Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **2 (2):** 68-80 (2014)

Review Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

A Review on Downstream Processing of Bacterial Thermoplastic-Polyhydroxyalkanoate

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ABSTRACT

Plastics are of wide range of synthetic or semisynthetic mouldable organic solids. Almost all aspects of daily life involve plastics. Plastics play a role in in transport, telecommunications, clothing, footwear, and as packaging materials that facilitate the transport of wide range of food, drink, and other goods. These plastics are nonbiodegradable and cause waste disposable problems, leading to environmental pollution. Therefore there is a need to study and to develop new biodegradable polymers with plastic like properties. This review demonstrate the upstream and downstream processing of bioplastic.

Key words : *Bioplastic, Extraction, Characterisation.*

INTRODUCTION

Since synthetic plastics marked their debut in the 1950s, they have emerged to be among the most needed material in our daily life[1]. The world's plastics production was estimated to be 260 milliontonnes in 2007 The world's plastics production was estimated to be 260milliontonnes in 2007[2].The extensive usage of petrochemical plastics due to their versatile properties especially durability is causing severe problem in waste management affecting the aesthetic quality of cities, water bodies and natural areas. The accumulation of plastic wastes has become a major concern in terms of the environment[3]. Problems concerning the global environment have created much attention in developing eco-friendly products .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[4]. Therefore, the development and use of biodegradable plastics is gaining more serious attention. The most extensively studied thermoplastic biopolymers are the polyhydroxyalkanoates (PHA) and polylactic acid LA)[5].Polyhydroxyalkanoates (PHAs) are biodegradable polyesters synthesized by various bacteria They represent products with biotechnological importance due to their special properties .They are accumulated intracellular as carbon and energy reserves under certain conditions[6,7,8,9]. PHA is attractive because of its biodegradability and physical properties that closely resemble some conventional plastics such as polypropylene (PP) and low-density polyethylene (LDPE)[10].

The various PHA monomers can be classified based on the number of carbon atoms as short-chain length PHA (scl-PHA), medium-chain length PHA (mcl-PHA) and long-chain length PHA (lcl-PHA). Scl-PHA refers to PHA comprised of monomers having 5 or less carbon atoms. These include 3-hydroxybutyrate and 3-hydroxyvalerate. The mcl-PHA is comprised of monomers having 6 to 14 carbon atoms. These include 3-hydroxyhexa- noate, 3-octanoate and 3-hydroxydecanoate. The lcl-PHA, which is uncommon and least studied, consists of monomers with more than 14 carbon atoms. Recently, it has also been made possible to synthesize a new type of PHA containing lactide as a co-monomer[11,12,13]. The completely biodegradable plastic is of recent origin and promising, because of its complete utilization by microbes in nature[14.]

Petroleum based synthetic plastics have found various industrial and domestic applications worldwide for the past seventy years due to their versatility and durability[15]. To produce biodegradable plastics resembling conventional plastics, bacteria are employed to make the building blocks for plastic polymers from renewable sources. Polyhydroxyalkanoates (PHAs), polylactic acid (PLA), polybutylenes succinate (PBS), polytrimethylene terephthalate and polyphenylene are the best studied polymers containing at least one monomer synthesized via bacterial transformation[16]. There have been reports of PHAs and their derivatives produced by and derived from a variety of microorganisms, over 300 different bacteria, including Gram-negative and Gram-positive species. Until recently, there were only few reports on marine PHAs producing microorganisms[17,18,19,20,21,22]. The production of PHAs can be from renewable carbon resources, whereby it is unaffected by the depleting fossil fuels, or rise in crude oil prices, in tum resulting in their neutrality with regard to CO_2 emission , leading to conservation of finite fossil resources like mineral oil and coal. The wider use of bioplastics in daily life will solve the increasing problem of organic wastes and decrease the count1y's dependence for fossil fuels[23.] When nutrient supplies are imbalanced, PHA accumulate as discrete granules in bacteria and act as carbon and reducing equivalents sink in microbes. This property helps bacteria to store excess nutrients invivo and the polymerization of these soluble intermediates into insoluble molecules prevents the leakage of this valuable nutrients out of bacterial cell [24]. Accumulation of PHA enhances the survival ability of microorganisms under adverse environmental conditions and the relation between PHA accumulation and stress were discussed by many researchers[25,26,27,]. As PHAs are insoluble in water, the polymers are accumulated in intracellular granules inside, the cells and the polymerization of these soluble intermediates into insoluble molecules prevents the leakage of valuable compound out of bacterial cell[28]. Phospholipids and proteins form a layer over the surface of a PHA granule and in the interface of a granule, the most dominant compound seen is Phasin, a class oi proteins known to influence the number and size of PHA granules[29,30.]

