The Ecoscan 10(1&2): 81-84, 2016
AN INTERNATIONAL QUARTERLY JOURNAL OF ENVIRONMENTAL SCIENCES
www.theecoscan.com

POLY-β-HYDROXY BUTYRATE PRODUCTION IN BACTERIA EMPLOYING ECOFRIENDLY AND RENEWABLE AGRIBYPRODUCTS

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ABSTRACT

In the present study, Efforts were made to optimize conditions for maximum growth of three bacterial strains B2, B3 and C6 in presence of nutrients like agribyproducts, using glucose and fructose as carbon sources and mustard cake and yeast extract as nitrogen source. Three bacterial strains B2, B3 and C6 exhibited a maximum harvestable PHB as 0.031g/l, 0.027g/l and 0.21g/l respectively. To determine the best growth potential, these strains were grown in different combinations of organic and inorganic carbon and nitrogen sources. The carbon sources glucose and fructose and nitrogen sources mustard cake and yeast extract yielded good growth and PHB production. The extracted PHA granules were analyzed with Fourier transform infrared (FTIR) spectroscopy which demonstrated the presence of standard Polyhydroxybutyrate (PHB).

KEY WORDS
Polyhydroxybutyrate
Agribyproducts
Biodegradable, Bioplastics, FTIR, PHA, Ecofriendly

Received : 18.10.2015
Revised : 08.02.2016
Accepted : 11.03.2016

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INTRODUCTION

Poly hydroxy alkanoates (PHA) are biodegradable and compostable bioplastics that have been used as a substitute to petroleum-based synthetic plastics which is the main reason of environmental pollution. The use of biodegradable plastics will help to clean environment by dropping off non-biodegradable solid wastes and will help to beauty the environment (Sudesh and Iwata 2008). It was first discovered in the bacterium *Bacillus megaterium* (Lemoigne, 1926). PHAs are a type of polyester polymer produced by many microorganisms and plants (Shamala *et al*., 2003). PHAs are stored as discrete granules to levels as high as 90% of cell dry weight as a when bacteria encounters environmental strain and nutrient imbalance (i.e. nitrogen and phosphorus restrictive conditions) and plays an important function as a sink for carbon and energy. These storage polymers are water insoluble, biodegradable and eco-friendly. These biopolymers exhibit thermoplastic properties like petroleum based plastics and can be produced from different renewable carbon sources (Brandl *et al*., 1990) like agri by products (molasses, cotton cake and mustard cake). PHAs are high molecular mass polymers with properties comparable to conventional plastics (Reddy *et al*., 2003). Therefore, they have a wide range of applications, such as in the manufacture of shampoo bottles, as hardeners in cosmetic products, for hygiene products, packaging products and golf tees packaging materials; films for agriculture and also in medical applications (Oliveira *et al*., 2004, Khanna and Srivastava 2005). Theses polymers are completely degraded to water, CO_2 and methane by anaerobic microorganisms in various environments such as soil, sea, lake water and sewage and hence, is ecofriendly (Brandl *et al*., 1988). But cost of PHB production is still very high and it is urgent need to reduce the cost and recovery of PHB production. The cost of carbon feed stocks can make a considerable influence on the PHA production cost. Therefore, the identification of alternative renewable and cost-effective substrates for the production of PHA has become an important objective for the commercialization of bio plastics. Several investigations suggested that microorganisms like *Alcaligens eutrophus* (Jian, 2001) and *Alcaligens latus,* Actinobacillus, Azotobacter, Agrobacterium, Rhodobacter, Sphaerotilius and Rhizobium have focused on converting organic waste to bacterial PHA and these microorganisms are able to slot in up to 150 different type's monomer into their storage polymer and form a material with a variety of characterstics. The majority of PHAs are composed of R(-)-3-hydroxyalkanoic acid monomers ranging from C3 to C14 carbon atoms with a range of saturated or unsaturated and straight- or branched chain containing aliphatic or aromatic side groups (Doi *et al*., 1992, DeSmet *et al*., 1983). The major drawback in the use of biodegradable plastic is their high cost as compared to the synthetic plastic. It is well established that the cost of production of any microbial product can be reduced significantly if these micro-organisms are harvested on low cost media having agro-industrial byproducts since media is the main component that add to cost of production

(Dhingra, 2012; Goyal and Dhingra 2011). Effect of cheaper carbon and nitrogen sources on PHB production was analyzed by using cheap substrates (Priya Kumari and Harish Kumar, 2013). In this study, several strains of PHA accumulating bacteria from different samples like soil, sewage, cropped lands were isolated and characterized for their morphological, cultural and physical properties. In addition, comparison of PHA production by the selected strains was done in Minimal Media with different combinations of carbon and nitrogen sources. The major objective of this study was to reduce the cost of production.

