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Isolation Purification and Screening of Biodegradable Polymer PHB Producing Cyanobacteria from Marine and Fresh Water Resources

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Abstract: Fifteen cyanobacteria species were isolated from fresh water and marine water resources from different parts of Tamil Nadu, India. Based on their morphological features they were identified through microscopic observations. The isolates were then screened for PHB production using nile red staining. It was found eleven of them were capable of producing PHB in their native forms. Further, PHB quantitative analysis by HPLC showed marine *Phormidium* sp with 7.60±0.005 mg/L (7.6%) of PHB, followed by *Synechococcus* sp with 4.59±0.012 mg/L (4.5%), *Synechocystis* sp with 3.74±0.007 mg/L (3.7%) and *Anabaena* sp with 2.31±0.012 mg/L (2.3%) of fresh water isolates. Among the isolates, *Phormidium* sp (VIT-BMN3) isolated from marine environments is reporting first time for its PHB production. In addition, biodegradable polymer extracted in hot chloroform is analysed by FTIR which showed strong bands near 1725 cm^{-1}, 2977 cm^{-1} and 3437 cm^{-1} wave numbers and confirms the presence of ester carbonyl group (C=O), methylene C-H vibration and terminal OH group of PHB, respectively. Overall, nile red staining, HPLC analysis and FTIR spectrums obtained for all cultures conforms the PHB production by our isolates in their native conditions. Further studies of media optimization for increased PHB production are under the process.

Key words: *Cyanobacteria* · Poly-β-hydroxy butyrate · Biopolymers; Biodegradable

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are microbial polyesters produced by various microorganisms [1] including cyanobacteria. Cyanobacteria, also known as blue-green algae are prokaryotic organisms which can carry out oxygenic photosynthesis under photoautotrophic conditions [2, 3]. PHAs are among the most investigated biodegradable polymers in recent years due to their similar chemical and physical properties to polypropylene and also some special properties like nontoxic, biocompatibility and biodegradability [4]. PHAs composition was first reported from bacteria as unknown material in the form of homopolyester of the 3-hydroxybutyric acid (PHB) [5] and the biodegradability of PHB was noticed since 1958 by Macrae, Wilkinson and others [6-8]. Today, more than 300 different microorganisms are found to synthesize and accumulate PHAs intracellular. The most well studied type of PHAs is PHB.

To reduce the production cost, it is more advantageous to use photosynthetic organisms such as plants and cyanobacteria as alternative host systems because of their minimal nutrient requirement for growth and biomass production and photoautotrophic nature [9, 10]. Recently, higher plants into which PHB synthesis genes have been introduced have been used to produce PHB from CO_2 by photosynthesis. The transformant of *Arabidopsis thaliana* harboring PHB genes in a plastid accumulated approximately 14% dry weight as PHB in its leaves [11]. Under appropriate growth conditions, PHB can be accumulated up to 85% of the cellular dry weight of *Alcaligenes eutrophus* [12] and 70-90% in recombinant *Escherichia coli* cells [13-15]. Nevertheless, the demands for plant products can be expected to increase, which certainly will require more fertile land to be used for agricultural activities. In such a scenario, using cyanobacteria to produce PHB may become more promising because the large-scale cultivation of cyanobacteria does not require fertile land [16].

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In addition, cyanobacteria grow faster than higher plants and some can accumulate PHB under nitrogen or phosphorus limited conditions [17]. Cyanobacteria have a mechanism for storing phosphate predominantly in the form of polyphosphate and during phosphate deficiency these organisms counter the paucity of ð by breaking the polyphosphate chains [18]. Various species of cyanobacteria accumulate considerable amounts of PHB. The presence of PHB inclusion bodies in cyanobacteria was first reported by Carr in 1966 [19] following extraction of PHB from *Chloroglea fritschii* [20]. To date, the occurrence of PHB has been demonstrated for several cyanobacteria such as *Spirulina* sp, *Aphanothece* sp, *Gloeothece* sp, *Synechococcus* sp etc. Since then, the occurrence of PHB has been reported for several other species of cyanobacteria, including *Gloeocapsa* sp., *Spirulina platensis, Aphanothece* sp [21], *Synechococcus* sp [22], *Synechosystis* sp [23]. However, *Synechosystis* sp PCC6803 has only been reported so far for highest amount of PHB accumulation nearly 38% of its dry cell weight (DCW) [14]. Industrial utilization of cyanobacteria as PHB producers has the advantage of converting waste carbon dioxide, a greenhouse gas to environmental friendly plastics using the energy of sunlight. Therefore, in the current situation, searching for novel cyanobacterial species for increased PHB production has become obligatory. In the present report, 15 cyanobacterial species from different environments were isolated and screened for PHB production in their native conditions using nile red staining method followed by HPLC and FTIR analysis.