Occurrence of polyhydroxyalkanoates (PHA) accumulating microbes have been reported from various environments including mangroves[31], marine sediments[32, ,33,34] antartic areas[35], soil [36] sewage sludges[37,38] ponds[39](, palm-oil mill effluent pond [40]. Sugars have been shown to be an effective feedstock for PHA production in Brazil, especially when the PHA production is integrated to the sugarcane-processing factory[41].

METHODS USED FOR DETERMINATION OF PHB PRODUCING ORGANISMS

PHB produced were extracted and described in the method of Ramsay et al, 1994. The most common available at present for analysis of PHA_S in bacterial cells is gas chromatography (GC)[42,43,44].

SUDAN BLACK B STAINING

The selected isolates were then identified on the basis of their morphological, cultural, physiological and biochemical characteristics. Hartman (1940) was the first to suggest the use of Sudan black B, as a bacterial fat stain[45]. Subsequently, Burdon et al , (1942a) confirmed the greater value of this dye and modified the procedure for demonstrating intracellular fatty material in bacteria by preparing microscopic slides of bacteria stained with alcoholic Sudan black B solution and counterstained with safranin[46]. Smibert and Krieg, 1981 also demonstrated that the isolates were screened for PHB by staining with Sudan black B stain (0.3 in 70% alcohol) and observed under microscope (X100x)[47]. PHB producing bacteria was further confirmed using Sudan black B staining method[48] with some minor modifications. Sudan black B stain was prepared as 0.3% solution (w/v) in 60% ethanol. The smear of cultures was prepared on glass slides and heat fixed. Nile blue A stained PHA granules in the cells fluoresce orange[49]. PHB producing lipid inclnsions were stained with Sudan Black[50] volutin by Albert's method as modified by Laybourne (1924)[51] and spores by the malachite green method of Ashby (1938)[52]. The lipid inclusions occurred predominantly at the centre of the cell and varied in size from large spherical bodies as wide as the cell, often glassy blue in colour, to small brown or black opaque bodies with well- defined outlines. Volutin granules occurred almost solely at the poles of the cells and were just visible as rather indistinct smudges. Juan et al. 1998 employed viable colony screening method for the rapid detection and isolation

of PHB producing exopolysaccharide deficient mutants from wild type of *Rhizobium meliloti*[53] . Nile Blue does not stain either glycogen or poly-P granules[44] but it does stain lipophilic storage materials other than PHA, such as waxes and fats[54] .

NILE RED FLUORESCENE STAINING

Nile Red is soluble in neutral lipids that are liquid at the staining temperature (55 °C) and it is therefore adsorbed in PHA inclusions.The PHB accumulation was monitored from 16 hours onwards by Nile Red fluorescence staining of PHB granules. Samples collected and were subjected to direct dilution and plating on LB medium supplemented with 2% glucose and 1% Nile red and the plates were incubated at 37^0c for 48hrs. Colonies with pinkish pigment were selected for further studies and maintained on LB – glucose slants.(Spiekermann 1999). Kranz et al. (1997) described the colony screening and selection systems to analyze the production of PHAs in *R. capsulatus* [55].

CARBOL FUCHSIN STAINING

Carbol Fuchsin staining is performed to determine the intracellular production of PHB by the isolate. A thin smear of all the isolates were stained with carbol fuchsin stain for 45 seconds. The isolates capable of producing PHB showed dark colored granules of PHB intracellularly[56].

CHARACTERIZATION OF PHB PRODUCING BACTERIAL ISOLATES

The selected, most efficient PHB producing bacterial isolates were subjected to a set of morphological, physiological and biochemical tests for the purpose of identification.

