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# Polyhydroxybutyrate (PHB) production by Halomonas boliviensis isolated from waste water

**Anuradha Singh**
Department of Biotechnology, Faculty of Engineering and Technology
Rama University, Kanpur, Uttar Pradesh
Corresponding author email: er.anuradha.6725@gmail.com

**Vivek Srivastava**
Department of Biotechnology, Faculty of Engineering and Technology
Rama University, Kanpur, Uttar Pradesh
Email: viveksrivastavabio@gmail.com

***Abstract*---**Plastic materials are often utilized as plastic packaging and have various uses since their longevity and consistency. Plastic products are generally not biodegradable or persist in our surroundings, posing major harm to the ecology. Biopolymers, or bioplastics, are developing as a blessing in the fight against waste buildup. Polyhydroxybutyrate (PHB) is a biopolymer that can be used instead of manufactured polymers. PHB is a fatty storage substance that accumulates inside microbes' cell walls when stressed. Halophilic microbes can be highly useful in manufacturing PHB since they are cheap, and PHB extraction is considerably easy in halo-tolerant species. As a result, our research emphasizes the identification of PHB-generating halotolerant species of microbes using untreated wastewater. Nile blue A and Sudan Black B staining were used to identify PHB favourable strains. The effective microbe generated PHB on a large scale utilizing sewage as the medium.

***Keywords*---**Halotolerant Spp., polyhydroxybutyrate (PHB), bioplastics.

## Introduction

Biopolyesters, a compostable polymer made by various microbes using natural materials, have also been proposed as viable alternatives to hydrocarbon-derived polymers (Lee 1996b; Steinbüchel and Füchtenbush 1998). Numerous microbes retain such biopolymers as polyhydroxyalkanoates (PHAs) inside cell

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membranes as carbon and energy storage. Polyhydoxybutyrate (PHB), the most widely researched PHAs, is reported to have physical qualities comparable to polyethylene and has possible usage as a reusable substrate surface in packaging films, vessels, or paper coatings, among other things (Lee 1996a; Reddy et al. 2003).

Very few microbial strains are considered viable alternatives for massive PHB synthesis. Such microbes are *Wautersia eutropha* and *pseudomonas*, which generate PHB in the vicinity of an overabundance of carbon source and nutritional component exhaustion (Lee 1996a); several good organisms include *Escherichia coli, Azotobacter vinelandii,* and *Alcaligenes latus*, that do not demand carbohydrate restriction for PHB. The polymer can be produced by the first two strains throughout its aggressive development stage (Lee 1996a; Lee et al. 1994).

Because the carbon component, fermentation method, and downstream sorting of the composite material evaluate the efficacy and economic history of the PHB production system, developing cultivation situations for microbes that support higher PHA concentration and efficiency from cheap and eco-friendly carbon materials is critical. As a result, *A. vinelandii* and *A. latus* may use glucose as a carbon resource to make PHB, meaning that less costly substrates like raw sugar can also be used (Choi and Lee 1999; Lee 1996a;). These cultures can store 75–85 wt. Percent PHB of total CDW as well as achieve some of the greatest cell concentrations ever observed. The exceedingly halophilic archaeon Haloferax mediterranei, on the on either side, can store huge quantities of PHA (65 wt. percent) from carbohydrates; however, it also generates extracellular glycoproteins that also may interfere with polymer purifying and needs high salt concentrations (30 percent w/v) for optimized PHA manufacturing. Researchers previously documented the generation of PHB by the halophilic microbes *Halomonas boliviensis* using a variety of carbohydrates, such as volatile fatty acids, mono-/disaccharides, and starches hydrolysate, including a combination of malto-oligosaccharides (Quillaguamán et al. 2006). This work describes the adjustment of cultivation parameters to increase H. boliviensis organic matter percentage throughout PHB synthesis (Wang and Lee 1997; Page and Cornish 1993).

