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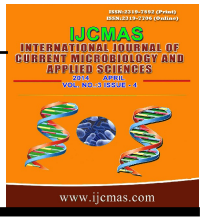

Original Research Article

Screening, optimization and characterization of poly hydroxy alkanoates (pha) produced from microbial isolates

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A B S T R A C T

Polyhydroxyalkanoates (PHAs) are polyesters of hydroxyalkanoates synthesized by various bacteria as intracellular carbon and energy storage compounds and accumulated as granules in the cytoplasm of cells. PHA producing bacteria from activated sludge and soil were isolated, characterized and screened by Sudan Black staining method. All the potential PHA producers were isolated, out of which two were of *Bacillus* sp. and one of *Pseudomonas* sp. and it was confirmed by their morphological and biochemical characters. PHA was extracted directly with solvent chloroform and estimated spectrophotometrically. Optimization of PHA accumulation was carried out at different temperature. The strain PHA-1 showed higher amount of PHA accumulation when compared to the other strains i.e. 52 mg/ml. The World wide dependence on petroleum by products for the manufacturing of plastics, scarcity of space for disposal and growing environmental concerns over non biodegradable synthetic plastics have fuelled research towards development of eco friendly biopolymer material. Hence, attention has been laid on production of Polyhydroxybutyrate, one of the most extensively studied PHA, is an intracellular microbial thermo plastic that is widely produced by bacteria present in soil.

K e y w o r d s

Polyhydroxy-butyrate, Activated sludge, Polyhydroxy-alkanoates, Sudan Black.

Introduction

Today, increasing industrial interest exists in the biotechnological production of polyhydroxyalkanoates (PHAs) from renewable resources to arrive at bio-based and biodegradable polymeric materials that can act as alternatives for common plastics derived from petrol (Braunegg *et al*., 1998, 2004; Brand, H *et al*., 1990; Luzier 1992; Steinbuchel and Valentin 1995). Finding an alternative way for responding to the problems associated with plastic waste and its effect on the environment, there has been an effort made in the production and development of biodegradable plastics.

Polyhydroxyalkanoates (PHAs) are regarded as new environmental friendly biodegradable plastics. Poly-hydroxybutyrate (PHB) and its copolymer

poly-hydroxybutyrate-co-hydroxyvalerate (P (HB-co-HV)) are the common types of PHAs, and other forms also exist. A wide variety of micro-organisms accumulate PHA within cells as an intracellular storage material of carbon and energy **(**Akar *et al*., 2006; Dionisi *et al*., 2006; Reis *et al*., 2003).

Activated sludge used as a mixed culture for PHA production has been studied by many researchers. When compared with pure culture fermentation processes, the merits of PHA production system by activated sludge are the cost reduction for producing bioplastics, simpler production facility, and wastes recycle. However, PHAs productivity and PHAs content during the use of activated sludge were much lower than those by pure culture (Lemos *et al*., 2003; Reis *et al*., 2003).

Polyhydroxyalkanoates (PHA) are polyesters that accumulate as inclusions in a wide variety of bacteria. Because of their inherent biodegradability, PHA are considered to be good candidates for biodegradable plastics, since they possess material properties similar to those of synthetic polymers currently in use and are completely biodegradable after disposal. Poly-hydroxyalkanoates (PHAs) are inclusion bodies accumulated by some bacterial genera as reserve material, when culture medium is unbalanced due to limited oxygen, nitrogen, phosphorous, sulphur or magnesium and an excess of carbon source (Lee, 1996). Lafferty *et al*., (1988) stated that the accumulation of PHA by microorganisms can be stimulated under unbalanced growth conditions, *i.e*., when nutrients such as nitrogen, phosphorus or sulfate become limiting, when oxygen concentration is low, or when the C: N ratio of the feed substrate is higher. PHB is accumulated by numerous microorganisms and is the best characterized PHA (Madison and Huisman, 1999). A number of bacteria such as *Azotobacter, Bacillus, Archaebacteria, Methylobacteria, Pseudomonas* have been found to synthesize PHA to varying levels. *Ralstonia eutropha* (formerly *Alcaligenes eitrophus*) has been the subject of much published research work because it can accumulate PHAs upto 80 per cent dry weight (Lee, 1996). The present study is focused on the isolation of PHA producing bacterial strains from activated sludge and forest soil, and their optimization.

