Bacillus thuringiensis Thuricin S (Bacteriocin) Efficacy against Bacterial Blight Disease Causative Agent of Xanthomonas sp. in Cowpea

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Abstract : ***Present study was focused the Bacillus thuringiensis isolates (RBL 10 and RBL 20) plant growth promoting activities, particularly for its antibacterial property against bacterial blight disease which was production of bacteriocin. Major plant growth promoting characteristics like IAA, siderophore production and phosphate solubilizations were analyzed and it was confirmed as it has the plant growth promoting capacity. Antibacterial activity was checked and bacteriocin was extracted and fractionated by column chromatography. Fractionated bacteriocin was analyzed and identified by FT-IR and GC-MS analysis. The fraction which having high antibacterial property was identified as the bacteriocin of Thuricin S by GC-MS. Further, controlling of bacterial blight disease was analyzed in cowpea plants in nursery trails. The nursery trails indicated that thuricin having good disease control ability in both preventive and curative treatments.***

Key words - Bacillus thuringiensis, Bacteriocin, Thuricin, Cowpea, Bacterial Blight disease, Xanthomonas sp.

I. INTRODUCTION

Bacteriocins are the ribosomally synthesized and post translationally modified peptides which is produced by most of the bacterial species (Elayaraja et al., 2014). These antimicrobial peptides are to inhibit or kill phylogenetically related or unrelated microorganisms (De la Fuente -Salcido et al., 2013). This was also provides them a competitive advantage in their environment, eliminating competitors to gains resources. Likewise, it initiates the opportunity for species specific communication through quorum sensing which leading to the stable microbiota (Nicholson et al., 2012). Generally, most of the bacterial species (~99%) produce at least one bacteriocin, with a length as short as 10 amino acids or as long as 688 residues (Hammami et al., 2010). These bacteriocins get attention by the increasing multidrug resistance among the pathogens. Bacteriocins are able to inhibit the animal and plant pathogens by causing bacterial cytotoxicity (Cleveland et al., 2001).

Over the past few decades, there has been a significant increase in the bacteriocins reported and classified. Particularly, Gram-positive bacteria are producing molecules classified into class I (modified peptides, antibiotics), class II (unmodified peptides, non-lanthionine) and class III (large proteins, heat unstable) (Yang et al., 2014). There are many reports on bacteriocins produced by lactic acid bacteria, which has wide application in food industry. However, bacteriocins produced by *Bacillus thuringiensis* also having much more potential in the pathogen control. Currently, 18 antimicrobial peptides of *B. thuringiensis* have been described from subspecies of *morrisoni, kurstaki, kenyae, entomocidus, tolworthi, tochigiensis* and *thuringiensis* (De la Fuente-Salcido et al. 2013; Pacheco Cano et al., 2014). In the present study, we purified thuricin S from *B. thuringiensis* and checked its antibacterial property against bacterial blight causing *Xanthomonas* sp. in cow pea.

II. MATERIALS AND METHODS

2.1. Bacterial Strains

Bacillus thuringiensis RBL 10 and RBL 20 strains are collected from own Rhizosphere Biology Laboratory, Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India.

2.1. IAA Production

The bacterial strains (RBL10 and RBL20) were inoculated in nutrient broth supplemented with 500µg of tryptophan and incubated at 27ºC. 1ml of bacterial culture was collected from 48 hours interval of 20ml broth and centrifuged at 6000rpm for 30 min. Indole acetic acid concentration in the culture supernatant determined Bric et al. (1991). 1 ml of the supernatant was mixed with 1 drop ortho phosphoric acid and 2 ml of Salkowski reagent (50ml, 35 % perchloric acid and 1ml, 0.5 M FeCl3). Development of pink colour indicates the IAA production and the amount of IAA were measured by spectrophotometric method at 530 nm. The IAA concentration in culture was determined using a calibration curve IAA as a standard.

