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Production of Polyhydroxybutyrate (PHB) by *Priestia* Sp. Using different carbon sources

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Abstract
Traditional, non-biodegradable plastics have many uses, which has expanded their use and environmental buildup. In the twenty-first century, this has emerged as one of the main reasons for concern over the environment globally. One biodegradable material that shows promise for replacing traditional non-biodegradable plastics is polyhydroxybutyrate (PHB). PHB is reported to have qualities that are similar to conventional plastics. PHBs are chosen as substitutes for fossil fuels in the manufacturing of biodegradable plastics due to their rapid degradation in ambient environments. In this present study cowpea rhizosphere soil sample was taken for the isolation of bacterial organisms. Pikovskaya agar media used for the process of isolation and screening PHB producing isolate was done by Sudan black staining. *Priestia* sp. shows maximum PHB production under the different carbon sources condition. The accumulation of polyhydroxybutyrate (PHB) granule in cells of *Priestia* sp. was significantly depended on the carbon sources in the production medium. PHB extraction was carried out by chloroform digestion method. The isolated *priestia* sp. can be used for feasible production of PHB by using various carbon sources. Among the various carbon sources, glucose was found to be the better carbon sources investigated.

Keywords: Bioplastic, carbon sources, Polyhydroxybutyrate (PHB), *Priestia megaterium*

Introduction
Plastics are preferred for a wide variety of applications and the amount of plastics manufactured and used worldwide is increasing with more than 380 million metric tons were produced and used annually. Approximately 80 million metric tons of plastic wastes were released annually, causing environmental and direct health problems (Haave *et al*., 2022) [5]. Since synthetic plastic degrades slowly and not fully, it contributes to severe contamination of the soil, water and atmosphere when it burns. According to Narayanan *et al*. (2021) [19], governments are searching for substitutes in order to curtail the utilization of synthetic polymers. Biopolymers, which are derived from biological sources, are the most effective substitute sources for polymers derived from petrochemicals. Microbial biopolymers, namely polyhydroxyalkanoates (PHAs), are noteworthy substances because they are thermoplastic, elastomeric, biodegradable and biocompatible polyesters. PHAs show promise results as petrochemical polymer substitutes (M. Koller, 2018) [10]. Microbially generated polymers are the most advantageous of all the biological sources since they are simple to produce and purify (Jiang Y *et al*., 2008) [8]. A wide variety of microorganisms produce a class of natural polyesters called polyhydroxyalkanoates (PHAs), which are stored as intracellular granules. The production of PHAs is typically initiated by restricting nutrients (such as nitrogen and phosphorus) while providing an excess of carbon (Blunt W *et al*., 2018) [4]. Polyhydroxybutyrates, polyhydroxybutyrate co-hydroxy valerates (PHBV), polyhydroxybutyrate co-hydroxyhexanoate (PHBHx), polyhydroxybutyrate co-hydroxyoctonate (PHBO) and other polymeric esters are members of the PHA family. The most well-known, prevalent and naturally occurring microbial polymer is poly-3-hydroxybutyric acid (PHB) (Verlinden *et al*., 2007) [27]. PHB is a carbon reserve compound that builds up as an insoluble granule inside the cytoplasm when there is an excess of carbon source and a shortage of nitrogen, phosphorus, and magnesium (Bhatia *et al*., 2019) [2]. Due to its characteristics, which closely resemble those of synthetic plastics but are biodegradable and break down into carbon dioxide and water in aerobic conditions and carbon dioxide and methane in anaerobic conditions (Hong *et al*., 2019; Pandian *et al*., 2010) [6, 20]. PHB is seen as a potential replacement for petrochemical-based plastics. A favorable habitat for PHB-producing organisms is the rhizospheric soil layer in terrestrial ecosystems, which is impacted