MATERIALS AND METHODS

Bacterial strains were isolated and screened from soils of different places like oil spilled soil, cropped lands, sewage water, garden soil and contaminated sites. One mg of different soil samples were dissolved in 1 ml of sterile distilled water and serial dilutions were made 5-6 times. 100 microliter of samples from 4th & 5th dilution were taken and spread on plates containing TY or LB media. The bacterial colonies appearing were purified by repeated streaking on fresh media and analyzed for their PHB production ability. Out of a total of 500 isolates based on orange/yellowish florescence produced by these strains under UV light using minimal media containing Nile blue dye (Ostle and Holt, 1982), three bacterial strains B2, B3 & C6 were selected for present study. These isolates were identified by using cultural, morphological and biochemical tests. These isolates were evaluated for PHB production on the basis of growth on minimal media having glucose as carbon source and KNO_3 as nitrogen source. Theses bacterial isolates were selected on the basis of presence of fluorescence on Nile BlueA containing media as Nile Blue get integrated with PHB granules and PHB producer bacteria fluoresce under ultraviolet light. The screening for intensity of fluorescence indicates the quantity of PHB produced. The biomass was harvested by centrifuging them at 6500 rpm and bacterial cells were lysed chemically by with sodium hypochlorite hydrolysis method (Law and Slepecky, 1961) and chloroform and PHB extracted using chloroform. An aliquot of mixture was heated at 65 °C for 3h and dried till a constant weight was obtained to calculate the cell biomass and PHB recovery. The PHB yields were determined and computed and expressed as micro grams per unit mass of cells. Selected strains were screened on different combinations of cost effective carbon and nitrogen sources and a low cost protocol has developed.

RESULTS AND DISCUSSION

To make the PHB production cost effective and expeditious, growth kinetics of bacterial strains were studied and to develop an economical protocol for PHB production formulations of media containing carbon and/or nitrogen from agri-byproducts were utilized as supplement for expansive carbon and nitrogen source. PHB production kinetics of three strains is shown in Fig.1. Keeping Minimal media as control media with several component substitutions were used to optimize PHB production. The optimum condition for maximum PHB productions were found when glucose minimal media with some modifications was used having the following

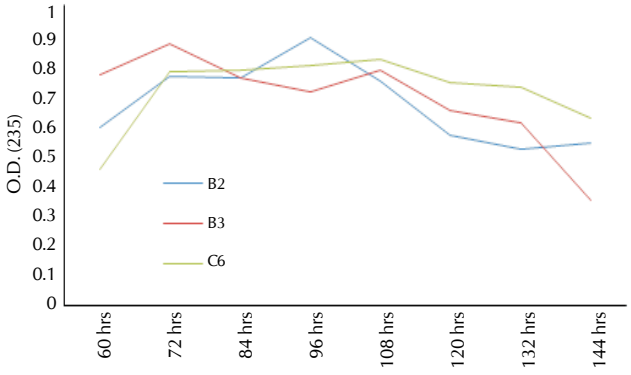

Figure 1: Kinetics of PHB production in three strains expressed as Crotonic acid (O.D. 235)

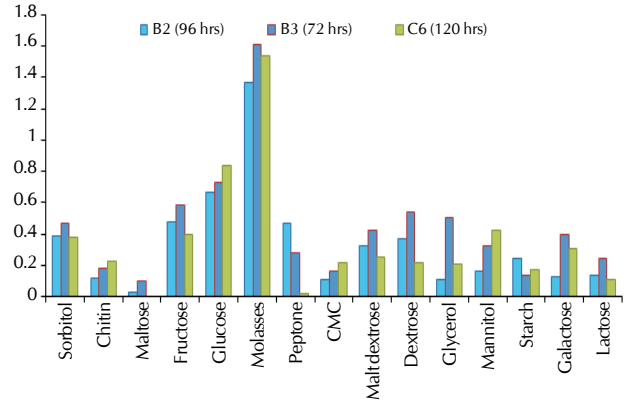

Figure 2: Effect of different carbon sources on PHB (expressed as Crotonic acid (O.D. 235)

