





RESEARCH ARTICLE

Extraction and characterization of polyhydroxybutyrate (PHB) from *Bacillus flexus* MHO57386.1 isolated from marine sponge *Oceanopia arenosa* (Rao, 1941)

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ABSTRACT

Polyhydroxybutyrate (PHB) is the most widely studied biodegradable plastic that does not release any toxins or residues in the environment like petroleum based plastics. This work has been undertaken to screen PHB accumulating microorganisms from marine sponges and a total of sixteen isolates were collected and purified. Screening of isolated strains was done by Nile blue staining and observed under Leica LSCM to confirm the production of PHB. Yellow pigmented AB8a isolate from *Oceanopia arenosa* scored positive for PHB accumulation and subjected to morphological, biochemical and phylogenetic characterization. The biopolymer was extracted by dispersion of sodium hypochlorite and chloroform solution and characterized by FT-IR and ¹H NMR for the confirmation as PHB. The highest PHB production (70.25% /100 ml) was achieved at pH 7.0 by applying dextrose as medium at incubation temperature 30°C and 150 rpm agitation speed. The FTIR spectrum of the PHB sample showed major peaks at 3457, 1692, 1550, 1454, 1420, 1190 and 1050 cm⁻¹, whereas the remaining peaks are closely laid between 3450 cm⁻¹ and 600 cm⁻¹. ¹H NMR spectrum of PHA isolated from dextrose media indicated characteristic signals of PHB. The spectrum also revealed the presence of three groups of signals characteristic of PHB by the doublet at 1.3 ppm attributed to the methyl group coupled to one proton; and the spectrum of the quadruplet at 2.57 ppm, the methylene group adjacent to an asymmetric carbon atom bearing a single proton and the multiplet at 5.28 ppm indicated signals of PHB. The PHB accumulated bacterium identified as *Bacillus flexus* strain based on characterization studies and 16S rRNA sequence analysis and confirmed the presence of intracellular accumulated polymer substantiated as PHB.

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Introduction

Bacterial polyhydroxyalkanoates (PHAs) are alternates for petroleum based polymers due to their eco-friendliness, which are produced and stored by prokaryotes as cytoplasmic inclusion bodies in response to environmental stress (Castilho et al., 2009; Chen, 2009; Rehm, 2010). PHAs are polyester biological macromolecules and reported to be outstanding because of their biodegradability and biocompatibility (Koller, 2018). In bacteria, PHA synthesis is triggered by stressful conditions and it can be produced industrially when the medium contains excess of carbon source with nitrogen limitation (Saharan et al., 2014). Polyhydroxybutyrate (PHB) is the most widely studied and best characterized derivative of PHA (Bhuwal et al., 2014).

Various bacteria from different environmental niches have been sourced for PHBs production and marine bacteria are rarely discovered for PHBs synthesis (Numata & Morisaki, 2015). The majority of PHB producing bacteria was isolated from soil and activated sludge (Getachew et al., 2016). Recently, new bioresources such as marine environments were also explored regarding their potential to harbour new PHB producers. In general, marine ecosystems are unique habitat of microbes which are exposed to a wide variety of environmental conditions including extremes in temperature, salinity, nutrient limitation and pressure (Poli et al., 2017).

To date majority of chemicals have been identified from marine invertebrates of which sponges predominate (Lie and Zhou, 2002). Considering its pharmaceutical and drug development prospective, they are known to produce excellent resource of novel bioactive secondary metabolites (Koopmans et al., 2011). As marine sponges are usually having symbiotic relationship with different microorganisms and the marine sponge-associated symbionts have been accepted as prosperous resource of biological macromolecules, research on sponge-associated bacteria will provide remarkable new avenues for biopolymer research in future (Lie & Zhou, 2002).

The first documented PHA producing bacterial genera was *Vibrio* isolated from different marine arenas (Baumann et al., 1971; Oliver & Colwell, 1973). In marine haloarchaea, the first PHB accumulation was reported in *Halobacterium* sp. from Dead Sea and it was authenticated through free-fracture technique (Kirk & Ginzburg, 1972) followed by the genera like *Halococcus*, *Halorubrum*, *Haloarcula*, *Haloquadratum*, *Haloterrigena*, *Haloferax*, *Natronococcus*, *Natrialba* and *Natronobacterium* which were also established as active producers of PHB (Poli et al., 2011). The notable problem in PHB production is the process of optimization to reduce the production cost. Hence, researchers are focusing on the

selection of cheap raw materials for the production of PHB from marine bacteria.

The present study describes isolation and identification of bacteria having PHB productivity and special emphasis has been given to optimize the most significant variables such as temperature, pH and substrates in order to optimize the production of PHB. The purified biopolymer was characterized by FTIR and NMR analysis by comparing with the standard PHB. Hence, the present research work has been aimed to screen marine sponge symbiotic bacteria as biological tools for production of PHB.

Material and Methods

Collection and Identification of Marine Sponges

Marine sponges were collected from the rocky shores of Kovalam (Lat. 8°22'0.01"N; Long. 76°59'48.01"E), Southern West coast of India at depth ranging from 6 to 7 m during November 2018 (Figure 1). Details of form, color, surface ornamentation, resiliency and biological associates were also recorded at the time of collection (Rachana et al., 2014). Sponges were identified by studying the spicule's nature using pertinent literatures and keys: Demospongiae of the Gulf of Mannar and Palk Bay (Thomas, 1986); Systema Porifera: A guide to classification of sponges (Hooper & Van Soet, 2002); Sponguide: Guide to sponge collection and identification (Hooper, 2003) and compared it with the original description of the species in World Porifera Database (<http://www.marinespecies.org/porifera>).

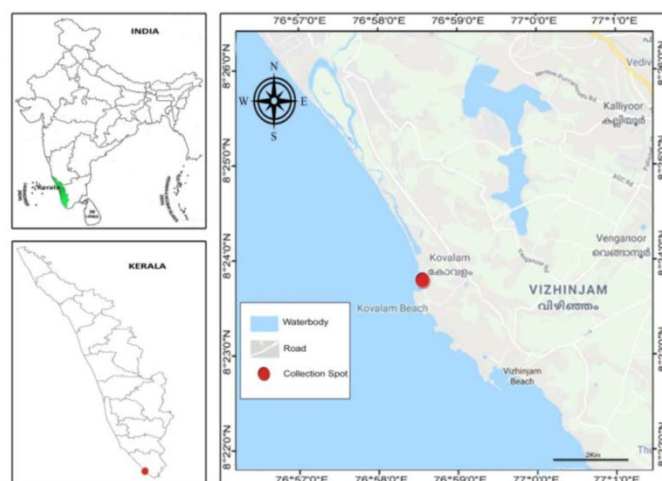


Figure 1. Map showing the collection site

Isolation and Screening of Sponge Associated Bacteria for the Production of PHB

The bacteria from sponges were isolated according to the method of Kim et al. (2006). Nearly 1 cm³ of sponge tissue was excised from the internal mesohyl area using sterile scissors in

aseptic conditions on a sterile ceramic tile. Sterile 0.85% saline (1 ml) was added to squeeze the specimen and homogenization continued till the sponge exudates were obtained (Selvin et al., 2009). The diluted sample was pour plated on modified culture medium of Zobell Marine Agar 2216 medium and incubated for 24 hrs at 32°C (Gandhimathi et al., 2008). The morphologically distinct colonies were re-isolated and maintained on ZMA (HiMedia) at 4°C. The pure cultures were maintained by sub-culturing.

