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PRODUCTION OF MEDIUM CHAIN LENGTH POLYHYDROXYALKANOATES FROM PALMITIC ACID USING *Ralstonia eutropha* BY FED BATCH CULTURE

Surbhi Jain*, A.K.Singh and Archana Tiwari

Department of Chemistry,
Rajiv Gandhi Proudyogiki Vishwavidyalaya Bhopal-Madhya Pradesh-462033
*corresponding author**:** surbhijchem@yahoo.com

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ABSTRACT:

Polyhydroxyalkanoates (PHA's) are carbon and energy storage compounds which accumulate intracellulary as granules in certain bacteria during unbalanced growth and in the presence of an excess carbon source. PHAs were well studied regarding their promising applications, physical, chemical and biological properties. PHAs are biodegradable, biocompatible, have good material properties, renewable and can be used in many applications. In this study, we have biosynthesized Poly(β-hydroxyalkanoate)s (PHAs) by *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*) by using pamitic acid extracted from palm oils. The bacterium Ralstonia eutropha accumulates high levels of PHA and can effectively utilize Palmitic acid. The *Ralstonia eutropha* H 16 successfully incorporated Poly 3-hydroxyhexanoate (PHB) monomer when fed with crude palm kernel oil (CPKO) as the sole carbon source. The experiments were carried out through submerged discontinuous cultures in agitated flasks and a bioreactor. The cultures of *R. eutropha* were grown for 24h at 30ºC. Approximately 63 ± 2 wt% of P(3HB-co-3HHx) copolymer with 4 mol% of PHB was synthesized from 5 g/L of oil after 48 h of cultivation. The results showed that *R. eutropha* growth in this carbon source was similar when compared with the standard medium (µm = 0.20 h-1). *R.Eutropha* demonstrated its ability to synthesize P(3HB-co-3HHx) copolymer from a readily available and renewable carbon source; CPKO, limiting the Nitrogen component in a fermentor without the addition of any precursors.

Keywords: Polyhydroxyalkanoates, biosynthesis, Ralstonia eutropha, Palmitic acid, fed batch cultures, bioreactor
Running Title: Production of PHA from *Ralstonia eutropha* using Palmitic acid as a carbon source

INTRODUCTION:

Polyhydroxyalkanoates (PHA) are bacterial storage compounds produced widely by many microorganisms under growth-limited conditions [16,21]. PHA can be synthesized and degraded in vivo to accumulate the excess carbon and energy sources and reutilize when needed [21]. PHA has good thermoplastic properties, biodegradability, biocompatibility and other excellent traits which

attract considerable academic and industrial interest in the past 30 years[16]. According to their side chain length, PHA was divided into short-chain-length PHA (scl PHA) and medium-chain-length PHA (mcl PHA)[30]. Many researches focused on how to reduce the PHA production cost, including adopting cheap carbon sources such as whey and bagasse hydrolysates[20,36] or mixed culture method [15].

PHAs possess exceptional stereochemical regularity. The chains are completely linear, and the chiral centers have been shown to possess only R-stereochemical configuration [17,2] Recently, PHAs attracted substantial industrial interest because of their potential use as biodegradable thermoplastics and sources of chiral monomers [26,28]. The monomer composition of a PHA depends on the microorganism as well as on the substrate used for its accumulation.

Production of PHA by both Gram negative and Gram positive bacteria have been investigated and well documented. Besides this, isolation and identification of new bacterial strains with better PHA production capabilities are also being continuously investigated. Pseudomonas spp.[6], for instance, can produce longer-chain copolyesters when grown on alkanoic acids that contain ≥6 carbon atoms. In a recent study, a locally isolated Cupriavidus strain from the Malaysian environment had been evaluated for the production of PHA copolymer from various carbon sources [32].