MORPHOLOGICAL TESTS

The PHB accumulating strains were examined for their colony morphology, pigmentation fluorescence, cell shape and gram reaction .

COLONY CHARACTERIZATION

The colony characters viz., shape, colour and polysaccharide production were observed on agar medium.

SIMPLE STAINING

Twenty four hour old culture was smeared on a clean glass slide and heat fixed. It was then kept on the staining tray and five drops of safranin stain was applied for few seconds. Stain was poured off and smear was washed gently with slow running water. The slide was air dried and observed under oil immersion.

GRAM STAINING

The overnight grown cultures were stained with gram reagents as per Gram (1884) to determine their Gram reaction. Twenty four hour old culture was smeared on a clean glass slide and heat fixed[57]. The smear was covered with crystal violet for 30 seconds and washed off with 95 per cent ethyl alcohol. The slide was washed with distilled water and drained. Safranin was applied on smear for 30 seconds as counter stain, washed with distilled water and blot dried. The slide was observed under microscope for gram reaction.

SPORE STAINING

Fourty eight hours old nutrient agar grown isolates were smeared on a glass slides, air dried and heat fixed. The smears were flooded with malachite green and steamed on water bath for 5 min adding more stain on the smear from time. The slides were washed with water and counter stained with safranin for 30 seconds. The smear was washed with distilled water, blot dried and examined under oil-immersion objective.

MOTILITY TEST

The motility test was done to determine the motility of the organism. Bacterial cultures were stabbed into the motility test medium (Himedia) and were incubated at 37 C for 48 hrs. Turbitity and observation of growth besides the stab line indicated a positive reaction whereas clear visibility with growth indicated a negative reaction.

BIOCHEMICAL TESTS

The PHB producing bacterial isolates were identified on the basis of classification schemes published in Bergey' s Manual of systematic bacteriology, based on the characters such as morphology, physiology

and nutritional and cultural characteristics and biochemical tests such as motility, indole,methyl red, voges proskauer, triple sugar iron test, citrate utilisation, Catalase test , Urease test, Oxidase test, Glucose oxidation test, Lactose utilisation, Nitrate reduction test[58,59] with 24 hr old cultures.

STARCH HYDROLYSIS

Production of amylase was determined on starch agar medium, incubated at 28ºC for 48h.The isolates were made a single streak on starch agar plate for 72-96 h at 25 ^{0}C in an inverted position. Grams iodine solution was flooded on the surface of the plates for 30 seconds. The plates were examined for the starch hydrolysis around the line of growth of each isolates i.e., for the color change of the medium. Clear zone surrounding the microbial colonies is a typical positive starch hydrolysis. Carbohydrate catabolism was determined by Hugh and Leifson's[56] medium deep tubes in both aerobic and anaerobic condition, incubated at 28ºC for 24-48h. To determine cellulase production, Czapek-mineral salt agar medium was inoculated and incubated at 28ºC for 2-5 days. The plates were flooded with hexadecyltrimethyl ammonium bromide and observed for formation of zone around the growth.

GELATIN LIQUEFACTION

The isolates were inoculated on Gelatin agar deep tubes and gelatin agar medium plates at 37 ^{0}C, for 4-7 days. After incubation, the tubes were placed in a refrigerator for 15 minutes at 4^{0}c for 15 min and observed for liquefaction of gelatin[60].

CASEIN HYDROLYSIS

Overnight grown cultures of the test isolates were spotted on skimmed milk agar plates and incubated at 28±2^{0}C for 48 hours. The production of halo zone around the colony was taken as positive for the test.

HYDROGEN SULFIDE TEST

Test cultures were stubbed into the tubes containing SIM agar and kept for in incubation at 37 ^{0}C for 24-48 hr. The tubes were examined for the presence or absence of black coloration along the lane of stab incubation[61].

CATALASE TEST

Nutrient agar slants were inoculated with overnight grown test organisms and were incubated at 30 0C for 24 hr. After incubation, the tubes were flooded with one ml of three per cent hydrogen peroxide and observed for gas bubbles. The occurrence of gas bubbles was taken as positive for catalse test[62].

