BIOSYNTHESIS OF POLYHYDROXYBUTYRATE BY *Pseudomonas spp.*

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Introduction

Polyhydroxybutyrate (PHB) is a polyhydroxyalkanoate (PHA), a polymer belonging to the polyesters class that was first isolated and characterized in 1925 by French microbiologist Maurice Lemoigne. PHB is produced by micro-organisms (like *Alcaligenes eutrophus* or *Bacillus megaterium*) apparently in response to conditions of physiological stress. The polymer is primarily a product of carbon assimilation (from glucose or starch) and is employed by micro-organisms as a form of energy storage molecule to be metabolized when other common energy sources are not available. Microbial biosynthesis of PHB starts with the condensation of two molecules of acetyl-CoA to give acetoacetyl-CoA which is subsequently reduced to hydroxybutyryl-CoA. This latter compound is then used as a monomer to polymerize PHB.

Polyhydroxybutyrate has attracted much commercial interest as a plastic material because its physical properties are remarkably similar to those of polypropylene (PP), even though the two polymers have quite different chemical structures. While PHB appears stiff and brittle, it also exhibits a high degree of crystallinity, a high melting point of about 180°C, but, most importantly, PHB is rapidly biodegradable, unlike PP[1]. There is no study conducted on PHB biosynthesis by bacterial species. It is necessary to provide biological production of PHB in order to understand the mechanism. Consequently in this study we conducted a biosynthesis of PHB by 3 Pseudomonas species for the first time.

Materials and Methods

Microorganism: Three strains of *Pseudomonas aeruginosa (*424), P. putida (*102) and P. fluorescens* (*103) were employed in this study.

Production of PHB: *P. aeruginosa, P. putida,* and *P. florescens* were used. The preinocula for the fermentation and shake flask experiments were prepared in Erlenmeyer flask containing 0.8% (wt/vol) nutrient broth and 0.5% (wt/vol) glucose and incubated at 34^0C for 18 hours. Five ml of each of the culture was added to 100 ml of the PHB medium. The preinocula thus inoculated into the PHB media was incubated for 8 days in orbital shaker at 37^0C with 110 rpm[2].

Recovery of PHB by Sodium Hypochlorite: Sodium hypochlorite solution was diluted with distilled water to give 10% concentration (vol/vol). The culture samples were centrifuged at 8500 rpm (4000 x g) for 5 min. and the supernatant was discarded and incubated at 30^0C for 1 hour. The procedure was repeated as above (centrifuge at 4000 x g for 5 min. and the supernatant was discarded and incubated at 30^0C for 1 hour). To the pellet 5 ml of distilled water was added and kept it for incubation at 30^0C for 1 hour and again centrifuged at same condition. Then, the supernatant was removed and to the pellet acetone was added and rinsed[3].

Determination of Biomass: Culture broth samples were centrifuged for 5 min at 4,000 x g (8500 rpm). The pellet was suspended in 5 ml of distilled water and centrifuged. The pellet was then dried at 105°C to obtain the total dry biomass[2].

PHA Analysis: The pellets thus recovered were analysed for PHA monomers. The analysis was carried out by GC method[3-4].

Determination of PHB by UV Spectrophotometer: Microbes *P. aeruginosa, P. putida,* and *P. florescens* were grown on nutrient broth medium at 30^0C for 48 h on a shaker. Suspensions of cultures were centrifuged at 6000 × g for 30 min. Then the pellets were suspended in 5 ml of sterile water and homogenized using ultrasonic treatment (5 min). Two ml of the cell suspension was added 2 ml of 2 M HCl and it was heated at boiling temperature for 2 h in a water bath and the tubes were centrifuged at 6000 ×g for 20 min. Five millilitre of chloroform were added to the resulting precipitate. The test tubes were left overnight at 28^0C on a shaker at 150 rev/min. Then the contents of the test tubes were centrifuged at 6000 × g for 20 min., and extracted with 0.1 ml of chloroform, and were dried at 40^0C. Five ml of concentrated sulphuric acid was added. The tubes were heated at 100^0C in a water bath for 20 min. After cooling to 25^0C, the amount of PHB was determined on a UV spectrophotometer, wavelength 235 nm[5].

