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Effective Utilization and Management of Coir Industrial waste for the Production of poly- β- hydroxybutyrate (PHB) using the Bacterium *Azotobacter Beijerinickii*

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ABSTRACT: Coir pith, a byproduct of coconut fibers and waste material from the coir industry, is stable and not easily degradable due to its high lignin content. Coir pith takes a decade to decompose thereby posing environmental hazard and disposal problem. Pollution by plastics creates an alternative solution to reduce problems. Hence the potential use of coir industrial wastes for production of bioplastics (Poly-β-hydroxybutyrate - PHB) as an alternative to plastics was studied. Commercial production of this polymer is limited, however, due to higher cost and longer fermentation process as compared to petrochemical plastics. These concerned make it necessary to use the cheapest and readily available raw materials. *Azotobacter beijerinickii* used coir industrial waste as a substrate and produced PHB. Production of PHB was maximized at pH 6.5 with 3% coir hydrolysate. The amount of PHB produced by *A. beijerinickii* was 2.4 ± 0.2 g/L. The yield was 48.19 %. Production of PHB was confirmed by Sudan black B staining under a light microscope, acridine orange staining under a fluorescent microscope and by an infrared spectrometer. This investigation showed that coir industrial waste could be effectively used for the production of PHB.

Key words: Biopolymer, Bioplastic, PHB, Coir waste, Azotobacter *beijerinickii*

INTRODUCTION

Industrialization, urbanization, improper agricultural practices and various anthropogenic activities are responsible for pollution and loss of environmental quality. Problems concerning the global environment have created much attention in developing eco-friendly products (Rawte and Mavinkurve, 2002). Biopolymers are one product that can help to overcome problems caused by petrochemical polymers. Biopolymers are generated from renewable natural sources and are often biodegradable and nontoxic (Poirier, 1999; Flieger *et al*., 2003). They can be produced by biological systems (microorganisms, plants and animals), or chemically synthesized from biological materials (sugars, starch, natural fats and oils, etc.) (Flieger *et al*., 2003).

Polyhydroxyalkanoates (PHA) are a good substitute for plastics and elastomers. The PHAs have characteristics similar to petrochemical plastics but are biodegradable (Anderson and Dawes, 1990; Doi, 1990a). PHAs are polyesters, which are accumulated as energy and/or carbon storage materials by numerous microorganisms, usually when a nutritional component such as nitrogen, phosphorus, sulfur, oxygen, and magnesium is limited in the presence of an excess carbon source (Anderson and Dawes**,** 1990**;** Lee, 1996; Page, 1992**;** Ribera, *et al*., 2001; Wang and Lee, 1997). At present, they are produced by microbial fermentation; in the future, production will also be possible by *in vitro* methods or by agriculture using transgenic plants (Steinbuchel and Fuchtenbusch, 1998). In case of microorganisms, PHA serves as a carbon and energy source during starvation and is rapidly oxidized thereby retarding the degradation of cellular components (Rawte and Marinkurve, 2001).Poly-β-hydroxybutyrate (PHB), the most commonly found member of the PHA family, is a thermoplastic with many desirable properties. It is biodegradable and the current market demand for a biodegradable thermoplastic is enormous (Lafferty, *et al*., 1988 and Khanafari, *et al*., 2006). The physical properties of the homopolymer of PHB are similar to those of polypropylene (melting point, crystallinity, glass transisition and temperatures), and represents a stiff and brittle material (Thakor *et al*., 2006). They can be used in a wide variety of products including containers, bottles, razors and food packaging materials. The latex of PHAs can be used

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to produce a water-resistant layer for paper, film or cardboard (Hocking and Marchessault, 1994). PHA/ PHB can be very useful in medical applications, such as implants, gauzes, suture filaments, osteosynthetic materials, and a matrix material for slow release drugs and *in vitro* cell cultures (Zinn *et al*., 2001; Sudesh, 2004; Chen, 2005; Chen and Wu, 2005a,b; Sudesh and Doi, 2005; Suriyamongkol *et al*., 2007). Because of the versatile applications as thermoplastic biopolymers, PHA can also be used for natural fiber composites, or as binder in paints, and various applications in medicine and pharmacy (Shanks *et al*., 2004).

