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REVIEW ARTICLE

Methods for identification, quantification and characterization of polyhydroxyalkanoates-a review

Suchitra Godbole
Department of Microbiology, Dr. D.Y. Patil Arts, Commerce and Science College, Sant Tukaram Nagar Pimpri, Pune, India.

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Abstract: Polyhydroxyalkanoates (PHAs) are the polymers of hydroxyalkanoates that accumulate as a carbon/energy storage material in various microorganisms. PHAs have been attracting considerable attention as biodegradable substitutes for conventional polymers, because of their similar material properties to conventional plastics. A number of review articles on the general features of PHAs, the physiology, genetics and molecular biology, the development of PHAs having novel monomer constituents, production processes, biodegradation of PHAs are available. Recently much effort has been devoted to develop a process for the economical production of PHAs. The isolation, analysis and characterization of PHAs are important factors for any process development. A number of methods have been developed for the analysis of PHAs. This paper is an effort to compile the methods available for the identification, quantitative estimation and characterization of PHAs. The methods described in this paper include- staining reactions, spectrophotometric methods, infrared and FTIR spectroscopy, HPLC, gas chromatography and GC-MS analysis, NMR spectroscopy, flow cytometry and spectrofluorometry, molecular weight determination and thermal analysis. The methods have been discussed with their advantages and disadvantages. Recent developments in the analysis of PHAs have also been discussed.

Key words: Polyhydroxyalkanoates, Characterization of PHA, Identification of PHAs.

Introduction

In response to the problems and harmful effects of plastic wastes on the environment, there has been considerable interest in the development of biodegradable plastic materials. Among the various biodegradable polymer materials, Poly-hydroxyalkanoates (PHAs) are attractive substitutes for conventional petrochemical plastics because they have similar material properties to various thermoplastics and are completely biodegradable upon disposal under various environmental conditions. With the aim of commercializing PHA, a great deal of effort has been devoted to reducing the production cost by the development of bacterial strains and more efficient fermentation/recovery processes.

Poly hydroxyalkanoates (PHA) are accumulated intracellularly as distinct granules by various microorganisms, usually when there is a growth limiting component in the presence of excess carbon source.

For success of any fermentation process, the identification and characterization, analysis of the product with a rapid, simple and reliable method is essential. A number of methods are available for screening the PHA producing bacteria, the quantitative estimation and characterization of PHAs, which include staining reactions, Spectrophotometric analysis, infrared-FTIR Spectroscopy, HPLC, flow-cytometry and spectrofluorometry, gas chromatography-GCMS, NMR spectroscopy, molecular weight determination and thermal analysis of the extracted polymer. In this paper the various methods for Identification, quantification and characterization have been reviewed.

***Corresponding Author:**
Dr. Suchitra. S. Godbole,
Dept. of Microbiology
Dr. D.Y. Patil Arts, Commerce and Science College,
Sant Tukaram Nagar Pimpri , Pune-411018, India.

Staining Reactions and Microscopy

Microbiologists have traditionally detected the presence of PHB granules in bacterial cells by staining with sudan black B. (1, 2, 3) Staining with sudan black B is considered as a presumptive test for the presence of PHB. Shlegel *et al.,* used sudan black B as a dye for the isolation and detection of P (3HB) producing colonies on a nitrogen limiting plate (4).

For selection of microorganism producing PHA, phase contrast microscopy is often used. When observed by phase contrast microscopy, the presences of intracellular refractive granules suggest the presence of PHB. This method is convenient for screening large number of isolates. (5)

The use of Nile blue, a water soluble basic oxazine dye, has been advocated by Ostle and Holt (6). Heat fixed smears are stained with Nile blue A (1% aqueous solution) at 55°C for 10 min in a coplin jar. The slides then washed with tap water to remove excess stain and with 8% aqueous acetic acid, for 1min. The stained smear blotted dry and remoistened with tap water and covered with No1 glass cover slip. The preparation examined with microscope with an episcopic fluorescence attachment with excitation wavelength of approximately 460nm, PHB granules show bright orange fluorescence. The other inclusion bodies,

such as glycogen and polyphosphate do not stain with Nile blue A emphasizing its usefulness.