OXIDASE TEST

The Oxidase test was done with the help of commercially available disc coated with a dye N- tetramethyl paraphenylene diamine dihydrochloride (Himedia), to detect the presence of cytochrome 'c' oxidase which is responsible for the oxidation of the dye. Rubbing a small quantity of bacterial culture by means of a sterile toothpick on the disc causes formation of purple colour within 10-30 sec indicating positive reaction whereas no colour change indicates a negative reaction.

MANNITOL TEST

This experiment is generally performed to determine whether the bacteria is capable of fermenting mannitol sugar or not. Whenever organisms ferment mannitol agar, the pH of media becomes acidic due to production of acids. The fermentation of the media form red to yellow which shows positive test result.

UREASE TEST

The overnight grown cultures were inoculated to the test tubes containing sterilized Urea broth and incubated for 24-48 hr at 28±2^{0}C. The development of pink color was taken as positive for the test[6,64].

CITRATE UTILIZATION TEST

Citrate utilization test was performed to find out the ability of the bacterial isolates to utilize or ferment citrate as the sole source of carbon. It was done on the Simmon's Citrate Agars slants and a change in the colour of the medium from green to blue was positive for the test[59].

INDOLE PRODUCTION

To the pre sterilized tryptone broth, the test cultures were inoculated. The tubes were incubated for 48 h at 28±2 ^{0}C. After incubation, each tube was added with ten drops of Kovac's reagent. The production of red colour was taken as positive for the indole production.

NITRATE REDUCTION TEST

This test was done to test if microorganisms are able to convert nitrate to nitrite or not by adding 1-2 drops of sulphanilic acid and 1-2 drops of N,N-Dimethyl-Napthylanine reagent to the kit medium. Immediate development of pinkish red colour there on addition of reagent indicates positive reaction. Negative reaction could be observed if there is no change in the colour.

METHODS USED FOR EXTRACTION OF PHA

Separation of particles (0.05-100μm) from biotechnological mixtures of particles, such as inclusion bodies, cell debris, and crystal, is gaining interest from industry because of an increasing number of production processes that yield a particulate product in a mixture with other particles[66]. Solvent extraction is the most extensively adopted method to recover PHA from the cell biomass This method is also used routinely in the laboratory because of its simplicity and rapidity. Two main steps are involved, first is the modification of cell membrane permeability thus allowing release and solubilization of PHA. This is then followed by non-solvent precipitation[67]. Solvent extraction has undoubted advantages over the other extraction methods of PHA in terms of efficiency. This method is also able to remove bacterial endotoxin and causes negligible degradation to the polymers. The use of a solvent to recover PHA is one of the oldest methods. The use of solvents destroys the natural morphology of PHA granules that is useful in certain applications such as the production of strong fibers. Another problem connected with the use of solvents is that it creates hazards for the operators and for the environment[68]. Extraction of PHA with solvents such as chlorinated hydrocarbons, i.e. chloroform, 1,2- ichloroethane or some cyclic carbonates like ethylene carbonate and 1,2-propylene carbonate is common[69]. Lower chain ketone such as acetone is the most prominent solvent especially for the extraction of mcl-PHA[70]. Chloroform and other chlorinated hydrocarbons dissolve all PHA from mixed culture biomass [71]. The solvent extraction is widely used to recover PHB with a high purity[72,73]. Another recovery method is the using of sodium hypochlorite for differential digestion of non-PHA cellular materials (NPCM)[74]. Most methods to recover intracellular PHA involve the use of digestion methods. Such a method can reduce the use of large quantities of solvent making the procedure economically and environmentally unattractive[75,76].

Table.1Various PHA recovery methods that have been reported

EXTRACTION METHOD	COMMENTS	STRAIN	RESULTS	REFERENCE
Solvent Extraction	Chloroform	*Bacillus cereus* SPV	Purity: 92%; Yield: 31%	Valappil *et al.* [77]
	Chloroform	*Cupriavidus necator* DSM 545	Purity: 95%; Yield: 96%	Fiorese *et al.* [78]
	1,2-Propylene carbonate	*C. necator* DSM 545	Purity: 84%; Yield:95%	Fiorese *et al.* [78]
	Acetone-water process		Yield: 80–85%	Narasimhan *et al.* [79]
	Methyl *tert*-butyl ether	*Pseudomonas putida* KT2440	Yield: 15–17.5 wt%	Wampfler *et al.* [80]
	Methylene chloride	*C. necator*	Purity: 98%	Zinn *et al.* [81]
	Non halogenated solventsisoamy propionate, propyl butyrate, isoamyl valerat etc.	*C. necator*		Mantelatto and Durao [82]
	Acetone, room temperature	*P. putida* GPo1	Yield: 94%	Elbahloul and Steinbüchel [83]

