Optimization and Production of Polyhydroxy Butyrate from a Marine Sponge Associated Bacteria MSI21

S. Priyadharshini[1*], Arya Sajayyan[1] and Amrudha Ravindran[1]
[1]Department of food Science and Technology,
Pondicherry University, Pondicherry, India

Abstract: ***Polyhydroxy butyrate is a biodegradable bacterial polymer used as a replacement of petro-chemically derived non degradable plastics. In this study a total of forty different bacteria were isolated from the marine sponge and were used for the screening of polyhydroxy butyrate (PHB) using Nile blue staining method. The isolate MSI21 was identified as a potent producer based on the intensity of fluorescence obtained under UV light. Morphological, biochemical and phylogenetic analysis showed the isolate MSI21 as Bacillus cereus. The maximum yield of PHB (3.4g/L) was achieved on optimization of the production media and its physical parameters such as pH and temperature. Maximum biomass of MSI21 was obtained in the minimal media incubated at a temperature of 30˚C and at a pH of 7.0. The isolate utilized 20% of glucose to increase the yield and production of PHB.***

Keywords: PHB, Bacillus cereus, Marine Sponge, Biomass

Introduction

Plastics are one of the main environmental issues faced by all the industries because of its non-degradable nature and disposal problems. Thus the emergence of idea of bio plastics gains its importance. They are biomass based plastics which are biodegradable in nature that can be produced from microorganisms, algae, bacteria and renewable substances. Poly hydroxyl alkonates (PHA) is a best degradable polymer and poly hydroxy butyrate (PHB) is the co-polymer of PHA. Polyhydroxyalkanoates (PHA) is a gathering of polyesters, integrated and normally gathered by prokaryotic living beings as intracellular granules produced under nutritional stress condition as well as in the presence of carbon source during its growth conditions (Kim, 2001). The attribute of PHB is the biodegradation and their thermal stability which made as important aspects for the mass production of PHB for industrial applications (Choi, 1997). Currently, PHAs are used as a replacer of petroleum-derived synthetic plastics (Nath *et al.*, 2008). PHA has gained increased attraction, as an eco-friendly polymer used in a broad range of applications such as medical, agricultural and industrial (Sudesh *et al.*, 2000). The increase in the price of PHB production restricts the economic benefits and use of PHB. Therefore, bioprocess using cheaper available carbon source and high yielding strains are important forbroadening PHB as an eco-friendly benign plastic for business applications (Li *et al.*, 2007). Marine Sponges (Porifera) are identified as a wealthy source of novel compounds which are of potential interest to mankind (Faulkner, 2000). Sponges are identified as an exquisite aid of bioactive compounds and sponge metabolites are known for the antimicrobial, antitumor and antiviral activity (Wang, 2000). Sponges are driving massive volumes of seawater through their aquiferous shape, which controls the pumping and food seizing choanocytes with a selection of canals, chambers and spaces (Taylor *et al.*, 2007). Marine sponges possess a first-rate array of microorganisms and the marine sponge-related endosymbiotic microorganism was recognized as a rich source of biologically important molecules that are of potential interest to numerous commercial sectors (Selvin *et al.*, 2008). Sponges provide the colonizing microorganism a nutrient-rich area of interest and the microbial communities confer upon their hosts an ability to exploit a variety of metabolic pathway (Steinert *et al*., 2000). PHAs can be converted into water and carbon dioxide by microorganisms observed in an extensive range of environments (Byrom, 1987).

Materials and methods

Collection of marine sponge and Isolation of sponge associated bacteria

Marine sponge *Dendrilla nigra* was collected from Gulf of Mannar, Rameswaram by Scuba diving. The sponges were collected at a depth of 2 meters and 1000 meters away from the seashore. The samples were immediately transferred into laboratory aseptically. Sponge associated bacteria were isolated using the serial dilution and spread plating method (Gandhimathi et al 2008). The samples were cut into pieces of 1 cm^3 cube size and rinsed several times with sterile sea water to remove outer contaminants. The sponge tissue was then homogenised with phosphate buffered saline. The homogenate was serially diluted with sterile distilled water and spread plated on different media including Zobell marine agar, nutrient agar supplemented with 2% salt and starch yeast peptone- sea water media and incubated at 28˚C for three days. Colonies were differentiated with morphology and forty different types were isolated and stored at 4ºC for further studies.