MATERIALS AND METHODS

Sample Collection: Samples were collected from both fresh water regions as well as from marine environments of different parts of Tamil Nadu which includes Trichy, Elagiri, Chennai, Mandapam, Vellore.

Isolation and Purification of Cyanobacterial Species: Cyanobacterial species were isolated and purified from different sources using basic microbial techniques primarily with serial dilution and followed by spread plating and quadrant streaking on BG11 agar plates. Bacterial and fungal contamination was avoided using antibiotics like; streptomycin and cycloheximide, each at 100 µg/mL [24]. Morphological identification of the cyanobacterial strains was performed through microscopic observations. In total 15 different strains were isolated, in which 12 strains from fresh water bodies and 3 strains are from marine environments.

Culture Conditions: The purified axenic cultures were grown in 250 mL Erlenmeyer flasks containing 100 mL of BG11 broth medium (Himedia). The medium constituents were citric acid: 0.006 g, ferric citrate: 0.006 g, EDTA: 0.001 g, Na_2CO_3: 0.02 g, $MgSO_4.7H_2O$: 0.075 g, $CaCl_2.2H_2O$: 0.036 g, K_2HPO_4: 0.04 g, $MnCl_2.4H_2O$: 1.81 mg, Na_2MoO_4: 0.039 mg, H_3BO_3: 2.86 mg, $CuSO_4.5H_2O$: 0.079 mg, Co $(SO_4).7H_2O$: 0.04 mg and $ZnSO_4.7H_2O$: 0.222 mg/L. The pH of the culture medium was maintained at 8.0. Experimental cultures were incubated at 25±2°C, 14/10 light/dark cycle with illumination of 3000 lux under cool white fluorescent lamps. Every day the cultures were mildly shaken by hand for 10 minutes.

Screening for PHB Producing Cyanobacterial Species

Nile Red Staining: PHB accumulation in cyanobacterial isolates was observed by staining with lipophilic dye Nile red. Staining solution was prepared by dissolving 1 mg Nile red in 1 mL of dimethyl sulfoxide (DMSO). Two drops of Nile red dye solution were added to 200 µL sterile culture medium and incubated for 10 min at 55°C. Cells were then centrifuged and washed with PBS solution to remove excess of dye. Slides were prepared with stained cultures and were observed under fluorescent microscope (Olympus-BX61) at an excitation wavelength of 450–490 nm [16].

Quantification of PHB using HPLC: A modified HPLC method [19] was used to quantify the PHB produced from different cultures. From each purified samples (O.D 1-1.5@ 730 nm) 5 mL of culture was taken in 15 mL screw cap centrifuge tubes and centrifuged (MPW-351R, Poland) at 10,000 rpm for 10 minutes. The resulting pellet was dried at 80°C at overnight. The dry pellets were boiled in 1 mL of conc. H_2SO_4 at 90°C for 30 minutes. Samples were then diluted with 4 mL of 5 mM H_2SO_4 and vortexed. From this, 200 µL of sample was taken in a fresh micro centrifuge tube and further diluted 10 times with 5 mM H_2SO_4 followed by membrane filtration using 0.22 µm filters (Millipore). 50 µl of this filtered sample was then analysed by HPLC with an ROA column 78 X 300 mm (Shimadzu CBM-20A, Made in Japan). Commercially available PHB (Sigma-Aldrich) was also processed in parallel with the samples.

Extraction of poly-β-hydroxybutyrate (PHB): Cells were harvested by centrifugation at 8000 rpm for 10 min and washed with distilled water followed by overnight methanol suspension at 4°C for the removal of pigments. The pellet obtained after centrifugation was dried at 60°C for 1 h and PHB was extracted into hot chloroform using

soxhlet extractor [25]. After evaporation of the solvent, PHB was obtained as a tough, translucent film which was further analyzed by FTIR spectroscopy.

