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Research Article

# The Effect on Poly-β-hydroxybutyrate Production the Presence of Different Carbohydrate Sources in *Bacillus ceresus* and *Cupriavidus necator*

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ABSTRACT

Polyhydroxybutyrates (PHB) are granular polyesters synthesized by many bacteria as a carbon and energy source in environments where substances such as nitrogen, oxygen, carbon, and phosphorus are limited. Polyhydroxybutyrates is biodegradable, consisting of hydrophobic long chains, and is non-toxic. It is classified as one of the basic polymers of polyhydroxyalkanoates. In this study, the Polyhydroxybutyrates production of *Bacillus cereus* (ATCC 10876) and *Cupriavidus necator* (formerly *Ralstonia eutropha* ATCC17699) in the presence of different minimal carbon sources was investigated under static and shaking (150 rpm) states. According to the results of the research, the highest PHB production was observed in *Bacillus cereus* PBS + 1% xylose medium (7.395 μg/ml) in static conditions; *Cupriavidus necator* exhibited the highest production of polyhydroxybutyrates under shaking conditions in PBS + 1% fructose medium (9.626 μg/ml). The lowest polyhydroxybutyrates production was observed in *Cupriavidus necator* in PBS + 1% maltose medium (0.027 μg/ml) under static conditions; however, under shaking conditions, it was carried out in PBS + 1% dextrose medium (0.122 μg/ml). Considering these results, it is evident that there is an increase in the production of polyhydroxybutyrates by microorganisms as the shaking speed.

## 1. Introduction

Polyhydroxybutyrates are a group of biodegradable storage polyesters produced by diverse prokaryotic organisms, especially during nitrogen or phosphorus restriction and in the presence of a greater quantity of carbon. These polymers have features similar to those of synthetic plastics and are considered good substitutes for petrochemical polymers such as polyethylene, polypropylene, nylon, and polyvinyl chloride [1].

Microorganisms manufacture these plastics. Many prokaryotic and eukaryotic microorganisms under suitable growth conditions synthesize Polyhydroxybutyrates. Polyhydroxybutyrates are the most important members of polyhydroxyalkanoates [2].

Polyhydroxybutyrates has been attracting significant interest as a raw material to make various medical devices such as bioresorbable surgical sutures, screws, plates for cartilage and bone fixation, and surgical meshes for hernioplasty surgery. The use of PHB in sustained drug delivery systems is also continuously increasing in pharmaceutical applications [3]. *C. necator* is the most widely used industrial strain for bioplastic manufacture because of its high biomass yield with 90% accumulation (as dry weight) [4].

Polyhydroxybutyrates is a storage material accumulated by many microorganisms as carbon and energy reserves under conditions where the carbon source is high but nutritive and essential elements such as N, P, S, O, or Mg are limited. This polymer is a product mainly resulting from

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the assimilation of carbon sources such as glucose and starch in the absence of other energy sources [5].

Some organisms that synthesize PHB include *Nostoc muscorum*, *Oscillatoria okeni tistr* 8549, *Synechocystis* spp., *Aeromonas hydrophila*, *Alcaligenes* spp., *Clostridium* spp., *Corynebacterium* spp., *Pseudomonas* spp., *Staphylococcus* spp., and *Streptomyces* spp. [6, 7].

*Bacillus cereus* is an aerobic-facultative anaerobic, endospore-forming Gram-positive soil bacterium in the *Bacillus* family. It is 0.5-2.5 µm wide, 1.2-10 µm long, rod-shaped, seen under the microscope in double or short filamentous forms [8]. Additionally, *B. cereus* strains can produce one or more enterotoxins in the intestine or emetic toxins in food [9].

*Cupriavidus necator* (*R. eutropha*) is a facultative chemolithotroph, non-pathogenic Gram-negative bacterium that can live in soil and water. It is rod-shaped, 0.5-1.0 µm wide, and 1.8-2.6 µm long, belonging to the *Alcaligenes* family. It can reduce nitrates to nitrogen gas [10]. *Cupriavidus necator* are capable of accumulating PHB under unstable growth conditions where protein synthesis reactions are limited, and carbon and energy are abundant [11].

This research aimed to investigate the production of PHB in static and shaking (150 rpm) environments in the presence of different minimal carbon sources for Gram-negative and Gram-positive microorganisms.

## 2. General Methods

### 2.1. Chemicals

Acetone, NaClO, NaCl, $H_2SO_4$, KH2PO4, $Na_2HPO_4$, Fructose (Merck), Chloroform, Glucose, Maltose (Sigma-Aldrich), Ethanol, KCl (Carlo Erba), Nutrient Agar, Nutrient Broth (Lab M), Sucrose (Alfa-Aeser), Xylose (Himedia). All chemical analytical grade use.

### 2.2. Bacteria and nutrient media

*Bacillus cereus* Gram-positive (ATCC 10876) and *C. necator* (*R. eutropha*) Gram-negative (ATCC 17699)) strains used in our study were incubated on nutrient agar medium for 24 hours at 37 °C [12].

