

Optimization and Characterization of Poly[R]hydroxyalkanoates of *Pseudomonas aeruginosa*

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DOI: <http://dx.doi.org/10.13005/bbra/1883>

(Received: 10 June 2015; accepted: 05 August 2015)

Polynucleotides, polypeptides, polysaccharides are the polymers seen in living organisms. Many biopolymers are important reserve compounds that are stored in the cytoplasm as insoluble inclusions. Likewise Polyhydroxyalkanoates are natural, renewable biopolyester accumulated in bacteria in the presence of limited nitrogen sources and higher concentration of carbon source. In this study, PHA accumulation pattern of *Pseudomonas aeruginosa* was studied. The PHA was optimized for various carbon sources, incubation time, pH and temperature. Analytical techniques like FTIR and GC-MS were done to determine the types of monomer present in the biopolyester.

Key words: PHA – Polyhydroxyalkanoates, *Pseudomonas aeruginosa*, GC-MS, FTIR

Polyhydroxyalkanoate(PHA) are biopolyesters accumulated in microorganism when bacterial cells are grown in excess carbon sources and other nutrients at limited conditions. Polyhydroxy alkanoates (PHA) are biopolymers accumulated when bacterial cells are grown in excess carbon source and other nutrients at limited conditions as energy source at late stationary phase and reduces mortality rate^{1,2}. PHAs are normally found as insoluble inclusions in cytoplasm thus known as PHA granules and these granules contain 97.5% PHA, 2% proteins and small amounts of lipids. PHA granule associated proteins plays an important role in PHA synthesis and granule formation³. The maximum yield of PHAs depends on several factors including stability and safety of the organism, growth and PHA accumulation rates, achievable cell densities

and PHAs content, extractability of the polymer, molecular weight of accumulated PHA, range of utilizable carbon sources, cost of carbon sources and other components of the medium and occurrence of the byproducts⁴. Major problem associated with the production of PHAs from bacteria are its cultural optimization studies. Optimization of the suitable carbon source in the medium is an important criteria for high production of PHA. By changing the carbon source and also the concentration of carbon source used in the fermentation process, it is possible to produce different PHAs with varied monomer content ranging from thermoplastic to rubbery polymers². The physical parameters like incubation time, temperature and pH are also to be considered in PHA production studies. Microbial PHA production is highly influenced by pH of cultivation medium⁵. In the earlier times the most common analytical technique used for PHA estimation was gravimetric method⁶. Several methods are now available for determination of the PHA contents of

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microorganisms, the composition of PHAs, and the distribution of the monomer units. The gas chromatographic (GC) identification of PHA components was later proposed by Brauneegg *et al.*,⁷. This method involves simultaneous extraction and methanolysis of PHA, in mild acid or alkaline conditions, to form hydroxyalkanoate methyl esters which are then analysed by GC. This method is rapid (4 h), sensitive, reproducible and requires only small amount of samples. Other analytical techniques used for confirmation of PHA are FTIR, UV spectrometry.

There are two groups among the PHAs producing bacteria on the basis of number of carbon atoms present in monomer units⁸ i.e Short-chain length PHAs(3-5 carbon atoms) and medium chain length PHAs(6-4 carbon atoms)⁹. Most organisms synthesize either SCL-PHA primarily having 3HB units or MCL-PHA having 3-hydroxy octanoate. The co-polymers of PHA are also produced which varies according to substrates used. These co-polymers of SCL-PHA and MCLPHA has got more tensile strength and other mechanical properties. PHA can be synthesized either by chemical methods or by biological synthesis. The biological synthesis alone yields much higher molecular weight than chemical method as these PHA can also be produced by chemical method. The biosynthesis of different types of PHAs significantly depends on the chosen microorganisms, fermentation condition and different carbon sources. The vast majority of microbes synthesize either SCL-PHA containing primarily 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers^{2,9,10}

MATERIALS AND METHODS

Isolation of the bacterial strain

The bacterial strain were isolated from the dairy waste and soil. 1g of soil sample were serially diluted. After serial dilution, 1 ml of the culture was introduced into sterile nutrient agar. Plates were incubated for 24 hours and observed for colony formation. The organisms grown were inoculated into cetrimide agar and incubated for 24 hours at 37°C. Thus obtained pure culture was stored in nutrient agar slant containing 25% glycerol solution at -20 °C for further use.