Though several organisms that accumulate PHA have been identified, commercial production of this polymer is limited. Several factors influence the economics of biodegradable polymer production. One main factor is cost of the substrate. The ability to produce biodegradable polymers from inexpensive and renewable carbon sources may improve the economics of the process and lower production cost (Hanggi, 1995; Khanfari *et al*., 2006). One of the cheapest raw materials is coir pith, byproduct of coir processing industry that produces coconut fibers. Large amounts of coir pith accumulate nearby coir processing units (approximately 7.5 million tones annually in India), causing severe disposal problems, fire hazards and ground water contamination due to the release of phenolic compounds (Namasivayam *et al*., 2001). Coir pith contains 87% of organic matter and 13% of ash content (Thampan, 1987). So recycling of industrial wastes is one way of disposal mechanism and another may be resource management. In the present study, to evaluate the potential value of coir pith, PHB has been produced from coir industrial waste applying a bacterial species, *A. beijerinckii.*

MATERIALS & METHODS

A. beijerinickii MTCC 2641 was procured from the Microbial Type Culture Collection and Gene Bank of Institute of Microbial Technology, Chandigarh, India for the production of PHB. *A. beijerinckii* was grown in the selective medium containing: 0.2g KH_2PO_4; 0.8g K_2HPO_4; 0.2g $MgSO_4$; 0.1g $CaSO_4$; 0.59g Yeast extract; 20g sucrose; $FeCl_3$ (trace) at 37°C for 24 h. Flasks containing 50 mL of specified medium were inoculated with a loopful of cells. After 48h of growth at 37°C, the medium attained cloudiness and used as inoculum for the production of PHB.

Coir industrial waste was collected from the coir pith processing industry located in Tenkasi (Tamil Nadu, India) in a sterile container. The waste material was washed water, sun dried for two days and dried at 80°C for over night in hot air oven. Then dried sample was ground to fine powder so the samples passed through a 35-mesh sieve. Concentration of cellulose, hemicellulose and lignin were estimated (Updegraff, 1969; Goering and Vansoest, 1970). Coir industrial waste was first partially delignified by autoclaving (120°C, 15 lb, 20min). After cooling, it was hydrolyzed by the enzyme (Cellulase, Hi-media). Twelve filter paper activity units (FPU) of cellulase were added per gram of substrate. The mixture was kept at room temperature (37 ± 2°C) for 24h. The amount of reducing sugar released in the coir waste hydrolysate was estimated spectrophotometrically using dinitrosalicylic acid (DNS) reagent (Miller, 1959).

Optimum concentration of the hydrolysate was determined by incubating 5% (V/V) of the culture with 1 to 5% coir waste hydrolysate. Optimum pH for the maximum growth of the organism was studied by incubating 5% (V/V) of culture into the specific medium with pH from 4.5 to 8.5. Growth of the organism was measured by determining the dry cell weight (DCW). Culture samples (10 mL) were centrifuged at 2,000 *g* for 10 min and the cell pellet was washed in deionized water (6,000 *g* for 5 min) and dried to constant weight (90°C, 24 h), cooled in a desiccator and weighed (Grothe *et al*., 1999). Culture of *A. beijerinckii* (5% V/V) was grown for the production of PHB in the medium containing the optimized concentration of hydrolysate and pH. The medium was supplemented with meat extract (1%) and NH_4Cl (0.5%). Flasks were incubated at 37°C for 48 hrs.

After incubation, PHB produced was extracted chemically. 10 mL of culture was centrifuged at 2,000 *g* for 10 min. The collected pellet was treated with 10 mL of sodium hypochlorite and the mixture was incubated at 30°C for 2 hrs. After incubation, the mixture was centrifuged at 2,000 *g* for 15 min and washed with deionized water, acetone, methanol respectively for washing and extraction. After washing, the pellet was dissolved in 5 ml of boiling chloroform; chloroform was evaporated by pouring the solution on sterile glass tray kept at 4°C. After evaporation the powder was collected for further analysis (Rawate and Mavinkurve, 2002; Kumar and Prabakaran, 2005). The dried PHB was weighed and the yield was defined as a percentage of the dried PHB to DCW.