Digestion method				
Surfactant	SDS	Recombinant *Escherichia coli*	Purity: 99%; Yield:89%	Choi and Lee [84]
Sodium hypochlorite	Sodium hypochlorite	*C. necator, Recombinant E. coli*	Purity: 86%; Purity: 93%	Hahn et al. [85]
Surfactant-sodium hypochlorite	SDS-Sodium hypochlorite	*Azotobacter chroococcum* G-3	Purity: 98%; Yield: 87%	Dong and Sun [86]
Surfactant-Chelate	Triton X-100-EDTA	*Sinorhizobium meliloti*	Purity: 68%	Lakshman and Shamala [87]
	Betaine-EDTA disodium salt	*C. necator* DSM 545	Purity: >#96%; Yield: 90%	Chen *et al*. [88]
Dispersion of sodium hypochlorite and chloroform	Chloroform- sodium hypochlorite	*B. cereus* SPV	Purity: 95%; Yield: 30%	Valappil *et al.* [77]
Selective dissolution by protons	Sulfuric acid	*C. necator*	Purity: >#97%; Yield: > 95%	Yu and Chen [89]
Enzymatic digestion	*Microbispora* sp culture-chloroform	*S. meliloti*	Purity: 94%	Lakshman and Shamala [90]
	Enzyme combined with SDSEDTA	*P. putida*	Purity: 93%	Kathiraser *et al.* [91]
	Bromelain; pancreatin	*C. necator*	Purity: 89%; Purity: 90%	Kapritchkoff *et al.* [92]
	SDS-High pressure homogenization	*Metylobacterium* sp V49	Purity: 95%; Yield: 98%	Ghatnekar *et al.* [93]
	Sonication	Baci*llus flexus*	Purity: 92%; Yield: 20%	Divyashree *et al.* [94]
Gamma irradiation	Radiation-chloroform	*B. flexus*	Yield: 45–54%	Divyashree and Shamala [95]

ANALYTICAL METHODS USED FOR PHA CHARACTERISATION

FIELD EMISSION SCANNING ELECTRON MICROSCOPY (FE-SEM)

The Field Emission Scanning Electron Microscopy (FE-SEM) was used to see the predominance of PHB granules in the bacterial cells. The PHB granules were found as electron dense granules of spherical to oblong shaped, while the bacterial cells were long and rod shaped. Furthermore, the PHB granules showed the highly crystalline morphology under FE-SEM. Due to freeze drying under vacuum, the nature of PHB granules were probably transformed from amorphous to crystalline form during lyophilization as reported earlier[96].

FTIR ANALYSIS

Fourier transform infrared spectroscopy (FTIR) has been applied to determine the content of PHA in cell suspensions[97,98.] However, all these methods lack the specificity to discriminate between different monomers and hence they cannot be used to determine the monomeric composition of PHA copolymers. The bands associated with PHA and other biomolecule markers , can be clearly distinguished . It is observed that each individual spectrum directly relates to the relative concentrations of the specific components of the sample[99.]

Methods have been applied to determine the content of PHA in biomass and to analyze the monomeric composition of PHA-copolymers, analysis of cell content, structure and composition of PHB and other PHAs have been reported, including gas chromatography (GC) after solvent extraction and hydrolytic esterification of the polymer[100]. GC is being used in the analysis of complicated mixtures of fatty acids. GC analysis of PHA offers quantitative information about the total amount and percent composition of PHA when combined with MS detection it also adds information about the mass and identity of the monomer involved. Combination of GC with other specialised detectors like atomic emission detector

(AED) gives relevant information about whether monomers contain atoms like chlorine, bromine or iodine.