Production and extraction of PHA

The organism was inoculated into minimal media containing (g l⁻¹) K₂HPO₄ – 3g, Na₂HPO₄ – 6g, NH₄Cl – 2g, NaCl – 5g, MgSO₄ – 1g, CaCl₂ - 0.1g and FeCl₃ - 0.06g and it was incubated at 37°C for 48 hours under aerobic agitation of 150 rpm. The fermented culture was centrifuged at 10,000rpm for 25 minutes at 4 °C. The cell pellet was washed twice with distilled water. Cell pellet was dried in Hot air oven. 3ml of 4% Sodium hypochlorite and 7ml of Chloroform was added to 1g dried biomass and agitated vigorously. The mixture was then centrifuged at 2500rpm for 5 minutes to get three separate layers. The bottom layer contains chloroform with dissolved PHAs. PHA was obtained by adding methanol drop-wise-drop to get white precipitate. The precipitate was collected and added with 1ml Chloroform and transferred to an eppendorf tube, air-dried to get pure PHA. PHA % was calculated using dry weight of PHA and Cell Dry Weight¹¹.

Optimization of PHA production

The production of PHA was optimized with respect to five different carbon source such as glucose, fructose, maltose, sucrose and lactose at various concentrations^{12,13}. The optimum conditions like incubation time, pH and temperature for PHA accumulation were also determined.

Characterization of PHA

UV spectroscopy

The PHA was added with 10 ml H₂SO₄ and heated at 100°C for 10 min. Absorbance between 200 and 400 nm was recorded in UV spectroscopy, peak between 230 and 240 nm confirms the presence of PHA¹⁴.

Thin layer chromatography

The presence of PHA was confirmed by thin layer chromatography based on the method of Panda *et al.*,¹⁵.

FTIR

The extracted PHA was dissolved in chloroform and Infrared spectra (IR) (4000 to 400 cm⁻¹) were recorded on polymer films cast from chloroform solution onto KBr plates using FTIR (Shimadzu, DR-800) at 27°C¹⁶.

GC-MS analysis

GC analysis of powdered PHA was carried out by the method described by Brandl *et al.*,¹⁷. The 2 methyl esters were analyzed by GC clarus 500 Perkin Elmer detector in a 30m x 0.25 mm ID x

lim df capillary column was used. Helium (1 ml/min) was used as carrier gas. The injector and detector are at 250 °C and 200°C respectively.

RESULT SAND DISCUSSION

Isolation and identification of organism

The isolated organism was found to be *Pseudomonas aeruginosa* as it was able to produce pigment on cetrimide agar (Fig. 1). A similar study was done by Bhuwal *et al.*,¹⁸ who isolated PHA producing organism from cardboard manufacturing industry waste water.

Pseudomonas aeruginosa accumulated more PHA in glucose containing medium (Fig.2). Nisha *et al.*,¹⁶ also found *Pseudomonas putida* to accumulate more PHA in glucose containing medium only. Highest accumulation was observed at 48 hours in minimal media. The organism was found to accumulate higher percentage of PHA in glucose containing medium. PHA producing bacteria can be divided into two groups based on the culture media .In the first group ,the bacteria accumulates PHA at exponential phase only if there is a limited amount of nutrients (eg, *Pseudomonas oleovorans*)¹⁹.The second group, accumulates PHA during growth and it does not requires nutrient limitation(e.g. recombinant *E.coli*)²⁰. All the organisms are tends to accumulate PHA at the stationary phase¹². Even Jenifer *et al.*,¹³ found *Agrobacterium tumefaciens* SU-11 to accumulate at 48h of incubation. But this was contradictory to the result of Nisha *et al.*,¹⁶ who found *Pseudomonas putida* SU-8 to accumulate in early log phase.