2.2. Siderophore Production

The bacterial isolates were grown at room temperature ±28°C in nutrient broth for 3 days and centrifuged at 10000 rpm for 10 min and the supernatant was collected. The siderophore content was quantified by Arnow assay. 0.5 ml of the sample was mixed with 0.5 ml of 0.5N Hcl (reagent A) 0.5 ml of 10g of sodium nitrite and 10g of sodium molybdate in a final volume of 100ml (reagent B) and 0.5 ml 0f 1NaoH (reagent C). A standard curve was prepared using catechol by Arnow assay. The assay performed quickly and sample mixed thoroughly after addition of each reagent. Samples containing catechol appeared pink in colour. Absorbance measured at 515 nm (Arnow, 1987).

2.3. Phosphate Solubilization Activity

Qualitative phosphate solubilization test was conducted in modified Pikovskaya medium (10g of glucose, 5g of tribasic phosphate ($Ca_5HO_{13}P_{13}$), 0.5g$(NH_4)_2SO_4$, 0.2gKCl, 0.1g$MgSO_4.7H_2O$,trace of $MnSO_4$ and $FeSO_4$, 0.5g yeast extract, 15g agar and 1000ml D.H_2O) by precipitation of tricalcium phosphate. Bacterial culture was streaked on the surface of replicated agar plates and incubated for 4 days, after incubation bacterial zone was formed around the inoculated area are positive test for phosphate solubilization. On the basis of diameter of clearing halo zones, solubilization efficiency (SE) and solubilization index (SI) were evaluated using following formula (Qureshi et al., 2012). SE = solubilization diameter / Growth diameter; SI = Colony diameter + halozone diameter \ colony diameter.

2.4. Antibacterial Activity of Culture Extracts

Agar well diffusion method is widely used to evaluate the antimicrobial activity of microbial extracts. Similar procedure was used in disk-diffusion method, the agar plate surface is inoculated by spreading a volume of the microbial inoculum over the entire agar surface. Then, a hole with a diameter of 6 to 8 mm is punched aseptically with a sterile cork borer or a tip, and a volume (20–100 µL) of the antimicrobial agent or extract solution at desired concentration is introduced into the well. Then, agar plates are incubated under suitable conditions depending upon the test microorganism. The antimicrobial agent diffuses in the agar medium and inhibits the growth of the microbial strain tested (Yang et al., 2012).

2.5. Extraction of Bacteriocin

The crude bacteriocin substance was recovered from the culture filtrate of isolates by solvent extraction with butanol. Butanol was added to the filtrate at a ratio of 3:1 (v/v) or equal volume of sample and shaken vigorously for 20 min. To obtain a crude extract, the organic layer was collected and evaporated to dryness in a vacuum evaporator at 40^{o}C (Barboza et al., 2007).

2.6. Purification of Bacteriocin

Column chromatographic technique using silica gel (60-120 mesh) of column chromatography grade were used for the purification of bacteriocin. Column (35×10 mm) was cleaned using water and rinsed with acetone. After drying, a small piece of glass wool was placed at the bottom of the column. Silica gel was then packed in the column by using petroleum ether: chloroform: methanol (60: 20: 20) as solvent system. The crude antimicrobial compound was loaded at top of the column and eluted with solvent system of petroleum ether: chloroform: methanol (60: 20: 20). Fractions were collected at 20 min interval each fraction were dried and dissolved in Di-Methyl Sulfoxide (DMSO). Further, each fraction antibacterial activity was checked by agar well diffusion assay (Ajay Kumar et al., 2008).

2.7. FT-IR Analysis

FTIR has proven to be a valuable tool for the characterization and identification of compounds or functional groups (chemical bonds) present in an unknown mixture of plants extract (Eberhardt et al., 2007; Hazra et al., 2007).

2.8. Gas chromatography-Mass Spectrophotometric Analysis

The fraction (F12) that showed high antibacterial activity against majority of the selected pathogens was further identified using, gas chromatography mass spectrometry (GC-MS) analyzer (Shimadzu QP-2000). ULBON HR-1 column equivalent to OV-1 fused with silica capillary 0.25mm X 30M with film thickness 0.25 micron was used for this purpose. The initial temperature maintained was 100°C for 6 min. and then heated at the rate of 10°C per minute up to 250°C at 70eV bombardment energy. Carrier gas helium was used at the rate of 2ml per minute.