### 2.3. Method

Glucose, fructose, xylose, maltose, rhamnose, ribose and sucrose 1% were used, respectively. The bacteria were incubated at 37 °C for 24 hours. A volume of 100 µl of bacterial cultures was inoculated into media containing Nutrient Broth, PBS (Phosphate Buffered Saline), and minimal carbon sources 1% (glucose, fructose, xylose, maltose, rhamnose, ribose, sucrose) in 25ml /125 ml Erlenmayer flask. A mixture of 75 µl NaClO + 75 µl $dH_2O$ was added to the pellet and incubated at 37 °C for 1 hour. Subsequently, centrifugation (13,500 rpm) was performed for 10 minutes. Washing was carried out first with water, then with pure acetone, and finally with ultra-pure ethanol.

A solution of 1 ml chloroform + 2 ml $H_2SO_4$ was added and kept in boiling water for 20 minutes. The amounts of PHB production by microorganisms were calculated as µg/ml by comparison with the standard graph [13, 14]. Commercially available pure PHB solutions, prepared at different concentrations (10, 7.5, 5, 2.5, 1, 0.5, and 0.125 µg/ml) in $H_2SO_4$, were measured at a wavelength of 235 nm (Shimadzu UV-Visible spectrophotometer).

A standard graph was obtained using these values. The amount of PHB production by microorganisms was then compared with this standard graph, and the amount was calculated as µg/ml (Figure 1) [5]. All experiments were performed in triplicate.

## 3. Results and Discussion

Polyhydroxybutyrates is produced by microorganisms under certain stress conditions (nutrient deficiency, extreme heat-cold, humidity, pH), in cases where growth conditions are not available and especially in cases where

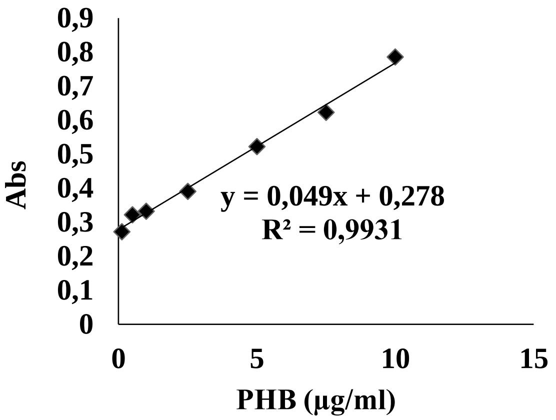


**Figure 1.** Polyhydroxybutyrate Standarts

the C/N ratio is unbalanced (excess carbon source in the medium, limited nitrogen source). The C/N ratio in the culture medium is of great importance in the synthesis of various polymers. Increasing the carbon ratio decreases the number of cells and increases PHB production [15]. The PHB standard with used and analysis methods, it was determined whether the product obtained was PHB or not. In this study, Nutrient Broth was used as a rich nutrient medium. Phosphate-Buffered Saline (PBS) was used as the minimal medium.

Phosphate-Buffered Saline is a buffer solution containing the chemical components NaCl, KCl, $Na_2HPO4$, $KH_2PO_4$, with a pH of 7.4, widely used in biological studies. It is observed that there is a 1.64-fold difference in ratio between the lowest (2.993 µg/ml in PBS + 1% glucose medium) (Table 1) and the highest (4.912 µg/ml in PBS + 1% sucrose medium) (Table 2) PHB production of *B. cereus* under static conditions. There is a 6-fold difference rate between the lowest (1.095 µg/ml in PBS + 1% maltose medium) (Table 1) and the highest (6.571 µg/ml in PBS + 1% xylose medium) (Table 2) PHB production of *B. cereus* under agitated states.

A 273.9-fold difference ratio is observed between the lowest (0.027 µg/ml in PBS + 1% maltose medium) (Table 1) and the highest (7.395 µg/ml in PBS + 1% xylose medium) (Table 2) PHB production *of C. necator* (*R. eutropha*) under static conditions. A 78.9-fold difference ratio is observed between the lowest (0.122 µg/ml in PBS + 1% dextrose medium) (Table 1) and the highest (9.626 µg/ml in PBS + 1% fructose medium) (Table 2) PHB production of *R. eutropha* under agitated states.

When *B. cereus* and *C. necator* (*R. eutropha*) were compared, at the lowest PHB production, there is a 110.8-fold difference in B. cereus compared to *C. necator* (*R. eutropha*) under static conditions. In shaking conditions, it is observed that B. cereus has an 8.9-fold difference ratio compared to *C. necator* (*R. eutropha*). When *B. cereus* and *C. necator* (*R. eutropha*) were compared, in the highest PHB production, it is observed that there is a 1.51-fold difference between *C. necator* (*R. eutropha*) and *B. cereus* under static conditions. It is observed that there is a 1.46-fold difference between *C. necator* (*R. eutropha*) and *B. cereus* under shaking conditions. In this study, it was shown that *C. necator* (*R. eutropha*), a Gram (-) bacterium, produced the highest PHB production under static and shaking conditions. In addition, advantageous minimal carbon nutrient sources for microorganisms are fructose, xylose, and sucrose; glucose, dextrose, and maltose are stated to be disadvantageous.