R. eutropha is the model organism for studying PHA synthesis and accumulation [27]. Ralstonia eutropha is one of the most widely used microorganisms with regard to PHA production, due to the ease of culturing them in renewable carbon sources, and also because this bacterium may attain up to 80% of its dry mass as a polymer [8]. P(3HB) production in R. eutropha is carried out in two phases: (1) cell growth under non-limiting nutrient conditions, with the aim of biomass generation; (2) nutrient (nitrogen,phosphate, oxygen, etc.) limitation with polymer accumulation according to the source of carbon offered [7].

Ralstonia eutropha is capable of producing the most common PHA, poly(b-hydroxybutyrate) (PHB), when glucose is used as the substrate. On the other hand, copolyesters that contain 3-hydroxypropionate [9], 3-hydroxyvalerate [12], 4-hydroxybutyrate [10], or 5-hydroxyvalerate [11] , in addition to 3-hydroxybutyrate, have been reported when the medium is supplemented with other carbon substrates.

Although it presents advantages in relation to the conventional plastics, such as being a biocompatible and biodegradable material, the industrial production of PHA still has a very high cost when compared to petrochemical-derived plastics. Some factors such as the development of improved bacterial strains, more efficient extraction/recovery processes and, most importantly, a reduction in the costs of the raw material, may contribute to making PHAs more competitive in the market [4].

The expense related to the raw material can represent up to 40% of the total polyhydroxyalkanoates production cost. A form of reducing these costs is the use of alternative carbon sources, that may be residues or by-products of the food industries such as whey or inverted sugar [23]. Starch is another source available in large amounts. Starch and its derivatives are the major components of biological oxygen demand (BOD) in wastewaters from industries such as textiles, paper, fermentation, beverage and food processing [35].

The use of fatty acids such as oleic acid, or of vegetable oils that contain these constituents, as nutritional supplements is an interesting alternative to increase the production and the productivity of the PHA obtaining process. These agents can interfere in the metabolic pathway

providing a greater production of cells and resulting in higher productivity. They can also act directly in the synthesis of biopolymers. The use of these fatty acids and vegetable oils has been demonstrated by several authors [14,3,22,19]. Palmitic acid (n-hexadecanoic acid) is a fatty acid obtained by the hydrolysis of triglycerides of kernel palm, palm, tallow, or coconut oil. The objective of this study was to investigate poly(3-hydroxybutyrate) production from *R.Eutropha* H 16 strain with the supplementation of the culture medium with palmitic acid as a solitary carbon source; limiting the Nitrogen component in a fermentor.

2. Material and Methods

2.1. Bacterial strain and culture medium

The bacterial strain used was *R. eutropha*, H16. It was obtained from IMTECH, Chandigarh, India and was subcultured in trypticase soy broth. Stock cultures were maintained on trypticase soy agar at 4°C with transfer every 14 days. The fermentation medium and process are described below.

2.2. Cultivation conditions

The experiments were carried out in 1000 mL agitated flasks, containing 300 mL of culture medium tryptic soy broth dextrose-free (TSB) medium (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and incubated at 150 rpm in a rotatory shaker. The temperature used was 30°C for all assays. The culture medium was inoculated with 10% of the seeded culture. The pH was maintained at 7.0 by the manual addition of NaOH (10%) or HCl (10%). Samples were collected every 2h. The cells were harvested and washed twice with sterile water, then used to inoculate 5 mL of PHB minimal medium, prepared as previously described [34] with a final NH4Cl concentration of 0.1 g/L. Antibiotics added to growth media to the final concentrations for R. eutropha, gentamicin (10 µg/ml), kanamycin (270 µg/ml), and spectinomycin (250 µg/ml).