by plant roots and has a high microbial activity. Native microorganisms in the rhizosphere have adapted to shifts in the levels of nutrients released by plant roots and to the shifting conditions of the soil environment. Numerous bacteria have been found to colonize plants and these bacteria could be able to accumulate polyhydroxybutyrate as a source of carbon and energy. This is because bacteria that are able to incorporate storage compounds have an advantage over other bacteria in the marketplace. The biodegradability of P(3HB) in numerous circumstances is an impressive feature. In order to protect our environment, there is a strong desire to switch from conventional plastics to biodegradable plastics because of their physical characteristics over conventional plastics and their added benefit of being biodegradable. However, the biggest obstacle to PHB's commercial manufacturing and broad use is its high production cost in comparison to synthetic polymers (Sangkharak & Prasertsan, 2008) [23]. This study focuses on the isolation and characterization of PHB-producing bacteria and has aimed to optimize the production of PHB by using different carbon sources such as Glucose, sucrose, fructose, maltose and mannitol. Morphological, biochemical and screening tests were helped to identify the PHB producing ability of the organisms. Analysis of the isolates for PHB production was the goal in order to identify an effective strain of bacteria.

Materials and Methods

Collection of soil sample

A soil sample from the cowpea rhizosphere (5–15 cm depth) was collected in the Cuddalore district of Tamil Nadu. After collection, a portion of the sample was immediately taken to the lab and kept there at 4°C for further analysis (Fig. 1).

Fig 1: Collected rhizosphere soil sample from Cowpea crop

Isolation of PHB-Producing Bacteria

Cowpea rhizosphere soil samples were collected from Cuddalore district and used for bacterial isolation. One gram of soil sample was added to 10 mL of sterilized distilled water. Samples were subjected to shaking for 30 min on a rotary shaker (150 rpm) at 30 °C. Then, serial dilutions were prepared up to 10^{-6} dilutions and the pikovskaya agar plates were inoculated with 10^{-4}, 10^{-5} and 10^{-6} dilutions. The inoculated plates were incubated at 35 °C for 24 h (Fig. 2). The isolated strain was purified and maintained on nutrient agar slant and stored at -4 °C.

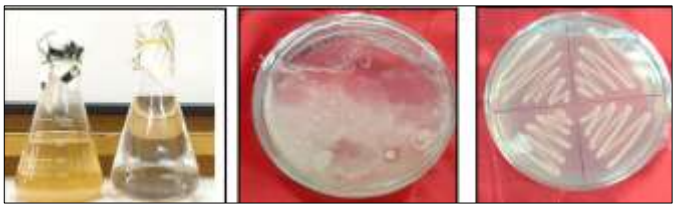

Fig 2: Isolation of PHB producing bacteria from the rhizosphere soil sample of cowpea

Screening of isolates for PHB production under petri plate

The isolated strain was screened for the presence of Polyhydroxybutyrate (PHB) granules using the Sudan Black B staining technique as per the protocol of Rendón-Villalobos *et al*., 2016) [22]. Pikovskaya agar medium was supplemented with 2% glucose. The plate was divided into equal parts and in each part, the bacterial isolates were spread. The petriplates were incubated at 30 °C for 24 h. Sudan Black B stain was prepared by dissolution of 0.02 g powdered stain in 100 mL of 70% ethanol. After spreading Sudan Black B dye over the plates, they were left alone for thirty minutes. Plates were washed with ethanol (96%) to remove the excess stain. The Colonies which are unable to incorporate the Sudan Black B appeared white, while PHB producers appeared bluish-black (Nishida *et al*., 2018) [18]. The promising isolate for PHB production was genetically identified by morphological and biochemical tests.

Fig 3: Screening of isolated strain for PHB production by Sudan Black B staining technique

Screening of isolates for PHB production under light microscope

For microscopic studies, smears of respective colonies were prepared on glass slides, heat fixed and stained with a 0.3% (w/v in 70% ethanol) solution of Sudan Black B for 10 min. After submerging the slides in xylene to decolorize the colonies, they were counterstained for 10 seconds with safranin (5% w/v in sterile distilled water). According to Legat *et al*., (2010) [13], bacterial organisms that appeared black under a microscope were regarded as PHB-producing strains, whereas other microorganisms were identified as negative.

