Analysis of Biopolymer Efficacy against *Xanthomonas* Causing Bacterial Blight Disease in Cowpea Under Nursery Experiment

[1]Ramya Rajendiran, [1]Madhubala Veeramuthu and [1]Rajesh Kannan Velu*

Rhizosphere Biology Laboratory, Department of Microbiology, Bharathidasan University,

Tiruchirappalli 620 024, Tamil Nadu, India

Tel.: +91 431 2407082; **Fax:** +91 431 2407045

Email address: ramyabdu@gmail.com

ABSTRACT

Polyhydroxyalkanoate (PHA) is the biodegradable plastic, which is shown the similar properties to the synthetic plastic. These synthetic plastics are eco-friendly, since they gets degraded in the environment within weeks to months. Apart from this PHA have several properties including antimicrobial activity. The major drawback of this polymer is, its production cost and brittleness. The present study aimed to identify the potential PHA producing bacterial strains from seaweeds. Based on PHA production and crotonic acid assay, two bacterial strains were screened and designated as A3 and S3, which were found to be efficient PHA producers. Potential PHA producers of A3 and S3 were identified as *Alcaligens* sp. and *Pseudomonas* sp. based on morphological and biochemical identification. The purified PHA had been chemically characterized by FT-IR. The purified biopolymer was applied to pathogen infected plant for the control of plant pathogen *Xanthomonas* sp. causing bacterial blight disease.

KEYWORDS: Seaweed associative bacteria, *Alcaligens* sp., *Pseudomonas* sp., Biopolymer, Phytopathogens, *Xanthomonas* sp, Bacterial blight disease.

I. INTRODUCTION

Polyhydroxyalkanoates (PHAs) are natural polymers which were synthesized and sequestered as intracellular granules by microorganisms belonging to the bacteria and archaea domains of life. PHAs as reserve material when carbon source is available in excess in the environment and there is limitation of nutrients essential for growth. It is a family of linear polyesters of 3, 4, 5 and 6-hydroxyacids they are found as discrete cytoplasmic inclusions that typically have a diameter from 0.2 to 0.8µm. The PHAs are recyclable and can be easily degraded to carbon dioxide and water (Doi *et al*., 1995). The PHA has attracted much commercial and research interests due to its biodegradability, biocompatibility, chemical diversity and its manufacture from renewable carbon resources. A PHA molecule is typically made up of 600 to 35,000 (*R*)-hydroxy fatty acid monomer units. PHA are commonly grouped in to two major categories the short chain length (scl) and medium chain length (mcl) PHAs. The repeat units of scl-PHAs are composed of hydroxyl fatty acids (HFA) having three to five carbon atoms, whereas, mcl-PHAs contain HFAs repeat units with six or more carbon atoms (Khanna, 2005).

In general, the scl-PHAs are more crystalline than the mcl-PHAs. These granules can be visualized by phase contrast microscopy due to their high refractivity or using staining dyes such as Sudan Black B and Nile red. The granules appear transparent, discrete, spherical particles with clear boundaries under a transmission electron microscope when thin sections of cells containing PHAs. The number and sizes of granules per cell differ depending on the PHA-producing microorganisms and their growth stage. Granules of PHA are surrounded by a phospholipids monolayer contains specific associated proteins. A PHA granule is not attached to structural protein, regulatory protein and cytosolic protein *via* hydrophobic interaction (Prieto *et al.*, 1999; Sudesh *et al.*, 2000).

The plant pathogens are considered economically important agricultural microorganisms around the world. They induce decay of large number of agricultural crops during the growing season and post harvest. The current agricultural practices increase the production, such as non-judicious application of chemical pesticides and fertilizers have imposed a long list of environmental and health hazards (Gunnell, 2007). The emergence, re-emergence and endemic plant pathogens pose a serious threat to the agricultural crops (Miller, 2009). In this present study, the PHA producing potential seaweed associative bacteria were screened and identified for the production of PHA. The plant pathogen was isolated from infected cowpea plant. Then the produced biopolymer were further purified and characterized through FT-IR analysis. Finally, the produced biopolymer was applied to *Xanthomonas* infected cowpea plant for the control of bacterial blight disease.

