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Research Article

OPTIMIZATION OF CULTURAL CONDITIONS FOR POLYHYDROXYBUTYRATE [PHB] PRODUCTION BY *ALCALIGENES SP.* FROM MANGROVE SOIL

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ABSTRACT

Polyhydroxyalkanoates [PHAs], which is produced by several bacteria, is a biodegradable polymer that has many industrial and medical applications. The present study was aimed for the isolation, characterization of biopolymer [PHB] producing bacteria from the mangrove soil and optimization of culture conditions for maximum PHB production in an attempt to reduce the production cost. PHB producing bacterium was isolated from mangrove soil and identified as *Alcaligenes sp.*on morphological, cultural, biochemical tests. Culture conditions for the organism were optimized by changing the parameters, viz. carbon source, nitrogen source, pH and incubation time. Results for optimized parameters for the isolated PHB positive species showed that synthesis of PHB was maximum with lactose and beef extract at pH 7 with 10% inoculum size.

Keywords: Polyhydroxyalkanoates, PHB, biodegradable, biopolymer.

1. INTRODUCTION

Plastic materials originated from petrochemicals cause significant environmental problems because of their non-degradable properties. Such synthetically produced polymers are generally cost-effective, but their persistence property has a huge environmental impact [1]. In a year the world plastic industry generates profits of around $600 billion, and with a market-driven by convenience and consumerism, along with the relatively low price, demand for plastic materials is increasing. However, recycling and recovery are inadequate, and every year, many large amounts of plastic end up in oceans and landfill areas.

With the impending fossil fuel crisis, the atrocious rate of petroleum prices, and the environmental impact associated with the products, the search for alternatives is essential in reducing mankind's dependencies on non-renewable resources [2].

The properties of PHB are almost like synthetic thermoplastic polymers, similar to polypropylene, making PHB a promising candidate for replacement for petroleum-based plastics in a wide selection of applications like paper coatings, packaging, and manufacturing of plates and bottles [3].

Biodegradable plastics provide the simplest solution to guard the environment against hazards attributed to conventional petroleum-based plastics as they are 'eco-friendly' in nature. There are varieties of biodegradable plastics with different degrees of biodegradability. Among them, polyhydroxybutyrate [PHBs] are the sole 100% biodegradable ones [4].

PHBs are macromolecules synthesized by bacteria as inclusion bodies and are stored as reserve material when the bacteria grow under nutrient-limited. PHBs are biodegradable polymers, which have the potential to substitute fossil-derived polymers. This makes them useful for extensive applications and future commercial production of biodegradable plastics that can replace plastic materials currently obtained from petroleum bases [5]. Despite the potential of PHB as biodegradable thermoplastic has been long appreciated, substantial information is not available on the cultural conditions regulating its production [6].

Currently, capital-intensive production and inefficient recovery have limited commercialization and vast applications of PHB [7]. High PHB content is important because the cost of polymer recovery increases significantly with low polymer content [8].

In the present study, an attempt was made to isolate PHB producer from a rhizobacterium *Alcaligenes* sp. isolated from mangrove rhizospheric soil sample and to study of the effect of different cultural conditions and physico-

chemical parameters on the growth of *Alcaligenes* sp and production of PHB.

2. MATERIAL AND METHODS

2.1. Sample collection

Soil sample was collected from the mangrove forest of Mansarovar, Navi Mumbai. Samples were collected from the rhizospheric region of mangrove plants, at a depth of 10-15cms. Collected samples were refrigerated at 4°C.

2.2. Enrichment and isolation of microorganism from soil

One gram of soil was mixed in 10 ml of sterile saline and was allowed to settle for 10-15 minutes. Then 1 ml supernatant was taken and mixed with 50 ml of sterile Nutrient broth [NB]. The flasks were kept for incubation at 28°C for 24 hours. After 24 hours one loopful of culture was streaked on respective media plates and incubated at 28°C for 24 hours. After incubation isolated colonies were purified and preserved on respective media slants for further use and were assigned with code numbers.