Screening for PHB producer

The sponge associated bacteria were screened for PHB production by viable colony staining method. The isolates were streaked on Zobell marine agar supplemented with 0.25 mg of Nile blue A (w/v) in DMSO (Ostle and Holt, 1982). The colonies were examined under UV light for fluorescence to examine the accumulation of Polyhydroxy butyrate. The PHB producing ability was further confirmed by Nile red staining.

Identification of the PHB producer

Active PHB producer MSI21was phenotypic ally identified using Bergey's manual of bacteriology. The isolate was further characterized with biochemical tests. Genomic DNA was isolated and amplified using the universal primers 8 F (5′- AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′- GGT TAC CTT GTT ACG ACT T-3′) and the amplified products were then purified using gel purification kit and then sent for sequencing (Macrogen, Korea). The sequences were then analysed using NCBI mega BLAST. More than one alignment of these sequences was achieved by means of Clustal W 1.83 model of EBI with 0.5 transition weight. Phylogenetic tree of the isolates was built using MEGA 6.0 model (www.Megasoftware.Internet) the use of most parsimony set of rules.

Optimization of culture conditions

Culture conditions were optimized for maximum PHB production using different fermentation media including Zobell marine broth, nutrient broth supplemented with 2% salt and minimal medium (Na_2HPO_4. $2H_2O$, KH_2PO_4, NH_4Cl, NaCl, $CaCl_2.2H_2O$ $MgSO_4.7H_2O$). All the media were adjusted to pH 7 and autoclaved. To test the effect of different carbon source on PHB production varying carbon sources was added to the M9 minimal medium containing 20.0% (w/v) of sterilized glucose, fructose, lactose and starch, and then incubated for 3 days at 28° C. PHB production by the isolate MSI21 was optimized for varying pH from 5-9 and temperature (4, 20, 30, 37 and 50˚C).

Extraction and quantification of PHB

PHB production was done by submerged fermentation in the optimized minimal media supplemented with glucose and incubated at 30°C for 72h**.** PHB accumulated in the cell pellet was collected by centrifugation at 10,000 x g for 20 minutes. The pellet obtained was digested and lyophilized with 30% sodium chlorite solution. Then the samples were centrifuged at 8000xg for 30min and washed, successively with distilled water, acetone and methanol. The produced PHB was then extracted using boiling chloroform and the filtrate obtained was treated with con. H_2SO_4, and then incubated at 100˚C for 30mins. The absorbance of the extracted PHB was recorded at 235nm (Kiran *et al*., 2014).

Results and Discussion

Collection of Marine sponge and isolation of Marine sponge associated bacteria

The Marine sponge Dendrilla nigra was collected from the Gulf of Mannar area (Mandapam cost, Rameswaram) through Scuba diving. The collected sponge sample was transported aseptically to laboratory for further experiments. Forty different types of phenotypically distinct bacteria were isolated from the sample and subcultured and they were further screened for PHB production (figure 1).

Figure 1: a) Marine sponge collected from Gulf of Mannar, Rameswaram: b) Isolated colonies on Zobell marine agar

Screening of PHB producers

Among the 36 different sponge isolates, isolate MSI21 was selected as a potent PHB producer according to the intensity of the orange fluorescence obtained in the Nile blue A staining (Figure 2). PHB accumulation was further confirmed by Nile red staining. The active producer MSI21 was identified by morphological, biochemical, cultural characterstics and Phylogenetic analysis. The isolate was characterized as Gram- positive, aerobic, flat, smooth, irregular and opaque colony and citrate & catalase positive. The isolate MSI21 showed maximum similarity with *Bacillus cereus* by neighbour joining method with maximum parsimony algorithm. Based on the morphological, biochemical and Phylogenetic characteristics, the strain MSI21 was identified as *Bacillus cereus* (Sajayan et al., 2016).