Fourier-transform Infrared Spectroscopy (FT-IR) Analysis: Chloroform was evaporated from the extracted PHB and KBr pellet was prepared with PHB from different cultures and standard PHB from Sigma. A PerkinElmer spectrum GX FTIR spectrometer was used with spectral range, 4000-450 cm^{-1} to record the IR spectra.

RESULTS AND DISCUSSION

The efficient production of PHB (bioplastics) using cyanobacteria is technologically challenging. Nevertheless, it remains as an attractive approach considering the fact that the carbon source comes directly from atmospheric CO_2 for PHB synthesis [13, 26]. The present study aimed to screen novel and potential cyanobacterial species from different environments for PHB production.

Cyanobacterial Isolates and Screening for PHB Production: A total of 15 strains were isolated from different regions of Tamil Nadu. Among the strains isolated, 3 species are from marine resources while rest of them are from freshwater regions. On the basis of their morphological features observed through microscopic studies, cultures were identified in genus level as *Anabaena* sp., *Synechococcus* sp., *Synechocystis* sp., *Spirulina* sp., *Nostoc* sp., *Phormidium* sp., *Oscillatoria willei, Lyngbya* sp. and summarised them in Table 1. All species were primarily screened for PHB production using Nile red staining method and found 11 out of them are capable of producing PHB which were seen as bright orange colour inclusions under the fluorescent microscope (Fig. 1). All the positive species were named as VIT-BMN1 to VIT-BMN11.

Quantification of PHB using HPLC: Eleven cyanobacterial isolates found promising for PHB production with Nile red staining, were further taken for PHB quantification using high performance liquid chromatography by converting PHB into crotonic acid. PHB analysis relies on measuring crotonic acid which is formed by acid-catalyzed elimination during chemical depolymerization of PHB. The crotonic acid from acid digested PHB (Sigma) showed one peak with retention time of 31 min and it served as standard to calculate the PHB produced by different cyanobacterial isolates. The cultures treated with con. H_2SO_4 have also shown single peak with no change in their elution pattern with respect to retention time. Among all species, *Phormidium* sp. isolated from marine environments was found to accumulate maximum PHB of 7.606±0.005 mg/L medium followed by marine *Synechococcus* sp., fresh water cultures *Synechosystis* sp. and *Anabaena* sp. of PHB 4.599±0.012 mg/L, 3.746±0.007 mg/L and 2.314±0.012 mg/L respectively. Stal [24] has reported that *Phormidium* sp. isolated from microbial mat are found negative for PHB accumulation but in oppose to Stal's statement, findings in the present study showed marine *Phormidium* sp. with maximum PHB accumulation among all isolates. Further to enhance the PHB production, media optimization and genetic manipulation studies on this marine isolate *Phormidium* sp. could results with high yield. The amount of PHB produced by different species of marine and fresh water cyanobacterial cultures were summarised in Table 1. However, the reason for higher production is yet to discover.

Qualitative Analysis of PHB using FT-IR Spectrometry: Fourier-transform infrared spectroscopy (FT-IR) has been proved to be a powerful analytical tool for studying microorganisms and their cell components in intact form [27-30]. It has been reported that PHB is observable in FT-IR spectra [31-33]. In the present study, PHB

Table 1: Quantitative analysis of PHB produced by different isolates using HPLC

S. No	Strain No.	Species	Source	PHB mg/L
01	VIT-BMN 1	*Anabaena* sp.	Fresh water	2.30±0.012
02	VIT-BMN 2	*Synechococcus* sp.	Marine environment	4.60±0.012
03	VIT-BMN 3	*Phormidium* sp.	Marine environment	7.60±0.005
04	VIT-BMN 4	*Synechosystis* sp.	Fresh water	0.83±0.001
05	VIT-BMN 5	*Spirulina* sp.	Fresh water	1.18±0.007
06	VIT-BMN 6	*Nostoc* sp.	Fresh water	0.54±0.008
07	VIT-BMN 7	*Phormidium* sp.	Fresh water	0.90±0.004
08	VIT-BMN 8	*Synechosystis* sp.	Fresh water	3.75±0.007
09	VIT-BMN 9	*Oscillatoria willei*	Marine environment	0.42±0.007
10	VIT-BMN 10	*Lyngbya* sp.	Fresh water	0.71±0.007
11	VIT-BMN 11	*Nostoc* sp.	Fresh water	0.58±0.021

