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ISOLATION, OPTIMIZATION AND PRODUCTION OF BIOPOLYMER (POLY 3-HYDROXY BUTYRATE) FROM MARINE BACTERIA

Muthezhilan R*[1], C.P. Kaarthikeyan[1,3], M. Jayaprakashvel[1], A. Jaffar Hussain[2]

[1]*Department of Marine Biotechnology, AMET University, Kanathur, Chennai 603112, Tamil Nadu, India,*
[2]*Centre for Marine Bioprospecting, AMET University, Kanathur, Chennai 603112, Tamil Nadu, India,*
[3]*Department of Aquaculture, James Cook University, Townsville, Australia* E-mail*: mycomuthu@gmail.com

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ABSTRACT

The synthetic polymer plastics become an integral part of contemporary life. Excess use of plastics and indiscriminate dumping of it in soil and water is polluting the environment and other living organisms. To overcome this problem, the production and applications of eco-friendly biodegradable products (such as bioplastics) from microbes are becoming inevitable from the last decade and also good alternatives of synthetic polymers. Keeping this point in mind the present study aimed at isolating and identifying the poly 3-hydroxybutyrate (PHB) producing bacteria from marine sources that can be effectively utilized for the synthesis of bioplastics. For isolation of poly 3-hydroxybutyrate producing bacteria, spread plate technique was followed using E2 mineral medium. After incubation, based on the morphological characteristics 32 strains were isolated and identified from the sand dunes plants of rhizosphere vegetation of Chennai coast. While staining with Sudan Black, six strains *viz.,* AMET 5103, AMET 5111, AMET 5113, AMET 5121, AMET 5124 and AMET 5128 were identified as poly 3-hydroxybutyrate producing bacterial strains. Based on the dry weight of total biopolymer content, the strain AMET 5111 showed the maximum accumulation and was selected for optimizing at different pH, temperature, salinity, carbon and nitrogen source, incubation period and peak time of poly 3-hydroxybutyrate accumulation. Based on the biochemical tests, the strain AMET 5111 was found to be *Pseudomonas* spp. The optimum pH, temperature and salinity were found to be 7.0, 30^{o}C and 5%. The peak time of poly 3-hydroxybutyrate accumulation was found to be 36hrs. The best carbon and nitrogen sources were found to be sucrose and $(NH_4)_2HPO_4$. The selected strain was massively cultured using the optimized media and poly 3-hydroxybutyrate was extracted by solvent extraction.

Keywords: Marine bacteria, PHB (poly-(3-hydroxybutyrate)), Biosynthesis, Biopolymer

INTRODUCTION

Growth in the human population has led to the accumulation of huge amounts of non-degradable waste materials in the environment. The disposal of no degradable (plastics) synthetic polymers is the environment are the major pollution around worldwide. Recently, some alternative synthetic plastics are developed by using cellulose and starch like sources, but the production cost was found to be high (Qinxue Wen *et al*., 2010). The researchers are searching a new source to develop a biodegradable polymers with plastic like properties as an alternative for synthetic polymers at the same time with ecofriendly and cost effective (Braunegg *et al*., 2004; Akiyama *et al*., 2003). So, there is an urgent need of biodegradable plastics (bioplastics) as an alternative with greater compatibility to the environment. Nowadays, the biodegradable plastics (group of biopolymers) from bacteria or archaea are usually defined as an existing new area of research (Das *et al*., 2004; Akar *et al*., 2006). In general, the poly hydroxybutyrate (PHB) found in prokaryotes, in which it acts as a reserve of carbon and energy. Especially bacteria have the ability to produce a broad range of polymers including co-polymers with varied functional groups and commonly found to be accumulating within the bacterial cell wall (Yavuz Beyatli *et al*., 2006). Recently, it is estimated that, 75 different genera of bacteria have been known to accumulate PHB as intracellular granules and its production has most commonly been studied with micro organisms belonging to the genera *Alcaligenes, Azotobacter, Bacillus* and *Pseudomonas* (Reddy *et al*., 2003; Chen and Wu, 2005a,b; Steinbuchel and Lu, 2003). The availability of nutrient, particularly phosphates, nitrates by marine bacteria which operates at high rate in summer and the ratio of die off rates of bacteria in the marine environment decreases. Mainly, the bacterial community from marine environment is not so far reported fairly compared to the terrestrial environment. Basically, the microbes belonging to marine environment will tolerate with the extreme conditions and they survive as long.