Figure 2: Orange fluorescence obtained in the Nile blue A staining under UV light

Optimization of PHB production

Culture conditions were optimized for maximum PHB yield. Maximum cell density of MSI21 was obtained in minimal media followed by nutrient broth supplemented with 2% salt, Luria Bertani broth and Zobell marine broth (Figure 3). Maximum PHB production was obtained in the minimal media supplemented with glucose. However the sucrose and maltose does not support the yield and cell biomass. Starch and glucose were readily utilized by bacteria and PHB production was higher. In the case of maltose and sucrose complexity of the moiety was higher and thus the bacteria may not be able to utilize it properly and hence the yield of PHB was low (Figure 3). The strain MSI21 grows at a wide range of pH from 5-9 and maximum PHB production was observed at a pH of 7.0 (Figure 4). The production of PHB was higher at 30°C at 72 hrs of incubation. The production was inhibited at 4°C and at a temperature above 50°C the production was drastically decreased (figure 5). The isolate MSI21 showed improved PHB production in the optimized culture medium and the PHB was extracted using standard chloroform method. 3.4 g/L of PHB was obtained from the production media by submerged fermentation.

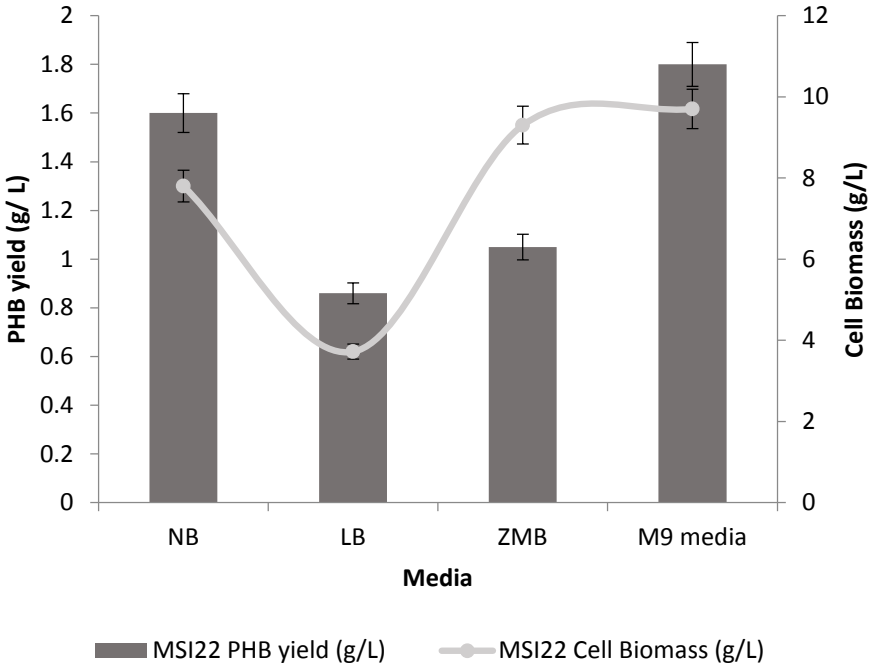

Figure 3: Optimization of media for maximum PHB yield and biomass

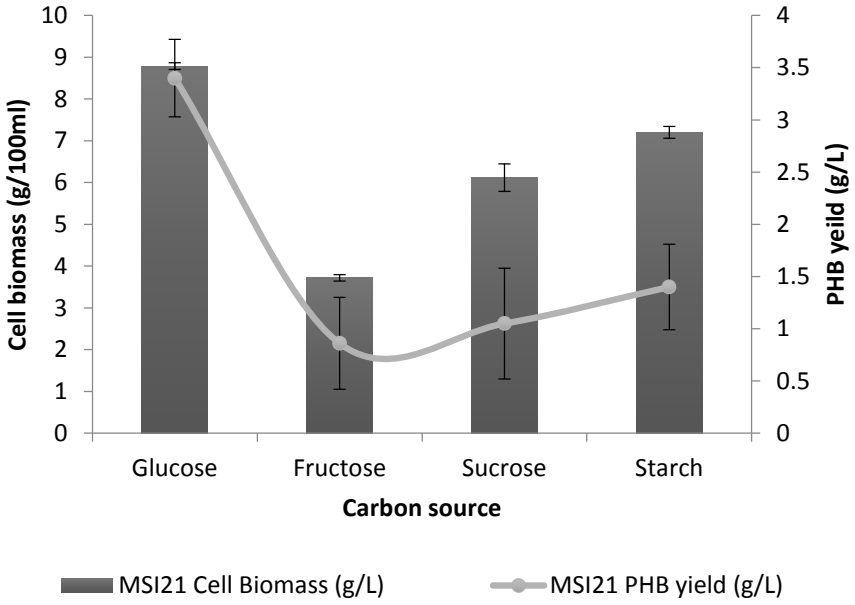

Figure 4: Optimization of carbon source for maximum PHB yield and biomass

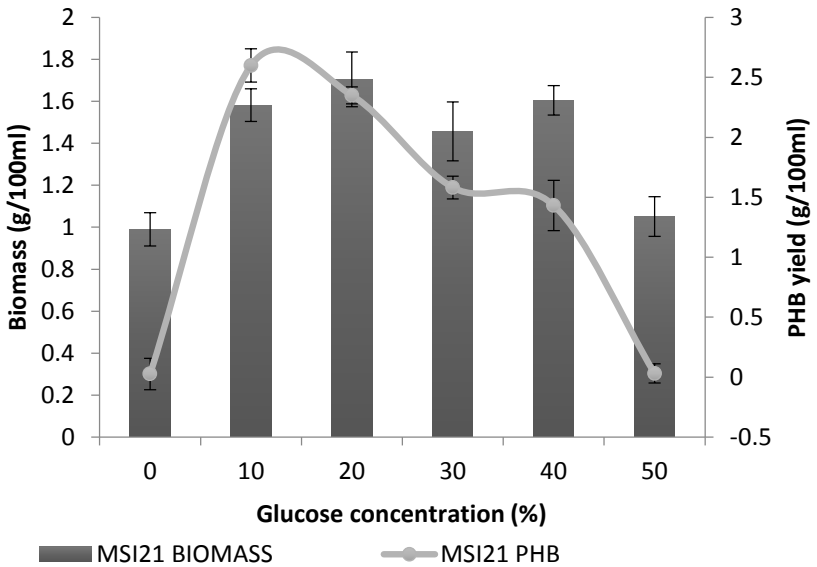

Figure 5: Effect of varying concentration of glucose for maximum PHB yield and biomass