Bacterial isolates were maintained in Minimal Davis Media and cultured for 2-3 days at 37°C supplemented with dextrose (10 ml of 10% in of Minimal Davis Media) as carbon source and screened by Nile Blue staining (Nile blue sulphate 90% dye content for microscopy, 1µg/ml) (Lillie, 1977) and observed under Leica LSCM - Laser Spectral Scanning Confocal Microscope, Model TCSSP 8 (Microscope Model Leica DM 18, software used-LASX). Those bacterial isolates showed bright yellowish-orange color were selected for further study (Ostle & Holt, 1982).

Characterization of Selected Bacterial Strain

Identification of bacteria were made by morphological observations of colony, shape of bacteria, catalase test, test oxidase, test the mannitol motility and the production of compound indole, test O/F (Oxidative/Fermentative), TSIA (Triple Sugar Iron Agar), Citrate test, Lysine, H₂S, Urease, Lactose, Glucose and Bile esculin reactions.

The isolate showed significant activity was characterized using 16S rRNA gene sequencing. The methodology and the primers for sequencing were adapted from Kamke et al. (2010). Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel, Düren, Nordrhein-Westfalen). A small portion of 16S rRNA gene were amplified with the primers 16S-UP-F (5'-CCGAATTCGTCGACAACAGAGTTTGTATCCTGGCTCAG-3') and 16S-UP-R (5'-CCCGGATCCAAGCTTACGGCTACCTTGTTACGACTT-3') (Enkicknap et al., 2006). The DNA sequence was edited and aligned using BioEdit sequence alignment editor V.7.0.9.0. (IbisBiosciences, Carlsbad, USA., Hall, 1999). Sequence similarity of specimen was done by using database GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Divergence in the sequence was analyzed using the Kimura 2-Parameter distance model of MEGA (Version 6.0) (Tamura et al., 2013). Maximum likelihood tree was selected for phylogeny analysis.

Disc diffusion method (Bauer et al., 1966) was carried out on Muller Hinton agar plates (Hi-media) to determine the in vitro antibacterial susceptibility test. A 100 µl of test strain (106 CFU/ml bacteria) was spread on the Muller Hinton agar media and 25 different types of antibiotic discs were placed on the medium and were incubated for 24 hrs. After incubation at 37°C in incubator (ROTEK), the area of inhibition zone (mm)

was determined using a Hi Antibiotic Zone Scale-C (PW-297, Hi-media).

Optimization of Cultural Parameters for PHB Production

PHB production were optimized by growing the culture with different pH ranging from 6, 7, 8 and 9 and the inoculated flasks were incubated at 30°C at 150 rpm for 48 hrs and PHB was quantified. Effects of different temperatures upon production were optimized by incubating the cultures on a rotary shaker at 20, 30 and 40°C and 150 rpm for 48 hrs and PHB was quantified. Effects of different media also optimized by dextrose, glucose and mannitol media and were sterilized at 121°C for 20 minutes. The inoculated flasks were incubated at 30°C at 150 rpm for 48 hrs and PHB was quantified.

Extraction and characterization of PHB from potent isolates for the extraction of PHB, the fluorescence displayed bacterial isolates were cultured in Minimal Davis Media supplemented with Dextrose as carbon source. The culture were kept in a rotary shaker at 37°C and 150 rpm for 3 days. Extraction of PHB was performed by sodium hypochlorite-chloroform method. After the centrifugation process the solution were appeared as three phases. The upper phase contains hypochlorite solution and the middle phase contains chloroform with cell debris. The bottom phase containing PHB with chloroform were collected and followed by extraction with hot chloroform and precipitated with ethanol and acetone (1:1). The precipitate was allowed to evaporate for dryness at 30°C to obtain PHB crystals (Singh & Parmar, 2011).

The extracted crystals were analysed qualitatively by using Thermo Fisher Scientific Nicolet iS50 FT-IR Spectrophotometer to know the presence of different functional groups at a range of 4000-400 cm⁻¹. IR spectra were recorded at 4 cm⁻¹ resolution (Kansiz et al., 2000). The ¹H NMR spectra of extracted PHB sample was also obtained at 400 MHz using a model Bruker Avance III HD NMR spectrometer and methanol is used as solvent. Chemical shifts (ppm) and coupling constants (Hz) were recorded.

Results

Ten species of marine sponges were collected and based on morphological features of spicules and other specialized characters the sponges were identified as *Callyspongia (Cladochalina) fibrosa* (Ridley and Dendy, 1886), *Callyspongia (Cladochalina) diffusa* (Ridley, 1884), *Tedania (Tedania) anhelans* (Vio in Olivi, 1792), *Myxilla (Ectyomyxilla) arenaria* (Dendy, 1905), *Sigmadocia carnososa* (Dendy, 1889), *Dysidea fragilis* (Montagu, 1814), *Ecionemia acervus* (Bowerbank, 1864), *Oceanopia arenosa* (Rao, 1941), *Mycale (Carmia) mytilorum* (Annandale, 1914) and *Mycale (Aegogropila) crassissima* (Dendy, 1905) (Figure 2).

