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Research Article

PRODUCTION AND OPTIMIZATION OF POLY (3-HYDROXYBUTYRATE) BIOPOLYMER BY ASCIDIAN ASSOCIATED BACTERIUM *VIBRIO PROTEOLYTICUS* DCM CAS2

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ABSTRACT

Polyhydroxybutyrate (PHB) is a biodegradable polymer produced by many bacteria. It can be used as an alternative to petroleum-based commercial plastics as they can be easily degraded by the soil microorganisms. The present work focuses on the screening of a potential bacterium for PHB production. In this study, ascidian associated bacterial colonies were isolated and screened for PHB production. They were grown in E2 mineral medium and screened for biosynthesis of PHB using Sudan Black B stain and the strain was identified as *Vibrio proteolyticus* DCM CAS2 using 16S RNA sequencing. *V. proteolyticus* shown highest production of PHB and it was selected to optimize its culture conditions using different carbon sources, nitrogen sources, pH and temperatures. After optimization, the bacterium *V. proteolyticus* yielded maximum amount of PHB at pH 6 and incubation temperature of 30°C using fructose and tryptone as carbon and nitrogen sources. The increased production of PHB after optimization indicates that the bacterium *V. proteolyticus* DCM CAS2 was a potent PHB producer.

Keywords: Ascidians, polyhydroxybutyrate, optimization

INTRODUCTION

Bioplastics are a special type of biopolymer defined by the American Society for Testing Materials as "degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae" (Mooney, 2009). They are polyesters, produced by a range of microoganisms, such as Bacillus, Pseudomonas, Aeromonas (Madison and Huisman, 1999), Aeromonascaviae, Burkholderia sp. (Chee *et al*., 2010), Comamonas sp. EB172 (Zakaria *et al*., 2010), and fungi, such as Rhizopus oryzae (Accinelli etal., 2012) cultured under different nutrient and environmental conditions. These polymers, which are usually lipid in nature, are accumulated as storage materials allowing microbial survival under stress *conditions* (Barnard and Sanders, 1989; Sudesh *et al*., 2000). The number and size of these granules, monomer composition, macromolecular structure, and physico-chemical properties vary, depending on the producer microorganisms (Anderson and Dawes, 1990). They can be observed as intracellular light-refracting granules or as electronlucent bodies that, in overproducing mutants, cause a striking alteration of the bacterial shape. Polyhydroxyalkanoates (PHAs) are a class of biopolymers formed as naturally occurring storage polyesters by a wide diversity of microorganisms (Murray etal., 1994). They are deposited as spherical intracellular inclusions with an amorphous hydrophobic PHA core which is mainly surrounded by proteins involved in PHA metabolism (Rehm, 2010; Grage *et al*., 2009). The weight of the polymer can range from 200 to 3000 kDa, depending on the organism and conditions under which it was produced (Jendrossek, 2009). PHAs can vary substantially in composition, as there are over 150 known constituents, resulting in a wide diversity of material properties.

Due to its useful properties, it has been found technical, commercial and biomedical significance (Anderson and Dawes, 1990). In spite of many useful properties, it has not been commercialized for large scale application due to the high cost of production and less efficient recovery methods involved in it. Therefore, the fermentation methodology remains essential. The present study was aimed towards the search for high PHB production method and to develop efficient production and recovery method. Agricultural sources are a good and renewable source of carbon and therefore it has been purposefully chosen for the production of PHB biopolymer. The present work was undertaken to study PHB production by *Vibrio proteolyticus* DCM CAS2 isolated from marine ascidian *Diplosoma listerianum.* Marine ascidians are filter feeders, having a good symbiotic relationship with various microbes and the research on ascidian associated bacteria may provide remarkable milestone in the future biopolymer research. At this study polyhydroxybutyrate producing ascidian associated bacterial strain was isolated, confirmed and used for the production of PHB biopolymer.

MATERIALS AND METHODS

Isolation of PHB producing bacteria:

Marine ascidian *Diplosoma listerianum* was collected from Palk Bay of Southeast coast of India by

SCUBA divers at the depth of 10 – 15 m. Ascidian associated bacteria was isolated using Zobell marine agar. 1g of ascidian tissue was ground with sterile distilled water and plated on Zobell marine agar and kept for incubation at 37°C for 48 hours. Morphologically dissimilar colonies were individually picked and sub cultured 4-5 times on zobell marine agar plates. The pure cultures isolated were maintained in zobell marine agar slants and preserved in 50% glycerol stock at -80°C.