## PHB production by Halomonas boliviensis

Halomonas spp may survive in a broad variety of NaCl solutions; the ideal range is 4.5 percent (w/v) NaCl (Rodriguez-Valera and Lillo 1992; Lillo and Rodriguez-Valera 1990). The mild amount of sodium necessary for optimum development of such microbes is sufficient to prevent the development of non-halophiles, allowing cultivations to be performed in non-sterile environments (Quillaguamán et al. 2005). Moreover, it is clear that most organisms may develop on a variety of carbon substrates from agriculture leftovers or commercial food wastes. These substances might be used as raw resources to manufacture PHA. The fluctuation in NaCl content in the media has been observed to provide no discernible influence on the PHB. Nevertheless, at larger doses (12-17 percent w/v), PHB production was slowed, and cell proliferation were severely impaired (Quillaguamán et al. 2009). H. boliviensis cannot develop when glucose is the only carbon compound. Nevertheless, in a minimal media with degraded carbohydrates as the carbon source and restricted nutrient uptake, this culture was developed and might generate PHB (to around 56 wt percent). This hydrolysate's usage revealed that the microorganism preferred maltose for PHB formation. If cheaper compounds are limited, greater carbohydrates could be used; although this results in a much decreased PHB concentration (Quillaguamán et al. 2005). Different inexpensive substrates materials, including wheat bran hydrolysate and processed vegetable garbage, could be used by H. boliviensis to produce PHB. After 18 hours of culturing in a batch fermentor containing sodium acetate, butyric acid, and wheat bran hydrolysate, a maximum PHB composition of 45% and a PHB value of 5 g/L were attained. The substitution of processed potato peel for sodium acetate and butyric acid led to a drop in PHB composition and intensity to 41 wt percent and 2.6 g/L, correspondingly. The utilization of discarded crop wastes has also significantly cut substrate costs. As a result, it is feasible to reduce manufacturing expenses.

## Materials and Methods

### Samples Collection

Water specimens have been gathered in several locations in North India. The specimens have been taken in sterile Schott Duran bottles, transported to the research lab, and then kept at 4°C.

### Isolation of PHB-producing Microbes

5 ml of the obtained water sample has been seeded in Nitrogen-deficient medium lacking agar. After serial dilution in sterile distilled water, the specimens were inoculation onto the Nitrogen Deficient Agar medium using the pour plate technique (Patnayak and Sree, 2005). The plates were incubated for 24 hours at room temperature. Single pure bacterial cells have been selected from the bacterial consortia and then spread on Nutrient Deficient Medium for two days of incubation.

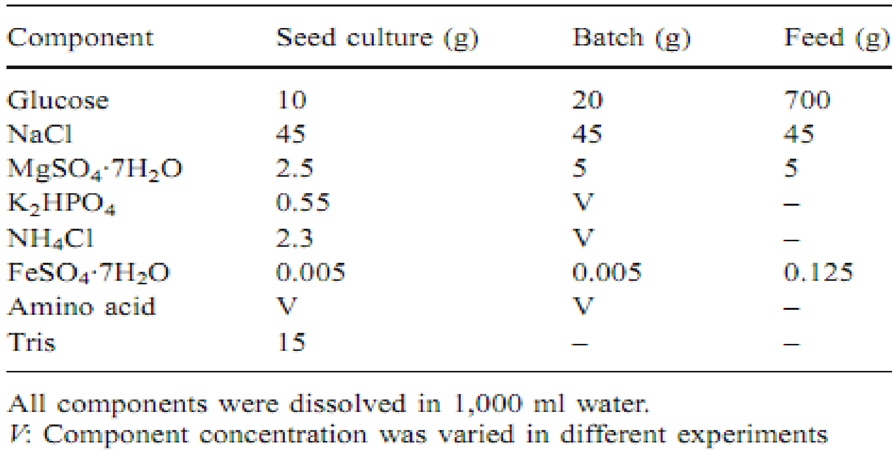
### Bacterial Strains Characterization

Gram's staining was used to identify the bacterial species, and several biochemistry assays like Starch Hydrolysis, IMViC, Catalase test, Triple Sugar Iron Agar, Carbohydrate Fermentation, and Oxidase were used to describe them better.

### Media composition for PHB production

*H. boliviensis* colonies have been cultivated in HM-2 media that included (percentage, w/v): $MgSO_4 \cdot 7H_2O$, 0.037; $KH_2PO_4$, 0.02; $CaCl_2 \cdot 2H_2O$, 0.012; NaCl, 4.5; KCl, 0.074; peptone, 0.025; NaBr, 0.02; yeast extract, 0.1. For numerous tests, the substrate has been supplied with numerous carbon compounds.