Thus the present study aimed to isolate and identify the potential PHB producing bacterial strains from the coastal sand dune rhizosphere soil, and to optimize and synthesize the biopolymer.

MATERIALS AND METHODS

Collection of samples: Totally 12 rhizosphere sediment samples of Coastal sand dune plants were collected from three different locations of Chennai Coast (Kanathur, Muthukadu, Kovalam). The samples were collected using a sterile spatula and aseptically transferred in to sterile polythene bags. To avoid further contamination they were stored in ice box (4^{o}C) and bought into the Laboratory, Department of Biotechnology, AMET University.

Isolation of poly **3-hydroxybutyrate producing bacteria:** Ten grams of rhizosphere soil along with root bits of coastal sand dune plants were suspended in 90 ml sterile 50% seawater blank. After vigorous shaken, it was serially diluted up to $10^{-6.}$ and 0.1 ml of sample was taken from the dilution 10^{-4}, 10^{-5} and 10^{-6} and spread over on sterile E-2 mineral medium (Norris and Ribbons, 1971). The plates were incubated at 28±2^{0}C for 48 hrs. Thus obtained isolates were inoculated into sterile E-2 mineral agar slants for further use.

Screening of poly 3-hydroxybutyrate producing bacteria using Sudan Black-B staining: Bacteria accumulating PHBs can also easily identified on solid medium, as they appear as more turbid gummy-like colonies than the cells not producing PHBs (Ostle and Holt, 1982). However, for the definite visualization of PHB producers staining methodologies were useful (Kim *et al*., 1996), the poly-β-hydroxybutyrate stain was used to stain granules present within the confines of the filamentous bacterial cells. Primary stain-Sudan Black (IV), 0.3% (w/v) in 60% ethanol: Secondary stain - Safranin O 0.5%(w/v) aqueous. Thin smears were prepared in glass slide using 18 hours bacterial cultures from E-2 mineral medium agar plates. It was stained for 10 minutes with primary stain and gently washed with distilled water for 30 seconds. Then it was again stained for 10 minutes with secondary stain and rinsed with distilled water and blot dried. After drying the stained slide was examined under oil immersion microscope at 100x magnification with direct illumination for the presence of PHB granules. These selected strains were inoculated into E2 mineral broth and incubated for 48hrs.

Extraction and purification of poly 3-hydroxy butyrate:

Harvest of cells: After the incubation period, cells were harvested by centrifuge at 8000 rpm for 15 minutes. The obtained pellets were washed with sterile distilled water and recentrifuged again at 8000 rpm for 12 minutes. The pellets obtained were collected aseptically into a sterile watch glass and they were dried at 60^{o}C and the constant weight was measured.

Extraction of poly 3-hydroxybutyrate: The potential bacterial strains were optimized in E-2 mineral broth, and the suspensions of cultures were centrifuged at 6000 rpm for 45 minutes. For homogenization, the pellets were suspended in 5ml sodium hypochlorite solution from this 2ml of the cell suspension was taken and 2ml of 2 N HCl was added and it was kept for 2 hrs at 100^{0}C in water bath. Then the tubes were centrifuged at 6000 rpm for 20 minutes. Then 5 ml of chloroform was added to obtain precipitate. The test tubes were left overnight at 28^{o}C on a incubation shaker at 150 rpm. Then the contents of the test tubes were centrifuged at 6000 rpm for 20 minutes. After evaporation, the powdery mass along the test tube wall was collected. Dry weight of the biodegradable polymer and percentage (w/w) of it against cell dry weight was measured (Anwar *et al*., 2005; Bonartseva and Myshkina 1985).

$$\text{Biopolymer content (\%)} = \frac{\text{Dry weight of the bio degradable polyester}}{\text{Total biomass}} \times 100$$

Identification of potential poly 3-hydroxy-butyrate producing strain: The screened PHB-producing potential strains were subjected to Gram staining and biochemical characterization, identification was done by using the manual described by Cappuccino and Sherman (2002) and the results were cross checked with Bergy's Manual of Determinative Bacteriology (Holt *et al*., 2004).