Screening of PHB producing strain:

The cultures were grown in zobell agar medium were heat fixed on a slide and immersed in 0.5% (w/w) Sudan black B staining with ethylene glycol for 5 min. Then the slide was air dried, the excess amount of stain was de-stained using xylene several times and blot dried with absorbent paper. Then the counter stain (0.5%w/v aqueous saffranin) was added for 5 to 10 seconds. The slide was washed with tap water and dried. The stained cells were observed under oil immersion microscope. The morphological and physiological and biochemical properties of the isolates were investigated according to Bergey's method of Determinative Bacteriology (Holt *et al.,* 1993).

Identification of PHB producing bacterium:

The morphological and physiological and biochemical properties of the isolates were investigated according to Bergey's method of Determinative Bacteriology (Holt *et al.,* 1993). For a brief confirmation the genomic DNA was extracted (Marmur, 1961) and 16S rRNA gene sequences were amplified using suitable primers and the 16S rRNA gene sequence obtained from the *Vibrio proteolyticus* DCM CAS2 was deposited into GenBank (NCBI).

Production and extraction of PHB:

Bacterium *Vibrio proteolyticus* DCM CAS2 was inoculated in to E2 mineral medium and allowed to grown at 37°C for 48 hours for the production of PHB biopolymer. Extraction of PHB from *Vibrio proteolyticus* DCM CAS2 was carried out using the hypochlorite digestion method with slight modification. For this, the isolate was grown in 250 ml Erlenmeyer flasks containing 50 ml of E2 mineral medium and incubated at 37°C for 48 hours at 160 rpm. After the incubation period, cell suspension was centrifuged at 6000rpm for 10 minutes. The cell pellet was washed with distilled water and then suspended sodium hypochlorite solution and incubated at 37°C for 10 minutes. This was again centrifuged at 8000rpm for 20 minutes and the pellet was again centrifuged at 8000rpm to get the purified PHB and was dried to get constant weight at 60°C.

Optimization of cultural conditions:

The production medium was inoculated with the bacterium *Vibrio proteolyticus* DCM CAS2 and incubated at 37°C for 48 hours in shaker under different conditions of growth. To optimize the culture

conditions, different carbon sources, nitrogen sources, pH and temperatures were tested at a fixed concentration of 1g/L. Ranges of pH from 5 to 9 and the temperature 20, 25, 30, 35, 40°C were studied.

RESULT AND DISCUSSION

Concern over petrochemical plastics in the environment, has created a renewed interest in biologically derived polymers. PHAs can serve as an efficient alternative to petroleum based non-degradable polymers (Verlinden *et al*., 2007). Many bacterial strains which have potential to produce PHA have been isolated and identified from different origins but, still screening of a novel bacterium remains untapped (Nisha *et al*., 2011). The identification of bacteria capable of producing, large amount of PHB by utilizing cheap sources is very needed for the large scale production of commercial PHB. This requires careful optimization and cultural conditions under which PHB synthesis is maximized. In this study, a potential PHB producer *Vibrio proteolyticus* DCM CAS2 was isolated from the ascidian *Diplosoma listerianum* from Palk Bay, Southeast coast of India. On the basis of molecular identification using 16*S* rRNA sequencing the candidate strain was identified as *Vibrio proteolyticus* DCM CAS2 (Accession number: KP794054) and submitted to Gen bank. Screening of biopolymer production was done by Sudan black B staining method. In the slide method, blue coloured granules were observed. Sudan black B is slightly basic dye and accumulates in the acidic groups of fat globules in the PHB producers. This stain is specific for lipids.

PHB production is largely dependent on the type of the carbon source utilized by the bacteria. In the present study, the culture was grown for 48 h at 37°C and the effect of carbon sources such as glucose, xylose, fructose, sucrose and starch are utilized at a fixed concentration of 4g/l, and the production of PHB was evaluated. Of all the various carbon sources, fructose positively affected PHB production with a concentration of 5.05g/l when it was the sole carbon source (Fig.1). The optimum PHB production was attained at 55 % of CDW, when the fructose was used as a sole carbon source. The PHB production was clearly decreased when fructose was replaced by glucose, sucrose, xylose and starch that were 37, 26, 43 and 38 % of CDW, respectively. However, PHB production and biomass were low, when the carbon source was glucose and sucrose. Similar results were observed in *Bacillus megaterium* and other *Bacillus* sp (Limpon, 2013).