Morphological and Biochemical Analysis

The isolate was morphologically characterized by observing the colony morphology such as shape, size, structure, colour and microscopic characteristics such as cell shape, cell size, motility and spore formation.

The biochemical characterization of the isolate was done by a series of biochemical tests which includes Catalase test, Methyl red test, Starch hydrolysis test, Gelatin hydrolysis test, Urease test, Voges proskauer test and Indole test were also examined in this research work. These morphological and biochemical characteristics helps to determine the genus level

of microorganisms.

Media composition

The isolated strains were grown on mineral salt medium (MSM) which was prepared according to Schlegel *et al*., (1961) [25] and supplemented with 20 g/l of glucose as carbon source (Aramvash *et al*., 2015) [1]. MSM consisted of 9.0 g/l $Na_2HPO_4.2H_2O$, 1.5 g/l KH_2PO_4, 0.4 g/1 NH_4Cl, 0.2 g/l $MgSO_4.7H2O$, 0.02 g/l $CaCl_2.2H_2O$, 1.2 mg/l Fe(III)NH_4 - citrate, 0.1 ml/l Trace elements solution 6 and 15 g/l agar. The trace element solution comprised of (per litre of distilled water): 10 mg/l $ZnSO_4.7H_2O$, 3 mg/l $MnCl_2.4H_2O$, 30 mg/l H_3BO_3, 20 mg/l $CoCl_2.6H_2O$, 1 mg/l $CuCl_2.2H_2O$, 2 mg/l $NiCl_2.6H_2O$ and 3 mg/l $Na_2MoO_4.2H_2O$ and it was sterilized with 0.22 µm sterilized filter system**.**

Effect of various carbon sources on PHB production

To accurately validate how carbon sources influence the PHB production, different carbon sources were employed and optimized by shake flask culture method. The optimization was done using the Mineral salts medium with each carbon source. The MSM broth with different carbon sources *viz*., glucose, sucrose, maltose, mannitol and fructose were prepared in 250 ml conical flasks containing 100 ml of broth and inoculated with 1ml of bacterial inoculums (1×10^7 cfu/ml). Uninoculated flask was kept as control. The flasks were incubated at 30 °C for 2 days. The cell biomass and PHB were quantified for each carbon source at three different incubation periods.

Effect of incubation period and carbon sources on the growth of *Priestia* sp.

To study the growth of the *Priestia* sp. on different carbon sources, 24 hours freshly grown culture was transferred to MSM broth without the addition of carbon sources. Different carbon sources such as glucose, mannitol, fructose and sucrose were added aseptically to the medium and the growth of the *Priestia* sp. was determined at different time periods such as 24, 48 and 72 hours by measuring the optical density at 235 nm.

Effect of different concentrations of carbon source on the growth and yield of PHB

The bacterial cultures were grown in 250 ml conical flask containing 100 ml MSM broth with 0.5, 1.0, 2.0 and 3.0 g/l concentration of the carbon source – glucose. Three replications were maintained for each concentration and inoculated with 1 ml of bacterial inoculum (1×10^7 cfu/ml). The flasks were incubated at 30° C for 2 days. The cell biomass and PHB were quantified in different concentration method.

Measurement of dry biomass

For dry biomass measurement the culture was centrifuged at 10,000 rpm for 15 min, and the pellet was dried in an oven at 55 °C to constant weight (Hungund *et al*.,2013) [7]. This value was used for determination of cell dry weight (CDW).

