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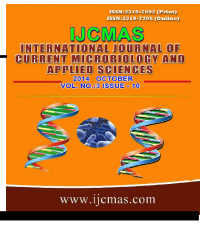

Original Research Article

Isolation and partial characterization of iturin like lipopeptides (a bio-control agent) from a *Bacillus subtilis* strain

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Keywords

Lipopeptides, *Bacillus*, Bio-control, Iturin, HPLC

A B S T R A C T

Most of the crops get disease and finally destroyed by the phyto-pathogens such as fungi, bacteria, yeast and other members of phyto-pathogens. Bacterial isolate was identified as *Bacillus subtilis* HQ851067 by 16s rDNA sequencing followed by phylogenetic analysis. In the present study, lipopeptide (iturin) from *Bacillus subtilis* HQ851067 is a prominent molecule active against various phyto-pathogens like *Aspergillus niger* and *Fusarium oxysporum* with % inhibition 83.3 and 60 respectively. Lipopeptide (iturin) is environmentally eco-friendly, biodegradable, and stable at high temperature. These iturin like lipopeptides in *Bacillus subtilis* are synthesized by NRPS (non-ribosomal peptide synthetase) enzyme complex. These are mega enzyme organized in an orderly functional unit called modules that catalyze the different reactions. These iturin like lipopeptides were partially characterized by thin layer chromatography and High performance liquid chromatography (HPLC) that is a sensitive technique used for purification as well as partial identification of any lipopeptide like bio-active molecules.

Introduction

Most widely use of chemicals to control plant diseases disturbed the ecological balance of eco-system that cause groundwater contamination, arising of resistant races of pathogens and health risks to humans. Because of the reluctance of most companies to test newer chemicals due to the financial and registration difficulties a lot of attention has been paid to plant growth-promoting rhizo-bacteria (Bashan, 1998). Search of an alternative for chemical control of plant pathogens, gained a revolution in recent years. Surfactin also consisting of distinct hydrophilic and hydrophobic moieties are easily bio-degradable and thus are particularly suited for environmental applications (Muller-Hurting *et al.,* 1993). Scientists have purified and characterized the diffusible anti-fungal metabolite (iturin) by applying various techniques. HPLC is the most common technique use for analyzing and separating partially purified extract from *Bacillus* strains. HPLC used to show the presence of interested molecules (iturin) from the mixture on the basis of peaks.

Among rhizo-bacteria, *Bacillus* sp. play vital role in the management of plant disease to increase crop production via various mechanisms by which bacterial strain can suppress plant diseases (Satpute *et al.,* 2008). *Bacillus subtilis* produce a number of cyclic lipopeptides, which are biologically active. For example, various strains of *Bacillus subtilis* produce more than twenty different molecules with antibiotic activity including many lipopeptides. Iturin A is a potent anti-fungal lipopeptides with many properties, out of which anti-microbial activity was the first, reported. (Besson *et al.*, 1976). Schematic diagram of iturin is shown in (Fig.1).

Materials and Methods

Screening of lipopeptides producing bacterial isolates

Bacterial isolate (VP) was procured from department of biotechnology, university of north Bengal, Siliguri (W.B) and screened for its anti-fungal activity against selected fungal pathogens (like *Fusarium oxysporum* and *Sclerotium rolfsii*. For the initial screening, in the middle fungus culture (*Fusarium oxysporum*, *Sclerotium rolfsii*) was transferred and outside the fungal culture, bacterial culture was streaked and anti-fungal activity (% inhibition) was calculated. For second screening, iturin was extracted and its methanolic extract was tested for the anti-fungal activity against above shown selected fungal pathogens.