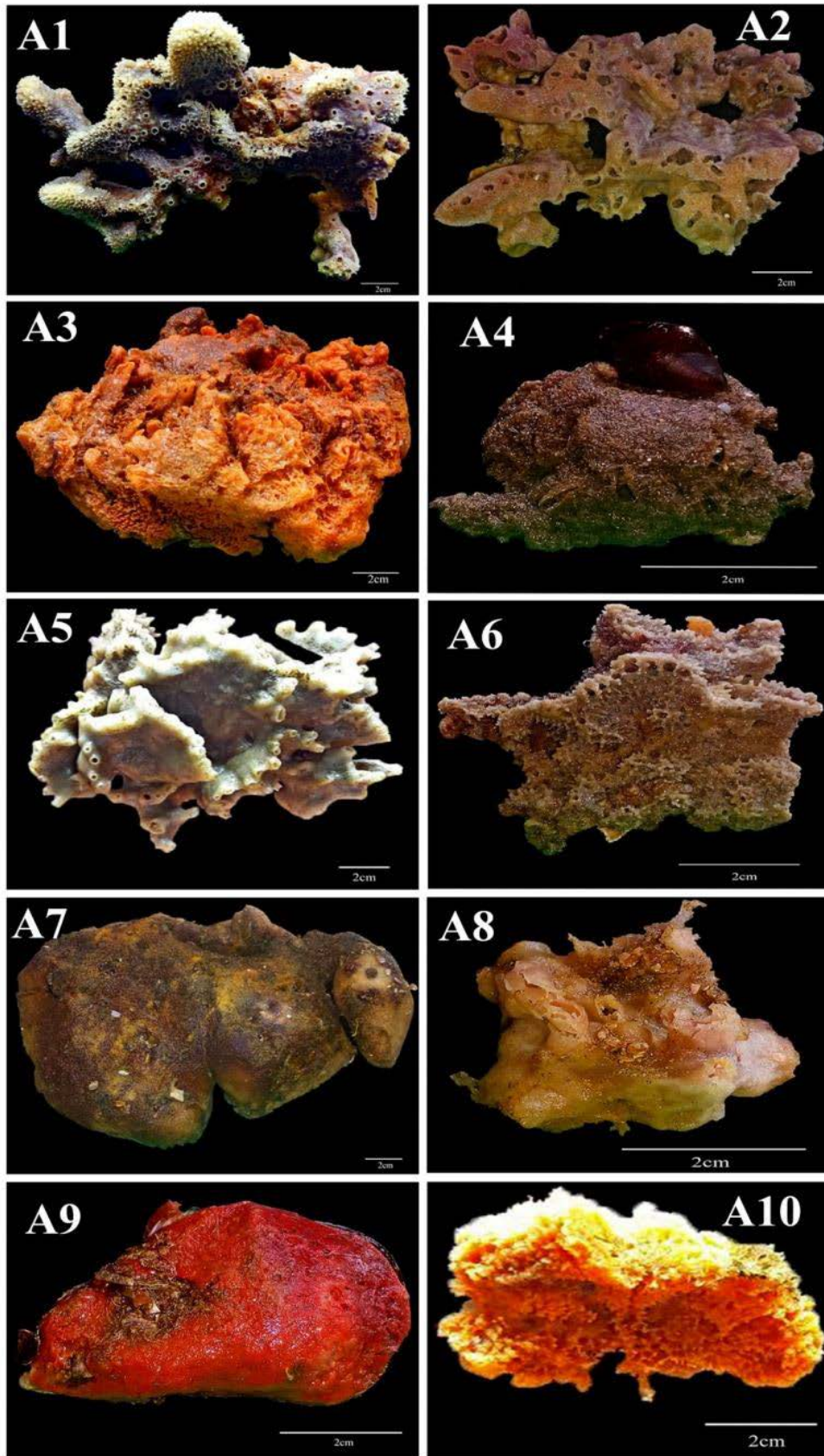


Figure 2. Collected marine sponges; **A1-** *Callyspongia (Cladochalina) fibrosa* , **A2-** *Callyspongia (Cladochalina)*, **A3-** *Tedania (Tedania) anhelans*, **A4-** *Myxilla (Ectyomyxilla) arenaria*, **A5-** *Sigmadocia carnososa*, **A6-** *Dysidea fragilis*, **A7-** *Ecionemina acervus*, **A8-** *Oceanopia arenosa*, **A9-** *Mycale (Carmia) mytilorum*, **A10-** *Mycale (Aegogopila) crassissima*

Screening of isolates for PHB production

The isolates obtained from marine sponges were screened for PHB production using Nile blue staining method and cultured in Minimal Davis Media (Figures 3 & 4). Ten isolates showed growth in Minimal Davis Media were further stained with Nile blue staining and observed under Leica LSCM to confirm the production of PHB. Five isolates such as two strains each from *Callyspongia diffusa* (AB2a, AB2b) (Figure 5), *Mycale mytilorum* (AB9a, AB9b) (Figure 6) and one isolate from *Oceanopia arenosa* (AB8a) (Figure 7) flourish bright yellowish orange color were assumed as the PHB producing colonies. In this study, the AB8a PHB granules were fluoresced as bright orange, and it was concluded that AB8a was the best PHB producer with bright orange fluorescence. So the yellow pigmented AB8a isolate from *Oceanopia arenosa* showed high intensity was selected for further observations and study.



