mycolytic enzymes such as chitinase, β-1,3-glucanase, and protease production (Ashwini et al., 2014). The colony morphology of *Bacillus amylloliquifaciens*, *Bacillus velezensis* and *Bacillus methyllotrophicus* are similar with the phyllobacterial strain LBF-01 as reported by earlier researchers. Growth curve and initial pH change assay was studies and showed that pH increase along with increases of cell biomass of *Bacillus* sp.LBF-01.Antibiotic sensitive test revealed that this bacterial strains sensitive to all antibiotics which was symptoms of non pathogenic or beneficial strains. Biochemical assay of *Bacillus amylloliquifaciens*, *Bacillus velezensis* and *Bacillus methyllotrophicus* showed similar results as reported by some investigators .

5. CONCLUSION: -

The antagonistic activity of *Bacillus* sp. LBF-01 against plant pathogens indicates as a good candidate for the development of biocontrol agents.

6. ACKNOWLEDGEMENT:

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