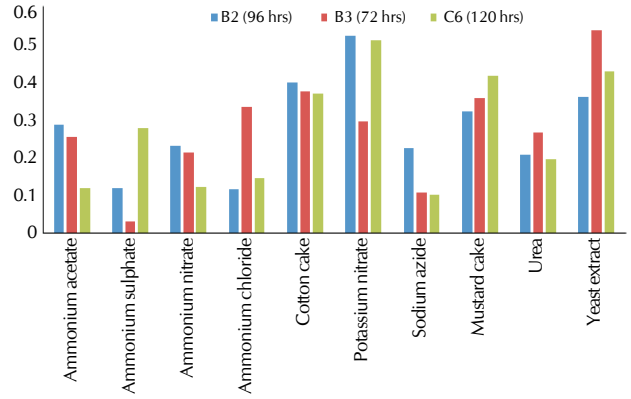

Figure 3: Effect of different nitrogen sources on PHB (PHB expressed as Crotonic acid (O.D. 235)

compositions: glucose (3 g/L), KNO_3 (0.3 gl/L), KNO_3 (0.3 gl/L), K_2HPO_2 (0.6 gl/L), $MgSO_4.7.H_2O$ (0.05 gl/L) and $CaCl_2$. $2H_2O$ (0.02 gl/L) and another media with potato peels (4 gl/L), yeast extract (0.6 g/L), K_2HPO_2 (0.6 gl/L), $MgSO_4.7.H_2O$ (0.05 gl/L) and $CaCl_2.2.H_2O$ (0.02 gl/L).

Kinetics of PHB production in three strains

The progress of PHB production is determined as growth phases in terms of time after inoculation during the total growth cycle. Kinetics of intracellular PHB synthesis was studied in

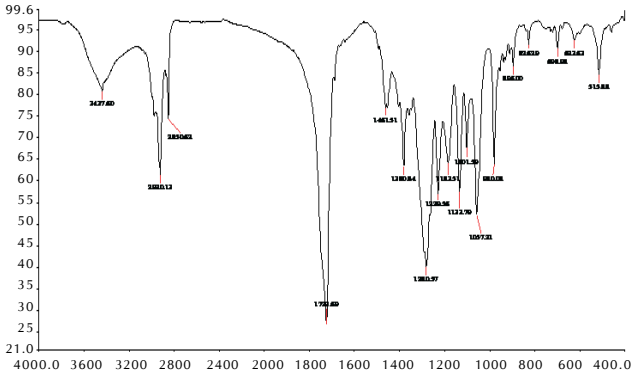

Figure 4: FTIR spectrum of PHB produced by strain B3 peaks at 29

bacterial isolates B2, B3 and C6 using one litre of minimal medium and incubated at 37ºC. Samples were drained at incremental time intervals spread over several days and the amount of PHB was extracted using chloroform and sodium hypochlorite method and quantified by Crotonic acid estimation and PHB recovery. As per the result shown in fig. 1, it was observed that strain B3 gave the maximum PHB production in just 72h while strain B2 gave maximum PHB production in 4 days and strain C6 took maximum 5 days.

Effect of different carbon sources on maximum PHB production

In B2 strain, the Crotonic acid formation (Fig. 2) was 51% more than glucose and 65% more than that of fructose. Crotonic acid formation with carbon source molasses also showed 54.5% more than glucose and 63.3% more than fructose in case of strain B3. In C6 strain, molasses showed 45.6% more Crotonic acid formation than glucose and 74.1% more than fructose.So, amongst the isolates, B2 was found to be a significantly higher PHB producer as compared to B3 and C6. Amongst different carbon sources, molasses (3gm/l) was found to be the best carbon source for PHB production. The next capable carbon source was glucose and fructose.

Effect of different nitrogen Source on PHB production

The Crotonic acid formation in B2 strain (fig.3) with nitrogen source KNO_3 found to be 30% more than yeast extract and 23% more than cotton cake. In case of B3 strain yeast extract was the best nitrogen source which gave 50% more Crotonic acid formation than KNO_3 and 30% more than that of cotton cake whereas in C6 strain, KNO_3 was the better nitrogen source than yeast extract and cotton cake. It was found that KNO_3 gave 16.3% more Crotonic acid formation than yeast extract and 27.7% more than cotton cake. Amongst the isolates B3 was found to be a significantly higher PHB producer compared to B2 and C6.

Amongst different nitrogen sources, potassium nitrate (1gm/L) for B2, yeast extract for B3 and KNO_3 for C6 were found to be the best nitrogen sources for PHB production. The next promising and cheap nitrogen sources were cotton cake and mustard cake.