Figure 3. Bacterial isolates - pour plate method

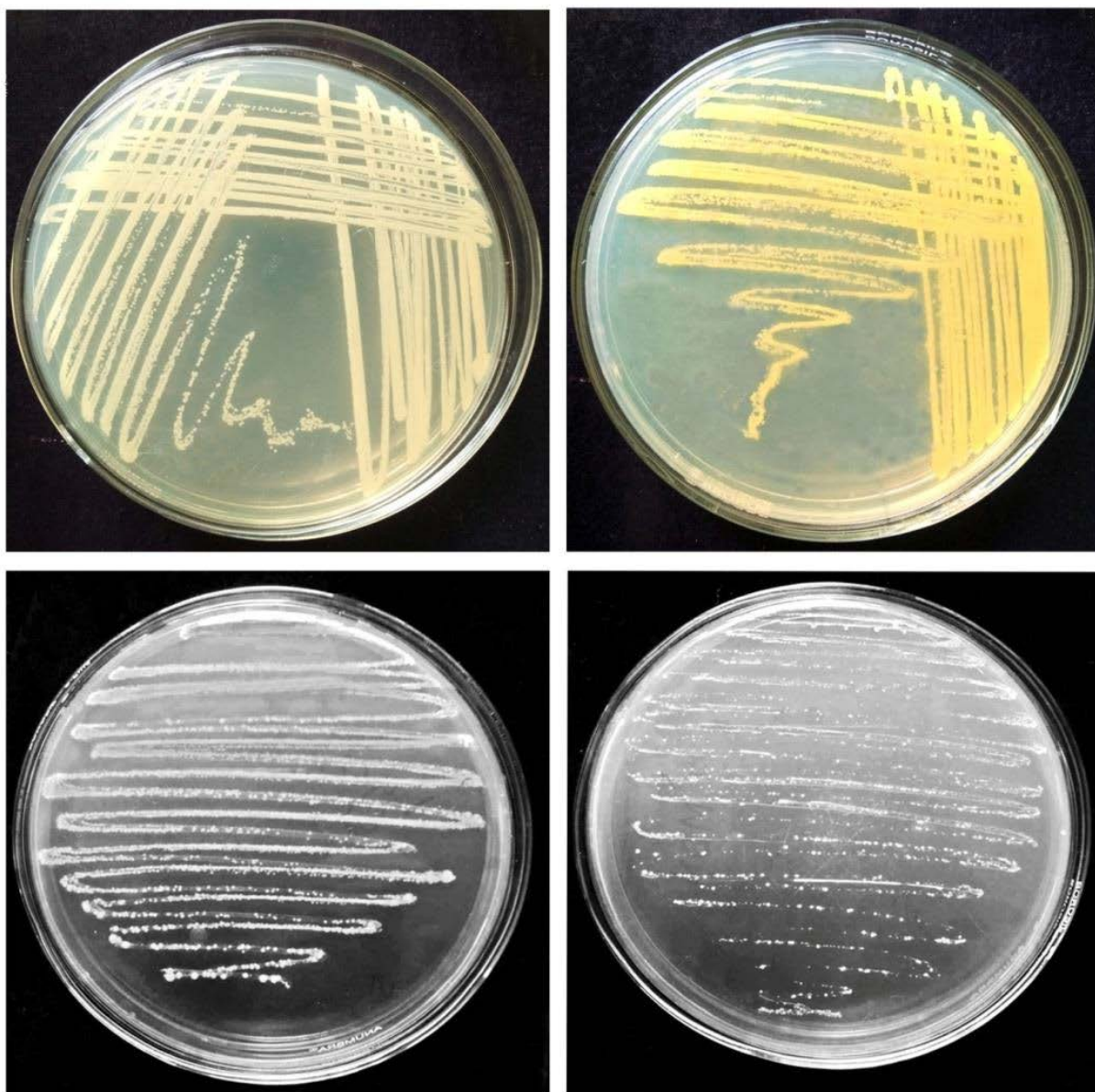


Figure 4. Pure culture of isolates from marine sponge

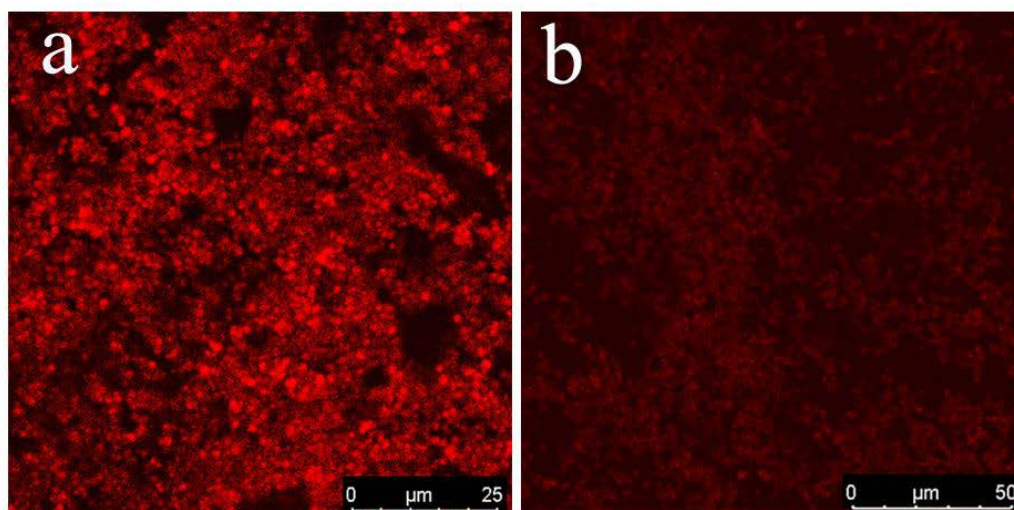


Figure 5. AB2a and AB2b isolates from *Callyspongia diffusa* showing positive result in confocal microscopy

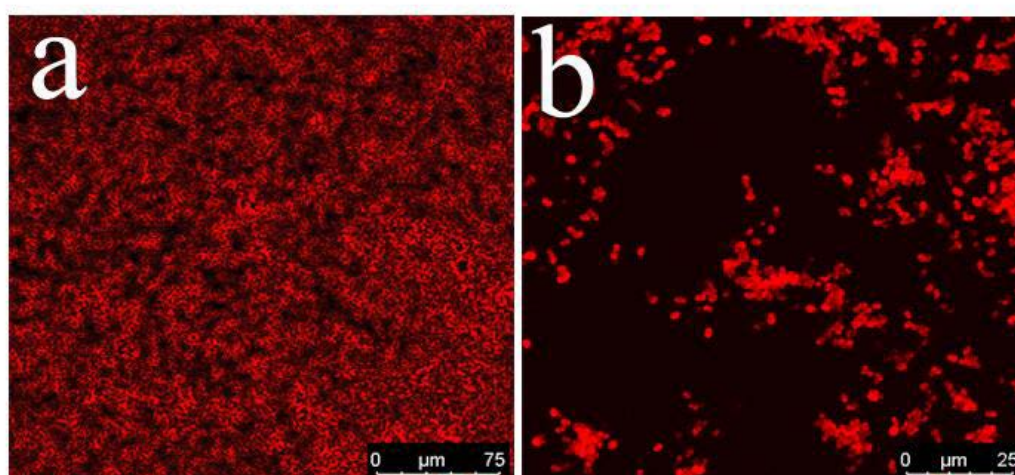


Figure 6. AB9a and AB9b isolates from *Mycale mytillosum* showing positive result in confocal microscopy

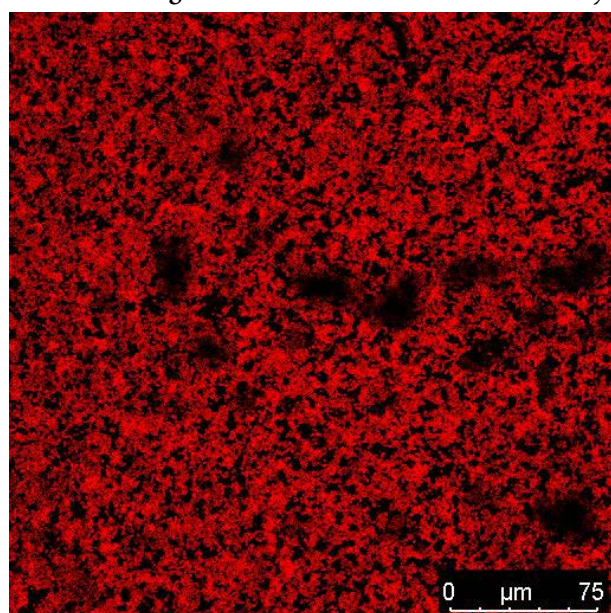


Figure 7. AB8a isolates from *Oceanopia arenosa* showing positive result in confocal microscopy

Characterization of Potent PHB Isolate

To characterize the potent isolate, Gram staining (Figure 8) and biochemical tests (Figure 9, Table 1) were performed. From that result the AB8a isolate was identified as a Gram-positive motile, sugar fermented rod-shaped *Bacillus sp.*

Antibiotic Sensitivity Test

Among twenty five different types of antibiotics used, AB8a was found to be resistant for Ampicillin, Cloxacillin, Cefuroxime, Bacitracin, Amoxyclav and Cephalothinand. The zone diameters of sensitivity of the organism to the antibiotics obtained were recorded (Figure 10, Table 2).

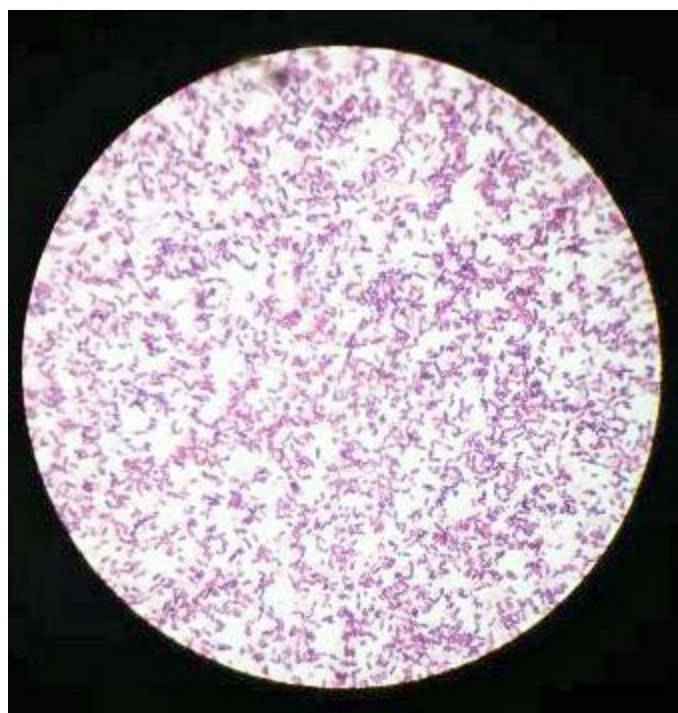


Figure 8. Gram staining of isolated bacteria



Figure 9. Biochemical tests

Table 1. Biochemical characteristics of AB8a

Test	Observation
Motility	Motile, fermented
Gram stain	Gram positive
Catalase	+
Oxidase	+
Mannitol motility	Fermented
Indole	+
Oxidative fermentative	-
TSIA	Sugar fermented A/A
Citrate	-
Lysine	-
H ₂ S	-
Urease	-
Shape of bacteria	Rod
Lactose	-
Glucose	+
Bile esculin	+