2.9. Nursery Trails

In order to evaluate the pathogen control in plants nursery trails was conducted by foliar spray on cowpea leaf foliage after 15 to 20 days of growth, which was bacterized with pathogen. The experiments were carried out in a completely randomized block design (CRBD) using two regimens, preventive and curative, and three doses 1, 10 and 100 µl of extract compound with five replicates of each treatment. Positive control plant was sprayed with *Xanthomonas* sp. and negative control plants with distilled water. Treatments were performed in two different timings for curative or preventive effect against bacterial blight disease. In the preventive regimen, different concentration extract compound was sprayed per plant 24 hours before spraying with same concentration of *Xanthomonas* sp. In the curative regimen, plants were sprayed first with *Xanthomonas* sp. and extract compound was sprayed after 24 hours. The number of lesions was determined after 21 days of last spraying. After that, symptoms of blight diseases were tested on the control plant, it would be compared with all other treated plants (Olivier *et al*., 2016).

III. RESULTS

3.1. Collection and Confirmation of *Bacillus thuringiensis*

Bacterial samples *B. thuringiensis* (RBL 10 and RBL 20) were collected from the culture repository in Rhizosphere Biology Laboratory, Department of Microbiology, Bharathidasan University. Then culture viability and colony morphology were observed for the confirmation of *B. thuringiensis* as creamy in colour, large (2-7mm diameter) variable shape from circular to irregular (Fig. 1). Further, Gram staining was performed for the identification of bacterial strains colour and shape. In Gram straining purple colour cells recognized as Gram positive and pink colored cells were recognized as Gram negative, shape may be cocci or rod shape. Among the two bacterial strains RBL 10 and 20 both was observed as Gram positive, long rod-shaped bacteria through Gram staining and it is identified as *Bacillus* (Fig. 1B). Spore production by the bacterial strains RBL10 and RBL 20 was identified by endospore staining method. In microscopic observation, the vegetative cells were in pink colour and spores were in green colour was observed (Fig. 7) and these strains RBL10 and RBL20 confirmed as spore producing bacteria. Based on the Gram staining and endospore staining results observations the organisms were confirmed as *Bacillus* sp.

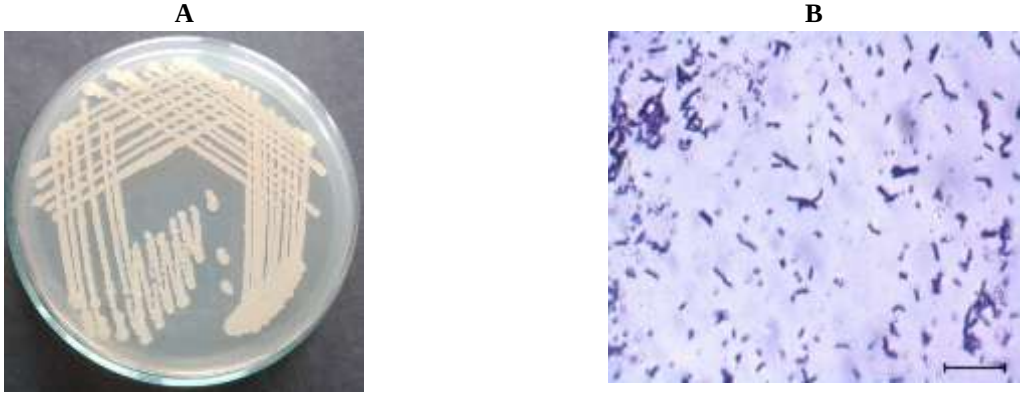

Figure 1. Colony morphology and microscopical confirmation

3.2. IAA Production

Indole acetic acid (IAA) production is one of the major properties of rhizosphere bacteria that stimulate and facilitate plant growth. At highest IAA concentration, the colour development was visible within a minute and it was continued to increases in intensity for a period of

30 min (Fig.2). Pink colour production was the indication of IAA production and it was confirmed by optical density reading. Then, the sample OD was compared with standard graph. When compared with RBL 10 and RBL 20, maximum IAA production was obtained from RBL20 (Fig. 2). Further, RBL 10 has produced a minimum amount of IAA and it was very low when compared to RBL20. RBL10 and RBL20 OD were recorded as 0.605 and 1.445 respectively. Therefore, we have concluded that RBL 20 strain was good IAA producer.