GC – MS ANALYSIS

The chloroform extracts of biodegradable polymer were dried and analyzed by GCMS The major compounds among the analyzed compounds were n-Hexadecanoic acid (Stearic acid), Oleic acid and Phenyl isobutyrate The n- hexadecanoic acid is an aliphatic polymer esters. This aliphatic biodegradable polyester family due to hydrolysable ester bonds was reported by Dawes (1988)[101] and others (Anonymous, 2002)[102]. Polymer content and PHA composition was determined with a gas chromatograph-mass spectrometer. The level of PHB recovery was calculated from the total amount of PHB in the cell powder as determined by gas chromatography and the amount of PHB recovered.

HPLC

In this method there is no need for lyophilising the sample material, so there is no loss of time needed for drying. Furthermore , as the HPLC methods analyse the dehydrated free fatty acids , no further derivatization is needed , thus reducing the total analysis time even more. The HPLC method is useful in the analysis of poly (3HB – 3hv) types of PHA. HPLC measures only PHB and is based on conversion of PHB to crotonic acid followed by UV detection at 210 nm. PHA detection by ionic chromatography is based on the conversion of monomers to alkanoic acids. The determination involves acid propanolysis followed by an alkaline hydrolysis with calcium hydroxide or acidic hydrolysis with concentrated sulphuric acid. The sample is then run on a HPLC having a conductivity detector. Ion-exchange HPLC with conductivity detection was applied for the analysis of digested poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) in activated sludge (Hesselmann et al., 1999)[103]. Karr et al.

1983) avoided a large part of the time-consuming sample purification After quantitative con- version of Poly(3HB) to crotonic acid, the obtained free acid was chromatographed on a ion-exchange HPLC column[104]. No further pretreatment was necessary and samples containing 0.01±14 µg of Poly(3HB) could be analyzed. The method was used to analyze the Poly(3HB) contentof *Rhizobium japonicum.* The HPLC method of was used to analyze the content of PHA in the biomass of phototrophic sulfur bacteria. An interesting phenomenon is the ability of the HPLC method to discriminate between cis- and trans-crotonic acid, formed by the hydrolysis of R- and S-Poly(3HB). Furthermore 3- hydroxybutyric acid was separated from 3- hydroxyvaleric acid. Study used the HPLC method of Karret al.(1983) to analyze Poly(3HB) which was synthesized in vitro by combining purified PHA synthase from *A. eutrophus* with synthetically prepared R-3- hydroxybutyryl coenzyme A. The study used the same method to investigate Poly(3HB) formed invitro by combining R-3- hydroxybutyryl coenzyme with purified re- combinant PHA synthase from Chromatium vinosum. Hesselmann et al. (1999) combined a propanol/sulfuric acid digestion with ion- exchange HPLC and conductivity detection to determine the PHA content of activated sludge. The method could be used with wet material, thus avoiding time-consuming lyophilization[103]. The relative yield was 100g 2% in wet material and 98g 7% in dry material.

NMR

NMR is very useful technique in analysing PHA containing double bonds. With the aid of homonuclear and heteronuclear techniques the exact location of double bonds in the monomer and the cis/trans configuration can be determined. It is also very useful in the analysis of all kinds of specialised PHA such as halogenated or acetylated PHA. It is essential in the analysis of epoxidised PHA, as epoxy groups will split into diols in the acidic hydrolysis of PHA.

CONCLUSION

Bioplastics can be isolated by centrifugation (cell-free extracts) or by solvent extraction (dried intact bacteria) with chloroform, trifluoroethanol, dichloroethane, propylene carbonate, methylene chloride or dichloroacetic acid[105,106,107,108] . Atomic force microscopy and confocal Raman spectroscopy are techniques currently used for poly(3-hydroxyalkanoate) (PHA)-granule analysis. Their molecular weights (ranging from 50,000 to 1,000,000 kDa) have been established by light scattering, gel permeation chromatography, sedimentation analysis and intrinsic viscosity measurements[109] and their monomer

compositions have been determined by gas chromatography (GC), mass spectroscopy (MS) and nuclear magnetic resonance (NMR) analyses[110] . Other physical properties, such as crystal structure, polydispersity, melting temperature, enthalpy of fusion, glass transition temperature and mechanical properties were established using different procedures[111,112,113].

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