2.3. Screening of isolated colonies for PHA production

Nutrient agar with 1% glucose was sterilized by autoclaving at 121°C for 20 minutes. The medium was poured into sterile Petri plates and allowed to solidify. The isolates were spot inoculated on the plate and incubated at 28°C for 24 hours. After incubation at 28°C for 24 hours, the plates were flooded with ethanolic solution of 0.02% Sudan black B stain and kept undisturbed for 30 minutes. After 30 minutes the excess stain from the plate was discarded and the plate was washed with absolute ethanol. The colonies showing blue-black colour were considered as PHA positive and used for further study [9].

2.4. Extraction of PHA by Acetone: Alcohol method

PHB extraction from the isolates was carried out by acetone: alcohol method [10]. After 24 hours incubation, 10 ml of culture medium was taken into a pre weighed sterile centrifuge and centrifuged at 10000 rpm for 10 minutes. The pellet obtained was treated with 10 ml of sodium hypochlorite and was incubated at 37°C for 1 hour. After incubation, the mixture was again centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded and the pellet was washed with distilled water and acetone: alcohol [1:1] respectively. The pellet was dissolved in boiling chloroform [5ml]. The chloroform was evaporated by pouring the solution in a sterile petri plate and kept at 40°C. After evaporation, a layer of white colored powder was obtained on the petri plate.

2.5. Quantification of PHA

After obtaining a layer of white colored powder, 1ml of concentrated sulphuric acid was added to a Petri plate and the solution was taken in a clean dry test tube which was then heated in a boiling water bath for 20 minutes. After boiling, 0.1 ml of the solution was diluted with 4.9 ml of distilled water. The UV absorbance was measured in a UV spectrophotometer [SHIMADZU UV-1800] at 235 nm with distilled water as blank. Amount of PHB in the sample was estimated by standard crotonic acid assay as PHB on heating with concentrated sulphuric acid was converted into crotonic acid. Standard solution of crotonic acid [10 to 100 ppm] was prepared by using distilled water. Absorbance of these solutions was measured at 235 nm with distilled water as a blank solution. Calibration curve was made with absorbance of crotonic acid at 235 nm versus known concentration of crotonic acid.

The PHB accumulation by the different isolates was compared to help in the identification of the best producer.

2.6. Characterization of isolate

The isolate was primarily characterized by Gram staining, biochemical tests including IMViC, catalase, nitrate reduction, TSI, urease, gelatin hydrolysis and carbohydrate fermentation to identify the isolate.

2.7. Optimization of cultural parameters for maximum PHA production

2.7.1. Effect of carbon source on PHA production

The selected isolate was grown in 250 ml of conical flasks containing 50 ml Nutrient broth with different carbon sources such as, fructose, glucose, lactose, mannitol, sucrose and xylose at 1% concentration. The flasks were incubated at 28°C for 24 hours on a rotary shaker [150 rpm]. After incubation, PHA produced by the isolate was quantified spectrophotometrically as per the above mentioned protocol.

2.7.2. Effect of nitrogen source on PHA production

The selected isolate was grown in 250 ml of conical flasks containing 50 ml Nutrient broth with different nitrogen sources like peptone, beef extract, sodium nitrite,

sodium nitrate, Na_2HPO_4, NaH_2PO_4, ammonium sulphate and ammonium nitrate at 1% concentration.

The flasks were incubated at 28°C for 24 hours on a rotary shaker [150 rpm]. After incubation, PHA produced by the isolate was quantified spectrophotometrically as per the above mentioned protocol.

2.7.3. Effect of pH on PHA production

The selected isolate was grown in 250 ml conical flasks containing 50 ml Nutrient broth with different pH such as 5, 6, 7 and 8. The flasks were incubated at 28°C for 24 hours on a rotary shaker [150 rpm]. After incubation, PHA produced by the isolate was quantified spectrophotometrically as per the above mentioned protocol.

2.7.4. Effect of inoculum size on PHA production

The selected isolate was grown in 250 ml conical flasks containing 50 ml Nutrient broth with different inoculums sizes *viz.* 1%, 2%, 4%, 6%, 8% and 10%. The flasks were incubated at 28°C for 24 hours on a rotary shaker [150 rpm]. After incubation, PHA produced by the isolate was quantified spectrophotometrically as per the above mentioned protocol.