3.3. Siderophore Production

Bacterial siderophore production was analyzed by the Arnow test with tetrazolium and NaOH. Formation of deep red colour is the positive result for the siderophore production. Development of deep red colour by the culture isolate RBL20 shows that the isolate able to produce siderophore and it was hydroxamate siderophore.

3.4. Phosphate Solubilization

Phosphate solubilization by PGPR was qualitatively identified in modified Pikovskaya's medium that utilized tricalcium phosphate as the sole source of phosphate and the plates were observed at 4th day. The phosphate solubilization efficiency (SE) and solubilization index (SI) of the bacterial strains were observed. After fourth day of incubation, zone was appeared around the inoculated area and it was measured (Fig.3), and it was recorded as 13mm and 16mm for RBL 10 and RBL 20 respectively.

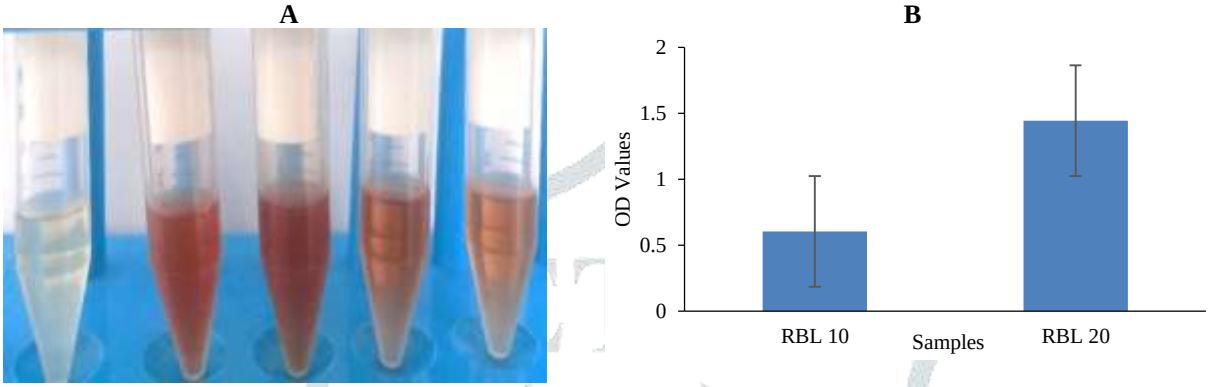

Figure 2. IAA Production. A- IAA production; B- IAA production in RBL10 and RBL20; error bar represents standard error.

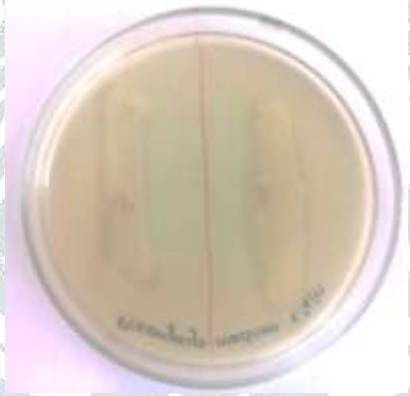

Figure 3. Phosphate Solubilization

3.5. Antibacterial Activity of Culture Extracts

Antibacterial activity was analyzed by agar well diffusion assay and determined by zone of clearance around the well. Antibacterial properties of *B. thuringiensis* strains (RBL 10 and RBL 20) were assessed against *Xanthomonas* sp, *Bacillus thuringiensis* inhibit the growth of *Xanthomonas* and halo zone was observed and it was confirmed as it has antibacterial property against *Xanthomonas* sp.

3.6. Extraction of Bacteriocin

From the mass cultivated *B. thuringiensis* RBL 20 nutrient broth medium (1000ml) antibacterial bacteriocin compound was extracted from bacterial culture by centrifugation. Further, antibacterial activity was checked against *Xanthomonas* sp. by agar well diffusion method and both strains RBL 10 and RBL 20 shows considerable level of zone of clearance at 14mm and 18mm respectively.