Kitamura and Doi used a dilute Nile blue A (0.05%) ethanolic solution to detect bacterial colonies on an agar plate by their bright orange fluorescent color on irradiation with UV light (254nm) (7). A replica plate technique was developed by Yasuo Takagi and Tsuneo Yamane; to improve the conventional method for screening poly-hydroxyalkanoate [P (3HA)] accumulating bacterial colonies on agar plates by Nile blue A staining. To avoid the damage due to ethanolic solution to the colonies in the conventional method the colonies on the agar plate were transferred on to a membrane by replica technique, the membrane stained with 1% Nile blue. An ethanolic solution and the dried membrane were irradiated with UV light. Cells that accumulated PHA exhibit bright-orange fluorescence, whereas the cells that do not accumulate P (3HA) appear as dark spots (8). A sensitive, viable colony staining method using Nile red for direct screening of bacteria that accumulate PHA and other lipid storage compounds has been developed by P. Spiekermann *et al.,* (9).

The Nile red colony staining technique is used by many workers for screening of various environmental niches for isolation and identification of PHA producing microorganisms. e.g. Mamoru Oshiki used it for PHA accumulating microorganisms in full scale water treatment plant. (10). Kung *et al.,* (11) used a viable colony staining method to screen PHA producing bacteria followed by PCR to confirm microbes from environmental samples, and could be readily detected by colony staining method. They were also examined by Sudan black B staining.

A combination of fluorescence in situ hybridization FISH, with staining techniques for cell viability and accumulation of PHA and polyphosphate in microorganisms in complex microbial systems was studied by Nelson *et al.,* (12)

Spectrophotometric Methods

The most widely used method for the quantitative determination of the polyesters content of the bacterial cells has been to measure the weight of polyester isolated from the lyophilized cells with a solvent extraction method. This method is applicable only to sample with a relatively large quantity of polyesters. Several analytical methods have been developed to determine the content of P (3HB) in the cells. A method developed by Williamson and Wilkinson (13) involves measuring the turbidity of P (3HB) granules produced by the digestion of bacterial cells with sodium hypochlorite solution.

Two different spectrophotometric methods have been developed, using the fact that the P (3HB) molecule is degraded to crotonic acid by heating in concentrated sulfuric acid and the polyester content of the cell is determined by the use of a crotonic acid Ultraviolet (UV) absorption band at 235nm. (14,15). These spectroscopic methods may be applicable only to the determination of the P (3HB) homo-polymer content.

Infrared Spectroscopy

This method uses the fact that the P (3HB) molecule has a strong carbonyl absorption peak at 1728cm^{-1} in the infrared spectrum. P (3HB) is extracted from dried cells with chloroform and its content determined from the absorbance of carbonyl band (16). The limit of sensitivity reported was 50μg/ml.

The identification of B-hydroxybutyric acid in intact bacterial cells by infrared spectrophotometry was reported by A.C. Blackwood *et al.,* (17). The culture broth was centrifuged, washed twice with saline, suspended in saline and lyophilized. The analyses were made by pressing a weighed amount (2.5mg) of the cells with 500mg of potassium bromide into a window in a die. The spectra were obtained with Perkin Elmer double beam recording infrared spectrophotometer. The cultures tested had spectra with a sharp absorption band at 5.80u associated with B-hydroxybutyric acid.