2.3 Fed-batch fermentation culture

Fed-batch cultures were carried out at 37°C in volumetric flask with a starting volume of 500 microliters semidefined medium (OM) containing 100 µg/ml ampicillin or 50 µg/ml kanamycin [25]. In the first stage of the cultivation, pH was controlled at 7.20 by automatic addition of 3 N KOH. The dissolved-oxygen concentration was maintained above 30% air saturation by automatic control of the agitation speed. Antifoam (0.02% [vol/vol] Antifoam 289; Sigma) was added at the onset of cultivation. The feeding solution used for fed-batch cultures was a concentrated and deproteinated whey solution containing 25% (wt/vol) lactose prepared as described by[1]. The pH-stat feeding strategy was employed. When the pH rose to a value higher than its set point (7.20) by 0.15 U due to carbon source depletion, an appropriate volume of feeding solution was added (up to 800 ml). Samples for biomass and PHB production and plasmid stability determination were withdrawn every 4 h.[24]

2.4. Addition of Supplements

At the beginning of the production phase, an amount of each selected supplement was added in order to obtain an equivalent concentration of 0.3 g palmitic acid/L in the culture medium with a concentration of approximately 25%.

2.5 Staining procedure

The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan black-B. After the complete production of PHA under suitable growth conditions, thin smear of strains were made on a clean glass slide and was heat fixed. This slide was immersed in a filtered solution of 0.3% (w/v) Sudan black-B (in ethylene glycol) for 15 - 20 min. Then, the slide was immersed in xylem and blot dried with absorbent paper. Finally, the microscopic slide was counter-stained for 10 s with (0.5% w/v) aqueous safranin. The slide was then rinsed with

tap water and blot dried and examined under a microscope.[31].

2.6. PHA extraction

Intracellular PHA polymers were isolated from lyophilized cells by hot chloroform extraction at 100 ^{o}C for 4 h, and filtration was used to remove the cellular debris. Then PHA were dissolved in chloroform and purified by precipitation with 10 volumes of ice cold methanol.

2.7 Extraction & Purification of PHA

Chloroform extraction has been widely used to recover PHA with high degree of purity without polymer degradation during the recovery[29].Therefore, PHA samples were first recovered by chloroform extraction of bacterial cells with different PHA contents. Cells were collected by centrifugation at 4,000X g for 20 min at 25^{o}C and were washed with hot acetone for 20 min. After being dried; the cells were mixed with volumes of chloroform for 48 h at 30^{o}C. A clear PHA solution was recovered by centrifugation: this was followed by non-solvent precipitation (five times the volume of chloroform) and filtration. The nonsolvent used was a mixture of methanol and water (7:3v/v).The chloroform extraction method was found to be efficient not only for the recovery of PHA with a high degree of purity but also for the removal of the endotoxin of gram negative bacteria. NaOH can be used for digestion purpose, since it has many advantages (i) NaOH is inexpensive and much more environmentally friendly, (ii) a high degree of purity (>98%) of PHA can be obtained, and (iii) there is no degradation of PHA during recovery [5].

2.8 Purification of PHB.

PHB produced in bioreactor cultures was extracted from lyophilized cells with hot CHCl3 using a Soxhlet apparatus, ethanol precipitated, and recovered by filtration. The precipitate was dried, dissolved in CHCl3, filtered to remove particles, and dried on a glass petri dish to obtain a thin film further.

2.8 Analytical Methods

During fermentation, around 50 ml of culture broth is periodically removed for analysis. The culture samples will be centrifuged to separate the culture supernatant and the cells. The supernatant will be used for analysis of nitrogen and the cells will be dried in oven at 105^{0} C, for cell dry weight determination. At the end of fermentation, the entire culture broth is centrifuged to collect all the cells. The cells will be then used for PHA extraction, after washing with saline solution, followed by methanol and dried at 105^{0}C. Concentration of ammonium in the medium is determined by Berthelot reaction [18] and expressed as NH 3 concentration. PHA content of the samples are analyzed by gas chromatography (GC) [18]. Residual cell concentration is defined as cell concentration minus PHA concentration

3. RESULTS AND DISCUSSION:

3.1 Biosynthesis of MCL PHA by *R. eutropha* PHB using palmitic acid as a carbon source & estimation of efficiency of PHA production

In order to find out the efficiency of the organism to produce PHA, the micro organisms was grown under nitrogen limiting condition and the sample was taken for estimation after 48 h. ***R. eutropha*** produced a higher amount of PHA. The concentrations of the polyhydroxy alkanote produced is summarized in Table 1.