3. RESULTS AND DISCUSSIONS

3.1. Screening of promising isolates for production of PHB and its characterization

Various PHB producers were obtained from rhizospheric soil samples of mangroves. The visual screening for PHA production using Sudan Black B was carried out to select PHA positive culture. PHA accumulators are stained dark blue, or black, whereas negative PHA accumulators remain white, or light-blue [11]. P-1 was selected for further studies as it showed maximum PHB production.

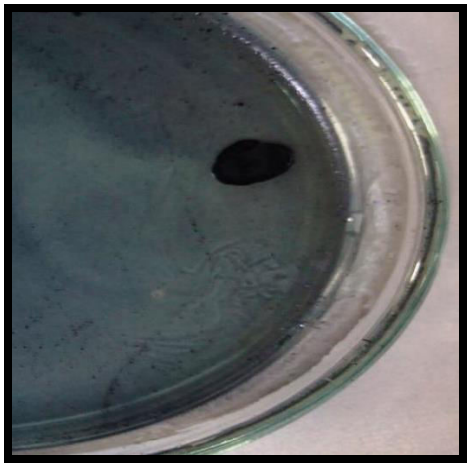

Fig. 1: Sudan black staining of the P-1

The morphological and physiological characteristics of P-1 were summarized as shown in Table 1. The morphological and biochemical characteristics indicated that the bacterium P-1 belongs to *Alcaligenes* sp. group as per Bergey's Manual of Determinative Bacteriology.

Table 1: Microscopic and Biochemical characteristics of *P-1*

Characteristics	P-1 isolate
Size	1mm
Shape	Circular
Margin	Entire
Opacity	Opaque
Elevation	Flat
Colour	Off white
Gram Nature	Gram negative short rods
Endospore	Absent
Oxidase	Negative
Catalase	Positive
Starch hydrolysis	Negative
Gelatin liquefaction	Negative
Citrate utilization	Negative
Indole	Negative
Methyl red	Negative
Voges-Proskauer test	Positive
Nitrate reduction	Positive
Growth in 6.5% NaCl	Positive
Urease	Negative
Sugar utilization	
Lactose	Positive
Mannitol	Negative
Xylose	Negative
Glucose	Positive
Fructose	Positive
Arabinose	Negative
Sucrose	Positive

3.2. Optimization of growth conditions

3.2.1. Effect of carbon sources on PHB production

PHA production has been previously shown to be affected by carbon sources [12]. In this study, six carbon sources were tested for maximum PHA production. These include glucose, fructose, sucrose, mannitol, xylose, and lactose. P-1 has been inoculated in 100ml Nutrient broth and grown overnight at 30°C for 48 hours at 150rpm. The absorbance of the culture was adjusted to 0.5 at 540 nm. 1ml of isolates was inoculated in 100 ml of Nutrient broth in a 250ml flask with 1% carbon sources and the flask was incubated at 30°C on a rotary shaker [150 rpm] for 24 hours. The yield of PHA by P-1 with glucose, fructose, sucrose, mannitol, xylose, and

lactose was 0.014, 0.0065, 0.032, 0.033, 0.0035, and 0.049 g/L respectively (Table 2). The result indicated that lactose is the preferred source of carbon for PHA production by P-1.

3.2.2. Effect of nitrogen sources on PHB production

Studies have shown that PHB accumulation is increased under limiting conditions of nitrogen and phosphorus /sulfur [13]. In order to investigate the effect of nitrogen on the PHB production, P-1 was grown in Nutrient broth with 1% glucose-containing 0.1 % of peptone, beef extract, sodium nitrite, sodium nitrate, Na_2HPO_4, NaH_2PO_4, ammonium sulfate, and ammonium nitrate, and flasks were incubated at 30°C on a rotary shaker (150 rpm) for 24 hours. The yield of PHA by P-1 with peptone, beef extract, sodium nitrite ($NaNO_2$), sodium nitrate ($NaNO_3$), disodium phosphate (Na_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), ammonium sulphate $\{(NH_4)_2SO_4\}$ and ammonium nitrate (NH_4NO_3) was 0.024, 0.059, 0.011, 0.037, 0.0195, 0.021, 0.0225 and 0.030g/L (Table 3). The result indicated that the beef extract is the preferred source of nitrogen for PHA production by P-1.