A rapid method for detecting bacterial poly hydroxyalkanoates in intact cells by fourier transform infrared spectroscopy has been described by K. Hong *et al.,* (18). The FTIR spectra of pure PHA containing short chain length monomers, such as hydroxybutyrate (HB), medium chain length polyhydroxy alkanoates (mcl-HA), monomers including hydroxyoctaoate (HO) and hydroxydecanoate (HD), or both HB and mcl HA monomers showed their strong characteristic band at 1728cm^{-1}, 1740cm^{-1}, 1732cm^{-1} respectively. The intensity of the methylene band near 2925cm^{-1}, provided additional information for PHA characterization. In comparison bacterial cells accumulating the above PHA also showed strong marker bands as 1732cm^{-1}, 1744 cm^{-1}or 1739 cm^{-1} corresponding to intracellular PHB, mcl PHA and P (HB+ mcl HA) respectively. The rapid non-destructive analysis involving minimum sample preparation offers a useful method that can complement existing procedures used for the analysis of PHA. The FTIR method is particularly suitable for screening large amounts of bacteria for their abilities to synthesize various types of PHA.

High Performance Liquid Chromatography (HPLC)

Ion-exclusion high pressure liquid chromatography (HPLC) was used by Karr *et al.,* (19), to measure poly-B-hydroxybutyrate (PHB) in *Rhizobium, Japonicum* bacterioids. The products in the acid digest of PHB containing material were fractionated by HPLC on Aminex HPX-87-H ion exclusion resin for organic acid analysis. Crotonic acid formed from PHB during acid digestion was detected by its intense absorbance at 210nm. The Aminex-HPLC method provides a rapid and simple chromatographic technique for routine analysis of organic acids. The method can measure crotonic acid in samples containing 0.01 to 14ug of PHB.

Gas Chromatography Mass Spectrometry (GC-MS)

The gas chromatographic determination of poly-3-hydroxybutyrate in cells was first described by G. Braunegg *et al.,* (20). The cells are subjected to direct mild acid or alkaline hydrolysis followed by GC of the 3 HB methyl ester in an accurate procedure that could be completed within four hours enabling concentrations as low as 10^{-5}g/L. Riis and Mai (21) have suggested that the method can be improved by the propanol and HCl which causes less degradation, and Comeau *et al.,* (22) have described a convenient GC method for PHA analysis of activated sludge samples. With flame ionization detector the sensitivity was estimated as 10^{-5}g/L.

Findley and white (23) quantitated chloroform extracted PHAs from *B. megaterium* and from environmental samples by acid (HCL) ethanolysis and GC-Mass spectrometric (MS) analysis of the resulting 3-hydroxyalkanoic acid ethyl esters, they were able to analyze routinely samples containing 100mg of purified polymer. Brandl (24) determined the PHA content and composition by GC- after methanolysis of lyophilized cells for 140min at 100°C to yield the methyl esters of the constituent 3-hydroxyalkanoic acids. Morikawa and Marchessault (25) used pyrolysis of PHA under nitrogen at controlled temperatures between 250 and 600°C., followed by GC-MS of trimethylsilyl derivatives and oligomeric products were identified as "dehydrated" monomer, dimer and trimer from both PHB and 3HB-co-3HV copolymers. Grassie *et al.,* (26, 27) have also applied pyrolytic techniques, and a fast and direct method of analysis of PHAs by rapid pyrolysis capillary GC. Heuller (28) developed a method wherein samples of PHA (50-100 ug) are pyrolyzed at 450°C for 10s and volatile products are subjected to GC. The techniques are applicable to lyophilized bacteria as well as to extracted PHAs. Lee E.Y. and Choi C.Y. (29) have described trimethylsilyl (TMSi) derivatizations and its application to a structural determination of PHA containing long chain saturated and unsaturated monomers. The characteristic fragmentation patterns of TMSi derivatives of saturated and monounsaturated 3-Hydroxyalkanoic acid methyl esters of purified PHA samples are discussed with respect to the structural determination of PHA. Their application to the screening procedure and some results of screening are also presented.

NMR Spectroscopy

NMR techniques have been applied successfully to the investigation of PHAs. The composition of the hydroxyalkanoate units in a copolymer can be determined by analyzing the nuclear magnetic resonace (NMR) spectra (30, 31, 32, and 33). The advantage of NMR analysis is that the hydrolysis step of the polymer can be avoided. In NMR analysis, 2-5mg of the copolymer sample is dissolved in deuteriated chloroform (CD Cl_3) (1.0ml) and high resolution 'H-NMR spectrum is recorded on a NMR spectrometer.