Table 1: Medium composition for PHB production



| Component | Seed culture (g) | Batch (g) | Feed (g) |
|---|---|---|---|
| Glucose | 10 | 20 | 700 |
| NaCl | 45 | 45 | 45 |
| $MgSO_4 \cdot 7H_2O$ | 2.5 | 5 | 5 |
| $K_2HPO_4$ | 0.55 | V | – |
| $NH_4Cl$ | 2.3 | V | – |
| $FeSO_4 \cdot 7H_2O$ | 0.005 | 0.005 | 0.125 |
| Amino acid | V | V | – |
| Tris | 15 | – | – |

All components were dissolved in 1,000 ml water.
*V*: Component concentration was varied in different experiments

### Quantification of PHB by Spectrophotometry

The crotonic acid assay has been used to quantify the isolated PHB (Lingayya et al., 2015). Crotonic acid granules were mixed in 4 mL of $H_2SO_4$ to make a stock mixture of 0.2g Crotonic acid/l $H_2SO_4$. $H_2SO_4$ functional limits of 5, 10, 15, 20, 25, 30, 40, and 50g/4ml have been produced. 4 mL of $H_2SO_4$ was added to make the negative. At 235 nm, the absorbance has been recorded. A reference concentration vs absorbance chart has been created.

### PHB production in rotating flasks

*H. boliviensis* has been cultivated at 25°C in 250 ml flasks with rotational stirring at 250 rpm in 50 ml of HM-2 medium containing 0.7 percent (v/v) butyric acid and 0.7 percent (w/v) butyric acid. After approximately 12 hours of development, 14 ml of the fermentation broth was seeded in 2 liter flasks, with 250 ml of the same media added with various NaCl concentrations (percent, w/v). Before cultivation, all pH levels were corrected to 7.5. During culture, samples were taken at standard intervals and PHB accumulation and dry cell weight (CDW) were evaluated.

### PHB production using fermentor

*H. boliviensis* was cultivated for 12 hours at 25 degrees Celsius in 1 liter flasks containing 250 ml of HM-2 media (pH 7.2) and a specified carbon substrate, stirring at 250 rpm. The cultured has been employed to seed a 3-liter fermentor container (Voyager, Luton, UK) holding 2.1 liters of the production media. When the media was inoculated, antifoam must have been introduced, and the pH was kept at 7.0 by applying 0.4 M HCl/NaOH. Throughout the fermentation processes, the air intake rate and agitation speed were originally set at 0.4 litres per min and 750 rpm, correspondingly. When the original DO content (i.e., 80%) was measured, the air intake was raised to prevent oxygen restriction in the cells.

For assessment, specimens (about 15 ml) have been taken at various subsequent intervals.

### Quantitative analysis

Cell dry weight (CDW) was calculated by centrifuging 4 ml of cultured specimens at 2500 g for 20 minutes, then washing the pellet twice with distilled water and drying it at 80 degrees Celsius until a consistent mass had been achieved. The Law and Slepecky (1961) technique was used to quantify PHB. Dried cell pellets carrying intracellular PHB were hydrolyzed for 1.5 hour in strong $H_2SO_4$ to yield crotonic acid, which was measured by measuring absorbance at 235 nm. Samples from shake flasks were analyzed in duplicate, while samples from the fermentor were analyzed in triplicate.

## Result and Discussion

### Screening and identification of halotolerant microbial isolates for PHB development

Microbial populations were enriched in Nitrogen Deficient Medium in halotolerant environments with 4 percent NaCl. A combination of 17 isolates has been collected, with 9 acquired from wastewater samples and 6 collected from river banks. By Sudan Black B staining, two of the 17 bacterial isolates (AST1 and AST2) tested positive for PHB formation. PHB aggregates are recognized to be accumulated in intercellular spaces by a wide range of microbes as an energy storage substance. It has been observed that microbial species from over 89 genera collect roughly 151 distinct hydroxy alkanoates as polyester granules (Steinbuchel and Schubert, 1988).

### Sudan Black B Staining Preliminary Detection of PHB Developing Microbial Strains

The bacterial strains' PHB generation has been determined using initial testing. These isolates were again utilised to analyse PHB polymerization. Both microbial strains AST1 and AST2 deposited PHB in the shape of cytoplasmic aggregates that could soak the Sudan Black stain, and the cell walls of both strains were coloured pink as a result of the counter stain utilized. The grains became dyed black upon a pink backdrop snapped up by the Safranin stain when magnified 100 times. These darkly colored granules influenced the bacterial strains' synthesis of PHB. Sudan Black B stain in 0.03 percent alcoholic mixture has been utilised to identify PHB-generating microorganisms quickly (Spiekermann et. al., 1999).