Identification of bacterial isolate by 6s r-DNA sequencing

The VP isolate shows the strong anti-fungal activity, was sequenced by 16s rRNA gene sequences of closely related strains were retrieved from server through the BLAST with the help of forward and reverse sequence and aligned using CLUSTAL-W program of MEGA version 5.0 (Tamura *et al.,* 2007).The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei (1987). The boots-trap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

Extraction of lipopeptides(s) from bacterial isolates *Bacillus subtilis*

For production of anti-fungal bio-active molecule (iturin), isolate was grown aerobically on potato dextrose broth maintained at pH 7.0. The culture was grown at 72 h at 30^{o}C with 200 rpm (shaking) in a shaker incubator. After incubation bacterial cells were removed by centrifugation (8,000 x g for 20 min) and the cell free broth was passed through 0.45µm Whatmann filter paper.The pH of the filtered cell free broth was adjusted to 2.0 by adding 6N HCl. The acid precipitates were recovered by centrifugation (10,000 rpm for 15 min at 4$^{\circ C)}$ and were extracted with methanol (Minakshi *et al.,* 2010). The methanol extract was bio assayed against the test pathogen *Fusarium oxysporum* and *Sclerotium rolfsii* by well diffusion method.

Bioassay of lipopeptides(s) (iturin)

Methanol extract of 100 µl lipopeptides was tested against *Fusarium oxysporum* and *Sclerotium rolfsii by* well diffusion method (Tagg, and McGiven, 1971) and growth inhibition (%) was calculated by the following formula:

Growth inhibition (%) = (DC-DT) / DC X100

Where, DC, diameter of control; and DT, diameter of fungal colony with treatment (Pandey *et al.,* 1982).

Biochemical characterization of bacterial isolates (VP)

Gram character

The Gram stain is a fundamental method to distinguish the bacterial sp. on the basis of phenotypic characterization. The staining procedure differentiates the organisms according to cell wall structure. Gram-positive bacterial cells have a thick peptidoglycan layer and stain blue to purple while Gram-negative bacteria cells have thin peptidoglycan layer stain to red to pink .

Characterization of lipopeptides

High Performance Liquid Chromatographic (HPLC) Analysis

Methanol extract was filtered by 0.45µmwhatman filter paper was analyzed by high performance liquid chromatography (HPLC) using UV-VIS wavelength detector and methanol as a mobile phase. The stationary phase consisted of silica on C-18 packed stainless steel column where Methanol at 1 ml/min flow rate was used as mobile phase. HPLC analysis was performed at wavelength of 240 nm, which was detected for absorption maxima using UV detector.20 µl of sample was injected into HPLC under standardized conditions and the detector response was measured in terms of peak areas.

Thin Layer Chromatography (TLC)

Thin layer chromatography was carried out by spotting the concentrated residue on TLC plates (Merck, Germany) and developed in different solvent systems, namely, chloroform-methanol-acetic acid (80:10:5)The silica containing the spot was air dried and sprayed with ninhydrin solution and heated at 80°C for 5 min after that color was observed under UV.

Results and Discussion

Gram staining

Rod shaped bacteria were observed under the microscope (Fig.1).

Phylogenetic analysis

A dendrogram analysis was performed with tree joining method involving 12 nearest neighbours presenting the maximum similarity to geneus *Bacillus*, species *subtilis* and strain HQ851067. The bacterial isolate (VP) identified as *Bacillus subtilis* HQ851067using 16s rRNA sequencing based on nucleotide homology and phylogenetic analysis (Fig.2).

Anti-fungal activity of Lipopeptide(s) extracted from *Bacillus subtilis* strain

The methanol extract (50 µl) was bio-assayed on agar plate against each of the test pathogen that included *Fusarium oxysporum and Sclerotium rolfsii* by well-diffusion method. The Petri plates were incubated at 30^{oC} in the incubator in inverted position and the zone of inhibition(s) against each of the fungal pathogen were recorded after 3 days (Table 1) and (Fig.3).

Characterization of lipopeptides(s) Thin Layer Chromatography

TLC of extracted lipopeptides produce blue violet colour indicates the presence of Protein part in the compound, spray reagent is the ninhydrin. (Fig.4).