Among the organic nitrogen sources studied, the beef extract significantly supported the maximum synthesis of PHA.

Table 2: Effect of carbon sources on PHA production

Sugar at 1% level	Incubation time in hours	Dilution factor	Absorbance at 235 nm.	Conc. from standard graph [ppm]	Conc. in g/L
Glucose	24	100	0.214	14.00	0.014
Fructose	24	100	0.106	6.50	0.0065
Sucrose	24	100	0.531	32.00	0.032
Mannitol	24	100	0.536	33.00	0.033
Xylose	24	100	0.049	3.50	0.0035
Lactose	24	100	0.804	49.00	0.049

Table 3: Effect of nitrogen sources on PHA production

Nitrogen source at 0.1% level	Incubation time in hours	Dilution factor	Absorbance at 235 nm.	Conc. from standard graph [ppm]	Conc. in g/L
Peptone	24	100	0.398	24.00	0.024
Beef extract	24	100	0.983	59.00	0.059
$NaNO_2$	24	100	0.175	11.00	0.011
$NaNO_3$	24	100	0.630	37.00	0.037
Na_2HPO_4	24	100	0.151	19.50	0.0195
NaH_2PO_4	24	100	0.344	21.00	0.021
$\{(NH_4)_2SO_4\}$	24	100	0.369	22.50	0.0225
(NH_4NO_3)	24	100	0.497	30.00	0.030