Extraction and quantification of PHB

Ten ml of culture was centrifuged at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was treated with 10 mL of sodium hypochlorite and the mixture was incubated at 30 °C for 2 h. The mixture was centrifuged at 10000 rpm for 15 min and then washed with distilled water, acetone and methanol respectively. Finally, 5ml of boiling chloroform was taken and the pellet was allowed to get dissolved in it. It was then poured in a sterile glass plate to evaporate chloroform and kept overnight at 4 °C. Then, the precipitate was dried at 100 ℃ for 24 h or until a constant weight was reached. Then, the weight of the extracted PHB was calculated (Kumar, 2017) [12]. This value was used for PHB–free cell dry weight determination. PHB accumulation % was calculated according to Munir *et al*., 2015 [17] and Sathiyanarayanan *et al*., 2017 [24]. The percentage of PHB accumulation was calculated using the formula:

PHB (%) = PHB weight (g/L) × 100/total cell dry weight (g/L)

Table 1: Morphological characteristics of *Priestia* sp.

Colony morphology	Observation
Shape	Round
Size	1.0–2.5 nm
Texture	Slimy
Colour	Creamy white
Microscopic characteristics	
Cell shape	Rod shape
Cell size	1.5–2.4 µm × 0.6–1.2 µm
Motility	Motile
Spore formation	+ve

Table 2: Biochemical characteristics of *Priestia* sp.

Biochemical Tests	Observation
Catalase test	+ve
Methyl red test	-ve
Starch hydrolysis test	+ve
Gelatine hydrolysis test	-ve
Urease hydrolysis test	-ve
Voges Proskauer test	+ve
Indole test	-ve

Table 3: Effect of different carbon sources on growth and PHB production of *priestia* sp.

S. No	Carbon Sources	At 24 hours			At 48 hours			At 72 hours		
		Cell biomass (g/l)	PHB (g/l)	%	Cell biomass (g/l)	PHB (g/l)	%	Cell biomass (g/l)	PHB (g/l)	%
1.	Glucose	2.55	1.52	59.60	3.75	1.98	52.80	3.85	1.99	51.68
2.	Sucrose	2.48	1.48	59.67	3.32	1.75	52.71	3.47	1.82	52.44
3.	Maltose	1.98	1.20	60.60	2.95	1.37	46.44	3.21	1.45	45.17
4.	Fructose	1.75	1.18	67.42	2.58	1.33	51.55	2.85	1.37	48.07
5.	Mannitol	1.48	1.10	74.32	2.35	1.30	55.31	2.37	1.32	55.69

Table 4: Effect of incubation period and carbon sources on the growth of *Priestia sp.*

Carbon Sources	OD at 235nm		
	At 24 hrs	At 48 hrs	At 72 hrs
Dextrose	0.727	1.321	1.396
Mannitol	0.421	1.062	1.213
Fructose	0.436	0.996	1.217
Sucrose	0.756	1.383	1.314

Table 5: Effect of different concentrations of Glucose (Carbon source) on PHB production of *Priestia* sp.

S. No	Glucose (g/l)	At 24 hours			At 48 hours			At 72 hours		
		Cell biomass (g/l)	PHB (g/l)	%	Cell biomass(g/l)	PHB (g/l)	%	Cell biomass (g/l)	PHB (g/l)	%
1.	0.5	2.38	1.35	56.72	2.72	1.51	55.51	2.98	1.61	54.02
2.	1.0	1.99	1.26	63.31	2.07	1.62	78.26	2.51	1.47	58.56
3.	2.0	2.97	1.62	54.54	3.38	1.81	53.55	3.43	1.89	55.10
4.	3.0	2.48	1.45	58.46	3.71	1.95	52.56	3.76	1.97	52.39

Results and Discussions

Collection of sample and isolation of PHB producing bacteria: The soil sample from the cowpea rhizosphere was collected and the PHB producing bacteria was isolated. The soil sample was taken from a site in rhizosphere region in Annamalai Nagar, Cuddalore district, Tamil Nadu, India (Fig. 1), as it is a reliable source for the isolation of powerful PHB-producing bacteria. The bacterial colonies were isolated and streaked over nutrient agar plates for additional morphological, screening, biochemical tests and optimization of carbon sources for PHB production.