PHB granules in the cells were determined by Sudan black B staining under a light microscope (Norris and Swain, 1971). PHB granules were determined by fluorescence staining method using acridine orange (Kumar and Prabakaran, 2005). For infrared analysis of PHB, 1 mg of sample was ground well with 10 mg of spectral pure anhydrous potassium bromide crystals and the powder was made into a pellet. The relative intensity of transmitted light energy was measured against the wavelength of absorption 4000 – 400 cm^{-1} using JOEL-FT IR-4000 plus double beam spectrometer.

Data were subjected to a one-way analysis of variance (ANOVA) and Tukey test using SPSS -11 package to determine the level of significance of variation in bacterial growth and PHB production caused by the variables, coir hydrolysate concentration and pH. All experimental data were expressed as mean ± SD. Probability (*P*) values of < 0.01was considered significant.

RESULTS & DISCUSSION

PHB, a biodegradable thermoplastic are accumulated by a wide range of bacteria as carbon/energy or reducing-power storage material (Salehizadeh and Loosdrecht, 2004). Their synthesis is seen as an attractive system for the sustained production of large amounts of biodegradable polymers at low cost (Poirier, 2002). Coir pith is a byproduct of the coir processing industry posing disposal problems (Christopher, *et al*., 2007). In the present study, we estimated the chemical composition of the coir pith. Coir contained 42.14 ± 3.2 % of cellulose, 43.26 ± 4.1 % of lignin and 0.86 ± 0.07 % of hemicellulose. Lignocellulose is the most abundant constituent of coir pith and provides simple sugars on hydrolysis. The reducing sugar content in the coir industrial waste hydrolysate was 30 ± 2.4 %.The composition and properties of coir industrial waste vary depending on the maturity of the coconut, method of extraction and disposal, period between extraction and disposal. Coir pith obtained from fully mature nuts has higher amounts of polysaccharides and fewer water soluble salts compared with younger nuts (Savithri and Khan, 1994). A study of Murugesan and Ruby (2004) revealed that cassava starch industrial waste could be used for the production PHB employing microbes such as *R. eutropha* and *Arthrobacter viscosus.* Kocer, *et al*. (2003) obtained PHB from corn oil acids and a mixture of glucose (15 g/L) and acetic acid (2.5 g/L) employing *Pseudomonas* sp. and *Alcaligenes eutrophus*. Poirier (2002) summarized that the application of autoretransgenic plants for the PHB synthesis. In the present study, among the different concentrations of coir hydrolysate tested, the maximum yield was noted in medium containing 3% of the coir hydrolysate (Fig. 1). The differences in DCW of *A. beijerinckii* due to hydrolysate concentrations were significant at the 1 % level (F=114.24; d.f=4; P<0.01). *A. beijerinckii* showed maximum growth at pH 6.5 in which the highest DCW (g/100mL), 0.498 ± 0.04 was observed, where as it was 0.280 ± 0.04, 0.31 ± 0.06, 0.296 ± 0.02 and 0.198 ± 0.04 at the pH of 4.5, 5.5, 7.5 and 8.5 respectively. Optimization of fermentation conditions has been used to substantially enhance yield and productivity of many bioprocesses (Chisti and Young, 1999). The pH values may have affected the bioavailability of some trace elements; pH values that differed even slightly from the optimum value reduced culture performance (Grothe, *et al*., 1999). When *R. eutropha* MTCC 1285 was grown at pH 8.0 with sago effluents and molasses as raw materials, more PHB was produced as compared to production at pH 9.0 (Kumar and Prabakaran, 2005). In this study, the amount of DCW was optimized at pH 6.5. Yield of PHB (%) was calculated only from the DCW of the bacterial cells determined. The differences in DCW of *A. beijerinckii* due to pH were significant at 1 % level (F=21.02; d.f=4; P<0.01).