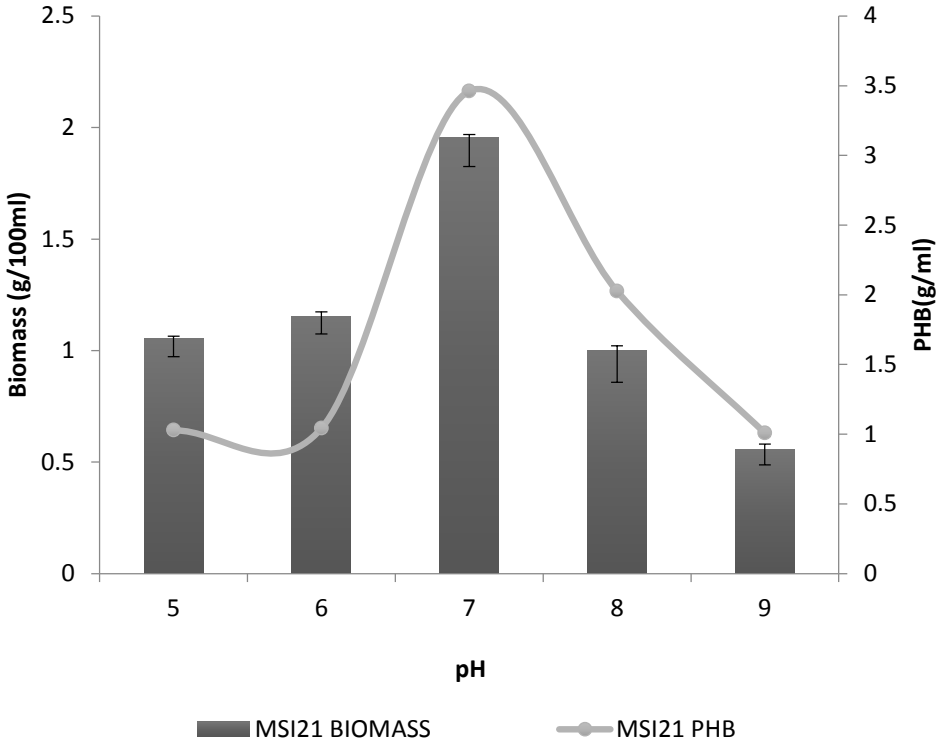

Figure 6: Optimization of pH for maximum PHB yield and biomass

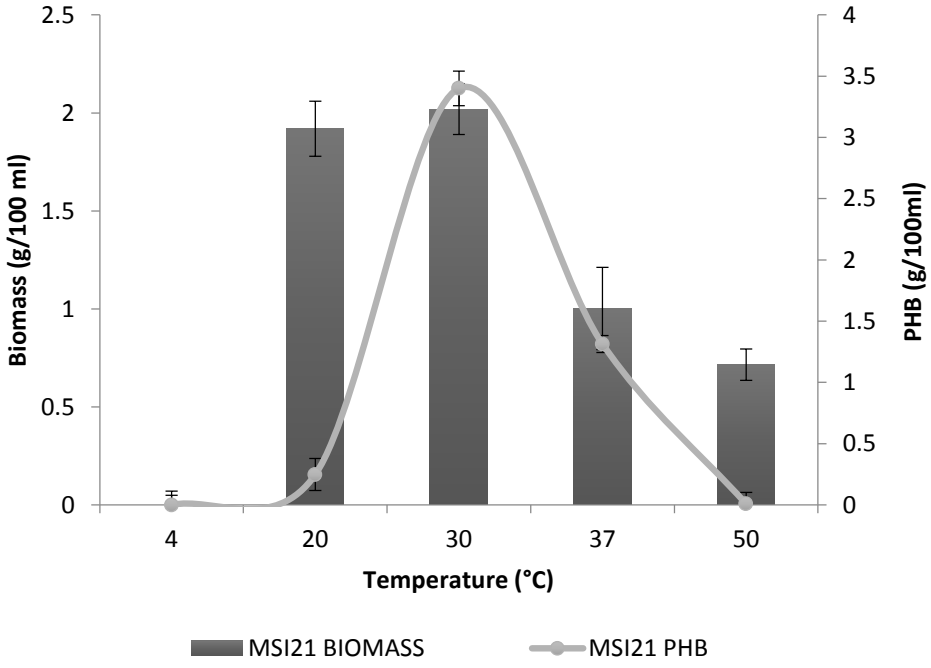

Figure 7: Optimization of temperature for maximum PHB yield and biomass

Discussion

In this study 36 distinct types of the colonies were isolated from the marine *Dendilla nigra* and screened for PHB production. Forty percent of the sponge biomass contributed by the bacteria, those bacteria exasperates only by outer pressure factors or they are permanently attached to the host sponges (Friedrich *et al*., 2001). Marine sponge-associated bacteria can be a potential source of PHB producers since the nutrient limitation in the sponge perhaps facilitates the synthesis of PHB granules (Sathiyanarayanan *et al*., 2013). Marine sponge-associated bacteria are reported for the production of exopolysaccharide, biopolymers and antibiofilm biosurfactants (Sathiyanarayanan *et al*., 2013). *Bacillus cereus* is known for the accumulation of PHB granules inside the cell under nutrient limiting conditions (Rohini et al., 2006). The production of PHB by MSI21 was optimized with a pH of 7.0, 20% glucose and incubation temperature of 30˚C. Further increase in the temperature affects the growth and yield of PHB. The experiment results obtained proved the

yield of PHB was growth associated; similar results were reported in the literature (Sayyed et al., 2009). Shah et al., (2014) reported glucose was found to be the best carbon source for the production of PHB.

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