World Journal of Pharmaceutical research

Volume 2, Issue 6, 2396-2404. Research Article ISSN 2277 – 7105

BIOSYNTHESIS OF POLYHYDROXYBUTYRATES FROM *AZOTOBACTER CHROOCOCCUM* USING CHEAPER SUBSTRATES UNDER DIFFERENT OPTIMAL CONDITIONS

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Article Received on
21 August 2013,

Revised on 29 Sept. 2013,
Accepted on 24 October 2013

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ABSTRACT

Polyhydroxybutyrate (PHB) is a biodegradable thermoplastic which can be extracted from a wide range of bacteria. PHB belongs to the class of polyesters collectively called polyhydroxyalkanoates (PHA). PHB have similar properties to polypropylene and are important due to their complete biodegradability and can be used as packaging material in drug delivery systems, since these polymers are immunologically inert. Biodegradable polymer which provides a reserve of carbon and energy accumulates as intracellular granules .Several factors influence the economics of biodegradable polymer production one such factor is cost of the substrate. The high cost of PHB can be decreased by strain development and improving fermentation process by using cheaper carbon source. Low-cost raw materials can be used to reduce significantly the production cost of PHB. Considering the industrial interest and its high production cost, work has been undertaken for the production of PHB by *Azotobacter chroococcum* isolated from *Azotobacter* biofertilizer. In this study, six various inexpensive materials were utilized to enhance PHB production. The influence of these additives were directly compared with cultivations on defined medium as well as on the medium supplemented with inexpensive source such as glucose, molasses, whey, wheat bran, starch and sugarcane bagasse under different optimal physical stress conditions. Among the various inexpensive sources tested, high concentration of PHB was obtained from glucose (72%) and molasses (68%) as substrates, the optimal pH was 7.5 (72.5%) as yield with

Glucose supplementation. The optimal temperature was 50^0 C for 30 min (64%) and UV exposure optimum was 10 min (68%). Sudan black staining and gas chromatography confirmed the PHB content in *Azotobacter chroococcum*.

Key words: Polyhydroxybutyrate, Biopolymer, *Azotobacter chroococcum*, Sudan black, Endopolysaccharide.

INTRODUCTION

Plastic materials have become an integral part of contemporary life because of their desirable properties including durability and resistance to degradation. These non degradable plastics accumulate in the environment at the rate of millions of tons per year. Recently problems concerning the global environment and social management have created much interest in the development of biodegradable plastics that still retain the desirable physical and chemical properties of conventional synthetic plastics. Poly-β-hydroxybutyrate (PHB) is the best known Polyhydroxy alkanoate (PHA) and recognized with this lipid inclusion being accumulated by many bacteria as they enter the stationary phase of growth to be used later as an internal reserve of carbon and energy under limited nitrogen and in presence of abundance of carbon source.

PHB have been attracting considerable attention as biodegradable plastics substituting conventional petrochemical plastics because they have similar properties to various thermoplastics and elastomers, which have been used in consumer products, and complete biodegradability upon disposal under various environments. [1] However, one of the major problems in commercializing PHB is the high production cost. This is mainly due to the cost of the substrate and the fermentation strategy used. In order to reduce their production cost, mixed cultures and cheap carbon substrates have been used instead of PHB production from expensive carbon sources and pure culture. [2] A more efficient fermentation process better recovery and purification and the use of inexpensive substrates can also substantially reduce the production cost.[3,4,5,6] Several inexpensive carbon substrates such as molasses, whey, cellulose, plant oils and hydrolysates of starch (Corn and tapioca) can be excellent substrates for the bacteria utilizing them to produce PHB. [1] Low cost (waste-based) substrates have only recently been recognized as PHB raw materials because industrial producers are currently working towards decreasing the cost of biopolymers by increasing the volumetric production capacity of fermentor systems and improving process technology. Moreover, so

far, there has been less attention being made on PHB production under mixed cultures and renewable resources. Hence, the present study attempts the production, using various inexpensive sources as carbon substrates and under optimizing conditions such us different temperatures, pH, time, exposing the organism to UV radiation and temperature shock in a suitable media to compare the production of PHB. The bacteria store their excess energy in the biopolymer PHB, much as humans do with fatty tissue. The levels of PHB extracted can reach a maximum of 70% of the bacteria's dry weight, with the organism being harvested and the polymer separated out.

MATERIALS AND METHODS

Initial culturing of organism

The bacterial isolates grown on selective medium (Mannitol Agar) were identified and confirmed using biochemical tests. *Azotobacter chroococcum* were grown in Mannitol broth at 35^0C for 24h in rotary shaker maintained at 160rpm.