Pseudomonas aeruginosa was found to accumulate higher concentration of PHA at pH 8. Samrot *et al.*,¹² and Nisha *et al.*,¹⁶ found *Enterobacter cloacae* SU-1 and *Pseudomonas putida* SU-8 to accumulate PHA near pH7 (Fig.4). *Pseudomonas aeruginosa* was found to accumulate higher concentration of PHA at 40°C (Fig.5). Optimal condition for PHA accumulation by *Pseudomonas aeruginosa* were at glucose containing medium, pH 8, temperature at 40°C. A similar study on optimization of PHA was done by Prasad. and Sethi²¹, where they found *Pseudomonas sp* to accumulate maximum PHA at pH- 7, temperature 30°C in 2% carbon source.

Peak was obtained between 220 and 240 nm and confirmed the presence of PHA which indicated the presence of ester group. Even Nair *et*

al.,²² who confirmed the presence of PHA by obtaining peak between 230 - 240 nm. The presence of PHA was confirmed by the retention factor 0.8 after running TLC.

The region at 1723 corresponds to the stretching of C=O in *Pseudomonas aeruginosa* was found. Other absorption bands at 1363, 1458, 1723, 2926-2954 and 3881 cm⁻¹ for CH₃, -CH₂, C=O, -CH



Fig.1. Growth of *Pseudomonas aeruginosa* on cetrimide agar

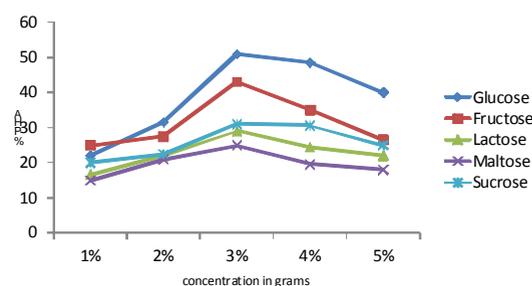


Fig.2. PHA accumulation in various carbon sources by *Pseudomonas aeruginosa*