3.7. Fractionization of Extract by Column Chromatography

Bacteriocin was extracted and purified by column chromatography by using ethyl acetate and methanol (70:30) as a solvent system. Totally 15 different fractions were collected at 5min of interval time for each fraction collection. Further, each 15 fractions were subjected to antibacterial activity by agar well diffusion and zone of clearance was measured. Among the 15 fraction 12th fraction showed high level of zone of inhibition and confirms high level of antibacterial activity against *Xanthomonas* sp. was recorded as 19mm. Further, the zone of inhibition was recorded for the fractions 3 and 15 as 10mm, 7 and 8 as 11mm, 4 as 12mm, 1, 5, 6, 8 and 14 as 13mm, 2 and 9 as 14mm, 11 and 13 as 15mm zone of inhibitions respectively.

3.8. FT-IR

Highest antibacterial activity was observed in 12th fraction so it was further characterized by FT-IR analysis using Perkin Elmer 1750 FT-IR spectrometer, the obtained inferred that, the band at 3435 corresponds to OH (Hydroxyl) group, whereas band at 1731 represents C=O (Carbonyl) and COO (ester) groups. The peak at 1741.28 corresponds to CH showing asymmetrical stretching and bending vibration in CH_3 group, whereas peak at 1242.12 representing COH bond. Stretch of bands ranging from 1057-1277 showed C-O bonding. FTIR studies has revealed that peak at 3435 corresponds to OH (Hydroxyl) group, whereas band at 1731 represents C=O (Carbonyl) and COO (ester) groups. The band at 1454 corresponds to CH showing asymmetrical stretching and bending vibration in CH_3 group, whereas band at 1379 representing COH bond. Stretch of bands ranging from 1057-1277 showed C-O bonding.

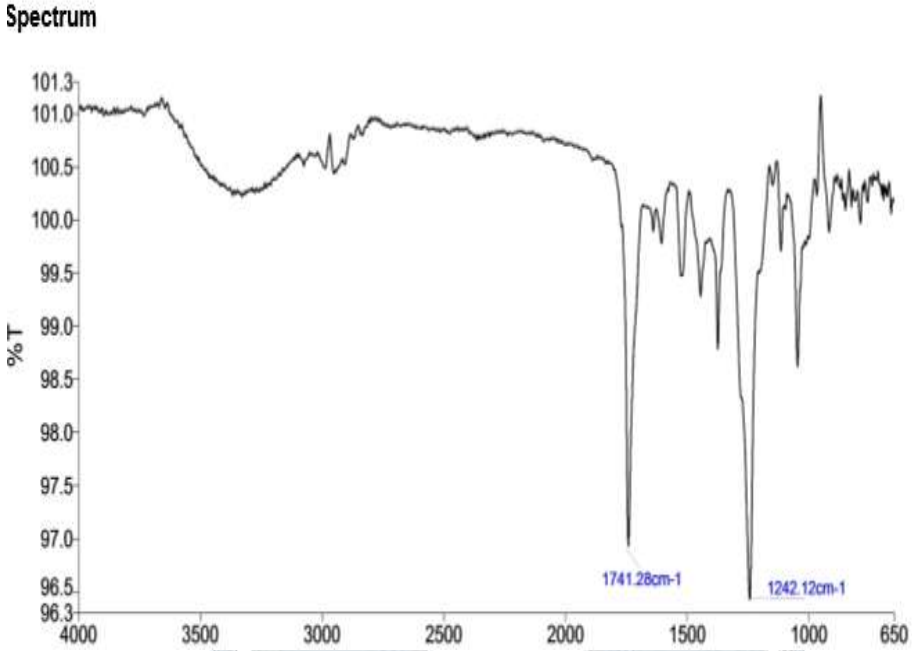

Figure 4. FT-IR Analysis

3.9. GC-MS Analysis

The GC-MS results has shows six peaks among the peaks highest area of percentage was observed in the retention time of 29.799 min, revealed the presence of hexadecane palmitic acid. Which was similar to the secondary metabolites bacteriocin present in the *B. thuringiensis.* Based on GC-MS library with its retention time and wave number the compound was identified as thuricin S.