Jacob *et al.,* (34) showed that it was possible to use cross polarization magic-angle spinning ^{13}C NMR spectra of lyophilized samples of *Pseudomonas sp.* Strain LBr to monitor directly and nondestructively PHB accumulated and utilized by this organism. A similar technique was used by Doi *et al.,* (35) for PHB isolated from *A. eutrophus*. Doi *et al.,* (36) have also analyzed the conformation of PHB in chloroform solution by 500 MHz^{1}H NMR spectroscopy.

Flow cytometry and Spectrofluorometry

Srienc *et al.,* (37) have used flow cytometry to measure distributions of single cell light scattering intensity, which increase as refractive PHB granules accumulate in *A. eutrophus*. This measurement clearly demonstrates potential advantage of single cell light scattering measurement by flow cytometry for analysis and control of fermentation processes. Flow cytometry -cell sorting technology should find significant application in strain improvement and mutant selection.

For optimization and control of the biotechnological production of PHAs, on-line monitoring of the product concentration during cultivation is desirable. Spectrofluorometric measurements were established; Muller *et al.,* (38) also described a flow cytometric approach for characterization and differentiation of bacteria during microbial processes. Groom *et al.,* (39) have described on-line culture fluorescence measurement during the batch cultivation of Poly-B-hydroxybutyrate producing *Alkaligenes eutrophus*. The results revealed a linear correlation between total culture fluorescence and total biomass concentration. The total fluorescence level was attributed to cellular fluorescence and total

biomass concentration. The total fluorescence level was attributed to cellular fluorescence and to the fluorescence of compounds presumably produced by cells and secreted into the culture medium. An increase in the specific cellular fluorescence during the course of the experiments indicated a shift in metabolism that favored the production of PHB.

An easier method for quantitative PHB detection by Nile Red fluorescence technique was described by Degelau *et al.,* (40). Nile Red is a fluorescent lipid dye which easily penetrates the cells in suspension, and measurements can be performed after a short time. The fluorescence intensity of the Nile Red Stained Cells results from PHB concentration, non-PHB staining is low. The relationship between the fluorescence data obtained by cytometric or (38) spectrofluorometric measurements were established.

These two methods for measurement of PHB are rapid and reproducible. The spectrofluorometric and flow cytometry PHB data were plotted against the GC data, showing a straight line having a correlation coefficient of 0.997 and 0.978 respectively. The method can have two advantages over the conventional PHB determination methods firstly, the PHB content can be measured soon after sampling and with less preparation time (approx. 30min vs up to 2 days) and secondly the PHB content can be determined with very small sample volumes (< 1ml vs 10ml). Thus staining with Nile red can facilitate good process control during cultivation.

Marose *et al.,* (41) have described two-dimensional fluorescence spectroscopy as a new tool for on line bioprocess monitoring. A method for quantitative estimation of bacterial polyhydroxyalkanoic acids by Nile Red has been described by Gorenflow *et al.,* (42). The method can be applied to follow PHA product concentrations during industrial processes on-line, hence facilitating the optimization of production times and product concentrations. It could be demonstrated that Nile Red has a high potential for the quantitative determination of hydrophobic bacterial PHA within living cells. The disadvantage of this method is that it is not capable of detecting the monomer composition within the accumulated polyester.

Berlanger *et al.,* (43) have shown that when bacterial cells were stained with Nile red suspended in water and subjected to fluorescent spectroscopy, the medium chain length and short chain length polyhydroxyalkanoates can be rapidly differentiated on the basis of their different excitation wave lengths.

PHA accumulated bacterial cells were stained with Nile red suspended in water and subjected to fluorescent spectroscopy at fixed excitation wavelength of 488nm. The scl PHA accumulated bacteria revealed max emission wavelength at 590 nm and for mcl PHA producers were seen at wavelength of 475 nm. Combining Nile red staining and fluorescence spectroscopy the accumulated bacteria revealed max emission wavelength at 590nm and for mcl PHA producers were seen at wavelength of 475nm combining Nile red staining and fluorescence spectroscopy the accumulated PHA granules could be rapidly differentiated into scl and mcl PHA from the intact cells. Recently Nile blue-A staining for *E. coli* in flow cytometry was also reported by Jose *et al.,* (44).