3.2 Effect of initial inoculum

The concentration of PHA polymer produced with 2% v/v initial inoculum, by ***R. eutropha*** was 3.96% production of PHA by different inoculum was compared as shown in Table 1.

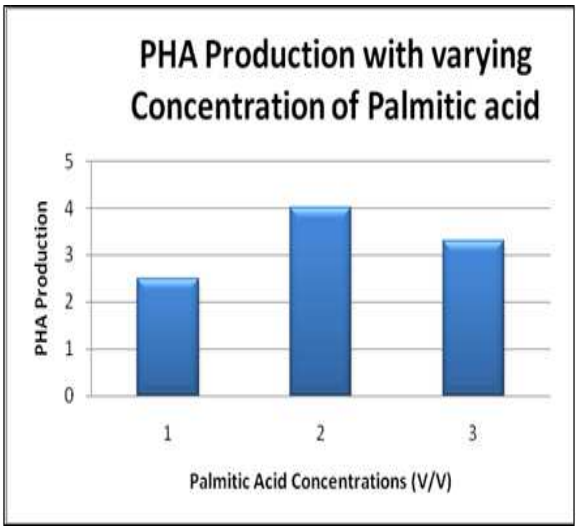
Table 1. Comparison of PHA production by ***R. eutropha*** with different concentration of initial inoculum.

Microorganism	**Polyhydroxyalkanoate (g/L)with different inoculum**		
	1% (v/V)	**2%(v/V)**	**5%(v/V)**
R. eutropha	1.85	3.96	2.41

3.3 Effect of carbon sources with varying concentrations:

To increase the yields of polymer PHA, palmitic acid was used as a sole carbon sources (2% to the nitrogen free medium with the inoculum of 2% (v/v). In this study, ***R. eutropha*** efficiently produced the maximum concentration of 4.14 g/l with palmitic acid.(Figure 1)

Fig 1. Comparison of PHA production by ***R. eutropha*** with different concentration of palmitic acid.



4.0 DISCUSSION:

PHA polymers were accumulated in the cells in nitrogen free mineral media containing palmitic acid as carbon substrates, and the accumulated PHA polymers were subsequently degraded after the carbon sources were exhausted. In this study, three different inoculum of microorganisms were studied to evaluate their PHA production under varying concentration of palmitic acid as a carbon source. Optimization of initial microbial load and suitable carbon source are very important for high production of PHA. Polyhydroxy alkanote obtained in this study were found to be sudanophilic, as confirmed by Sudan black-B staining procedures. Previous reports [13] have confirmed the sudanophilic nature of PHA granules. At high concentration (5% v/v) of inoculums, *R.etropha* was not able to produce high amount of PHA. At low concentration of initial inoculum (1% v/v), *R.etropha* produced low amount of PHA as compared to 2% v/v initial inoculum. This showed that, the higher inoculum of bacterial cells rapidly utilized the already accumulated intra cellular PHA granules as carbon and energy source [33]. *R.* eutrophus produced the maximum concentration of PHA using palmitic acid as carbon substrate compared to sucrose, fructose and lactose. This proved that palmitic acid may act as a better substrate for production of PHA by *R.* eutrophus and it may be compared to other carbon sources as a future studies.

5.0 CONCLUSIONS

These results have proven that palmitic acid can also be used as carbon source to produce PHA by Ralstonia eutropha. A higher formation and accumulation of PHA can be achieved by feeding of an excess carbon source while limiting other essential nutritions through fed batch cultivation method. Fedbatch cultivation strategy can increase both product concentration and specific product yield. In conclusion, this study has led to the preliminary finding of bacterial strains of *R.Eutropha* capable of producing PHA from palmitic acid and that commercial palmitic acid was one of the better substrate for PHAs production by *R. eutrophus.* This preliminary comparative analysis of three inoculum of bacteria with varying concentrations of palmitic acid has led to the novel invention of a biodegradable environment friendly polymer with a high potential for regular use.

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Conflict of interest: NIL

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