In the present study, production of PHB was qualitatively confirmed by the formation of a thin layer on extraction with chloroform which may be due to the presence of interlinked PHB granules. In the absence of PHB production, such a film was not formed. It was weighed (dry) and the amount of PHB produced was

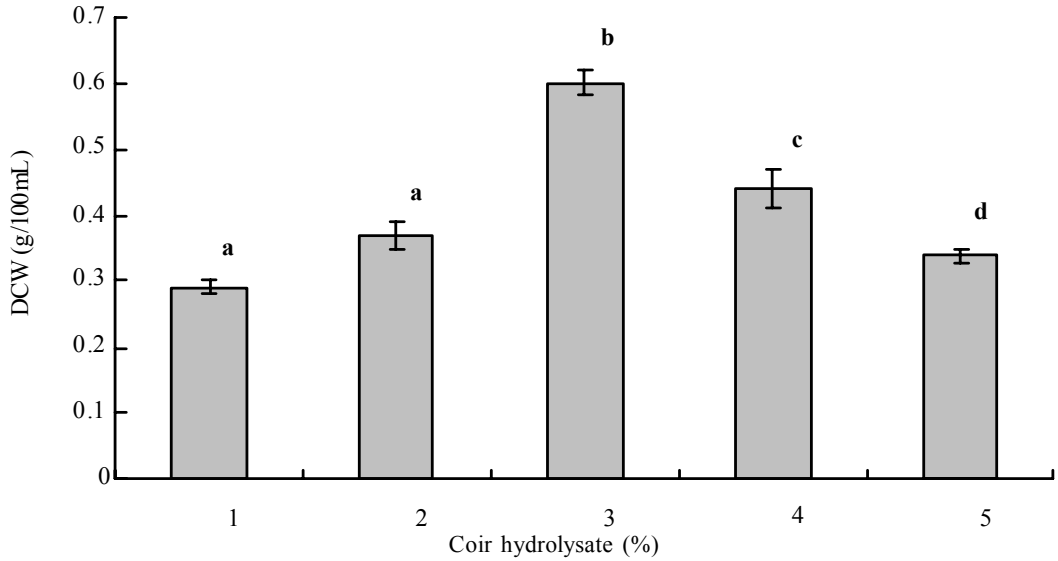

Fig.1. Influence of coir industrial waste hydrolysate on growth of *A. beijerinckii*. Means (±SD) followed by the same letters above bars indicate no significant difference (P<0.01) according to Tukey test

calculated. Chloroform extraction has been widely used to recover PHB with a high degree of purity without polymer degradation (Ramasamy *et al*., 1994). PHB can be extracted with hypochlorite solution, which is a highly degradable procedure and decreases the molecular weight of the isolated granules presumably by the removal of their outer layer (Winfered and Robards, 1973). In this study, *A. beijerinckii* produced 2.4 ± 0.2 g/L of PHB with the yield of 48.19 %.

In the present study the coir waste hydrolysate was supplemented with meat extract for the maximum production of PHB because Khanafari, *et al*. (2006) obtained maximum production of PHB in cultures of *A. chrococcum* 1735 grown in meat extract supplemented whey broth medium under suitable conditions. In this study, for PHB production, cultures were not shaken because O_2 values increased and decreased yield (Katie, *et al*., 2003; Khanafari, *et al*., 2006). The increasing O_2 values by shaking at 122 rpm decreased PHB yield from 4.43 to 0.04 g/L by *A. chroococcum* (Khanafari *et al*., 2006). In the present study, Sudan Black B staining also clearly showed the presence of PHB granules. Light microscope investigations of the cells, stained with Sudan Black B provide easy detections of PHA in the cells. In Sudan black B staining, lipid inclusion granules are stained blue – black or blue-grey, whilst the bacterial cytoplasm is stained light pink (Collee *et al*., 1989). Discrete granules of PHA generally occurred in the cytoplasm as inclusion bodies of irregular morphology with a diameter of about 0.2 – 0.5 nm (Vincenzini *et al*., 1990). Proteins and lipids associated with PHA granules apparently originate from a 2 nm thick coat surrounded the granules.The proteins associated with PHA possess PHA synthease and depolymerase activities (Doi, 1990b). Production of PHB was identified with fluorescence; cells became enlarged and the granules were observed. Normal cells were not enlarged. PHB granules exhibited a strong orange fluorescence which was stained by the Nile Blue A (Oste and Holt, 1982). FTIR spectrum of PHB was recorded in the range of 4000-400 cm^{-1} and spectroscopic analysis showed the presence of broad bands responding to the groups OH, C-O and C=O indicating the structure similar to PHB (Fig. 2).