Screening of PHB producing bacterial strains: Selecting the most effective bacteria for PHB synthesis, requires screening method. In screening by Sudan Black B staining on isolate grown petri dish results revealed that after the pouring of Sudan Black B stain into the petri dish, the color of colonies changed as dark bluish-black color. According to Mostafa *et al*. (2020) [16], this indication is positive for PHB accumulations in test isolates. Similar to this, Sudan Black B staining was used as the screening method by Kalaivani and Sukumaran (2013) [9] to isolate the novel strains with the potential to produce biopolymers. Then, the microscopic observations were performed to confirm the PHB producing competence of test isolate. The isolated bacteria was Gram-positive. The presence of PHB granules in the cytoplasm of isolated bacterium cells was confirmed using Sudan B black staining. PHB granules were seen in the bacterial cells after being observed under a microscope (1000 times magnification) using Sudan B black stained bacterial slides. Early research by Mostafa *et al*. (2020) [16], using Sudan B black staining to examine bacterial colonies revealed the presence of black PHB granules inside the bacterial cells which was similar to our work.

Morphological characteristics of *Priestia* sp. isolate

Priestia sp. bacteria was isolated using the dilution plate method from a soil sample on Pikovskaya agar media. Clear zones were created around the microbial colonies in media by phosphate solubilizers. The media contains insoluble mineral phosphates such hydroxyapatite and tricalcium phosphate. This result of the present study was similar to the result obtained by Patel *et al*., 2016 [21]. The isolated strain was analysed using morphological characteristics regarding to "Bergey's Manual of Systematic Bacteriology" (Krieg *et al*., 1984) [11]. The isolated bacteria have the morphological characteristics such as rod shape colony with 1.0-2.5 nm size and the isolated *Priestia* sp. colony has the slimy texture and creamy white in colour. Microscopic observation of the isolated strain helps to study about the various characteristics such as shape of the cell, size of the cell, motility and spore formation nature. Our research work revealed the microscopic observation of isolated microorganism as a rod shape cell with 1.5–2.4 µm × 0.6–1.2 µm size, motile and also a spore forming bacteria (Table 1).

Biochemical characteristics of *Priestia* sp. isolate

For biochemical examination, the bacterium showed positive results for the catalase test, starch hydrolysis test and Voges Proskauer test. The isolated bacterium showed negative results for the tests such as Urease Test, Methyl Red Test, Indole Production Test and Gelatin hydrolysis test. The morphological and biochemical characters were summarized in Table 1 and Table 2. From these results, the isolated bacterium was identified as *Priestia* sp. according to the Bergey's manual of determinative bacteriology and the obtained results were compared with the earlier findings of Patel *et al*., 2016 [21].

Effect of different carbon sources on the growth and PHB production of *Priestia* sp.

The effect of different carbon sources *viz*., glucose, sucrose, maltose, fructose and mannitol on the growth and yield of PHB was given in Table 3. Among the various carbon sources used, Glucose was found to be better against the other carbon sources investigated. Sucrose was found to be next preferred carbon source. Use of glucose as carbon source gave the maximum cell biomass and PHB in 24 hrs by recording 2.55 g/l and 1.52 g/l respectively followed by 2.48 g/l and 1.48g/l in sucrose. The minimum cell biomass and PHB was observed with mannitol (1.48 g/l and 1.10 g/l). After 48 hrs of growth, the cell biomass and PHB yield were 3.75 g/l and 1.98 g/l in glucose, 3.32 g/l and 1.75 in sucrose, 2.95 g/l and 1.37 g/l in maltose, 2.58 g/l and 1.33 g/l in fructose and 2.35 g/l and 1.30 g/l in mannitol respectively. After 72 hrs of growth the cell biomass and PHB were found higher again in glucose (3.85 g/l and 1.99 g/l) followed by sucrose (3.47 g/l and 1.82 g/l), maltose (3.21 g/l and 1.45 g/l), fructose (2.85 g/l and 1.37 g/l) and mannitol (2.37 g/l and 1.32 g/l) respectively. This result of the present study was similar to the result obtained by Mohanrasu *et al*., 2020 and Yüksekdağ *et al*., 2004) [15, 28].