Growth conditions

1ml of culture was inoculated in100ml-Erlenmeyer flask containing production medium. This culture was incubated on a rotary shaker at 300rpm and 35^0C for 48h. From the production medium kept in the incubator, optical density (OD) was taken at 650 nm, to ensure the growth of organism. Simultaneously, optical density at 400nm assay (CARY 50 scan UV/V is spectrum spectrophotometer) was observed for PHB production at the regular intervals of 24h (Fig 1 Growth of *Azotobacter chroococcum* with respect to PHB production). [7].

Comparative evaluation of *Azotobacter chroococcum* with various substrates for optimal production of Polyhydroxybutyrate (PHB)

To the production medium, different substrates like Whey, Molasses, Starch, Glucose, Wheat bran and sugarcane bagasse were added separately with 1% concentration in six 250ml conical flasks respectively.

Optimization

Production medium with varying pH values 5.5, 6.5, 7.5, 8.5 and 9.5 were prepared and inoculated with test organism. The effect of pH on the production of PHB was determined. The inoculated production medium was maintained at varying temperatures ranging from 28^0 C to 40^0 C and their productivity was analyzed to determine the effect of temperature.

Similarly samples were analyzed on 24hr basis for a week and the time of maximum production was determined. The organism was then subjected to UV shock and temperature shock (50^0 C) for 10 min and 30min respectively. The productivity was determined after 48h. In order to optimize the effect of substrate, concentration of various substrates were tested ranging from 1% to 8% under optimized conditions of temperature, pH and time.

Sudan black staining

Cells were air dried and heat fixed in a clean slide .The heat fixed cells were flooded with Sudan black stain for 15 min. To this xylene was added for 10s and allowed to dry. Safranin counter stain was added and allowed to stand for 10s. The counter stain was washed under running tap water, air dried and viewed under microscope.

Biomass harvesting

After preliminary staining, cells were harvested by centrifugation at 10^0 C for 15 min at 5000 rpm and washed twice with distilled water. Pellet obtained was suspended in 5 ml of distilled water and subjected to lyophilization.

Lyophilization

Freeze drying was done at -50^0 C for 10 h at suitable vacuum to remove the bound moisture and to retain the cell in its original form.

Cell disruption

Lyophilized cells were blend with chloroform and were subjected to ultrasonication at 750 Watts for 30min with pulse on and off for 30s.

Extraction of PHB

Viscous solution obtained on ultrasonication was filtered using Watt man filter paper No.1 to separate the biomass. To the filtrate 10 volumes of 95% ethanol was added. A white precipitate was seen at the interface between the filtrate and ethanol which was later separated by centrifuging at 10^0 C for 20min at 8000 rpm. White precipitate of PHB was seen on the sides of the centrifuge tubes which were later air dried for 24h.

Gas chromatography(GC)

The PHB obtained was dissolved in methanol and converted the fatty acids to corresponding methyl esters. These methyl esters were then subjected to GC analysis. GC was equipped with a Carbowax 20M 25 m × 0.25 mm, df = 0.25 µm. The cell density and the peak area

ratios of the monomers and the internal standard allow the determination of the amount of polymer accumulated by the cells and its composition.

RESULTS AND DISCUSSION

Azotobacter chroococcum is the only species that can hydrolyze starch among the *Azotobacter*. [8] It is well adapted to the laboratory conditions of growth and was able to produce significant amount of PHB at a variety of physical conditions when grown on the mannitol containing growth medium (Table 1). There was insignificant growth observed up to 48h in mannitol containing agar at 32^0 C. The colonies were mucoid due to the secretion of polysaccharide. From the results obtained *Azotobacter chroococcum* is regarded as an efficient producer of PHB biopolymer when subjected to different fermentation substrates of carbon sources like glucose, molasses, whey, starch, wheat bran, bagasse respectively. The biopolymer production was comparatively lesser in bagasse and starch containing media (60%), while maximum was obtained when glucose (70%), Molasses (68%), Wheat bran (64%) and whey (62%) were used as substrates (Table 2). Similar results using inexpensive substrates for PHB accumulation have been reported. [9,10,11]