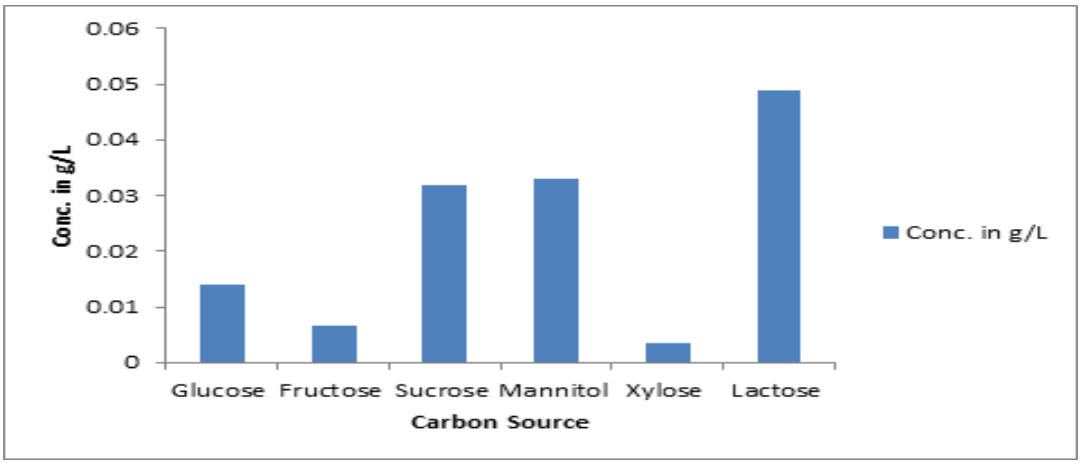

Fig. 2: Effect of carbon sources on PHA production

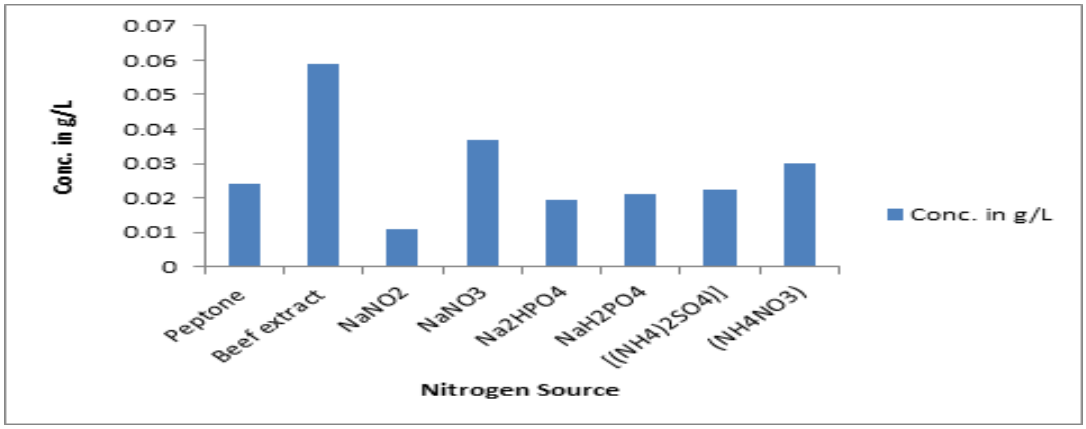

Fig. 3: Effect of nitrogen sources on PHA production

3.2.3. Effect of pH on PHA production on PHB production

The metabolic processes are highly susceptible to even slight changes in pH. Therefore, proper control of pH is critical. Different pH were maintained in the Nutrient broth prepared using the best carbon and nitrogen sources. Their effects on PHB production were evaluated [Table 4] In order to investigate the effect of pH on the PHB production, P-1 was grown in Nutrient broth with 1% sucrose maintaining pH at 5, 6, 7, and 8, and flasks were incubated at 30°C on a rotary shaker [150 rpm] for 24 hours. The yield of PHA at pH 7 and 8 was found to be 0.0785 and 0.053g/L respectively. No growth was found at pH 5 and 6. It indicated that pH 7 is more preferred for PHA production by P-1 [Table 4].

Ranveendra Sindhu *et al* [2011] also studied the effect of initial pH on PHB production by *Bacillus sphaericus* NII 0838 and study showed that pH 7.0 was optimum for maximum PHB accumulation. Studies on the influence of pH of the medium on PHA accumulation showed that the medium with initial pH of 7.0 resulted in the maximum production of PHA [83.56 and 77.94% by *B. subtilis* and *E. coli* respectively]. pH 7.0 is the most favorable pH for the growth of bacterial and hence, would have resulted in higher PHA production. Tavernier *et al*. [1997] reported a decrease in PHA accumulation in the medium with an acidic pH.

Table 4: Effect of nitrogen sources on PHA production

pH	Incubation time in hours	Dilution factor	Absorbance at 235 nm.	Conc. from standard graph [ppm]	Conc. in g/L
5	24	100	0.000	00.00	0
6	24	100	0.000	00.00	0
7	24	100	1.297	78.50	0.0785
8	24	100	0.888	53.00	0.053

