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# Poly(3-hydroxybutyrate) Synthesis by *Cupriavidus necator* DSMZ 545 Utilizing Various Carbon Sources

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**Abstract;** Polyhydroxybutyrate (PHA) synthesis was based on pure culture of *Cupriavidus necator* DSMZ 545. Due to the high production cost of PHAs, the use of cheaper and available carbon sources was considered. The aim of this research was to investigate the impact of sugarcane molasses as a low cost substrate and acetate as media supplements in mineral medium for the cell growth and poly(3-hydroxybutyrate), P(3HB). *C. necator* was cultivated in a 500ml shake flasks with 100 ml of the medium at 30°C for 96h, with agitation rate of 250 rpm. Samples for product analysis were taken at 12h time interval. Sodium acetate was used as supplementary substrate, it was added to molasses for the production of P(3HB). The obtained results showed that with addition of acetate as supplementary carbon source, enhanced the biopolymer concentration to 2.85g/l.

**Key words:** Acetate · Biopolymer · *Cupriavidus necator* DSMZ 545 · Poly(3-hydroxybutyrate) · Molasses

## INTRODUCTION

Due to thermoplastic properties and biodegradability of biopolymers, Polyhydroxyalkanoates (PHAs) have attracted industrial interests. PHAs have been extensively studied over the last two decades [1]. The environmental negative impact caused by the disposal of plastics as well as the progress of biotechnology has motivated the research on biodegradable and biocompatible polymers [2]. PHAs are thermoplastic polymer synthesized by a wide variety of bacteria as intracellular compounds and energy storage materials when the nutritional elements are limited and the carbon source is in excess. *C. necator* is one of the best known bacteria among PHA producing microorganisms. The most common investigated biopolymer of PHAs is poly(3-hydroxybutyrate), P(3HB), that is synthesized and deposited as intracellular inclusion bodies to a content of 80% of cellular dry weight [3]. A remarkable characteristic of P(3HB) is its biodegradability in different environments. A number of microorganisms in environments excrete PHA depolymerises able to hydrolyze PHA into water-soluble oligomers and monomers that can be utilized, as nutrients by the living cells [4]. Due to the high production cost of PHAs, the use of PHAs as substitutes for the conventional synthetic polymeric material with a wide range of applications has been compared [5, 6].

The use of a cheap carbon sources and nutritional supplements with feeding substrate strategies are required in order to reduce the production cost of biopolymers such as P(3HB) [7-11]. For production of PHA, molasses originated from sugar cane and olive oil mill wastewater have been utilized in acidogenic fermentation [12]. High concentration of PHB has been produced with fed-batch culture of recombinant *Escherichia coli* [13]. Several organic compounds as carbon sources and potential substrates for the production of P(3HB) using *Pseudomonas guezennei* were investigated [14]. Moreover, all of the existing literatures on PHAs have discussed about submerged culture of *C. necator* on a sole carbon source but not much elaborated on the media supplements. The exceptional reported literature on PHB production was addition supplementary media. The PHB has been accumulated inside the strains of *Rhizobium etli*

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and other *Rhizobium* species using supplements of biotin and thiamine with carbon sources of fumarate and malate [15].

The purpose of present investigation was to synthesis P(3HB) in a submerged culture of *C. necator* with cheap and various carbon sources locally available. The supplementary media was also investigated in P(3HB) synthesis.

## MATERIALS AND METHODS

**Microorganism:** The microorganism used in the present study was *Cupriavidus necator* DSMZ 545 (Deutsche Sammlung von Mikroorganismen und Zellkulturen) for culture propagation. It was stored and maintained on Luria Agar slants at 4°C. The organism was sub-cultured every 15 days to maintain its viability.

**Media:** Molasses was locally obtained from sugarcane molasses Industry (Sharivan, Iran). Molasses was treated with sulfuric acid solution (0.75 wt%, pH 1.1) and heat treated at 100, 115°C and 130°C for 15min. The solution was neutralized with 5 M NaOH solutions and the pH was adjusted to 7.0. Then, the neutralized solution was filtered, autoclaved at 121°C for 15min. Other sets of experiments with 10 g/l acetate as supplement to molasses were used.

**Experimental Conditions:** Stock culture in slants of *C. necator* was incubated at 30°C for 24h. The resultant cultures were transferred into 500 ml flasks contained 100 ml medium. The flasks were incubated at 30°C and agitated at 200 rpm for 10, 15, 20 and 30 h, to investigate the effect of the seed age on biopolymer production. The inoculum size was 5% of the medium. Then, the incubation temperatures were set at the following conditions: temperature at 25, 27, 30 and 33°C, agitation rate of 150, 200, 250 and 300 rpm. For the above experimental conditions the dry cell weight (DCW) and PHAs accumulation inside the cells were investigated.

**Cell Dry Weight:** The cell concentration in the cultured media was determined by the cell optical density at wavelength of 620 nm using spectrophotometer (UNICO2100, USA) with distilled water for suitable dilution rate. The cell dry weight was also measured based on standard calibration curve for cell optical density, absorbance as a function of cell dry weight for the pure cultured of *C. necator*.