### Quantification of PHB by UV – Spectrophotometric Analysis

Crotonic acid has been employed as a reference to measure PHB synthesis at levels varying between 6 to 49 g/ml. The optical intensity of the medium increases from 0.432 at 50g/ml level to 0.871 at 500g/ml level of Crotonic Acid in Table 2.

Table 2 Absorbance of Crotonic Acid at 235 nm

| S.NO | Crotonic acid µl | Sulphuric acid ml | Concentration g/ml | O.D value at 235 nm |
|---|---|---|---|---|
| 1 | - | 3 | - | 0 |
| 2 | 50 | 2.850 | 50 | .432 |
| 3 | 100 | 2.800 | 100 | .587 |
| 4 | 150 | 2.750 | 150 | .690 |
| 5 | 200 | 2.700 | 200 | .778 |
| 6 | 250 | 2.650 | 250 | .791 |
| 7 | 300 | 2.600 | 300 | .834 |
| 8 | 400 | 2.500 | 400 | .859 |
| 9 | 500 | 2.400 | 500 | .871 |

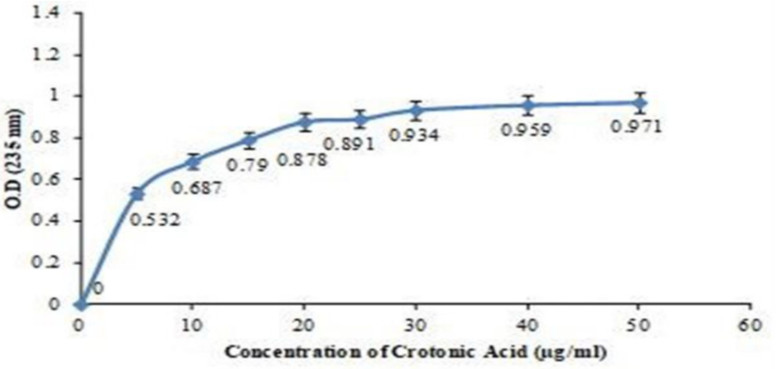


Figure 1: Concentration of Crotonic Acid

### PHB production by *H. boliviensis* in a defined medium

The experimental investigation has been conducted to evaluate the necessary nitrogen supply needed for the overall development of *H. boliviensis*. The microorganism has been cultured in such a manner by adding specific amino acids to a basic media previously developed for *H. elongata*. Only three amino acids, glycine, glutamine, and aspartic acid, were discovered to stimulate the development of *H. boliviensis*. CDW of 4.9 g per liter was achieved at 0.3 percent (w/v) glutamine level, which was substantially greater than that (3.2 g per liter) achieved at 0.4 percent (w/v) yeast extract composition (Fig. 2a).

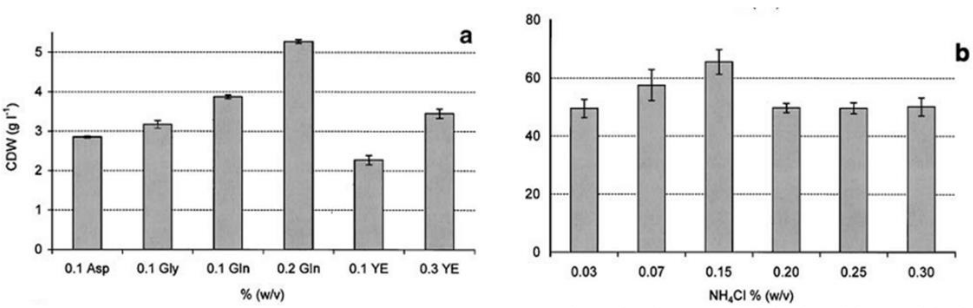


Figure 2: Cell Growth and PHB accumulation; a. CDW of H. Boliviensis; b. PHB production

Following that, utilizing a solution including 0.2 percent (w/v) glutamine, the impact of altering the basal NH4Cl level on initiating PHB formation was examined. Utilizing 0.14 percent (w/v) $NH_4Cl$, a maximal PHB level of 61 wt. percent has been achieved, whereas increasing the $NH_4Cl$ level to 0.2 percent (w/v) culminated in a decrease in PHB concentration to 49 wt. percent (Fig. 2b). Throughout 28 hours of development, the CDW in such cultivation practices has been in the ranges of 2.5–3.9 g per liter.