Optimization of media for poly 3-hydroxy-butyrate production: For optimization one parameter at one time method was followed, to optimize the growth conditions for PHB production the selected strain was inoculated in E-2 mineral medium with different pH (3.0, 5.0, 7.0 and 9.0), temperature (25, 30, 35 and 40^{o}C), saline concentrations (3, 6, 9 and 11%), different carbon sources at 0.3% (Sucrose, Maltose, Lactose, cellulose and Glucose) and

nitrogen sources at 0.3% ($(NH_4)_2HPO_4$, KNO_3, NH_4Cl, Tryptone and Peptone). The determination of peak time of PHB accumulation was determined at different time intervals (24h, 36h and 48hrs).

Mass scale culture of the selected strain: For mass culture, a total volume of 1000ml of above optimized E-2 mineral medium was prepared and sterilized. The selected strain was inoculated into the sterilised medium and incubated with gentle shaking for 36hrs. After the incubation period the above mentioned extraction method was followed to obtain a biopolymer.

RESULTS AND DISCUSSION

The biopolymers from bacteria will helps to produce biodegradable products in large quantities at a very low cost. Microbes belonging to more than 90 genera including aerobes, anaerobes, photosynthetic bacteria, archaebacteria and lower eukaryotes are able to accumulate and catabolise these polyesters (Bucci *et al*., 2005). So, focusing research in these areas will help to completely avoid the usage of non biodegradable products in future. In this present study, totally 12 rhizosphere sediment samples were collected from Chennai coastal sand dune plants. The PHB producing strains were isolated from coastal sand dunes plants from different area using E-2 Mineral medium, among the 12 samples, the maximum colonies were 1.27 X 10^5*c.f.u*/g from Muttukadu Coastal area and the minimum were observed in 4.9 X 10^4*c.f.u*/g from Kanathur Coastal area (Fig 1). From this, morphologically 48 different strains, were isolated from 12 different samples, based on the turbid gummy like appearance 32 strains were selected for Sudan black staining and Gram staining and they were named as AMET 5101 to AMET 5132.

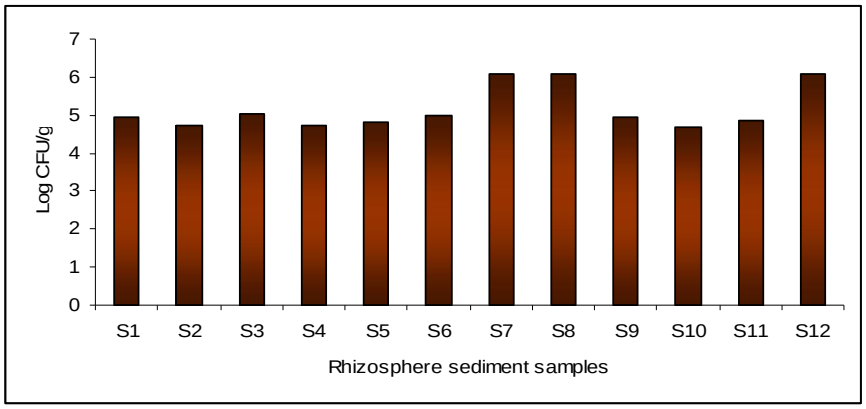

Fig. 1: THB population in different sand dune rhizosphere sediment samples.