II. MATERIALS AND METHODS

2.1. Isolation of PHA Producers

Seaweeds of *Eucheuma denticulatum* and *Kappaphycus alerversii were* collected from Manapad seashore in Tuticorin District of Tamil Nadu, India (Fig.1). Each seaweed samples were cut into small pieces using sterile scissor for the purpose of isolating seaweed associative bacteria. The homogenized samples were spread on Vaatanen Nine salt solution (VNSS) agar medium and the plates were incubated at 25˚C for 48 hrs (Egan *et al*., 2001). The seaweed associative bacterial colonies were characterized according to their shape, color, margin, etc. The morphologically distinct colonies were selected and purified by repeated subculturing for at least three times on VNSS medium and maintained at 4˚C (Ramya *et al*., 2017).

Eucheuma denticulatum (A) ***Kappaphycus alerversii (S)***

Fig. 1.Seaweed sample collection

2.2. Screening of PHA Producers

2.2.1. Plate Assay Method

The bacterial colonies were examined for PHA accumulation by staining with Sudan Black-B (0.3 g in 70% ethanol) by using rapid screening method. The bacterial isolates were grown on nutrient agar medium supplemented with 1% glucose. The plate was divided into equal parts and in each part a bacterial isolate was spotted. Then plates were incubated at 30ºC for 72 hrs after incubation the ethanolic solution of Sudan black B was spread over the colonies and the plates were kept for 30 min. Finally the plates were washed

with ethanol (96%) to remove the excess stain from the colonies. The PHA producing colonies giving dark blue coloured were taken as positive for PHA production (Mohamed *et al*., 2012).

2.2.2. Screening of Bacterial Strains in Liquid Medium

1.5ml of bacterial culture was taken and centrifuged at 10,000 rpm for 10 min, and then the sedimented cells were washed with distilled water and stain with acridine orange (1mg/ml). Vortex the stained solution incubated for 5 min and centrifuged at 10,000 rpm for 10 min. After centrifugation discarded the dye, added water and again centrifuge remove water. Mix the cells with water placed on slide fixed cover slip and observed under Confocal Scanning Laser Microscope (Ostle *et al*., 1982).

2.3. Identification of Biopolymer Producers

2.3.1. Morphological and Biochemical Characteristics

The screened bacterial isolates were carried out for their identification on the basis of their colony characteristics, Gram staining, biochemical tests, sugar utilization tests and enzymatic activities test required by Bergey's manual of systematic bacteriology (Holt *et al*., 1994).

2.4. Production of Biopolymer

The efficient isolates were used to produce the PHA. A loopful of A3 and S3 culture were transferred to the 50 ml conical flask containing 20 ml LB broth and incubated at 37°C for 24 hrs. This 24 hours grown culture was used as inoculum and it was transferred to 500 ml of conical flask containing 200 ml of mineral salt medium and incubated at 37°C for 72 hrs at 100 rpm (Kumbhakar *et al*., (2012).

2.5. Extraction of Biopolymer

72 hrs old bacterial cultures was transferred to centrifuge tubes and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellet was transferred into pre-weighted petriplates by dissolving it into distilled water. Then, the plates were dried by keeping it in hot plate at 80°C. The dried weight of the pellet was taken in order to know the biomass weight.

Sodium hypochlorite solution was added to the dried pellet then transferred to the centrifuge tubes and kept in the shaker at 37°C for half an hr. In this step, the cell wall of the bacteria gets distrupted and the PHA gets released. After incubation, the centrifuge tubes were centrifuged at 8000 rpm for 15 min. The supernatant was discarded and the pellets were washed with distilled water and add acetone to remove the hypochlorite solution by centrifugation. After add cold methanol and chloroform there could be three phases were formed the bottom phase containing PHA was transferred into fresh tubes (Ojumu *et al*., 2004).