At low frequency, the functional groups can be identified by their characteristics vibration transmittance makes the FTIR spectrum the simplest, most rapid and often most reliable means for assigning a compound to a class. The FTIR absorption peak values of PHB from *A. beijerinckii* ranged from 4000 - 3122.19/cm (Intra molecular H bond), 2238/cm – 2924/ cm (O – H stretching), 1710/cm (C = O stretching), 1401.03/ cm (O=H tertiary alcohol), 1076/cm (C-O stretching). PHB production from the organisms was confirmed under the FTIR spectroscopy (Mazarevica *et al*., 1984). The native PHB granules possess definite structural features and their crystal structure could be revealed by FTIR absorption and under an electron microscope (Merrick *et al*., 1965). The FTIR spectrum analysis of the PHB product clearly reveals its purity. The absorbance peak values obtained were compared with the available literature values and confirmed the product as PHB. The peak values obtained in this study coincides with previous results (Ramsay, *et al*., 1994;

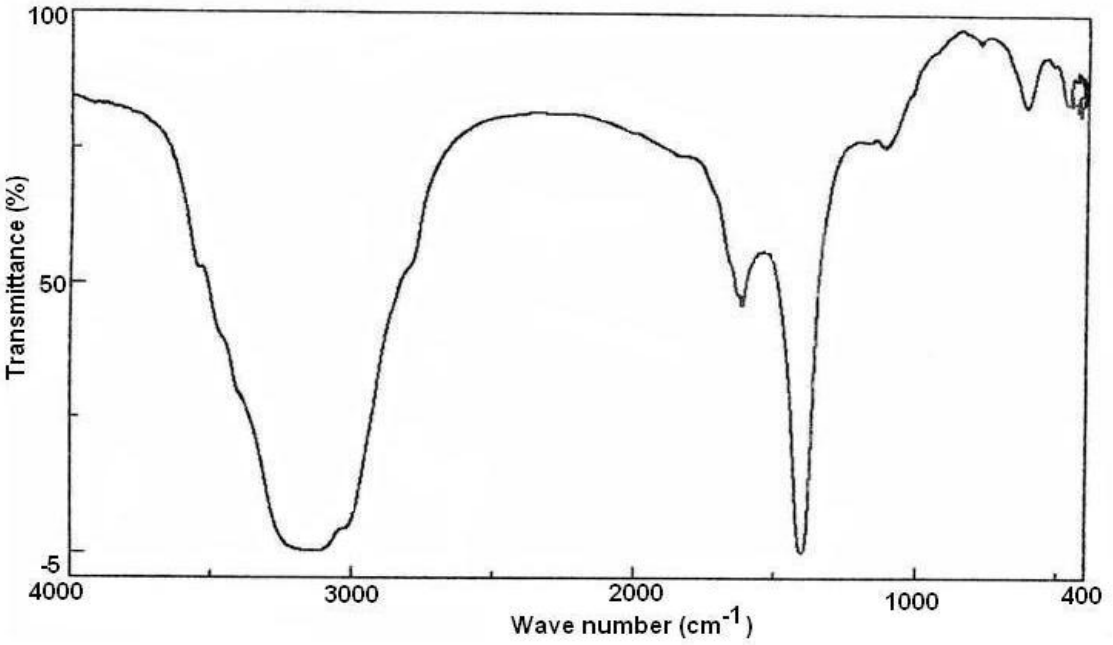

Fig. 2. FTIR spectrum obtained for PHB extracted from *A. beijerinckii*

Pal and Paul, 2002; Kumar and Prabhakaran, 2005; Oliveira *et al*., 2007).

CONCLUSION

The two factors increasing production cost of PHB are the substrate used for the polymer production and the down stream process. There are a number of coir industries in India. The waste material generated from their processing causes serious environmental hazards. Use of cheap carbon sources would bring down the polymer cost. Agricultural wastes like beet and cane molasses, malt extract, corn syrup, wheat bran and dairy wastes like cheese whey could be used for cultivating bacteria accumulating PHA. The cost of the substrate can be lowered by using the lignocellulosic wastes as a substrate for PHB production. However, in the production of PHB, the cost of the enzyme to be used for hydrolysis (highly preferred process) must be reduced and their efficiency increased in order to make the process economically feasible. This study provided valuable information about the coir industrial waste utilization and as an inexpensive potential substrate for the production of eco-friendly plastic.

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