## Discussion

The findings indicate that *H. boliviensis*, a severe halophile, may create substantial levels of PHB in the vicinity of excessive sources of carbon as well as a minimal quantity of yeast extracts in the substrate. In contrast to very halophilic archaea, low salinity levels give the ideal habitat for *H. boliviensis* to produce PHB. This is attributed mostly to decreased cell proliferation at elevated NaCl levels. Furthermore, unlike halophilic archaea (Hezayen et al., 2000), the ultimate PHB level of *H. boliviensis* appears unaffected by NaCl level.

Moderate halophiles vary physically from very halophilic archaea such as organisms producing and storing suitable salts, a procedure considered metabolically costly for the cells (Oren, 2002). It is anticipated that the overall quantity of ATP necessary for halophilic heterotrophs to generate suitable solvent is equivalent to that essential for producing cellular constituents (Oren, 1999). As a result, a drop in dry cell weight (CDW) of intermediate halophiles must be predicted as saline contents increase (Oren, 1999). Furthermore, the decreased diameter of H. boliviensis colonies at high salinity might imply a constraint in cellular structure growth. A comparable individual will develop larger coccoid forms with decreased diameters and cell thickness when NaCl content increases have been earlier seen in *Holomonas variabilis* and *Halomonas elongata* (Vreeland et al., 1984; Fendrich et al., 1988). Considering the cultivation conditions employed to induce PHB deposition by *H. boliviensis*, it appears that the physiological activity created by the organisms is allocated to expansion or adjusting their metabolic activity to salt potential preceding the beginning of PHB production.

The amount as well as the volume of PHB aggregates discovered in cell types, on the other side, is reliant on the microbial organisms; *Wautersia eutropha* microbes hold somewhere around 8 and 12 PHB particles with differing average diameters of 0.24–0.50 mm (Anderson et al., 1990), whilst *Azotobacter vinelandii* could acquire >40 aggregates (Page et al., 1995). When fed with an extra carbon substrate for the formation of exopolysaccharides, *Halomonas spp.* exhibits a wide range in the quantity and size of PHB aggregates (Martinez-Canovas et al., 2004; Niven, 1978; Quesada et al., 2004). In comparison, *H. boliviensis* typically synthesizes 1 or 2 granules (0.20–0.64 mm) per cell, while production of up to five aggregates in extended cells has been seen, regardless of the C-source used. The production of big and homogenous PHB aggregates, as demonstrated in *H. boliviensis*, is thought to be beneficial to polymeric refinement and durability (Steinbiiuchel et. al., 1995).

The cell envelope surrounding the PHB aggregates is thought to be made up of enzymes, non-enzymatic proteins and/or phospholipids, with the organization varying based on the microbial strain. The organized shape of the cell envelope carbon sequestration of the PHB aggregates is similar to that seen in *Rhodococcus spp.*, pseudomonas, and *A. vinelandii*, but it varies from that observed in recombinant *E. coli* and *W. eutropha* strains.

Using butyric acid and sodium acetate as carbon feed, the quantity of stored PHB was greatly increased by cultivating *H. boliviensis* in a fermentation process within monitored circumstances. The continuous remaining biomass is seen during growing areas strongly suggests that PHB is formed throughout the idophase phase of development, as seen in comparable microbes (Lee, 1998). The microorganism may also collect huge quantities of PHB from various energy carbon sources - carbohydrates, which were claimed to be relatively cheap sources (Choi and Lee, 1999). The maximal PHB concentration achieved *by H. boliviensis* with various carbon sources is equivalent to the most documented by non-halophilic microorganisms, such as, *Azotobacter vinelandii, Wautersia eutropha, Alcaligenes latus,* and *pseudomonas* strains (Rodriguez-Valera and Lillo, 1992; Steinbuchel, 1998; Lee et al., 2000). More research is being conducted into diverse culture procedures to increase cell growth and polymer output in H. boliviensis cultured on carbohydrates.

## Conclusion

PHB is the most well-known and well-studied plastic material in polyhydroxyalkanoates. It might be employed in uses comparable to ones of ordinary plastics and in innovative waste-management techniques. Relative to eubacteria, recovering PHB through the cell membrane of severe halophiles is significantly simpler. In this work, halotolerant microbial isolates were cultured through wastewater, and PHB synthesis in both bacterial strains was examined. The effective bacterial strain was utilized to produce PHB on a wide scale utilizing wastewater. This study's prospective aims include employing revolutionary ways of PHB manufacturing that are both cost-effective and ecologically beneficial.

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