Table 2. List of antibiotics used along with zone diameter in mm

Antibiotics	Quantity	Code	Zone (mm)
Ampicillin	10 mcg	AMP	-
Cefotaxime	30	CTX	18
Carbenicillin	100	CB	15
Cloxacillin	1	COX	-
Azithromycin	15	AZM	25
Norfloracin	10	NX	30
Tobramycin	10	TOB	16
Nalidixic acid	30	NA	22
Cefuroxime	30	CXM	-
Bacitracin	SD 105-1 CT	BT	-
Ceftazidime	30	CAZ	24
Amoxyclav	30	AMC	-
Piperacillin	100	PI	25
Tigecycline	15	TGC	20
Cephalothin	30	CZ	-
Erythromycin	15	E	25
Meropenem	10	MRP	33
Amikacin	30	AK	20
Vancomycin	30	VA	17
Gentamycin	10	GEN	19
Ciprofloxacin	5	CIP	14
Cotrimoxazole	25	COT	20
Nitrofurantoin	300	NIT	17
Pip-Tazobactam	100/10	PIT	19
Cefaperazonesulbactam	75/30	CFS	22

Molecular Analysis for Amplification of the Genes and Phylogenetic Analysis

Amplification and gel electrophoresis of 16S rRNA (Figure 11) showed that AB8a bacteria isolates had approximately 1500bp and it was belonged to bacteria groups. The phylogenic relationship of bacterial isolate (AB8a) was studied using Maximum Likelihood Method. The 16S rRNA strain sequence following pair-wise alignment exhibited 100% similarity at the DNA gene level with the members of the genus *Bacillus*. The ML tree was prepared (Figure 12) and clade stability was estimated using 1000 non-parametric bootstrap replications. Phylogenetic tree revealed that *Bacillus flexus* of the present study has got clustered with the identical reference sequence of the *Bacillus flexus* (MHO57386.1) from GenBank with highest boot strap value (100). The isolate of our present investigations was thus identified as *B. flexus*.

Optimization of Cultural Parameters for PHB

Production

Effect of different pH on PHB production indicated that, out of different pH of media tested, pH 7.0 was found to be optimum for maximum PHB production by *B. flexus*. Effect of

initial pH studies also showed that as the pH in the medium increases, PHB production increased up to pH 7.0. *Bacillus flexus* showed maximum PHB production (2.85 ± 0.08 g/100 ml) at pH 7.0, and at alkaline pH sharp decrease in the production of PHB were found. No PHB production was observed at pH 6.0 by the isolate. At pH 8.0, all the isolates were found to produce lower yields showing that pH 6.0, pH 8.0 and pH 9.0 were not suitable for PHB accumulation.

Effect of different incubation temperature on PHB accumulation was studied over a range of 20°C to 40°C. The

result indicated that the range of 30–35°C was suitable for the PHB production (2.82 ± 0.09 g/100 ml). Even though 30–35°C range was found to be suitable for PHB production, 30°C was selected as optimum temperature for further studies. Yield and PHB production was low at 20°C and 40°C.

Among the different carbon sources tested to evaluate their effects on PHB yield, dextrose was found to be the best carbon source. It yielded a mean PHB of 2.83 ± 0.08 g/100 ml. This was followed by glucose with a mean PHB of 1.55 ± 0.10 g/100 ml.

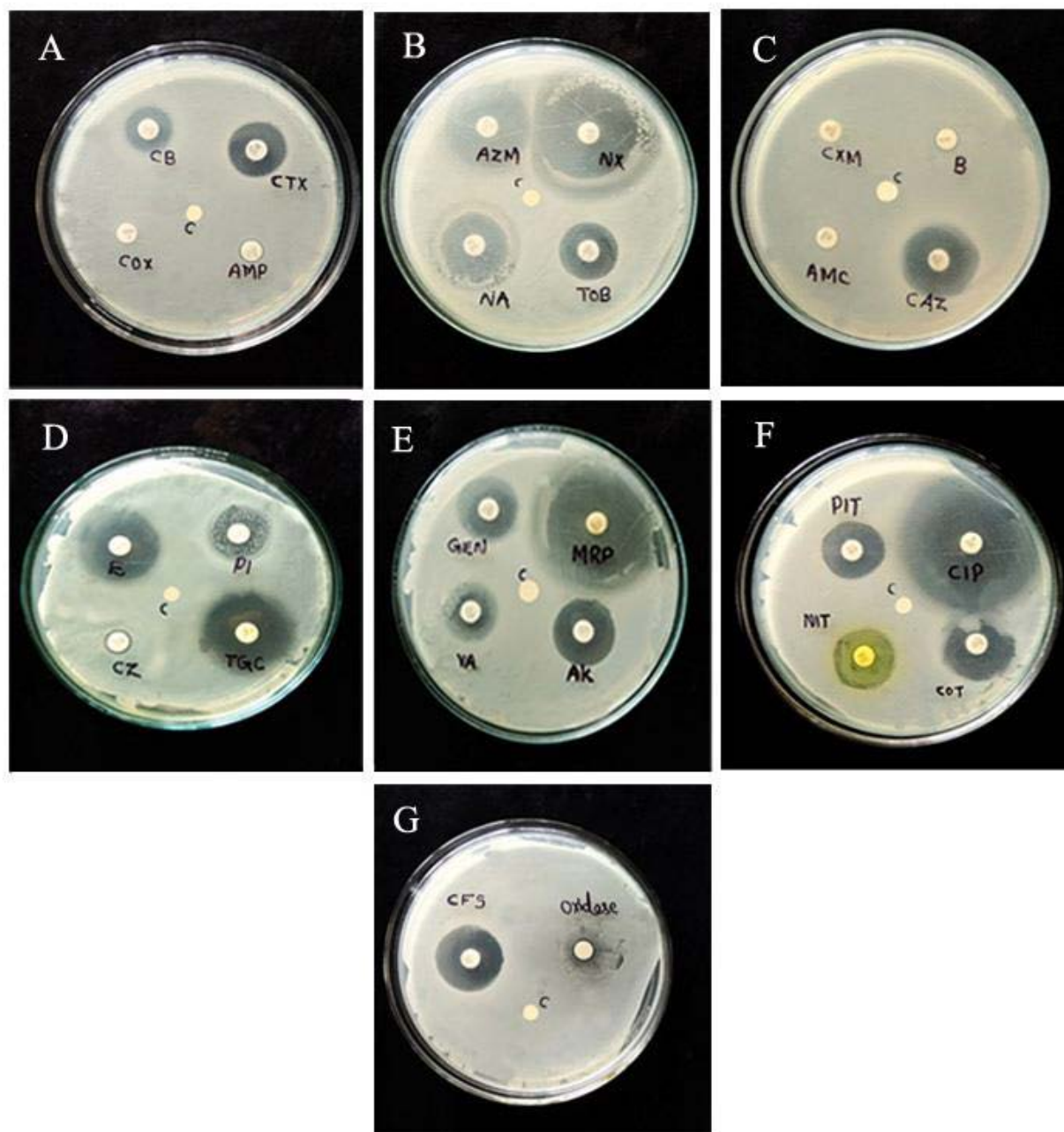


Figure 10. Antibiotic sensitivity test of the isolate; A: CB- carbenicillin; CTX-Cefotaxime; COX-Cloxacillin; AMP- Ampicillin; B: AZM- Azithromycin; NX-Norfloracin; NA-Nalidixic acid; TOB-Tobramycin; C: CXM- Cefuroxime; BT-Bacitracin; AMC-Amoxyclov; CAZ Ceftazidime; D: E- Erythromycin; CZ-Cephalothin; PI-Piperacillin; TGC-Tigecycline; E: GEN- Gentamicin; MRP-Meropenem; VA-Vancomycin; AK-Amikacin; F: PIT- Pip-Tazobactam; NIT-Nitrofurantoin; COT-Cotrimoxazole; CIP-Ciprofloxacin; G: CFS- Cefaperazone sulbactam