Materials and Methods

Sampling

Activated sludge and forest soil sample were collected from Sewage treatment plant and Banneraghatta National park respectively for the isolation of bacteria.

Isolation of Bacterial Strains

Isolation of bacterial strains was carried out by serial dilution of fresh activated sludge sample and soil sample in 0.85% sterile saline solution followed by plating of the samples on a nutrient agar media. The plates were incubated at 37°C for 24–48hrs.

Screening and characterization of PHA accumulating strains

Bacterial strains were screened for PHA production by Sudan black staining (Burdon, 1942; Lee, 1996). The isolated bacterial colonies were subsequently analyzed for haemolysis on blood agar. Identification of isolates was carried out by performing biochemical tests (Holt, 1994).

Production of PHA in shake flask

The selected, most efficient PHB producing bacterial isolates were inoculated in Mineral Salt Medium (MSM) comprised of following 5g Sodium chloride, 1.5g K_2HPO_4, 1.5g KH_2PO_4, 1g $MgS0_4$, 5g Sucrose, 0.5g Ammonium nitrate in 1L of distilled water. The pH of the media was maintained to be 7±0.1.The culture flask was kept in shaker at 150 rpm at 35 °C for two days (Amirul *et al*., 2008, Du *et al*., 2001 and Yamanka *et al*., 2010).

Extraction of PHA

The PHA was directly extracted using the solvent chloroform. The bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The cell pellet was suspended in sodium hypochlorite solution and incubated at 37°C for 1 - 2 hrs for complete digestion of cell components except PHA, where by lipids and proteins were degraded. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with 10 ml of distilled water and centrifuged. The PHA granules in the sediment were washed twice with acetone, methanol and diethyl ether (1:1:1) respectively. The polymer granule was dissolved with boiling chloroform and was evaporated by air drying, to yield dry powder of PHA (Santhanum and Sasidharan, 2010).

Quantification of PHA

The bacterial culture was centrifuged at 6000 rpm to obtain the cell pellet and dried to estimate the dry cell weight (DCW) in units of g/L (Du *et al*., 2001). Residual biomass was estimated as the difference between dry cell weight and dry weight of extracted PHA (Zakaria *et al*., 2010). This was calculated to determine the cellular weight and accumulation other than PHAs. The percentage of intracellular PHA accumulation is estimated as the percentage composition of PHA present in the dry cell weight.

Residual biomass (g/L) = DCW (g/L) – Dry weight of extracted PHA (g/L)

$$\text{PHA accumulation (\%)} = \frac{\text{Dry weight of extracted PHA (g/L)}}{\text{DCW (g/L)}} \times 100\%$$

Optimization of PHA

PHA producing isolates were optimized at 37 ºC and 45ºC in Minimal Salt Broth, the sediments were collected which was further evaporated at 60 ºC with chloroform. The cell dry weight was treated with conc. H_2SO_4 at 100ºC for 10 minutes. And the absorbance was taken under UV spectrophotometer at 320 nm (Law and Slepecky, 1961).

Results and Discussion

Isolation and screening of bacterial isolates for PHA production

Several bacteria were isolated from activated sludge and soil sample by serial dilution method. From this, six isolates were selected for PHA production and based on Sudan Black staining, three potential PHA producers were screened out of six isolates.