Molecular Weight Determination

The absolute molecular weight of P (3HB) molecules has been determined by light scattering or osmometric analysis in chloroform, 1, 2-dichloroethane or trifluoroethane (45-48). These absolute data are used to determine the Mark-Houwink parameters relating the intrinsic Viscosity to the molecular weight of P (3HB). Gel permeation chromatography (GPC) in chloroform at 30°C with a set of 5 microstyragel columns was used by Suzuki (49) *et al.,* to determine the viscosity average molecular weight (MW) of PHB from *A. eutrophus*. Polystyrene standards were used and results were corrected by using the Mark-Hownink relationship, $(n) = KM^{\dot{\alpha}}$, When (n) is the intrinsic viscosity, M is the molecular weight and K and ά are constants for a particular solute-solvent-temperature combination. Scandola *et al.,* (50) and Mimoto (51) carried out similar analyses to determine the molecular weight distribution of PHB from the *Methylotroph* and *Protomonas extroquens*. Scandola *et al.,* (52) have used viscometry to compare the MW of the PHB samples extracted by different methods from *Rhizobium*.

Thermal Properties

PHAs are partially crystalline polymers, and the definition of their thermal and mechanical properties is normally expressed in terms of the glass to rubber transition temperature (Tg) and the melting temperature Tm. Differential scanning calorimeter (DSC) is used for these determination. (52). The thermal stability of PHB granules studied with Thermogravimetric analyzer TGA operated at Nitrogen flow rate of 20ml/min and a scanning rate of 10°C/min.

Other Methods

Hesselmann *et al.,* (53) have described two other methods for the determination of poly-B-hydroxybutyrate (PHB) and –valerate (PHV) in activated sludge. Both methods are based on depolymerization of PHB/PHV to 3-

hydroxybutyrate (3HB) and 3-hydroxyvalerate 3(HV). For quantification of 3HB and 3HV directly, a method based on ion-exchange chromatography and conductivity detection was developed. (IC- method). Alternatively, 3HB & 3HV was quantified using a commercial enzymatic test kits and colourimetric detection. (Enzyme method). Both detection methods are easier to perform than previous methods and are suitable for complex matrices such as activated sludge. The IC method is recommended for high sample throughputs or if distinction between PHB and PHV is essential. Enzymatic detection is recommended if a few samples per day have to be measured immediately. The new methods presented increase the applicability of routine PHB/PHV measurements in wastewater treatment plants and complex matrices. The methods will be useful since more efforts are now made to produce PHAs from more complex and renewable resources such as activated sludge.

A rapid and simple analysis method for PHB content has been developed by S. Pavol. *et al.,* (54) PHB was converted to crotonic acid by H_2SO_4 and measured by isotachophoresis after neutralization by $CaCO_3$. This method can be used for rapid and routine analysis of fermentation process in samples containing 0.001 – 20mg of PHB.

Concluding Remarks

Research on PHAs has been encouraged by their potential use as biodegradable alternatives to petrochemical plastics. The commercial use of these materials will no doubt intensify research in this field. With the focus on reducing the production cost by development of efficient fermentation/recovery process, use of renewable resources for PHA production.

The estimation of PHA content and composition is most important factor because it has multiple effects on the PHA yield and recovery efficiency. However, for any process development a combination of various analytical methods has been applied for initial screening, then for the quantification and composition of the polymers, newer and newer microorganisms have been identified for the process. Wanner *et al.,* (55) have used a combination of staining with Sudan black B, Nile blue A or Nile red coupled with electron microscopy and NMR techniques for identification of polyhydroxyalkanoates in Halobacteria and other Haloarchaeal species. The development of newer, simple and rapid methods for detection and quantification of PHAs will certainly facilitate the efficient economic production processes for PHAs.

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