Bioplastics are plastics acquired from renewable biomass and have been manufactured from first-generation feedstocks like corn, sugar beet, or second-generation feedstocks like lignocellulose substance [16]. The functionalization of biodegradable polymers is beneficial. PHB, one of them, is broadly researched as a member of the polyhydroxyalkanoate family and has shown biocompatibility for *in vitro* and *in vivo* studies [17]. It is considered an important polymer because it can be manufactured from renewable and sustainable sources with the advantage of being degraded by some aerobic and anaerobic microorganisms [18].

Fathima and Krishnaswamy conducted a study on PHB production at 150 rpm for 72 hours by adding 2% of various carbon sources (glucose, lactose, maltose, and sucrose) to the Minimal Salt medium in different conditions with halotolerant bacterial strains, and they found the highest PHB production at 500 µg/ml in the presence of sucrose [19]. In our study, *B. cereus;* in static conditions, the lowest PHB production was achieved in PBS + 1% glucose medium (2.993 µg/ml) (Table 1).

Park et al; *Halomonas* spp. conducted a study on PHB production by adding 2% of various carbon sources (fructose, glucose, xylose, sucrose) to the Marine Broth nutrient medium under different conditions with YLGW01. They found the highest PHB production of 9150 µg/ml in 2% fructose [20]. In our study, *C. necator* (*R. eutropha*) showed the highest production of PHB under shaking conditions in PBS + 1% fructose medium (9.626 µg/ml) (Table 2).

Hagagy et al; *Haloarcula* spp. strain NRS20, added various carbon sources (10 g/l glucose, glycerol, sucrose) to the HSM medium under different conditions, and they found the highest PHB production at 2.946 µg/ml in sucrose medium [21]. In our study, *B. cereus*; was maintained in static conditions in a medium of PBS + 1% Sucrose (4.912 µg/ml) (Table 2).

Mi Lee et al; *Bacillus* spp. carried out a study with SM01 and *C.necator* NCIMB by adding 1% of various carbon sources (fructose, glucose, xylose, sucrose) to the Marine Broth nutrient medium under different conditions, at 200 rpm for 72 hours, and they found the highest PHB production 3410 µg/ml in xylose medium [22].

In our research, *B. cereus*; under shaking conditions, the highest PHB production was achieved in PBS + 1% Xylose medium (6.571 µg/ml). Khanna and Srivastava (2005) showed the highest PHB production in *Ralstonia eutropha* in Mineral Salt medium + fructose medium, 1400 µg/ml at 150 rpm for 48 hours.

However, it showed 42 µg/ml in sucrose medium, 31 µg/ml in glucose medium and 3 µg/ml in xylose medium. In our thesis study, R. eutropha showed 9.626 µg/ml in PBS + 1% fructose medium, 37 ºC, 150 rpm for 24 hours. It is thought that one of the main reasons for the higher values in the study of Khanna and Srivastava compared to our study is the time difference (48 hours). Our study was carried out after 24 hours [23] (Table 2).

**Table 1.** Lowest PHB production in bacteria in the presence of different carbon sources

| Bacteria | 0 rpm | 150 rpm |
|---|---|---|
| ***B. cereus*** | Glucose 2.993 µg/ml | Maltose 1.095 µg/ml |
| ***C. necator*** | Maltose 0.027 µg/ml | Dextrose 0.122 µg/ml |

**Table 2.** Highest PHB production in bacteria in the presence of different carbon sources

| Bacteria | 0 rpm | 150 rpm |
|---|---|---|
| ***B. cereus*** | Sucrose 4.912 µg /ml | Xylose 6.571 µg /ml |
| ***C. necator*** | Xylose 7.395 µg /ml | Fructose 9.626 µg /ml |

## 4. Conclusion

In this study, it has been shown that *B. cereus* (Gram (+)) and *C. necator* (Gram (-)) bacteria produce more PHB in the presence of minimal carbon sources and in agitated conditions. These organisms can produce PHB under difficult conditions and thus meet their energy needs. We consider that this research will pave the way for scientific work.

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***Authors' Contribution***
The authors contributed equally to the study.

***The Declaration of Conflict of Interest/ Common Interest***
No conflict of interest or common interest has been declared by the authors.

***The Declaration of Ethics Committee Approval***
This study does not require ethics committee permission or any special permission.

***The Declaration of Research and Publication Ethics***
The authors of the paper declare that they comply with the scientific, ethical and quotation rules of

SAUJS in all processes of the paper and that they do not make any falsification on the data collected. In addition, they declare that Sakarya University Journal of Science and its editorial board have no responsibility for any ethical violations that may be encountered, and that this study has not been evaluated in any academic publication environment other than Sakarya University Journal of Science.

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