2.6. Purification of Biopolymer

2.6.1. Estimation of PHA

The extracted polymer granules were dissolved in concentrated sulphuric acid (1mg/ml). It was heated at 100°C for 10 min, the PHA were converted to crotonic acid. The absorbance was read at 235nm against a concentrated sulphuric acid as blank (Law and slepsky, 1991).

2.7. Characterization of Biopolymer

2.7.1. Characterization of PHA using FT-IR Analysis

The extracted PHA was analyzed for its functional groups through FT-IT. The PHA was dissolved in chloroform and added to KBr pellets and then the solvent was evaporated. The infrared spectra of the samples were recorded in the wave number range from 400 to 4000 cm^{-1} using a Perkin Elmer FT-IR spectrophotometer using KBr disc (Shamala *et al*., 2003).

2.8. Isolation of Plant Pathogen from Infected Cowpea Leaf

The infected cowpea leaf was collected from nursery of the field research facility, Department of Microbiology, Bharathidasan University, Trichirappalli. The surface sterilization of the infected leaf was carried out by using 70% ethanol for 30 sec and 0.1% mercuric chloride for 30 sec. After that prepared the nutrient agar, yeast dextroxe calcium carbonate agar, yeast tryptone sucrose agar (YTSA) and inoculate the plant leaf sample incubate the plates at 37°C for 24 hrs.

2.9. Physiological and Biochemical Characters of Plant Pathogen

The bacterial isolates were identified on the basis of their colony characteristics, Gram staining, biochemical tests, motility test, enzymatic test such as catalase, oxidase test, colony morphology in the YDC Agar, YTSA and nutrient agar as per Bergey's Manual of Systematic Bacteriology (Schaad, 1988).

2.10. Pathogenicity Test for the Pathogen

The cowpea plant was grown in nursery at suitable conditions using plastic bags, transfer a small quantity of selected pathogenic colonies to a culture tube with 5-10ml sterile saline water. The colonies must not be more than 3 days old. Infiltrate a leaf of an assay plant with the suspension of pathogenic bacteria by gently forcing the liquid into the lower surface of the leaf using a sterile syringe without a needle. Infiltrate a leaf with sterile saline water served as control. The inoculated plants were incubated at 90% humidity and at 37°C. The plants were observed daily and recorded the *Xanthomonas* sp. could develop a water soaked lesion in 48-96 hrs on the surface of leaf were inoculation done (Schaad *et al*., 2001).

2.11. Application of Biopolymer under *in vivo* Method

2.11.1. Nursery Experiment - Seed Coating Method

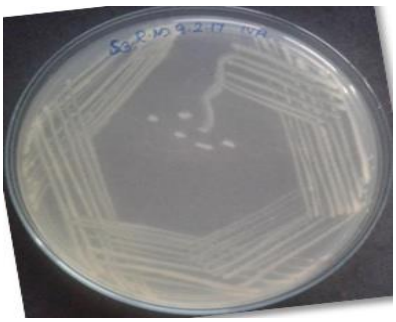
The nursery bag experiment for the plant growth promoting and disease control study was assessed in the month of March 2017. The soil was collected from "The Field Research Facility", Department of Microbiology, Bharathidasan University, Tiruchirappalli, India. The collected soils were sieved (2mm) and sterilized by steaming and packed in polythene bags. The seeds were surface sterilized using 0.1% of mercuric chloride for 3 min and rinsed 3 times with sterile distilled water, then rinsed with 70% ethanol for 3 mins. Cowpea seeds are soaked in extracted PHA compound along with carboxyl methyl cellulose for 12hrs and air dried overnight. Dried seeds transplanted in plastic bags (top diameter 300mm, 160mm bottom and height 16cm) containing 500g of sterilized soil. Treatment was arranged in complete randomized block design (CRBD) and in the nursery condition for biopolymer control phytopathogen experiment. The position was altered once in every 5 days to expose the seedlings to uniform environmental conditions. Bags were watered using sterile water regularly and care was taken. 20 days old plants observed for the infections on the leaf and stem. The extracted PHA was spray on the infected areas in the range of 10, 50 and 100 µl and watered regularly and care was taken after 48 hrs monitor the plant facts and observes the disease severity. It could be compared with the other treated and control plants (Campbell *et al*., 1980).