**Carbohydrate Concentration:** The supernatant obtained from centrifuged solution was used for residual nutrient analysis including total carbohydrates according to the method developed by reduced sugar analysis using 3, 5- dinitrosalicilic acid (DNS) method [16].

**Biopolymer Analysis:** For P(3HB) quantification, a 5ml of culture broth was centrifuged at 3600rpm for 20min. A 2 ml solution of chloroform and 2ml of acidified methanol (3% sulfuric acid) were added to the cell pellet in a vial with Teflon screw cap and heated at 100°C for 3.5h. The developed extraction method was based on experimental method developed by Braunegg *et al.* [17].

Gas chromatography (GC) was performed using a gas chromatograph (Philips PU4400, US) equipped with flame ionization detector (FID) and data acquisition system with computer software (Clarity 4.2, Data Apex, Czech Republic). The GC was used for the methyl-3-hyroxybutyrate (3HB) analysis. The GC was equipped with capillary column (BP20 SGE, Australia), 0.33 mm internal diameter and 25m length. The column temperature was initially maintained at 80°C for 4 min, followed by the temperature programming at a rate of 8°C/min till it reached to 160°C, maintained for 3 min and then at a rate of 30°C/min increased to 200°C. The detector and injector temperatures were 280 and 250°C, respectively. The carrier gas used were helium with a flow rate of 1.5ml/min. Hydrogen and air flow rates were 30 and 300 ml/min, respectively. The injection volume size was 1µl of the prepared samples.

## RESULTS AND DISCUSSION

Batch experiments were performed in shake flasks for production of the biopolymer. The flasks were incubated at 30°C and agitated at 250 rpm. The effect of seed age on cell growth and P(3HB) production was investigated and the obtained data are shown in Figure 1. The maximum cell growth and P(3HB) accumulation were resulted in 24 h of the seed age. There was a slightly decrease in amount of P(3HB) for the longer incubation of the seed age. The observed in concentration of P(3HB) and cell density were probably due to substrate depletion and the formation of intracellular by-products which caused inhibition. Also, the growth curve for *C. necator* showed that the microorganism at 24h was at the mid-exponential phase, with the inoculum contained live and very active cells. Under the above experimental condition, CDW and biopolymers concentration were 8.3 and 1.6 g/l, respectively.

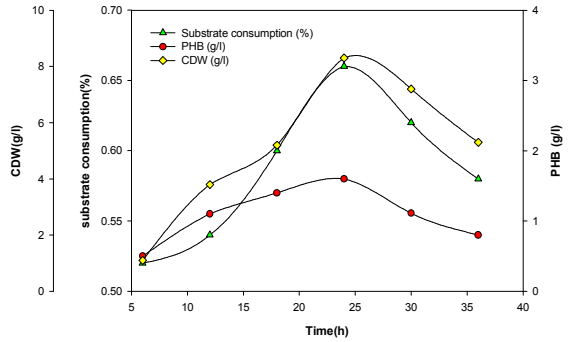


Fig. 1: Effect of seed age on cell growth and P(3HB) production at 30°C, agitation rate of 250 rpm

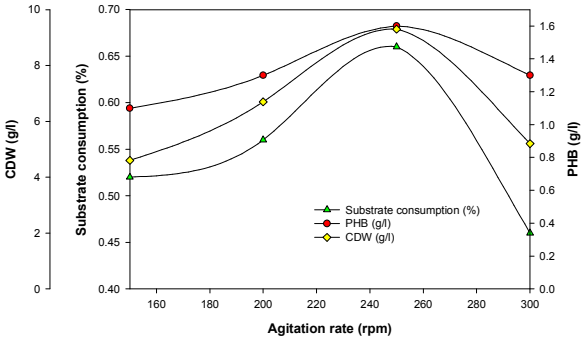


Fig. 2: Effect of agitation rate on cell growth and P(3HB) production at 30°C and the seed age of 24 h

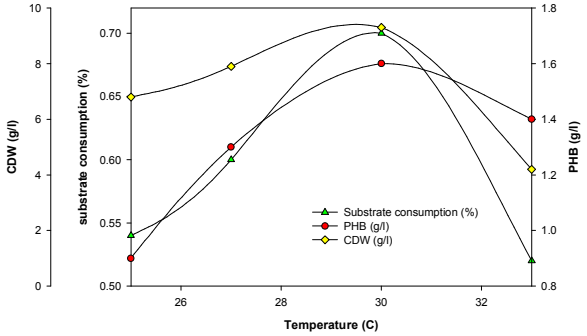


Fig. 3: Effect of temperature on cell growth and P(3HB) production at fixed agitation rate of 250 rpm and seed age of 24h

Effect of agitation rate on cell growth and P(3HB) production is shown in Figure 2. Both, the cell growth and P(3HB) production had an increasing trend with the agitation rate of up to 250 rpm. For the agitation rate of higher than 250 rpm cell autolysis occurred and the productivity started to drop.