Effect of incubation period and carbon sources on the growth of *Priestia* sp.

The results of growth phase study conducted by turbidimetric (OD) on different carbon sources *viz*., glucose, mannitol, fructose and sucrose were investigated and the results were reported in Table 4. Glucose showed the higher growth during the stationary phase followed by sucrose, mannitol and fructose.

Effect of different concentrations of glucose on the growth and PHB production of *Priestia* sp.

The effect of different concentrations of glucose *viz*., 0.5, 1.0, 2 and 3 g/l on growth and yield of PHB was given in Table 5. Among the various concentrations of glucose tested, 2 g/l was found to be optimum for the growth. The cell biomass and PHB yield of *Priestia* sp. at different concentrations as 0.5, 1.0, 2.0, and 3.0 g/l after 24 hours of growth were 2.38 g/l and 1.35 g/l, 1.99 g/l and 1.26 g/l, 2.97 g/l and 1.62 g/l, 2.48 and 1.45 g/l respectively. The corresponding percent PHB to cell biomass were 56.72%, 63.31%, 54.54% and 58.46% respectively. After 48 hours growth, the cell biomass and PHB were 2.72 g/l and 1.51 g/l, 2.07 g/l and 1.62 g/l, 3.38 g/l and 1.81 g/l, 3.71 g/l and 1.95 g/l respectively. The corresponding percent PHB to cell biomass were 55.51%, 78.26%, 53.55% and 52.56% respectively. After 72 hours growth, the cell biomass and PHB were 2.98 g/l and 1.61 g/l, 2.51 g/l and 1.47 g/l, 3.43 g/l and 1.89 g/l, 3,76 g/l and 1,97 g/l respectively. The corresponding percent PHB to cell biomass were 54.02%, 58.56%, 55.10% and 52.39% respectively. This result of the present study was moreover similar to the result obtained by Mohanrasu *et al*., 2020 [15]. According to the results, there may be a strong correlation between PHB production and higher and lower carbon loading ratios. Lower concentrations may be utilized for essential metabolic processes, while higher concentrations may be related to osmotic pressure acting on cells and influencing bacterial cell proliferation, which in turn reduces PHB production and modifies PHB's molecular weight and properties (Luo *et al*., 2002) [14].

Conclusion

The rhizosphere ecosystem is a potential resource for PHB accumulating microbes due to its high diversity and extreme environmental conditions (High or low temperature, unbalanced nutrition, pH and other environmental stresses) are favourable for PHB accumulating microbes. In this report PHB-producing bacterium *Priestia* sp. was isolated from cowpea rhizosphere soil sample collected from Annamalai Nagar, Cuddalore district. The screening process is done for the identification of PHB producing isolates using differential and viable staining techniques. In screening method, the isolated bacteria was positive for both gram staining and Sudan B black staining techniques. PHB granules were stained with Sudan black B, which causes them to appear dark blue or black. Sudan black is a lipophilic solution that has affinity for membranes and the biopolymer itself. As evidence that the isolated strain is positive for the production of PHB, black streaks and granules were also seen on petri dishes with naked eye and on glass slides under a microscope, respectively. The bacterial isolate was screened, identified, characterized and finally from the morphological and biochemical results, we conclude that the isolated organism belongs to the genus of *Priestia* sp. In contrast to other carbon sources, it was discovered in the current study that the bacteria efficiently metabolized glucose to produce larger levels of PHB.

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