The Gram negative bacteria harboured more than the Gram positive bacteria, the dominance of Gram-negative bacteria in aquatic environment is due to their cell structure. Marine environments are nutritionally dilute when compared with terrestrial environment. Under such conditions the outer membrane, especially the lipopolysaccharide (LPS) of Gram-negative bacteria helps to absorb nutrients. In general, poly 3-hydroxybutyrate is produced in an industrial scale using Gram negative bacteria like, *Cupriavidus necator, Alcaligenes latus* and *Escherichia coli* (Vandamme and Coenye, 2004). In this study, Sudan Black staining method was followed, to confirm the PHB accumulation of the each bacterial strain. When observed under phase contrast microscopy, granules were found to fill the cells almost entirely. The granules were spherical to oval shaped the results well confirmed with the findings of earlier workers Pal and Paul, (2002). Among the 32 strains the six strains, such as, AMET 5103, AMET 5111, AMET 5113, AMET 5121, AMET 5124 and AMET 5128 are PHB positive. It clearly indicates the all the colonies isolated from E2 mineral medium were not producing poly 3-hydroxybutyrate, and a cloudy (or) turbid nature of colonies may also be due to the production of biopolymers other than PHB. To confirm the poly 3-hydroxy butyrate production, the six potential strains were inoculated in E2 mineral broth and kept for incubation, after that a total biomass and degradable diester weight was estimated, based on their biopolymer content the strain AMET

5111 has showed maximum accumulation and it was selected for further study (Fig 2).

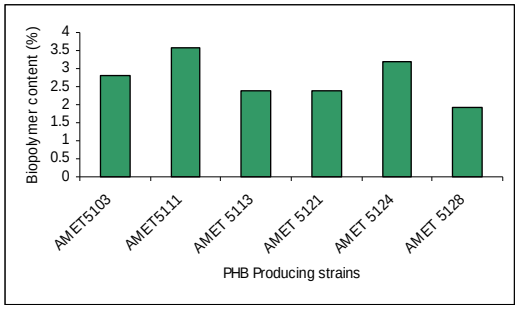

Fig. 2: PHB accumulation of the bacterial strains.

Based on their biochemical characteristic results, the most important characteristics such as gram negative, rod shaped, motile, catalase, oxidase positive and grows well in cetrimide agar has clearly indicated that the strain as belongs to *Pseudomonas* spp. The strain fails to produce yellow or green pigments, when streaked in King's B agar (KBA) medium (King *et al*., 1954) and viewed under UV transillu-minator. This clearly indicates that they might be non fluorescent *Pseudomonas* spp. The production of poly (β-hydroxybutyrate) (PHB)-accumulation in *Pseudomonas fluorescens* isolated from the soil in Alaska of USA (Jiang *et al.,* 2008). Many workers reported the ability of biodegradable polymer production by various members of the genera *Pseudomonas* (Taylor *et al*., 1989). For optimization of poly 3-hydroxybutyrate one time at a parameter was followed. Based on the incubation period, the incubation period, the maximum biopolymer was observed in 36 hours and minimum was showed on 24 hrs (Fig. 3).

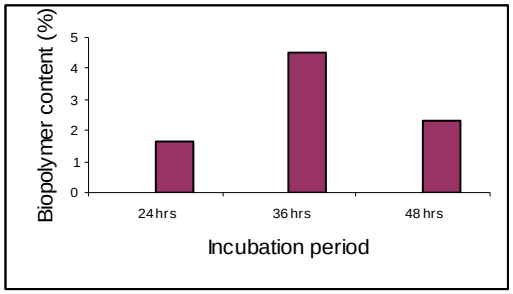

Fig. 3: PHB production in different incubation period.

When optimizing with different pH and temperature, pH 7 and temperature 30°C showed maximum activity (Fig. 4). Similar results were pointed out by Tamdogan and Sidal, (2011) they reported that poly 3-hydroxy butyrate production decreased at temperature extremes due to low enzyme activity at such temperatures.

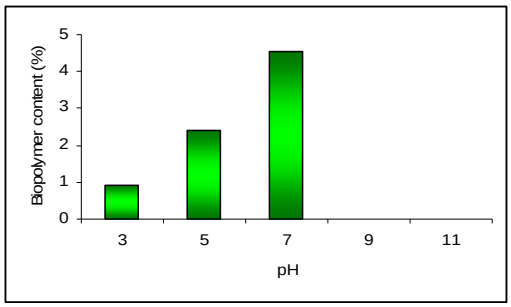

Fig. 4: PHB production in different pH.