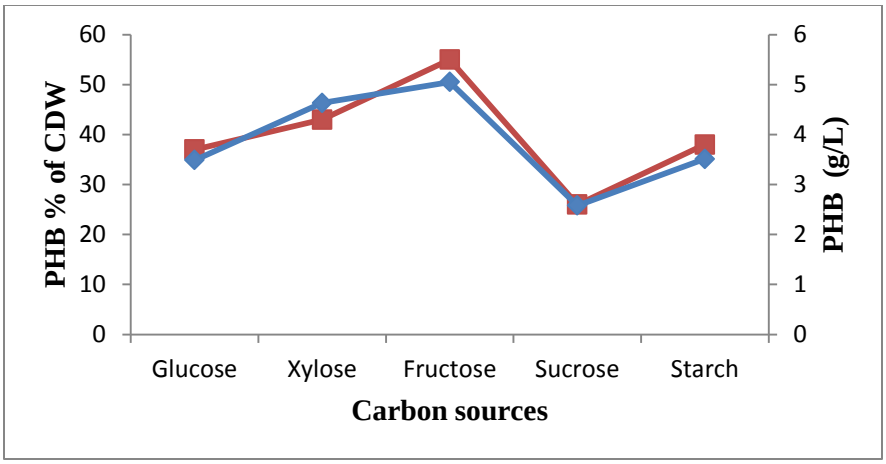

Figure 1: Effect of carbon sources in the production of PHB

Supplementation of different nitrogen sources such as yeast extract, beef extract, tryptone, ammonium chloride and peptone in E2 mineral medium clearly shows the influence of nitrogen sources in the production of PHB. PHB production was also significantly affected when different nitrogen sources were added to the production medium. The nitrogen sources were taken at a fixed concentration of 1 g/l and the isolate was grown for 48 h at 37°C in the E2 mineral medium. According to the results obtained, almost all the nitrogen sources except peptone enhanced PHB production and tryptone shown the optimum PHB production of 8.15 g/l PHB in the 75 % of CDW (Fig.2). Santimano *et al*, (2009) observed that the complex nitrogen sources increased the yield of PHB. Whereas the better yield of PHB was obtained by *Bacillus*, *Staphylococcus* and *Pseudomonas* using ammonium sulphate and ammonium phosphate as nitrogen sources than that of yeast extract (Borah *et al*, (2002). In the present study, strain *vibrio proteolyticus* DCM CAS2 showed optimum PHB production of 75% when compared with *Bacillus tequilensis* NCS-3 (67.3%) (Chandani *et al*., 2012).