Results and Discussion:

The fresh weight of the test organisms were ranged from 0.18 g to 0.14 g, respectively (Table 1). The maximum biomass was observed in *Pseudomonas aeruginosa* when compared to other two organisms which was about 0.27g/l. This is similar to that of Yilmaz *et al*.[6] reported that some of the *Alcaligenes eutrophus* strains used for commercial PHB production have a PHB concentration which is approximately 80% (w/w) of the dry cell weight. Chen *et al.*[7] studied PHA in 11 different *Bacillus spp*. and found PHB consisting 50% (w/v) of dry cell weight of the bacteria.

PHA analysis

The polymer content and composition were determined by methanolysis. The resulting methyl esters were characterized by GC has been listed in Table 2. Different kinds of butyrate in the GC result indicate the presence of PHB (Table 2). The result of present investigation is similar to Huisman *et al*.[5] which demonstrate several well-established prototype strains. *P. aeruinogsa, P. fluorescens, P. lernonnieri, P. testosteroni,* and the *P. putida* strains described above were grown on 10 mM octanoate or 0.7% 3-hydroxybutyrate in 50-ml 0.5*E2 cultures. The cells were harvested, and PHA content was determined for whole cells. The *P. putida* strains were grown in E2 medium on 30 mM octanoate or 0.7% 3-hydroxybutyrate in 1-liter fermentors, and the polyesters were isolated. The composition of the polymer was determined and shown to be identical to that obtained after whole-cell analysis. The PHAs from octanoate- grown cells were always composed of 3-hydroxy hexanoate, 3-hydroxyoctanoate, and small amounts of 3-hydroxydecanoate.

Determination of PHB by UV Spectrophotometer

The amount of PHB in *P. aeruginosa, P. putida and P. fluorescens* were analyzed using UV spectrophotometer (Table 3). The highest amount was found in *P. aeruginosa.* This is similar to the study of Arun *et al*.[8] who reported in his work that maximum PHB production by *A. eutrophus* observed was in the synthetic medium (0.44%) and also in 40% sesame oil medium under aerobic condition.

PHB and its copolymers have been drawing considerable industrial interest as biodegradable and or biocompatible. They are used in packaging, medicine and agriculture for a wide range of applications. PHB is accumulated as intracellular granules by many prokaryotic organisms. Due to the importance of PHB, the present study involves the production. Three strains, *Pseudomonas aeruginosa, P. putida, and P. fluorescens* were included in which PHB production by these strains were recovered by sodium hypochlorite and determined by spectrophotometric method. The value ranged for *P. aeruginosa, P. putida, and P. fluorescens* were 2.346 g/l, 1.581 g/l, and 1.063 g/l respectively. The highest PHB production was found in *P. aeruginosa*.

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Table 1. Microbial biomass (g/L) values.

Test organisms	Fresh weight (g/L)	Dry weight (g/L)	Total biomass (g/L)
Pseudomonas aeruginosa	0.18	0.09	0.27
Pseudomonas putida	0.23	0.01	0.24
Pseudomonas fluorescens	0.14	0.02	0.16

Table 2. Name of PHA components analyzed in this study.

S.No	Components
1	Isoamyl – 2 – methyl butyrate
2	Tetrahydrogeranyl butyrate
3	Decyl Iso butyrate
4	Methyl – 15 oxohexadecanoate
5	Methyl – 5 oxohexadecanoate
6	Heptyl hexanoate
7	Methyl ester of 3- hydroxy decanoic acid
8	4- Ethyl octanoic acid

Table 3. Concentration of PHB (g/L) by ultraviolet spectrophotometer.

Test Organism	PHB (g/L)
Pseudomonas aeruginosa	2.346
Pseudomonas putida	1.581
Pseudomonas fluorescens	1.063