III. RESULTS

3.1. Isolation of PHA Polymer Producing Bacteria

A lot of potential microorganisms especially bacteria for biopolymer production were obtained from marine environment and it was mainly focused to isolate a diverse range of PHA producers from seaweed associative bacteria with the hope of producing different classes of PHAs (Fig.2). From, the total number of isolated colonies, only 2 isolates was responded in positive for Sudan Black-B and Nile Red staining based on the intensity of the fluorescence observed in agar plate method. The highest PHA production was observed in S3 and A3 isolates. Based on the dry weight of the extracted PHAs, an effective producer of PHAs was selected for further study.

A3



S3

Fig. 2.Isolation and purification of biopolymer producing bacterial isolates

3.2. Screening of Biopolymer Producers

The isolates were initially screened by Sudan Black-B staining for PHA production using to investigate their ability to synthesis PHA granule. On microscopic observation, the 24 hrs grown culture showed the bluish black granules of PHA in (Fig. 3A). The PHA producing bacterial isolates were confirmed by Sudan black-B staining method, in order to found that the PHA granules present in the cytoplasm of bacterial cells.

A3 S3

Fig. 3A.Sudan black-B staining microscopic observation

3.3. Plate Assay Method

The biopolymer producers were screened and confirmed by plate assay method using Sudan Black staining. In plate assay method blue blackish granules were appeared and it was confirmed that the organism had the ability to produce the PHA (Fig. 3B).

A3 S3

Fig. 3B. Sudan black – B staining macroscopic observation on agar plate

3.4. Screening of PHA Producer in LB Liquid Medium

Confocal laser scanning fluorescence microscopy is a powerful technique for the detection of PHA granule formation at a wavelength of 543 nm. This microscopy was employed to investigate the PHA granule abilities of the A3 and S3 strains and the results revealed that both strains shows green fluorescence PHA granule in black background (Fig. 3C).

A3 S3

Fig. 3C.Confocal scanning laser microscopic observation of PHA producing bacterial cells

3.5. Identification of Biopolymer Producers

3.5.1. Morphological and Biochemical Characteristics

The potential PHA producing bacterial strains A3 and S3 were identified by colony morphology and biochemical characteristics analysis and the strains were confirmed through biochemical analysis according

to the Bergey's manual of determinative bacteriology. The potential PHA producing A3 and S3 were tentatively identified as *Alcaligens* sp and *Pseudomonas* sp. respectively.

3.6. Production and Extraction of Biopolymer

PHA production by the bacterial strains A3 and S3, which has indicates that PHA is a growth associated product and its accumulation significantly increased on stationary phase. The maximum growth was observed after 72 hrs of incubation based on the turbidity of the culture (Fig. 4). The organic solvents were investigated to determine their efficiency to recover PHAs and separation of them from cell debris after extraction. The maximum efficiency of solvent recovery of PHA was attained by chloroform - hypochlorite extraction (Ramsay *et al*., 1990).