3.10. Nursery Trails

Disease control ability of the isolated bacteriocin thuricin S was analyzed by nursery trail experiment in green shed in nursery trail research facility at Department of Microbiology, Bharathidasan University, Tiruchirappalli, Tami Nadu, India. The cowpea seeds were purchased from the local market and it was sterilized, viability of the seeds was checked before its sowing. Polybags were folded in black polybags with red soil, manure and sand at 1:1:1 ratio and seeds were sown into the polybags. After sowing the bags were watered regularly and the place of the bags were changed in two days interval in order to get the equal environmental exposure. Spray method was followed for this experiment and it was carried out in two different regimens, the first one is preventive and the second one is curative with three different doses like 1, 10 and 100ml along with five replicates in each treatment. In preventive method of treatment bacteriocin was sprayed 24h before the induction of the phytopathogen *Xanthomonas* sp. In curative method of treatment bacteriocin was sprayed 24 h after the induction of the pathogen. Further, a positive control, the plants induction was done with *Xanthomonas* sp. alone and a negative control was maintained without induction of any organism. After the bacteria blight symptoms development in positive control, the treatment plants also checked for the symptoms of disease development and it was compared with negative and positive control. The bacterial blight symptoms are very less in the both preventive and curative method of treatment (Fig.6).

IV. Discussion

Well known fact that *B. thuringiensis* act as a biopesticide, however it is also having antibacterial activity against wide variety of pathogens. Moreover, the *B. thuringiensis* also have plant growth promoting characteristics by producing IAA and siderophore, and phosphate solubilization. In first the production of IAA offers the great promise for sustaining increase in crop productivity. A low level of IAA production promotes primary root elongation by stimulating the plant cell elongation or cell division (Glick *et al*., 1998), where as a high level of IAA stimulates lateral and adventitious root formation but it inhibits primary root growth (Xie et al., 1996). The present study results have reveals that the *B. thuringiensis* strains RBL 20 produced a high amount of IAA, which would definitely favor the growth of the plants.

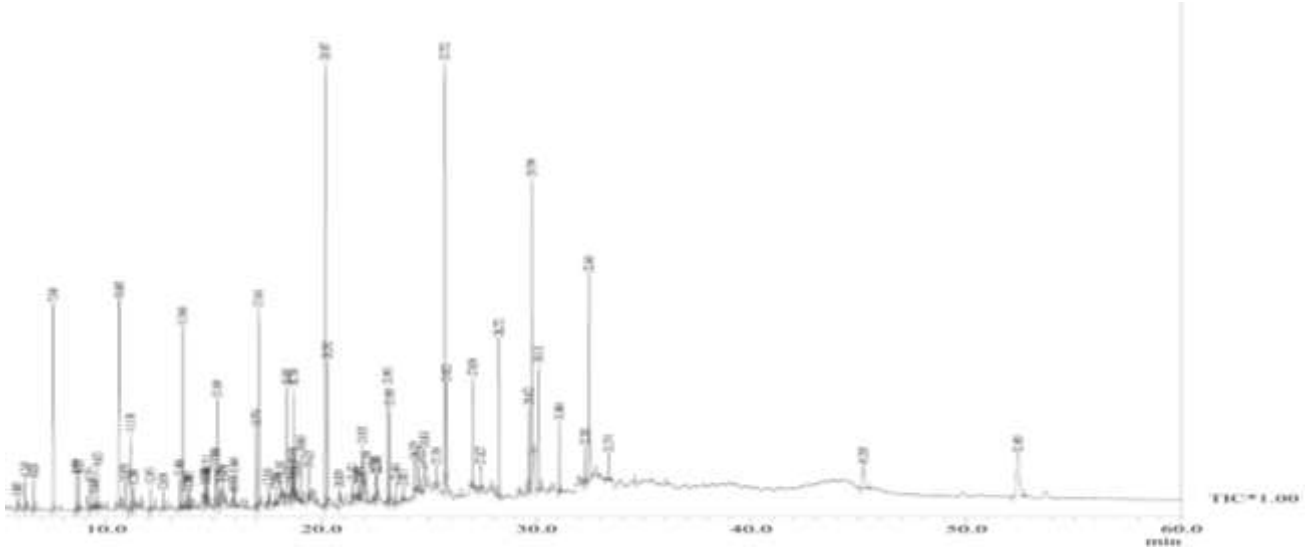

Figure 5. GC-MS Analysis

Figure 6. Nursery Trail Experiment. **A-** Curative method of treatment; **B-** Preventive method of treatment.