Moreover when testing with different salinity, 5% salinity showed maximum accumulation and increasing of salinity also reverse to poly-3-hydroxybutyrate poly-3-hydroxy butyrate production (Fig 5). In normal, it is well known that any bacteria capable of producing poly 3-hydroxybutyrate needs excess carbon source in addition to a limited other source such as nitrogen or phosphate (Naranjo *et al*., 2013; Santhanam and Sasidharan, 2010). So, in this present study, we optimized by

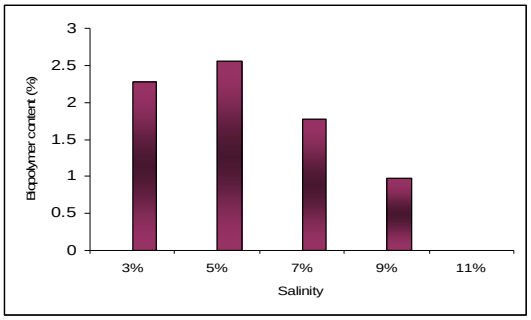

Fig. 5: PHB production on different salinity

different carbon sources (such as, glucose, lactose, sucrose, maltose and cellulose) and nitrogen sources ($(NH_4)_2HPO_4$, KNO_3, NH_4Cl, Tryptone and Peptone) at 0.3% were tested, in that sucrose and diammonium hydrogen phosphate showed maximum poly 3-hydroxy butyrate production (Fig 6 and Fig 7). Comparable results were observed in previous studies especially using glucose as carbon source (Pal *et al*., 2008; Rohini *et al*., 2006).

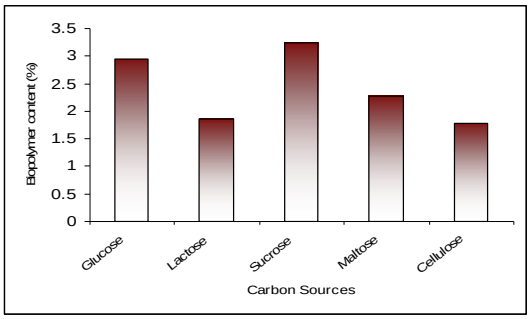

Fig. 6: PHB production on different carbon sources

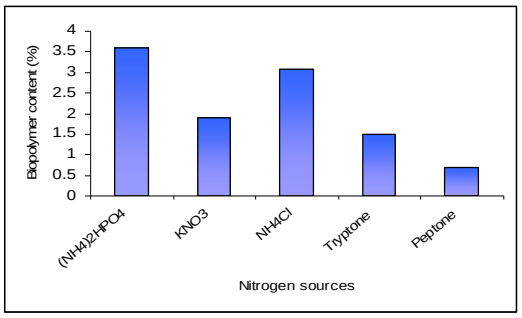

Fig. 7: PHB production on different nitrogen sources

For mass scale culture, optimized E2 mineral broth was prepared and *Pseudomonas* spp., of AMET 5111 was inoculated, incubated at 30ºC for 36hrs with the potential carbon and nitrogen sources. After incubation period extraction was done with chloroform. Many researchers have reported the extraction of biodegradable polymer with chloroform from bacterial biomass (Anwar and Hakim, 2002). During extraction the production of poly 3-hydroxybutyrate was noticed by the formation of thin layer. The extraction with chloroform may be carried out due to the presence of poly 3-hydroxybutyrate granules that interlinked with each other and form a thin layer (Senthilkumar and Prabakaran, 2006). Totally obtained content of PHB from AMET 5111 (*Pseudomonas* spp.) was calculated. The total cell dry weight was 1.93g and the biodegradable polyester was 0.04 g and the total biopolymer content was 2.07%. Naheed *et al*., (2011), stated that in their studies, 66% PHB per dry weight was achieved by *Enterobacter* after 24 hrs of incubation.

From the results, the work highlighted the mass scale production of biopolymer (poly (3-hydroxybutyrate) from marine bacteria of AMET 5111 *Pseudomonas* spp. The results from the optimization maximum and minimum production of poly 3-hydroxybutyrate achieved by using one parameter at a onetime method had shown the potential temperature, salinity, pH, carbon and nitrogen source for the production of biopolymer. Hence, this study clearly demonstrated the production of biopolymer from marine bacteria and the biopolymer is suggested to use and make as an ecofriendly biodegradable products.

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