S3 A3

Mother inoculum

S3 A3

Mass production of PHA

Fig. 4.Production of biopolymer of A3 and S3

3.7. Characterization of PHA using FT-IR Analysis

The FT-IR spectra of the PHA produced by two strains were analyzed. An intensity band was observed at around 1230.73 cm^{-1}, which was assigned to the stretching vibration of the C-O-C group. The region of 1625.14 cm^{-1} and 1633.13-1724.04 cm^{-1} were associated with the C=O stretching of the ester carbonyl bond. The bond at 1531.78 cm^{-1} and 1535.67 cm^{-1} were characteristic of the stretching and deformation vibration of the C-H group and those at 2924.12 cm^{-1} and 3280.06 cm^{-1} were characteristics of the stretching and deformation vibrations of the O-H groups (Fig. 5A and 5B). The FT-IR peaks were identical to those displayed by commercial PHA. The functional groups of the polymer PHA were confirmed as C=O groups by FT-IR spectroscopy. Hence, the FT-IR was analysis confirmed the presence of PHA in the extract by showing the presence of functional groups of PHAs.

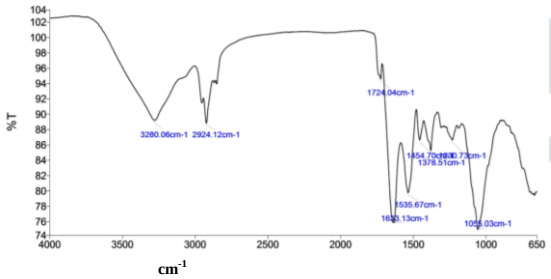


Fig. 5A. FT-IR Analysis of PHA from *Alcaligens* sp.

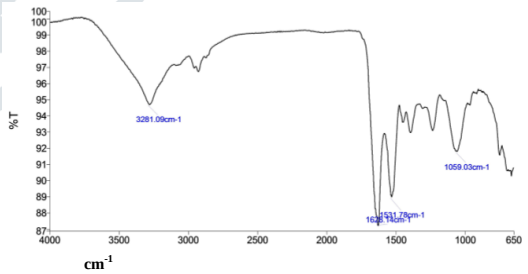


Fig. 5B. FT-IR Analysis of PHA from *Pseudomonas* Sp.

3.8. Isolation and Identification of Plant Pathogen

The leaf samples were placed on YTSA plates showed the bacterial colonies after 48 hrs of incubation at 30ºC. The colony colour of isolate was variable from yellow to light yellow. The size and shape of colony was found to be small, convex and mucoid (Fig. 6A). The pure culture was done by streaking method on nutrient agar plate. From a single colony, a loopful of culture was streaked on slants to preserve the isolate for a longer period of time. Selective growth on the YTSA agar plate and colony morphology that the bacterium as *Xanthomonas* sp. (Fig. 6B).

(A) Yeast dextrose carbonate agar

(B) Pure culture in nutrient agar

Fig. 6. Isolation of plant pathogen from infected cowpea leaf

3.9. Pathogenicity Assay

The strain of *Xanthomonas* sp. was inoculated to 30 days old cowpea plant, by spraying the bacterial culture to pre-injured leaves. The plants started producing small water soaked lesions on the leaves. The lesions became necrotic leading to blighting of infected leaves. The first symptoms of the disease observed 15 days after inoculation of pathogens. Re-isolations of the bacterium made from inoculated plants yielded yellow coloured colonies on YTSA and the strain was confirmed as *Xanthomonas* sp. (Fig. 7).

Uninfected cowpea leaf

Infection on the cowpea leaf

Fig. 7. Pathogenicity assay

3.10. Application of Biopolymer under *in vivo* Method

3.10.1. Nursery Experiment - Seed Coating Method

Cowpea seeds were inoculated with potential *Xanthomonas* sp. and seeds without inoculum were act as the control. After plants have been cultivated and observe the symptoms of bacterial infection with lesions on leaves. The biopolymer spray to the plant with infection of *Xanthomonas* sp. and after 48 hrs it control the infection on leaves (Fig. 8A and 8B).