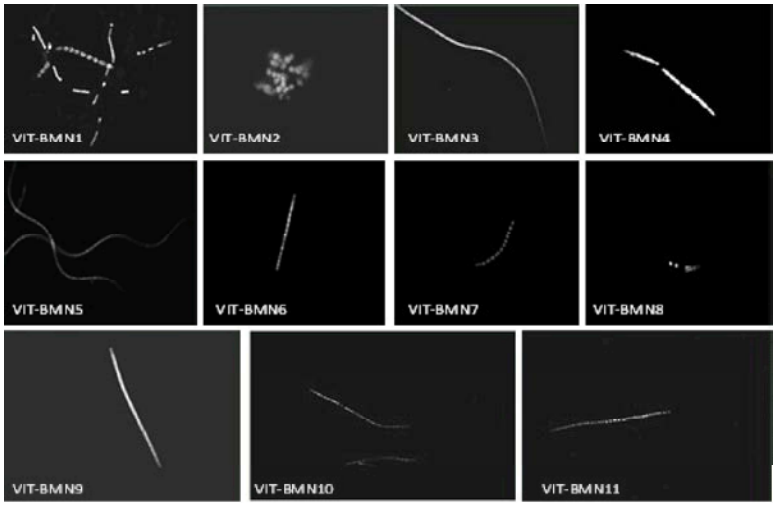

Fig. 1: Nile red staining of different Cyanobacterial isolates showing PHA granules as bright inclusions under fluorescent microscope (Olympus-BX61).

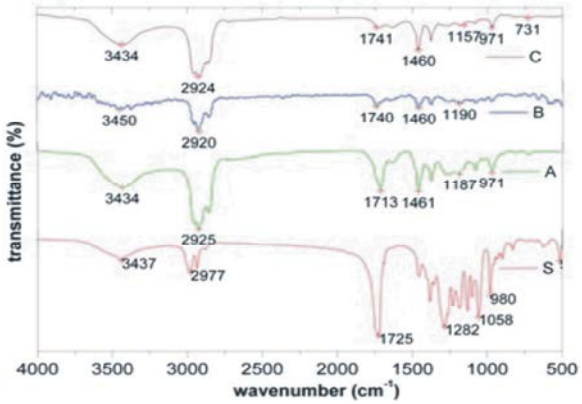

Fig. 2A: FT-IR analysis of PHB extracted from different isolates showing similarities with standard PHB obtained from sigma. (S) Standard, (A) *Anabaena* sp. (B) *Synechococcus* sp. (C) *Phormidium* sp.

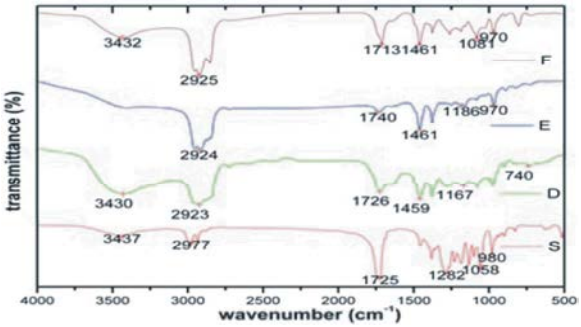

Fig. 2B: FT-IR analysis of PHB extracted from different isolates showing similarities with standard PHB obtained from sigma. (S) Standard, (D) *Synechosystis* sp. (E) *Spirulina* sp. (F) *Nostoc* sp.

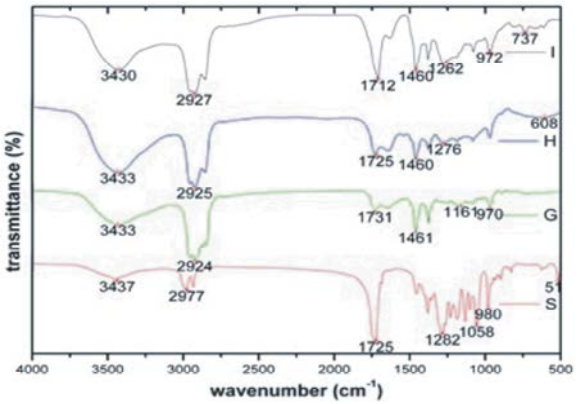

Fig. 2C: FT-IR analysis of PHB extracted from different isolates showing similarities with standard PHB obtained from sigma. (S) Standard, (G) *Phormidium* sp. (H) *Synechosystis* sp. (I) *Oscillatoria willei*.