PHB production using carbon and nitrogen from agri-byproducts

Different carbon sources from agri-byproducts and conventional carbon sources such as molasses, potato peels, fructose, glucose and their combination along with defined nitrogen source were used for comparison of PHB production in two selected strains B2 and B3. In B2 strain, it was observed that when molasses was used as a carbon source in combination with nitrogen sources KNO_3 and yeast extract, then the cell pellet was 39.2% more than Potato peels + fructose and 27.2% than potato peels + glucose whereas Crotonic acid formation was only 1.23% more than glucose and 35.7% more than glucose when molasses were used as carbon source. On the dry weight basis, it was found when molasses were used in media as carbon source, the PHB weight was 25% more than fructose and 32.1% more than glucose. So, molasses was best and cheap carbon source when used in combination with potassium nitrate and yeast extract. In another observation,

Table 1: Optimized PHB production using different combinations of carbon and nitrogen sources B2 strain

S. No.	Carbon Source	Nitrogen source	Pellet wt (g/l)	PHB recovery (Crotonic acid)	PHB wt. (g/l)
1.	Fructose(2g) + potato peels(1g)	KNO_3 (0.65g)	1.968	0.626	0.019
2.	Glucose(2g) + potato peels(1g)	KNO_3 (0.65g)	1.669	0.986	0.019
3.	Molasses(3g)	KNO_3 (0.3g) + YE(0.1g)	2.705	0.974	0.028
4.	Molasses(3g)	Mustard cake (1g)	2.851	1.039	0.034
5.	Glucose(3g)	Mustard cake (0.6g) + YE (0.2g)	2.667	1.075	0.037
6.	Fructose(3g)	Mustard cake(0.6) + YE (0.2)	1.410	0.959	0.026

Table 2: Optimized PHB production using different combinations of carbon and nitrogen sources B3 strain

S. No.	Carbon Source	Nitrogen source	Pellet weight (g/l)	PHB recovery (Crotonic acid)	PHB wt. (g/l)
1.	Fructose(2g) + potato peels(1g)	KNO3 (0.65g)	1.968	0.986	0.019
2.	Fructose(2g) + potato peels(1g)	YE(1g)	2.022	0.725	0.014
3.	Molasses(3g)	KNO3 (0.3g) + YE(0.1g)	2.705	0.893	0.028
4.	Molasses	Mustard cake + KNO3	2.565	0.881	0.028
5.	Glucose	Mustard cake	1.651	0.661	0.025
6.	Fructose	Mustard cake + YE	2.225	0.814	0.031

It was found that when mustard cake was used as nitrogen source, cell pellet was 6.45% more than glucose + mustard cake + YE and 50% more than fructose + mustard cake + Yeast Extract. Crotonic acid estimation showed that when mustard cake + YE were used in combination, then it was 3.4% more than mustard cake when it was used alone. Dry weight of PHB was about 29.7% more when mustard cake + YE used in combination than the mustard cake alone. So, it was found that in case of B2 strain nitrogen sources mustard cake and yeast extract were better nitrogen sources than cotton cake and KNO_3. In B3 strain, it was observed that when molasses was used as a carbon source in combination with nitrogen sources KNO_3 and yeast extract, then the cell pellet was 25% more than Potato peels + fructose + YE and 27.2% than potato peels + glucose + KNO_3 whereas Crotonic acid formation was only 9.4% more than Potato peels + fructose + YE and 27.2% more than potato peels + glucose + KNO_3. On the dry weight basis, it was found when molasses were used in media as carbon source, the PHB weight was 32.1% more than Potato peels + fructose + YE and 50% more than potato peels + glucose + KNO_3 So, molasses was best and cheap carbon source when used in combination with potassium nitrate and yeast extract (Table 2). Thus combination of agri- byproducts as a carbon sources proved as better carbon source than conventional carbon sources employed. It was also observed that when mustard cake was used in combination of KNO_3 as nitrogen source, cell pellet was 35.6% more than glucose + mustard cake and 13.2% more than fructose + mustard cake + Yeast Extract. Crotonic acid estimation showed that when mustard cake + KNO_3 were used in combination, and then it was 24.4% more than mustard cake when it was used alone 7.6% more than mustard cake + yeast extract. Dry weight of PHB was about 10.7% more when mustard cake + YE used in combination than the mustard cake alone and 9.6% more than mustard cake + KNO_3. So, it was found that in case of B3 strain, nitrogen sources mustard cake and yeast extract were better nitrogen sources than cotton cake and KNO_3.

FTIR spectrum analysis

Fourier-transform infrared spectroscopy (FTIR) has been demonstrated to be a powerful tool for screening various types of PHAs. It is used to detect functional groups in an organic compound. FTIR spectra of PHBs that were produced using different carbon and nitrogen sources were recorded in KBr (GJUS & T, Hisar). The FTIR spectroscopic analysis of B3 strain was done using Fourier Transform Infra Red (FTIR) spectrum of the PHB sample revealed 4 major peaks at 2920, 1722, 1461, 1250 cm-1, whereas the remaining peaks are closely lying between 3430 cm-1 and 649 cm-1. The predominant peak at 2921 and 1461 which represents the methane groups, followed by a peak at 1722 corresponds to C = O stretch of an ester group present in highly ordered crystalline structure whereas the peak at 1276 correspond to CH group. The presence of these marked peaks demonstrated the presence of PHB in strain B3.

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