High Performance Liquid Chromatographic (HPLC) Analysis

Analysis was performed to know the presence of a lipopeptides in the culture of *Bacillus subtilis*. The peak was obtained at retention time of 2.06 (Fig.5) which show that iturin or iturin may be present as compared to data obtained from literature**.**

Fig.1 visualization of rod shaped bacteria under the microscope

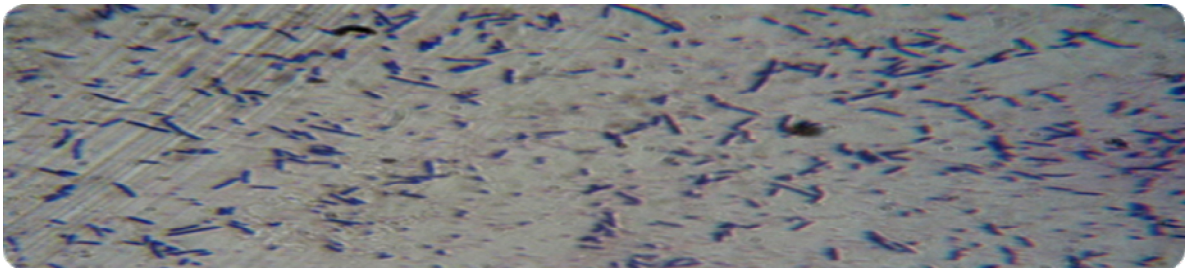


Fig.2 Phylogenetic tree of HQ851067 generated by software MEGA 5.0 version showed the similarity of the investigated amplified DNA (for rRNA) to 12 nearest neighbours

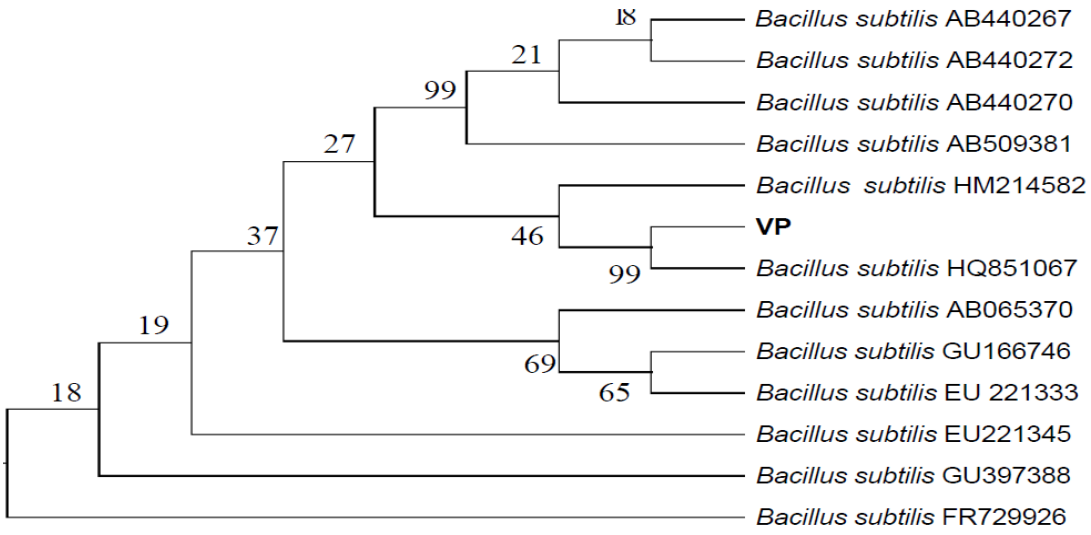
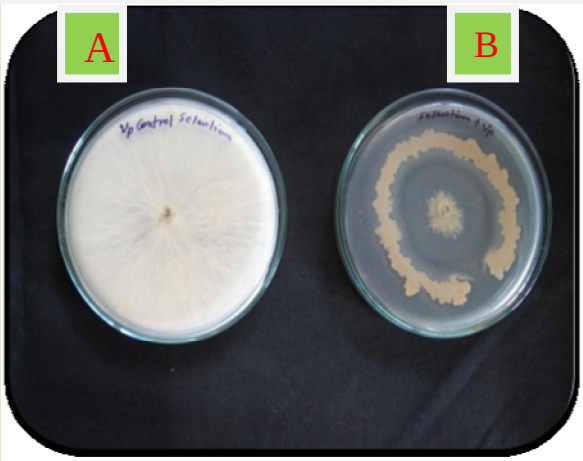


Table.1 anti-fungal activity of extracted lipopeptide(s)