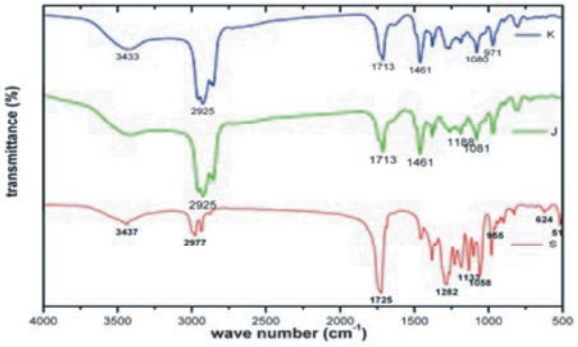

Fig. 2D: FT-IR analysis of PHB extracted from different isolates showing similarities with standard PHB obtained from sigma. (S) Standard, (J) *Lyngbya* sp. (K) *Nostoc* sp.

produced by different strains were observed by FTIR analysis. The standard PHB (sigma) showed its strong bands at 1725 cm^{-1}, 2977 cm^{-1} and 3437 cm^{-1} which corresponds to ester carbonyl group (C=O), methylene C-H vibration and its terminal OH group respectively [34-36]. Similar band patterns were observed in spectrum obtained for all extracted PHB samples of different cyanobacterial isolates and correlated to standard PHB spectrum. The spectrums obtained for standard PHB as well as extracted PHB's are given in a comparative manner in the Figs. 2A-2D.

CONCLUSIONS

At present, PHB production from photosynthetic microorganisms to produce biodegradable plastics as an alternative to conventional plastics is considered to be cost effective and eco-friendly compared to bacterial production of PHB [37, 38]. In this context, as a first report from Tamil Nadu, India this study reports 11 cyanobacterial species for PHB production in their native forms. Among the isolates *Phormidium* sp. (VIT-BMN3) is reporting first time for its PHB accumulation. The poly-β-hydroxybytyrate production of each individual was confirmed by Nile red staining method, HPLC analysis in addition with FT-IR spectrum analysis. Further studies on these isolates for improved PHB production will improve the ability to discover unexploited industrially important cyanobacteria present in the environment.

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چکیده

پانزده گونه سیانو باکتری از آب شیرین و منابع آب دریایی از نقاط مختلف تامیل نادو، هند ایزوله شد. این پانزده گونه بر اساس ویژگی های مورفولوژیک و از طریق مشاهدات میکروسکوپی شناسایی شدند. سپس گونه های ایزوله شده برای تولید PHB با استفاده از رنگ آمیزی قرمز نیل غربال شدند. مشخص شد یازده گونه از آنها قادر به تولید PHB در حالت طبیعی خود بودند. علاوه بر این، تجزیه و تحلیل کمی PHB توسط HPLC، وجود گونه دریایی سودوموناس پرمیدیوم با mg/L ۰/۰۰۵± ۷/۶ (۷/۶٪)، و وجود سودوموناس سینکوکوکوس با mg/L ۰/۰۱۲± ۴/۵۹ (۴/۵٪)، سودوموناس سینکوسیستیس با mg/L ۰/۰۰۷± ۳/۷۴ (۳/۷٪) و سودوموناس آنابنا با mg/L ۰/۰۱۲± ۲/۳۱ (۲/۳٪) از آب شیرین را نشان داد. در میان گونه های ایزوله شده، گونه دریایی سودوموناس پرمیدیوم (VIT - BMN3) برای اولین بار برای تولید PHB گزارش شده است. علاوه بر این، پلیمر زیست تخریب پذیر استخراج شده در کلروفرم داغ توسط FTIR آنالیز شد که باندهای قوی نزدیک به عددهای موج ۱۷۲۵ ، ۲۹۷۷ و ۳۴۳۷ cm^{-1} را نشان داد و به ترتیب حضور گروه کربونیل استر (C = O) ، ارتعاش C-H متیلن و گروه OH ترمینال از PHB را تایید کرد. به طور کلی، رنگ آمیزی قرمز نیل، تجزیه و تحلیل HPLC و طیف FTIR به دست آمده برای تمام کشتهای میکروبی، تولید PHB از گونه های ایزوله شده را تایید کرد. مطالعات بیشتر بر روی بهینه سازی محیط کشت برای افزایش تولید PHB تحت فرآیند می باشد.