Although *Azotobacter chroococcum* could produce biopolymer at 28^0C incubation, maximum yields were obtained from fermentation done at 34^0C. The formation of endopolysaccharide found to be maximum at temperature 34^0C (72%) (Table 3). This strain was able to produce biopolymer under uncontrolled pH condition also. Maximum production was seen in fermentations where the pH was maintained at 7.5 (72.5%) (Table 4). It is been observed that there was an improvement in polymer production when fermentations were done under aerated condition. Experimental results show that, PHB production was decreased at pH 5.5 (23%). The pH, time and temperature play an important role in both bacterial growth and biopolymer production. When the bacterial cells are exposed to pH beyond their optimum range, maintenance energy is used for pH control. This reduces the energy available for biopolymer production, thus the bacterial ability to produce the biopolymer is reduced. The media pH also affects the permeability of the bacterial cell membrane thus affecting the biochemical activities of the cell required for biopolymer production. The optimized time period showing high production of PHB is 48hr of incubation (Table 5) with gradual decrease in the production. Under UV stress condition and temperature shock PHB production was found to be 68% and 63.6% respectively (Table 6). After optimizing environmental factors

such as pH, incubation time, temperature, UV stress treatment and temperature shock (50^0C). *Azotobacter chroococcum* was able to produce PHB greater than 6mg/mL.

Table 1. Growth of *Azotobacter chroococcum* with respect to PHB production

Time (h)	Growth (650nm)	Phb produced (235nm)
24	2.3154	0.9653
48	3.5698	2.5421
72	2.5264	2.1045
96	1.6985	2.0021
120	1.4298	1.9563
144	1.0215	1.0047

Table 2.Effect of different Substrate on PHB production

Substrate (1%) (g)	Pellet weight (g)	Dry Weight (g)	PHB(g)	Yield %
Glucose	6.2314	5.7081	3.9956	70%
Molasses	5.9241	5.3315	3.6254	68%
Whey	5.7210	5.2152	3.2334	62%
Starch	5.4518	5.1932	3.1159	60%
Wheatbran	5.5123	5.4020	3.4572	64%
Bagasse	5.4782	5.2321	3.1392	60%

Table 3. Effect of temperature on PHB production

Temperature (ºC)	Pellet weight(g)	Dry weight(g)	PHB(g)	Yield%
28 ºC	5.0591	4.8724	3.0208	62%
30ºC	5.3958	5.0621	3.3156	65.5%
32ºC	5.7085	5.3806	3.7664	70%
34ºC	5.8001	5.4542	3.9270	72%
36^0 C	5.5891	5.1758	3.5195	68%
38^0 C	5.2185	4.9670	3.1937	64.3%

Table 4 .Effect of pH on PHB production using glucose as a standard

pH	Pellet weight(g)	Dry weight(X)(g)	PHB(g)	Yield %
5.5	4.6232	4.0215	0.92494	23%
6.5	5.2173	4.9261	2.9556	60%
7.5	5.7392	5.5219	4.0033	72.5%
8.5	5.6321	5.2163	3.5470	68%
9.5	4.7925	4.4137	1.4123	32%

Table 5. Effect of time on PHB production

Time (H)	Pellet weight (g)	Dry weight (g)	PHB(g)	Yield %
24	3.9321	3.6895	0.7579	20%
48	5.9634	5.6437	3.8775	68.7%
72	5.8321	5.5305	2.2122	40%
96	5.7632	5.4021	1.2424	23%
120	5.7891	5.4691	0.5469	10%

Table 6.PHB production with respect to different stress conditions

Stress conditions	Pellet weight (g)	Dry weight (g)	PHB(g)	Yield %
UV shock for 10min	5.4329	5.2172	3.5581	68%
Temperature shock(50 ºC) for 10 Min	5.1980	4.7316	3.0092	63.6%

The white powder that was obtained under optimized conditions of temperature, time, pH and different substrates by *Azotobacter chroococcum* was quantitated using gas chromatography. Therefore the production of biopolymer with commercial importance by different substrates utilization under different stress conditions ensures that not only value added products are

obtained, but the products such as whey, molasses, wheat bran and starch can also be fruitfully utilized.

CONCLUSIONS

Bioplastics are becoming more and more useful in daily life. Though a wide variety of microbes are able to produce it, their commercial production is remained expensive. Strain development, improved fermentation process and alternate substrates are different options to overcome this. Considering the medical and pharmaceutical interest of this compound, the present study *Azotobacter chroococcum* isolated from *Azotobacter* biofertilizer as an agent. Further, six various inexpensive materials such as glucose, molasses, whey, wheat bran, starch and sugarcane bagasses were utilized to enhance PHB production. The influence of these additives were directly compared with cultivations on defined medium as well as on the medium supplemented with inexpensive source under different physical conditions. It has been found that glucose and molasses as a substrate, at pH 7.5 was the most optimal condition for maximum yield.

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