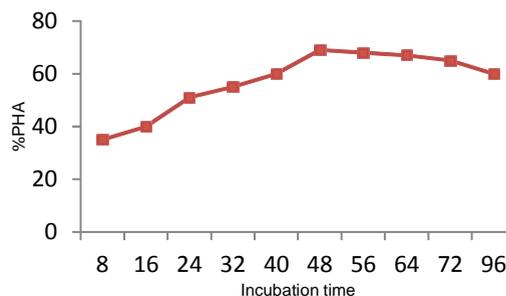


Fig. 3. PHA accumulation at various incubation time

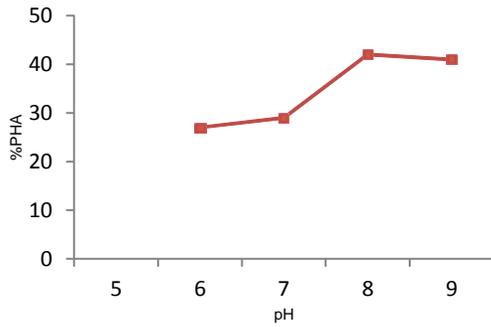


Fig. 4. PHA accumulation at different pH

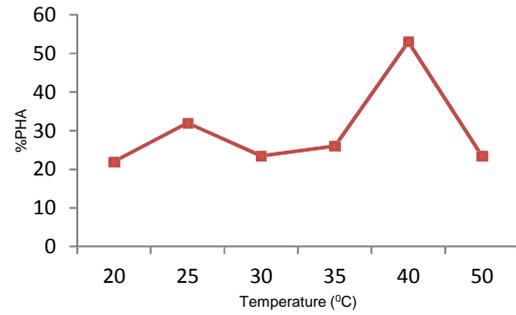


Fig.5. PHA accumulation at different temperature

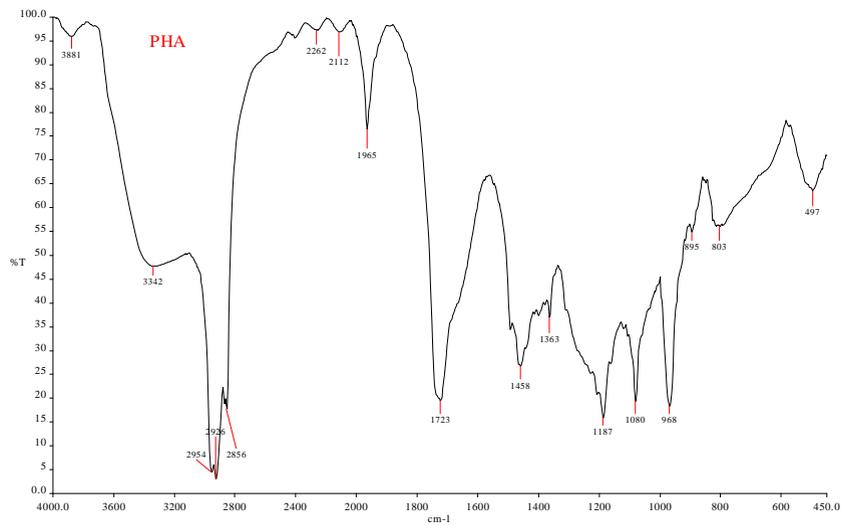


Fig. 6. FTIR analysis of PHA obtained from *Pseudomonas aeruginosa*

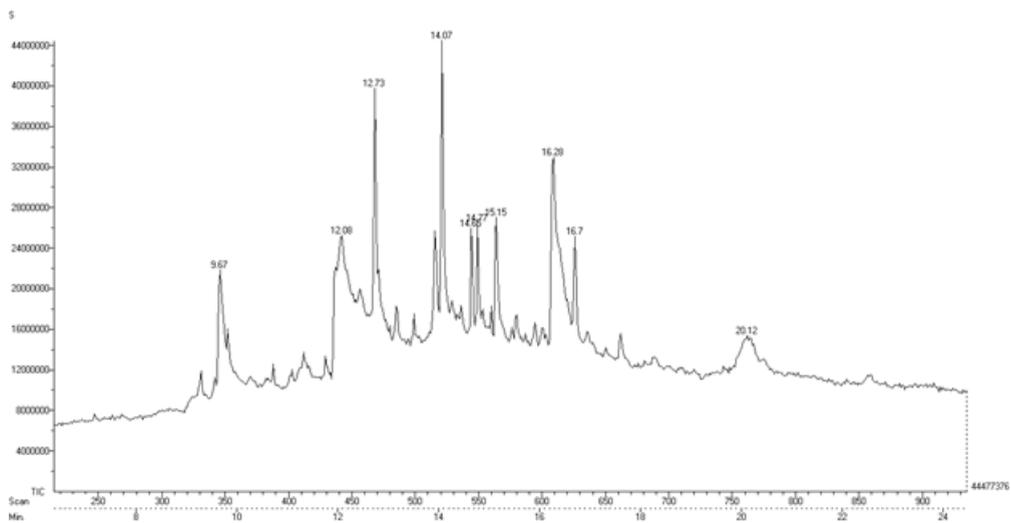


Fig.7. GCMS analysis of PHA obtained in sucrose containing medium from *Pseudomonas aeruginosa*

and OH groups (Fig.6). This is on par with the result of Nisha *et al.*,¹⁶ who found the peaks with the PHA isolated from *Pseudomonas putida* SU-8. The peaks at 14.07, 15.15 and 16.28 represents the presence of 3-hydroxytetradecatrienoate and 3-hydroxyhexadecadienoate in *Pseudomonas aeruginosa*. (Fig 7). Mostly microbes accumulate either scl-PHAs with 3HB units or mcl-PHAs containing 3-hydroxyoctanoate (HO) and 3hydroxydecanoate (HD) as the major monomers^{2,9,10,17}

CONCLUSION

Pseudomonas aeruginosa was found to accumulate more PHA at 48h incubation at 40°C in 3g/100ml in glucose containing medium at pH-8 GC analysis of PHA of *Pseudomonas aeruginosa* to possess monomers of 3- hydroxytetradecatrienoate and 3-hydroxyhexadecadienoate.