Characterization of PHA producing bacterial isolates

Screened bacterial strains were characterized by Gram staining and biochemical tests. Out of three isolates, 2 were Gram positive bacteria and 1 was Gram negative bacteria (Table.1)

Table.1 Microbiological Biochemical characteristics

S. No	Biochemical Characteristics	PHA 1	PHA 2	PHA 3
1	Gram's Reaction	Gram positive rods	Gram positive rods	Gram negative rods
2	Motility Test	Motile	Motile	Motile
3	Oxidase Test	Positive	Positive	Positive
4	Catalase Test	Positive	Positive	Positive
5	Endospore staining	Positive	Positive	Negative
6	Nitrate reduction test	Positive	Positive	Positive
7	Starch hydrolysis	Positive	Positive	Negative

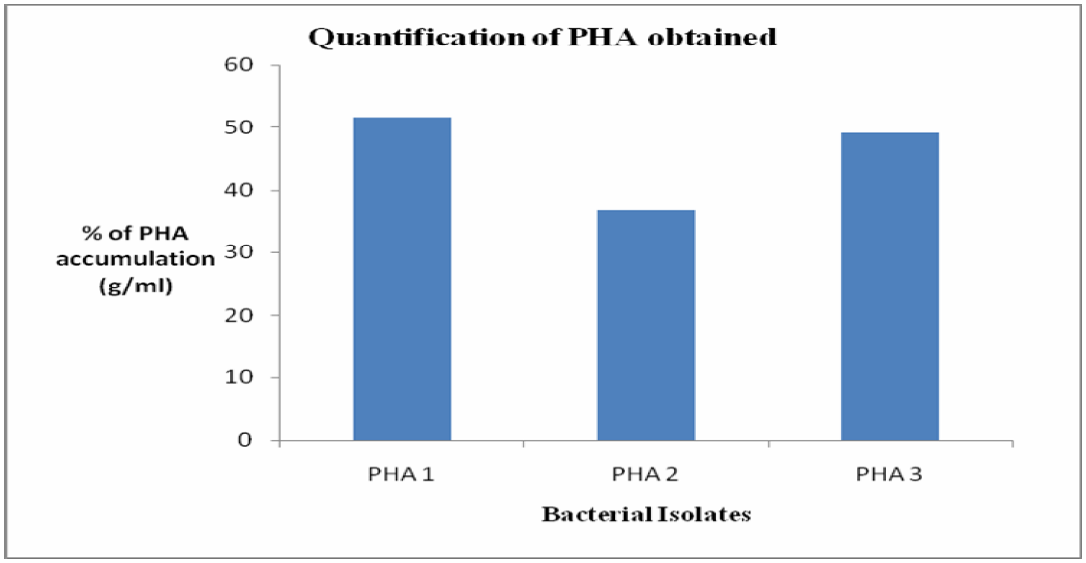
Table.2 Sugar Fermentation Test

Isolates	Glucose	Fructose	Lactose	Maltose	Sucrose	Mannitol	Galactose
PHA 1	**A/G+**	**A/G+**	-	**A/G+**	-	-	-
PHA 2	**A/G+**	**A/G+**	-	**A/G+**	**A/G+**	-	**A/G+**
PHA 3	**A**	**A/G+**	-	-	-	-	-

Table.3 Quantification of PHA obtained

Isolates	Dry weight of PHA (g/ml)	Cell Dry weight (g/ml)	Residual Biomass (g/ml)	% of PHA accumulation (g/ml)
PHA 1	0.052	0.101	0.049	51.49
PHA 2	0.041	0.112	0.071	36.61
PHA 3	0.052	0.106	0.054	49.06

Figure 1: Quantitative analysis PHA



Extraction of PHA

From the screened organisms, dry weight of PHA was high in PHA-1 and PHA-3, compared to PHA-2, grown in Mineral Salt Medium. The extracted PHA was an ivory white colored powder and it was found to be sparingly soluble in water. The extracted PHA is shown in (Table 2).

Quantification of PHA

Extracted PHA was quantified and compared with other isolated organisms. From the results it was found that PHA accumulation was in proportion to the dry cell weight which was similar to the studies of Du *et al*., (2001) and Zakaria *et al*., (2010). (Table: 3) (Figure: 1 & 2)

References

Akar, A., Akkaya, E. U., Yesiladali, S. K., Celikyilmaz, G., Cokgor, E. U., Tamerler, C., Orhon, D and Cakar, Z. P. 2006. Accumulation of polyhydroxyalkanoates by Micro lunatus phosphovorus under various growth conditions, Journal of Industrial Microbiology and Biotechnology 33(3):215-220.