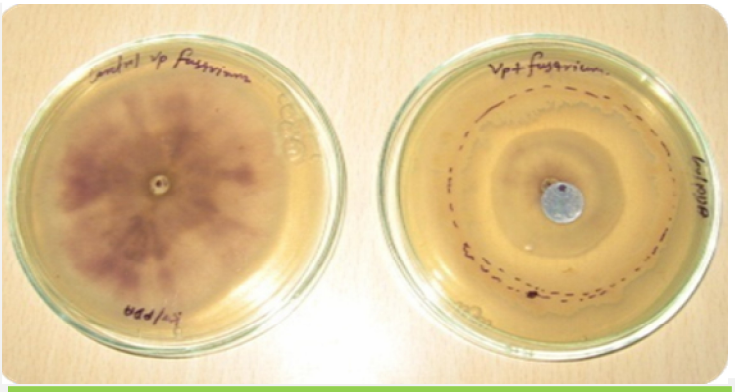
Bacillus subtilis strain	% Mycelial growth inhibition of tested fungal pathogens	
	Fusarium oxysporium	*Sclerotium rolfsi*
VP	60	83.3

Fig.3 anti-fungal activities of lipopeptides(s) [Fig.A–D] against *Fusarium oxysporum* and *Sclerotium rolfsii*



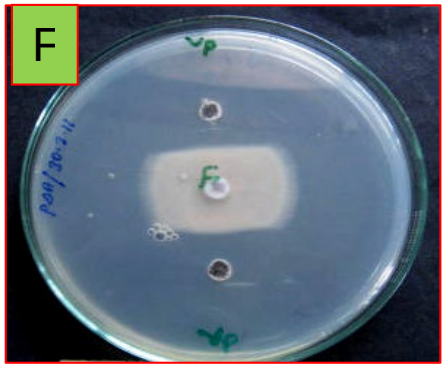
(A) positive control of Sclerotium rolfsii .

(B) Tested where *Bacillus subtilis* culture inhibiting the growth of fungal strain *Sclerotium rolfsii*.



(C) Positive control of *Fusarium oxysporum*

(D)Tested plate where *Bacillus subtilis* culture is inhibiting the growth of fungal strain *Sclerotium rolfsii*.



(E&F). Anti-fungal activity- Dual culture test (by extracted lipopeptides)

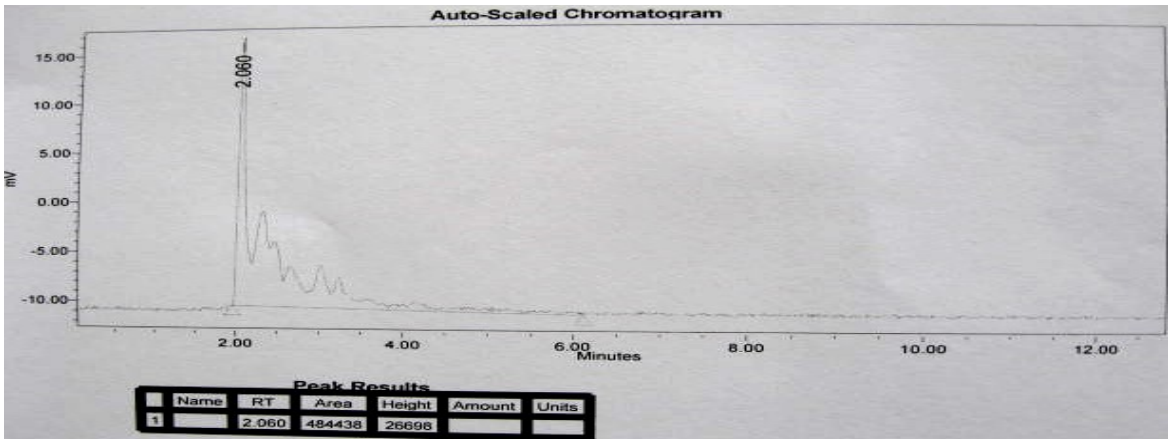
(E) *Fusarium oxysporum* positive control with methanol.

(F) *Fusarium oxysporum* growth inhibition by extracted lipopeptides

Fig.4 Thin layer chromatography sheet after ninhydrin spraying reagent

Blue violet

Fig.5 HPLC chromatogram



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