REFERENCES

1. Macrae RM, Wilkinson JF. Poly-beta-hydroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *J Gen Microbiol.*, 1958; **19**(1): 210-2.
2. Anderson, A. J., & Dawes, E. A. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. *Microbiological reviews.*, 1990; **54**(4): 450-2.
3. Griebel, R. J., and J. M. Merrick. Metabolism of poly-hydroxybutyrate: effect of mild alkaline extraction on native poly-hydroxybutyrate granules. *J Bacteriol.*, 1971; **108**: 782-9.
4. D. Byrom, "Production of poly-β-hydroxybutyrate: poly-β-hydroxyvalerate copolymers," *FEMS Microbiology Letters.*, 1992; **103**: 247-0.
5. Lillo, J.G., and Rodriguez-Valera, F. Effects of culture conditions on poly(β-hydroxybutyric acid) production by *Haloferax mediterranei*. *Appl. Environ. Microbiol.*, 1990; **56**: 2517-1.
6. Lemoigne M. Production of β-hydroxybutyric acid by certain bacteria of the *B. subtilis* group. *Ann. Inst. Pasteur.*, 1925; **39**: 144-6.
7. BrauneGG G, Bona .R, and Koller. M *Polymer Plastics Technology and Engineering*, 2002; **43**: 1-15.
8. Steinbuchel A. PHB and other polyhydroxyalkanoic acids: In G.R. H. J. Rehm, A. Puhler, and P. Stadler (ed.), *Biotechnology: products of primary metabolism*, 1st ed., vol. 6. Wiley-VCH, Weinheim, Germany. 1995; 432-434.
9. Lee SY. *Biotechnol. Bioeng* 1996; **49**: 1-14.
10. Steinbuchel A. and Valentin H E, *FEMS Microbiology Letter*, 1995; **128**: 219-228.
11. Law, J.H., Slepecky, R.A. Assay of poly-β-hydroxybutyric acid. *J. Bacteriol.*, 1961; **82**: 33-6
12. Jennifer S. A, Ahmed F, Jyotsana, P. Senthilkumar, and Antony V. Samrot. Accumulation Pattern of Polyhydroxyalkanoates by *Agrobacterium tumefaciens* SU-11 in Glucose Containing Medium. *Journal of Pure and Applied Microbiology.*, 2014; **8** (6): 4817-1.
13. Samrot, A. V., Avinesh, R. B., Sukeetha, S. D., Senthilkumar, P. Accumulation of poly [(R)-3-hydroxyalkanoates] in *Enterobacter cloacae* SU-1 during growth with two different carbon sources in batch culture. *Applied Biochemistry and Biotechnology.*, 2011; **163**(1): 195-3.
14. de Rijk, T. C., van de Meer, P., Eggink, G., & Weusthuis, R. A. Methods for Analysis of Poly (3 hydroxyalkanoate)(PHA) Composition. *Biopolymers online*. 2005.
15. Panda, B., Sharma, L., Singh, A. K., & Mallick, N. Thin layer chromatographic detection of poly-β-hydroxybutyrate (PHB) and poly-β-hydroxyvalerate (PHV) in cyanobacteria. 2008.
16. Nisha, J., Mudaliar, N., Senthilkumar, P., & Samrot, A. V. Influence of substrate concentration in accumulation pattern of poly (R) hydroxyalkanoate in *Pseudomonas putida* SU-8. *African Journal of Microbiology Research.*, 2012; **6**(15): 3623-0.
17. Brandl, H., Gross, R. A., Lenz, R. W., & Fuller, R. C. *Pseudomonas oleovorans* as a source of poly (β-hydroxyalkanoates) for potential applications as biodegradable polyesters. *Applied and Environmental Microbiology.*, 1998; **54**(8): 1977-1982.
18. Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V., & Yadav, A. Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes. *International Journal of Biomaterials*, 2013.
19. Durner, R., Witholt, B., & Egli, T. Accumulation of poly [(R)-3-hydroxyalkanoates] in *Pseudomonas oleovorans* during growth with octanoate in continuous culture at different dilution rates. *Applied and environmental microbiology.*, 2000; **66**(8): 3408-4.
20. Khanna, S., & Srivastava, A. K. Recent advances in microbial polyhydroxyalkanoates. *Process Biochemistry.*, 2005; **40**(2): 607-9.

21. Prasad M.P. and Sethi R. Production and isolation of polyhydroxyalkanoates from *Pseudomonas* sp using waste cooking oil as a sole carbon source. *International Journal of Advanced Biotechnology and Research.*, 2013; **4**(4): 527-2.
22. Nair, A. M., Annamalai, K., Kannan, S. K., Kuppusamy, S. Characterization of polyhydroxyalkanoates produced by *Bacillus subtilis* isolated from soil samples. *Malaya J Biosci.*, 2014; **1**: 8-12.