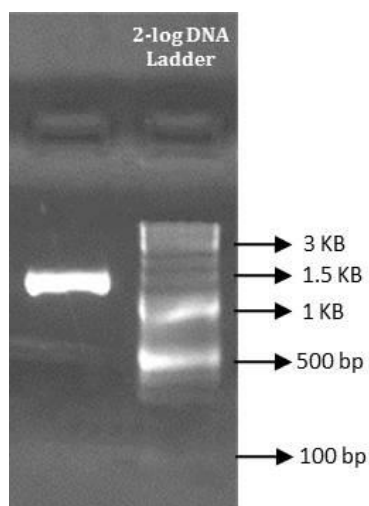


Figure 11. Details of partial 16S ribosomal RNA of AB8a bacteria isolates

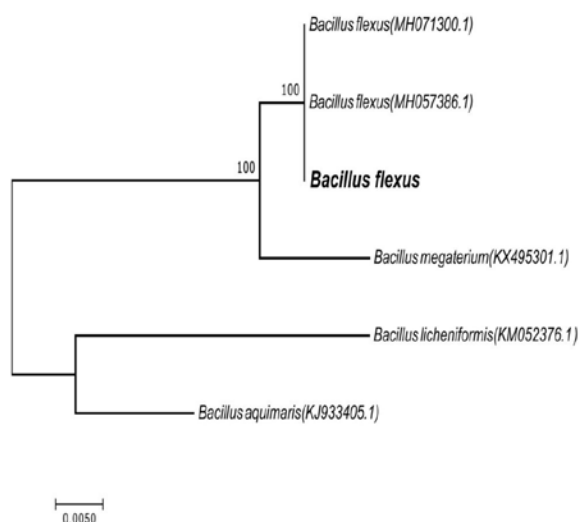


Figure 12. Phylogenetic tree of isolate *Bacillus flexus* and their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences

Extraction and Characterization of PHB

The sodium hypochlorite method was selected for the extraction of PHB. The precipitate was allowed to evaporate to obtain PHB crystals (Figure 13).

Fourier Transform Infrared Spectroscopy (FTIR)

The IR spectrum of each sample represents its total chemical composition, because every chemical compound in the sample makes its own specific contribution to the absorbance spectrum. The FTIR Spectra (Figure 17) were recorded at 4000 cm^{-1} to 400 cm^{-1} range. From the spectrum obtained it was inferred that the band at 3457 cm^{-1} corresponds to OH (Hydroxyl) group, whereas band at 1692 cm^{-1} represents C=O (Carbonyl) and COO (ester) groups. The band at 1454 cm^{-1}

corresponds to CH showing asymmetrical stretching and the band at 1550 cm^{-1} indicating bending vibration in CH₃ group, whereas band at 1420 cm^{-1} representing CH₂ bond. Stretch of bands ranging from $1050\text{--}1190\text{ cm}^{-1}$ showed C-O bonding.

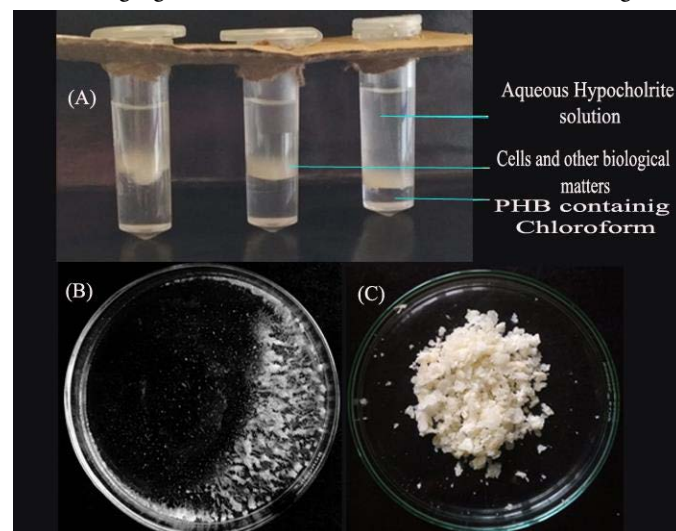


Figure 13. (A) Three different phase appeared after centrifugation, (B) PHB crystals obtained after the evaporation of chloroform by Sodium - Chloroform method, (C) Extracted PHB crystals

Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectrum (Figure 18) showed a triplet at 1.3 ppm which is attributed to the methyl group (-CH₃) coupled to one proton. Doublet peak ranging between 2.06-2.6862 is attributed to the methylene group (-CH₂) adjacent to an asymmetric carbon atom bearing a single atom. The multiple peak at 4.88 ppm is characteristic of methine group (-CH). Two other signals are observed, a broad one at 3.88 ppm which is due to water and another at 7.93 and 8.599 ppm is may attributed to the solvent used i.e. methanol.

Discussion

The marine environment provides a real untapped resource for novel bacteria and possibly the biopolymers they produce and this study was aimed to isolate a diverse range of PHB accumulating bacteria from marine sponges and the parameters for maximum PHB production were also optimized (Arun et al., 2009; Madison & Huisman, 1999). Based on morphological features and nature of spicules the collected marine sponges specimens were identified as *Callyspongia* (*Cladochalina*) *fibrosa* (Ridley & Dendy, 1886), *Callyspongia* (*Cladochalina*) *diffusa* (Ridley, 1884), *Tedania* (*Tedania*) *anhelans* (Vio in Olivi, 1792), *Myxilla* (*Ectyomyxilla*) *arenaria* (Dendy, 1905),