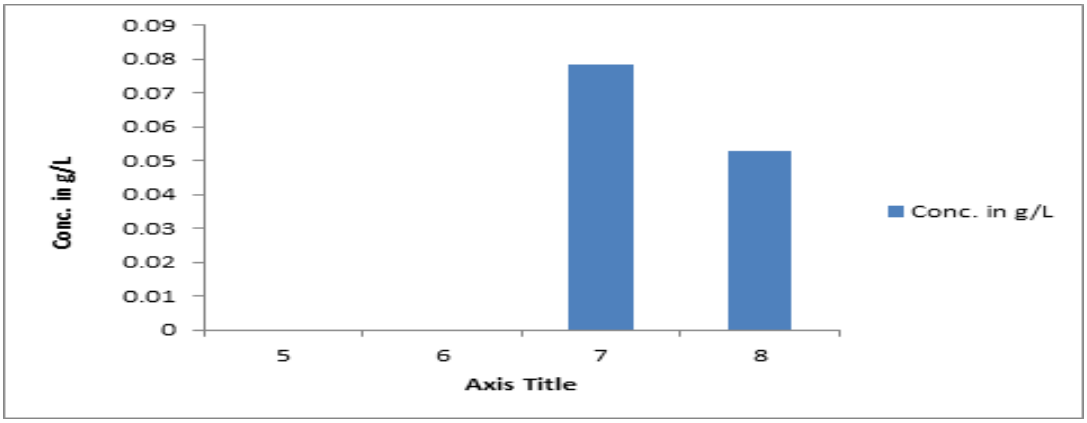

Fig. 4: Effect of pH on PHA production

3.2.4. *Effect of inoculum size on PHA production on PHB production*

The effect of different inoculum sizes on the production of PHA was studied for P-1. P-1 was inoculated in 100 ml Nutrient broth and grown overnight at 30°C for 48 hours at 150 rpm. The absorbance of the culture was adjusted to 0.5 at 540 nm. Then the different inoculum size viz. 1%, 2%, 4%, 6%, 8% and 10% culture was added in 250 mal flasks containing 100 ml Nutrient broth. The flasks were kept for incubation at 30°C on a rotary shaker [150 rpm] for 24 hours.

The yield of PHA at 1%, 2%, 4%, 6%, 8%, and 10% was found to be 0.015, 0.02, 0.025, 0.038, 0.058, and 0.080 which indicated that the production of PHA was highest at 10% and an increase in PHA production was observed with an increase in inoculum sizes..

Sushma Shenoy, *et al.*, 2007 reported that 10% [v/v] of the initial inoculum size of *Klebsiella* species NCCP- 138 was able to accumulate high amounts of PHA [1.55 g/l]. *Bacillus* spp was reported to require 10% inoculum to produce maximum PHA [17].

Table 5: Effect of inoculum size on PHA production

Inoculum size	Incubation time in hours	Dilution factor	Absorbance at 235 nm.	Conc. from standard graph [ppm]	Conc. in g/L
1%	24	100	0.019	15	0.015
2%	24	100	0.026	20	0.02
4%	24	100	0.027	25	0.025
6%	24	100	0.042	30	0.038
8%	24	100	0.088	50	0.058
10%	24	100	0.130	80	0.08

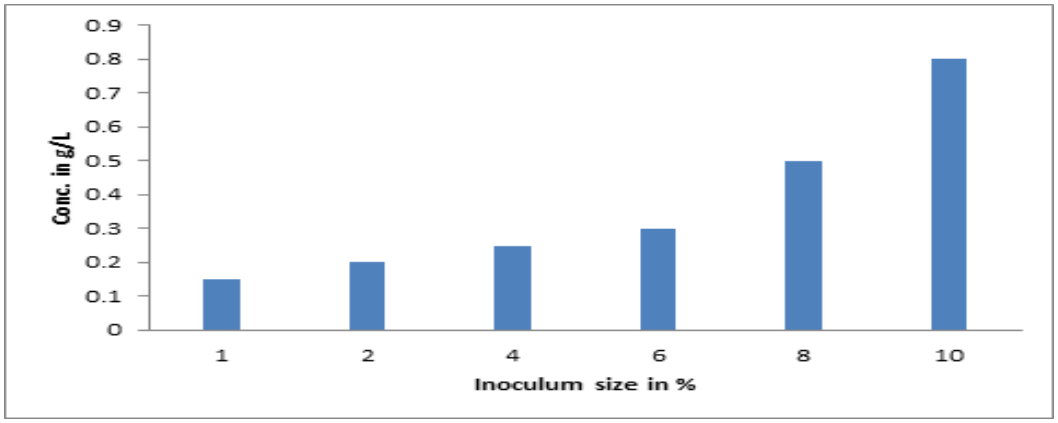

Fig. 5: Effect of inoculum size on PHA production

4. CONCLUSION

The present study was conducted for the isolation of effective polyhydroxybutyrate-producing strains from mangrove soil to yield maximum PHB under optimized conditions. *Alcaligenes* sp. isolated from Mangrove rhizospheric soil is capable of producing PHA bio-polymer. The yield of accumulated PHA was optimized for various physico-chemical parameters and the production of the PHA was quantified by UV-Vis-Spectrophotometer.

5. ACKNOWLEDGEMENTS

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