Second, siderophore production promotes plant growth by indirectly sequestering the available iron in the soils, especially neutral and alkaline soils and thereby, reduce it is availability for the growth of pathogen (Alexander and Zuberer, 1991; Subbarao, 1999). In this present study quantitative analysis of siderophore by Arnow assay method (Arnow, 1937) was performed for the bacterial strains and its pink colour confirmed its production of catechol type siderophore. Third, phosphate solubilization enhances the plant growth promotion and it have been shown to solubilize or precipitate phosphate and enhance phosphate availability to plant that represent a possible mechanism of plan growth promotion under field conditions. Free living phosphate solubilizing bacteria release phosphate from spare soluble inorganic and organic phosphate compounds in soil and so contribute available phosphate for the plants (Ashrufuzzaman et al., 2009; Yadav et al., 2010; Gopala Krishnan et al., 2011). In the current study, phosphate solubilizing bacteria was grown on pikovskaya's medium and it forms a clear zone around the colony, due to phosphate solubilization in the vicinity of the colony.

In the present study, antibacterial compound was extracted with different solvent like butanol, chloroform, N-hexane and ethyl acetate. After separation of compounds were screened by ager well diffusion against *Xanthomonas* sp. Among all the solvents butanol shows high antibacterial activity which it was recorded as 17 mm of zone of inhibition. For further, purification column chromatography was performed and 15 fractions were collected among them fraction No.12 has shown good antibacterial property as 19mm.

The FT-IR spectrum clearly showed peaks at 1739.8 cm^{-1}. The FT-IR spectrum observed at 1739.8 and 1373cm^{-1} revealed the presence of aliphatic C-H stretching of fatty acids at 1045.35 and 1240 cm^{-1} bared the presence of amide **I** and amide **II** respectively; at 3203.79cm^{-1} showed the presence of aromatic hydrocarbon; at 3468.35cm-1revealed the presence of primary and secondary amine (hydroxyl functionality); at 356.63 exposed the presence of free hydroxyl group. Likewise, Vijayalakshmi and Suseela (2016) characterized thuricin from *B. amyloiquifacians* through FT-IR analysis and the results showed that clear spectrum peaks at 3278, 1655 and 1544 cm^{-1} wave number which was provides a concentrate evidence for the presence of peptide bonds. The band at 1655 cm^{-1} indicates Amide I and the band at 1544 cm^{-1} indicates Amide II which was closely resembles to our present study. Further, the compound was identified using GC-MS analysis and it showed that peat at 1242.12 cm^{-1} which clearly resembled the compound thuricin S. In the present study thuricin S bacteriocin was identified from the *B. thuringiensis* were similar studied have been conducted by Chehimi et al. (2012) who has reported the identification of bacteriocin thuricin S from three novel *B. thuringiensis*.

In the present study, the pathogen suppression study also confirmed in the foliar spray method in the cowpea plant. Preventive and curative regimens followed for the study, the preventive regimen showed better control over the number of lesions formed, than the curative regimen. The applied compound over the surface of infected plant which protect the plant from the pathogen. At the 100 µl of compound spray treatment showed better control of lesions than the other treatment and control treatments.

V. Conclusion

The bacteriocins took major role in the disease control; mostly it acts as a probiotics. But, in the present study we described about the antibacterial activity of *B. thuringiensis* for the control of plant pathogen with plant growth promoting capability. These bacteriocins are produced by wide variety of bacteria but we have chosen *B. thuringiensis* in order to explore its antibacterial potential. In generally, most of the previous study reports show that *B. thuringiensis* used for the control of insecticide. To our knowledge we have used *B. thuringiensis* bacteriocin for the control of plant pathogen. Because, in disease control bacteriocins can solve the most challenging problems like multi drug resistance and pesticide resistance.

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