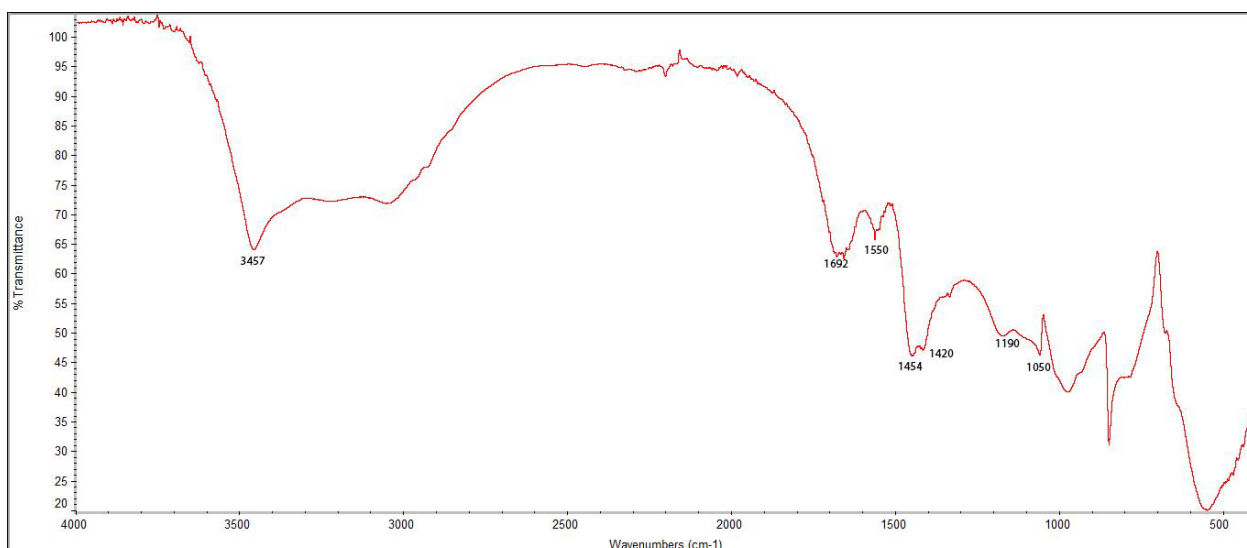


Figure 14. FT-IR spectrum of extracted PHB

PHB PLYMER H1 MeOD

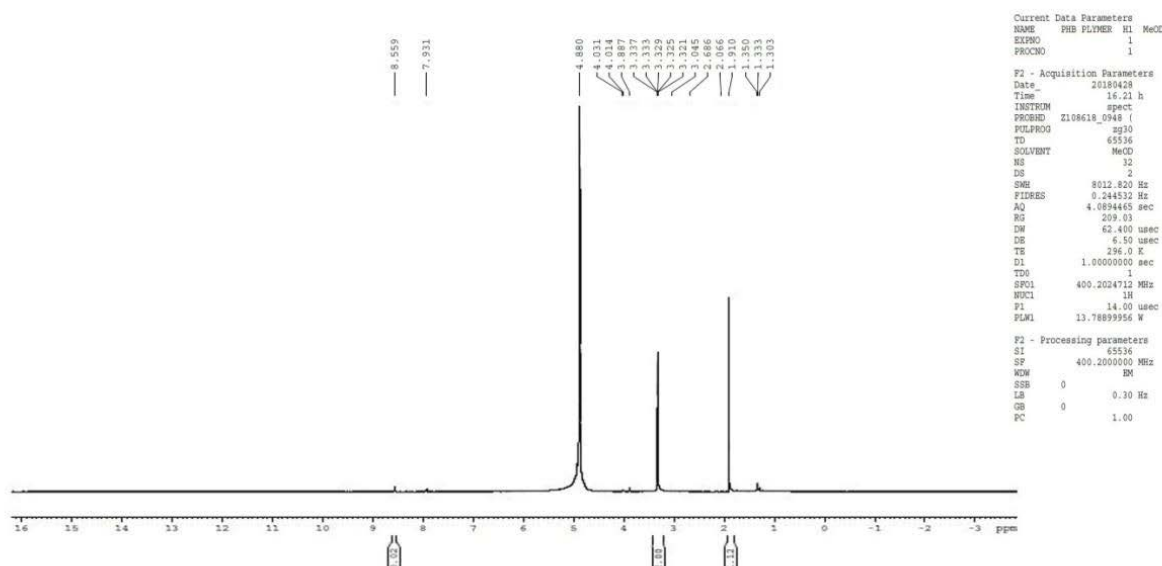


Figure 15. ^1H NMR spectrum of extracted PHB

Sigmadocia carnosa (Dendy, 1889), *Dysidea fragilis* (Montagu, 1814), *Ecionemia acervus* (Bowerbank, 1864), *Oceanopia arenosa* (Rao, 1941), *Mycale* (*Carmia*) *mytilorum* (Annandale, 1914) and *Mycale* (*Aegogropila*) *crassissima* (Dendy, 1905). Many reports reveals that these types of marine sponges are very common in Indian coastal areas including Tuticorin coast (Singla et al., 2013), Lakshadweep archipelago, Kavaratti Island (Gopi et al., 2012) and Gulf of Mannar and Mandapam bay (Velho-Pereira & Furtado, 2012). Selvakumar and Dhevendaran (2016) reported the occurrence of *Callyspongia diffusa*, *Mycale mytilorum*, *Tedania anhelans* and *Dysidea fragilis* from South West coast of India.

The result of the work indicated that five bacterial strains afforded fluorescence signals with Nile-blue test. Moreover, one of them (AB8a) gave a very strong fluorescence signal, whereas

the other strains showed faint signals. In addition, the high fluoresced strain was predicted as one of the effective producer of PHB based on the growth rate, the high intensity of fluorescence in viable Nile blue A staining and the presence of lipophilic inclusions. Spiekermann et al. (1999) reported that the Nile-blue stain emitted strongly positive fluorescence signals only with a hydrophobic compound like PHAs and lipids and could be detected by fluorescence spectroscopy or flow cytometry.

Microbiological properties were investigated according to the methods described in Bergey's Manual of Systematic Bacteriology (Kreig & Holt, 1984) and the organism was identified as a member of the genus, *Bacillus*. The Gram-positive bacteria, such as *Bacillus* sp. could be considered as ideal candidates for the industrial PHB production due to the

lack of LPS layer. *Bacillus* sp. has pronounced importance in industry due to their advantages of low nutritional requirements, rapid growth, having machinery enzymes and for utilization of several sugars (El-Sheekh, 2015). Members of this genus are known to grow rapidly, possess various hydrolytic enzymes and produce copolymers from structurally unrelated carbon sources (Halami, 2007; Valappil et al., 2007) and accordingly these characteristics of *Bacillus* sp. can be considered for the production of PHB with desirable material properties from various low-cost agricultural feed stocks.

Further characterization was confirmed with 16S rRNA sequence. The phylogenetic relationship of bacterial isolate (AB8a) was studied using Maximum Likelihood Method. The 16S rRNA strain sequence following pair-wise alignment exhibited 100 % similarity at the DNA gene level with the members of the genus *Bacillus*. Phylogenetic tree revealed that *Bacillus flexus* of the present study has got clustered with the identical reference sequence of the *Bacillus flexus* (MHO57386.1) from GenBank with highest boot strap value (100). The isolate of our present investigations was thus identified as *B. flexus*. In prokaryotes PHB accumulation property is broadly distributed among the Gram-negative organisms such as *Cupriavidus*, *Pseudomonas*, etc., and Gram-positive organisms such as *Bacillus*, *Clostridium*, *Corynebacterium*, *Nocardia*, *Rhodococcus*, *Streptomyces*, *Staphylococcus* etc. and certain archaeal strains of *Halobacterium*, *Haloarcula*, *Haloquadratum* and *Haloferax*. *Bacillus* spp. are well known for their ability to accumulate poly-3-hydroxybutyrate (PHB) (Balakrishna Pillai et al., 2017). According to Beveridge (2001) marine environment have reported that around 36% of the symbiotic isolates are Gram-negative rods. In our study, a Gram positive *Bacillus* was isolated from *Oceanopia arenosa*. Generally, bacteria belonging to the genera *Bacillus* accumulate short chain length polyhydroxyalkanoates such as PHB (Valappil et al., 2007).