Fig. 8A.Seed Coating Method

Leaves sprinkled with 10µl of PHA

Treated with 10µl of PHA

Leaves sprinkled with 50µl of PHA

Treated with 50µl of PHA

Leaves sprinkled with 100µl of PHA

Treated with 100µl of PHA

Fig. 8B. Polybag experiment in nursery

IV. DISCUSSION

The present study was mainly focused on production of PHA to minimize the production cost. There is no easier report of PHA production from seaweed associative bacteria. The initial study was carried out to confirm the viability of the bacterial strains using nutrient agar plate. During the starvation period excess carbon stored in the cytoplasm of bacteria cell is converted into the form the PHA granules confirmed by Mohamed *et al*. (2012).

Bacteria cells are utilized the dextrose as carbon source and they also used a mixture of different salts as mineral source. It has been observed that the composition of trace elements in solutions also play important roles in the metabolic regulation of PHB accumulation in bacterial cells (Grothe, 2000). The most common methods for PHA recovery from bacterial cells involve the use of solvents such as sodium hypochlorite and chloroform distrupted the cell wall of the bacteria and the PHA gets released during extraction of PHA was confirmed by Ojumu *et al*. (2004). After the solvent modifies the cell membrane and dissolves PHA, separation of the polymer from the solvent is necessary. This can either be mediated by evaporation of the solvent or precipitation of PHA by a non-solvent, such as ethanol, methanol or even water (Hanggi, 1990; Zinn *et al.*, 2003). In contrast, PHA separates from lysed cell matter in an aqueous sodium hypochlorite solution through sedimentation because it is insoluble in water.

The functional groups of PHA were determined using the FT-IR analysis. The FT-IR spectra showed high absorbance at 3280.06, 3281.09, 2924, 1724 and 1625.14 cm^{-1}, which were resulted in the vibration functions of O-H, C-H and C=O-C, respectively. Likewise, Phukon *et al.* (2011) analyzed the P(3HB-co-3HV) biopolymer produced by *Alcaligens* sp. and *Pseudomonas* sp., by FT-IR. The results were described above are congruent with the findings previously reported by several studies in the literature (Shah, 2012), particularly 2924.12 cm^{-1} (CH, CH_2, CH_3), 1724.04 cm^{-1} (ester C=O valence) and 1633.13 cm^{-1} (thioester C=O valence). Acros (2010) also characterized the functional group of the P (3-HB-co-3HV) produced by a mixed culture of bacterial systems by FT-IR. The FT-IR spectra showed high absorbance at 1724 and 1230.73 - 1055 cm^{-1}, which were resulted in the vibration functions of C=O and C-O-C, respectively.

The plant pathogen was isolated from the infected leaves based on the morphological, biochemical and molecular identification the belongs to the genus *Xanthomonas* sp. (Schaad *et al*., 2001). Pathogenicity

studies are usually conducted on shoots (leaves and stem) of young plants growing in bags, however this usually takes at least three weeks. Using cotyledons considerably shortens the period in which results can be obtained by as much as 80% as these results can be ready within 1 week. The polybags experiment of the cowpea seed coated with *Xanthomonas* sp. and transfer to the plastic pot appropriate cultivation period the show the symptoms in the plant leaves, the PHA were sprinkled to the plant in different concentration due to its innate immunity and defence mechanism. The PHA has antimicrobial activity and it act against *Xanthomonas* sp. and slowly it control the infections within 48 hrs.

V. CONCLUSION

The present study was confirmed on production of PHA and its impact in phytopathogen control. Consequently, much effort has been devoted to reduce its production cost by improving bacterial strains, efficient fermentation and recovery processes. In this study, the PHA accumulation through seaweed associative bacterial strains of *Alcaligens* sp. and *Pseudomonas* sp. was confirmed. Further, the produced PHA was well characterized to P (3HB-co-HV) functional group by FT-IR.

From the nursery trial, we concluded that the characterized PHA had been used to treat the plant pathogen as well as good growth promoting agent in the field of agriculture. Further, the investigations leads to the commercial production practices of these PHA and enhanced the crop yield. Hence, due to these findings, we hope that the biodegradable plastics could play a major role as ecofriendly in environment and agriculture.

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