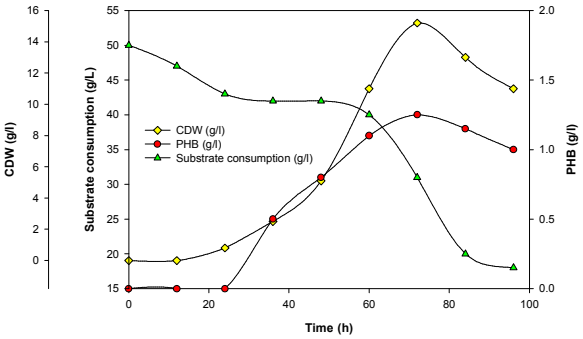


Fig. 4: The concentration profiles of cell growth, consumption of molasses and biopolymer P(3HB) production at 30°C and agitation rate of 250 rpm

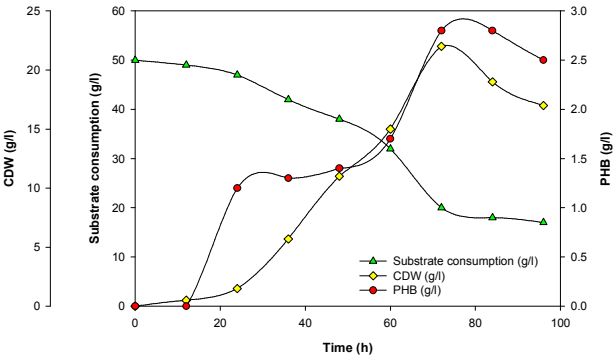


Fig. 5: The concentration profiles of cell growth, dual substrates (molasses and acetate) utilization and biopolymer P(3HB) production at 30°C and agitation rate of 250 rpm

Figure 3 shows the effect of the culture temperatures on cell growth and P(3HB) production. The temperature effect was investigated in the range of 20-35°C. The results indicated that at culture incubation temperature of 30°C, maximum CDW and P(3HB) biopolymer concentrations obtained, were 26 and 1.63 g/l, respectively.

The substrate used as carbon source for the production of biopolymer was the treated molasses. Normally, the dark brown thick syrup remained as residues of invert sugar crystallization is known as molasses. The solution had dark brownish pigments color of melanoidins. Since molasses are obtained from circulation of sugar solution in series of evaporation, it may have caramelized and invert sugars. If high substrate concentration is implemented, it may cause cell toxicities. In a batch experiment with 50 g/l of carbohydrates in media with diluted molasses, production of 1.35 g/l of P(3HB) was obtained. Figure 4 represents the

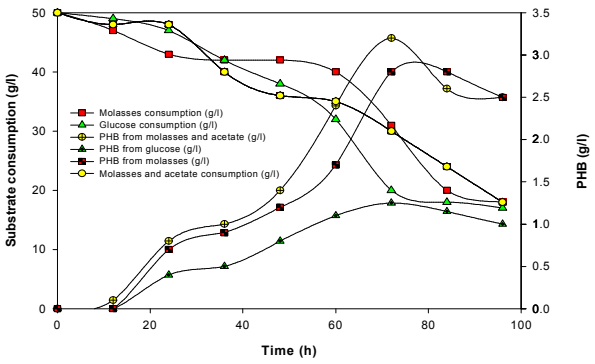


Fig. 6: Comparison for various substrate utilization and P(3HB) production at 30 °C and agitation rate of 250 rpm

concentration profiles of substrate consumption, cell dry weight and the biopolymer P(3HB) production at 30°C and agitation rate of 250 rpm. At 72 hours of incubation time, about 67% of substrate was consumed and maximum amount of P(3HB) produced was 1.35 g/l, while the mass of cell was about 2 g/l.

Addition of sodium acetate as supplementary carbon source to molasses solution was experimented, while total amount of carbon source was maintained at the same level. The ratio of acetate to molasses sugar content was 1:4. The use of supplementary media was investigated in the literature [15]. Addition of 20% acetate to the media created an environment for extra production of P(3HB). The concentration of P(3HB) had reached about 2.85 g/l. That was more than double the amount of P(3HB) produced in a media without acetate. Figure 5 shows, the concentration profile of the dual substrates (molasses and acetate). The profile for production of biopolymer P(3HB) is as shown (Fig. 3). Addition of acetate enhanced the cell dry weight by 25 %. According to obtained experimental result, it is also shown (Figure 5), addition of acetate as a supplementary substrate absolutely improved the cell growth and the concentration of the biopolymer to 21.8 and 2.85 g/l, respectively.

Figure 6 depicts P(3HB) production and substrate utilization profiles. The data compares P(3HB) production with and without addition of acetate, while the total carbohydrate concentration was fixed. The effect of 20% additional acetate showed great improvements on biopolymer production. The maximum P(3HB) production was obtained at 72 h incubation, 30 °C and agitation rate of 250 rpm.

## CONCLUSIONS

*C. necator* was grown in the media with and without acetate, as the total amount of carbon sources for both cases were fixed at 50 g/l. The batch culture was operated for maximum production of P(3HB) at optimum media conditions at 72 h incubation, 30 °C and agitation rate of 250 rpm. It was concluded that *C. necator* was suitable organism for production of P(3HB). Addition of 20 % acetate to media doubled the biopolymer production and enhanced P(3HB) concentration to 2.85 g/l.

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