Effect of incubation temperature on yield and PHB accumulation was studied over a range of temperature 20°C to 40°C. The result indicated the range of 30-35°C was suitable for the biomass and PHB production (73.40% yield). The incubation temperature ranging from 27 to 30°C favored PHB production and the maximal yield was attained at 30°C. The most adverse effect of incubation temperature on both PHB was recorded above 30°C. This result accorded to a great extent with those obtained by Divyashree et al. (2009a, b), who grew *B. flexus* strain at 30°C for PHA production. Higher or lower temperatures showed inferior results. This result also coincides with that represented by Aslim et al. (2002), who reported that optimum incubation temperature for PHB production by *Bacillus thuringiensis*, *Bacillus subtilis*, and *Bacillus pumilis*

was at 35°C. According to Tamodgan & Sidal (2011) higher and lower temperatures than 30°C lead to decrease in PHB synthesis by *Bacillus subtilis* ATCC 6633, as well as cell mass, probably due to the low enzymes activity. In this study, the optimum temperature for PHB production was found to be in 30°C. These results are similar with Grothe et al. (1999) that incubation temperature affects polymer accumulation at a range of 30-35°C and over this range, the effect of temperature is negligible. Interestingly, our isolated strain AB8a could accumulate the highest amount of PHB within 36 hrs that is very short time compared to previously reported *Bacillus* strains. Maximum PHB production from molasses was obtained after 72 hrs by *Bacillus flexus* ME-77 (El-Sheekh, 2015) and *Bacillus thuringiensis* (Desouky et al., 2014). Kalaivani & Sukumaran (2015) also reported maximum production of PHB from molasses observed after 76 hrs by *Bacillus* sp. KSN5.

Dextrose among all the other carbon sources led to the highest PHB production (70.25%) compared to glucose and mannitol. In addition, the enhancing effect of dextrose is probably due to its additional nutrients, such as trace elements, minerals and vitamins as thiamine and riboflavin (Oliveira et al., 2004). Gouda et al. (2001) studied the effect of different carbon sources on the production of PHB using *Bacillus megaterium*. In that study maximum PHB production was obtained from glucose and maximum cell dry mass was obtained from maltose. In this study among the carbon compounds used, dextrose was found to be best for PHB accumulation and mannitol was found to be poor carbon source.

It has been reported that pH in the range of 6.0-7.5 was the best for microbial growth and PHB production of *Alcaligenes eutrophus* was reported at optimum pH of 6.9, and the growth declined at pH below 5.4 (Grothe et al., 1999). Even a slight change in pH will cause malfunctioning of metabolic processes (Wei et al., 2011), and drastic changes in PHB production seems to be due to the effect of initial pH on the bioavailability of trace elements (Ramadas et al., 2009). In the present study, maximum PHB production obtained at pH 7.0 (73.55%). Flora et al. (2010) revealed that the maximum PHB production (25%) by *Bacillus sphaerius* was at pH range from 6.5-7.5. Earlier reports of Sivaprakasam et al. (2008) concluded that the optimum pH for growth of *B. flexus* was 8.0, while Priest et al. (1988) revealed that *B. flexus* can tolerate pH range from 4.5 to 9.5.

The biopolymer was extracted from the bacterial pellet using dispersion of sodium hypochlorite and chloroform solution. The extracted PHB was characterized by FTIR and ¹H NMR for the confirmation as PHB. The PHB sample extracted

was analyzed qualitatively by FTIR Spectrophotometer to know the presence of different functional groups. In the present study, the FTIR spectrum of the PHB sample shows major peaks at 3457, 1692, 1550, 1454, 1420, 1190 and 1050 cm^{-1} , whereas the remaining peaks are closely lying between 3450 cm^{-1} and 600 cm^{-1} . The IR spectrum reflects both monomeric units in addition a strong absorption band at 1714 cm^{-1} was detected in G1S1 (*Bacillus subtilis*), as is expected for the C=O (Shah, 2014). All absorptions due to the PHB moiety appeared in the spectrum, and in addition a strong absorption band at 1639 cm^{-1} was detected a thioester bond (Shah, 2012). As an evidence of this finding, the work done by Rohini et al. (2006) can be equated. They identified the polymer with the spectrum which revealed the presence of three groups of signals characteristic of PHB by the doublet at 1.3 ppm attributed to the methyl group coupled to one proton and the spectrum of the quadruplet at 2.57 ppm the methylene group adjacent to the asymmetric carbon atom having single proton and the multiplet at 5.28 ppm to the methylene group.

The results of this study demonstrates that the bacterium, which is isolated from marine sponge *Oceanopia arenosa*, identified as *Bacillus flexus* could be an effective and interesting bacterial sp., for production of PHB from dextrose. However, Use of inexpensive substrates could contribute to reducing the PHB production cost and further studies are needed for large scale production of the PHB.

Conclusion

The present investigation provides basis for assessing a potential for using sponge symbiotic bacteria for PHB (a biodegradable plastic) production, which is an economically and environmentally important product. In this study, sixteen marine bacterial strains associated with ten species of sponges *Callyspongia (Cladochalina) fibrosa*, *Callyspongia (Cladochalina) diffusa* (Ridley, 1884), *Tedania (Tedania) anhalens* (Vio in Olivi, 1792), *Myxilla arenaria* (Dendy, 1905), *Sigmatocia carnosa* (Dendy, 1889), *Dysidea fragilis* (Montagu, 1814), *Ecionemia acervus* (Bowerbank, 1864), *Oceanopia arenosa* Rao, (1941), *Mycale mytilorum* Annandale and *Mycale crassissima* were isolated. Ten isolates showed growth in Minimal Davis Media were further stained with Nile blue staining and observed under Leica LSCM - Laser Spectral Scanning Confocal Microscope to confirm the production of PHB. Five isolates such as two strains each from *Callyspongia diffusa* (AB2a, AB2b), *Mycale mytilorum* (AB9a, AB9b) and one isolate from *Oceanopia arenosa* Rao (AB8a) flourish bright yellowish-orange color were assumed as the PHB producing colonies. Yellow pigmented AB8a isolate from *Oceanopia*

arenosa showed high intensity was selected for further observations and study. In addition, AB8a was subjected to morphological, biochemical and phylogenetic characterization. The results of the tests showed AB8a to be a Gram-positive, sporulating, motile, catalase, oxidase positive and rod shaped bacteria. The highest PHB production (70.25% /100 mL) was achieved at pH 7.0 by applying dextrose as medium at incubation temperature 30°C and 150 rpm agitation speed. The biopolymer was extracted from the bacterial pellet using dispersion of sodium hypochlorite and chloroform solution. The extracted PHB was characterized by FT-IR and ¹H NMR for the confirmation as PHB. Phylogenetic analysis based on comparative analysis of sequenced 16s rRNA of the active strains indicated a preponderance of bacteria belonging to *Bacillus flexus* with 100% sequence similarities.

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Compliance with Ethical Standards

Authors' Contributions

DA and VSP conceived and designed the study, collect specimens, analyze the data and wrote the manuscript. DA carried out the experimental work, interpret the data, reviewed the results. VSP guided the research work, performed editing, critical revision and supervised the findings of the study. Both authors read and approved the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical Approval

For this type of study, formal consent is not required.

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