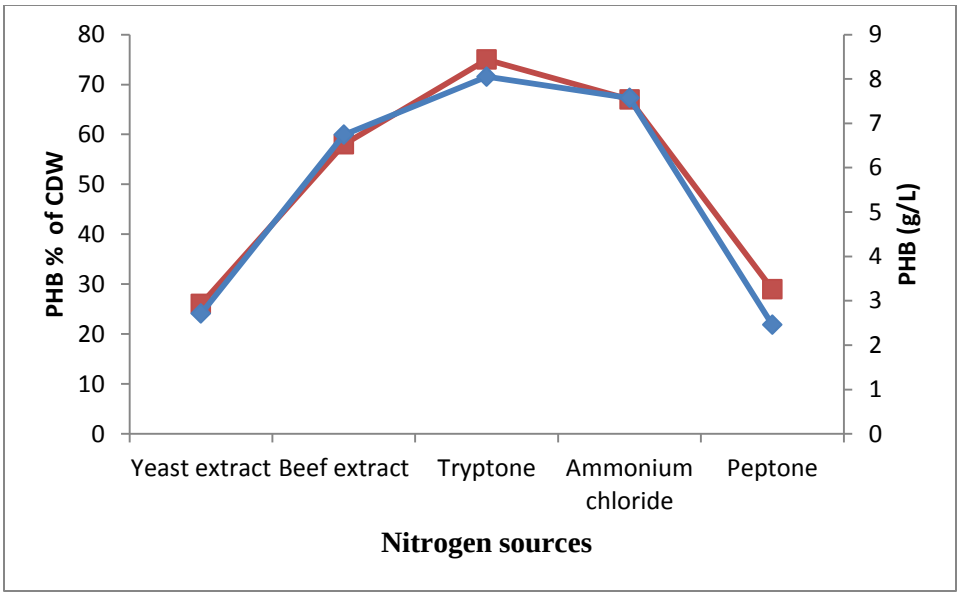

Figure 2: Effect of nitrogen sources in the production of PHB biopolymer

Cultural conditions such as pH and temperature plays a vital role in the production of PHB. The maximum PHB (4.92 g/L) was observed at pH 7 (Fig.3). Gorthe *et al*, (1999) reported that the optimum PHB production ranging from 6 to 7 pH by using *Alcaligenes latus.* According to Nakata (1963) it is observed that the lack of polymer accumulation at higher pH value may be due to an effect on the degenerative enzymes of polymer breakdown, so that the PHB is utilized at the rate almost equal to the rate of its synthesis.

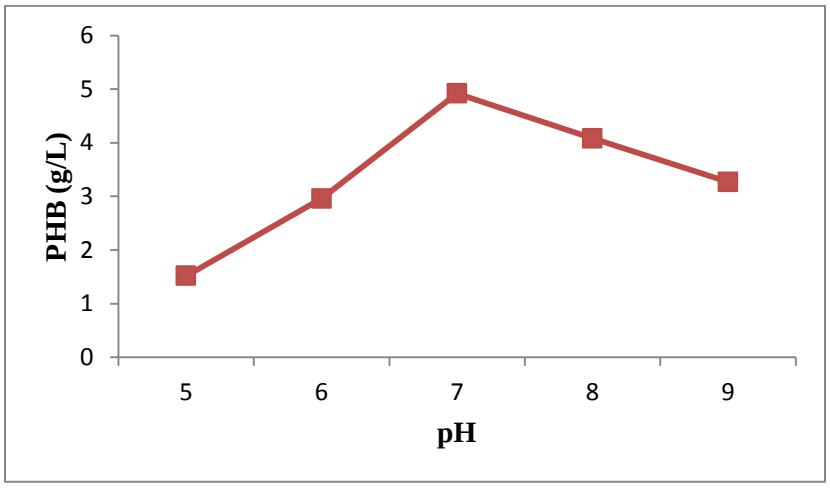

Figure 3: Effect of pH in PHB production

Yield of PHB at different temperature conditions were examined and the optimum temperature was obtained as 30°C, and the PHB yield is 5.57 g/L (Fig.4). Similar results were obtained by Yu-Hong *et al*, (2011)

using *Bacillus sps*. And it is observed that 30°C is a favourable temperature for PHB production.

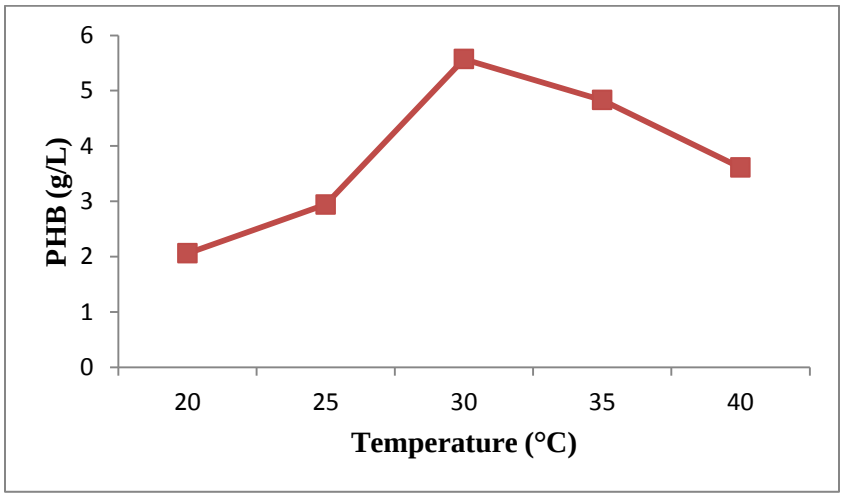

Figure 4: Effect of temperature in PHB production

CONCLUSION

Based on the above results, it could be concluded that optimum cultural conditions for an effective PHB biopolymer production for the strain *Vibrio proteolyticus* DCM CAS2 was pH 7 at 30°C by utilizing fructose and tryptone as carbon and nitrogen sources. Therefore selection of efficient PHB producing bacteria and on optimizing the most favourable conditions for PHB production is very important for successful production of biodegradable plastics.

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