Amirul, A. A., Yahya, A.R.M., Suresh, K., Azizan, M.N.M and Majid, M.I.A. 2008. Biosynthesis of poly (3-hydroxy butyrate-co-4-hydroxybutyrate) copolymer by *Cupriavidus* sp. USMAA1020 isolated from lake Kulim Malaysia. Bioresour Technol., 99: 4903-4909.

Aravind, J., Sasikala, P., Preethi, R and Aravind. 2012. Production of Polyhydroxyalkanoate (PHA) Using Hydrolyzed Grass and Syzygium Cumini Seed As Low Cost Substrates. Journal of Microbiology, Biotechnology 13: 2 (3) 970-982 Food Sciences 970.

Biochem Eng Biotechnol 41:77–93.

Brand, H., Gross, R.A., Lenz, R.W and Fuller, R.C. 1990. Plastics from bacteria and for bacteria:

Braunegg, G., Bona, R and Koller, M., 2004. Sustainable polymer production. Polym Plast Technol Eng43:1779–1974.

Braunegg, G., Lefebvre, G and Genser, KF. 1998. Polyhydroxyalkanoates, biopolyesters from renewableresources: physiological and engineering aspects. J Biotechnol 65:127–161.

Burdon, K.L., Stokes, J.C and Kimbrough, C.E. 1942. Studies of the common aerobic spore-forming *Bacilli* staining for fat with Sudan Black B- stain. Journal of Bacteriology, 43, 717-724.

Dionisi, D., Majone, M., Vallini, G., Di Gregorio, S and Beccari, M. 2006. Effect of the applied organic loadrate on biodegradable polymer production by mixed microbial cultures in a sequencing batch reactor, Biotechnology and Bioengineering 93(1):76-88.

Du, G., Chen, J., Yu, J and Lun, S. 2001. Continuous production of poly-3-hydroxybutyrate by *Ralstonia eutropha* in a two stage culture system. J. Biotech., 88: 59-65.

Holt, J.G., Krig, N.R., Sneath Pefer, H.A., Staley, J.T and Williams, S.T. 1994. Bergey's Manual of Determinative Bacteriology 9th edition, Williams and Wilkins, Baltimore.

Lafferty, R.M., Korsatko, B and Korsatko, W. 1988. Microbial production of poly -hydroxybutyric acid.Biotechnology Special microbial Processes, 136-176.

Lee, S.Y. 1996. Bacterial Polyhydroxyalkanoates. *Biotechnology and Bioengineering*, 49: 1-14. Microbiology Review, 103, 131-140.

Lemos, P.C., Serafim, L.S., Santos, H and Reis, M. A. 2003. Production of polyhydroxyalkanoates by a mixed culture in a sequencing batch reactor: the use of propionate as carbon source, Communications in agricultural and applied biological sciences 68(2 PtA):109-114.

Luzier, W. D. 1992. Materials derived from biomass/biodegradable materials. ProcNatlAcadSci U S A. 89:839–842.

Madison, L.L and Huisman, G.W. 1999. Metabolic engineering of poly (3 hydroxyalkanoates): from DNA to plastic. Microbiology Molecular Biology Reviews. 63, 21–53.

Nandini, P., Amruta, C., Bhavesh, P., Pragya, R., Priti, V and Mital, P. 2011. Screening of PHA (poly hydroxyalkanoate) producing bacteria from diverse sources International Journal of Biosciences (IJB) ISSN: 2220-6655 Vol. 1, No. 6, p. 27-32.

Poly (beta-hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. Adv

Reis, M. A. M., Serafim, L. S., Lemos, P. C., Ramos, A. M., Aguiar, F. R and Van Loosdrecht, M. C. M. 2003. Production of polyhydroxyalkanoates by mixed microbial cultures, Bioprocess and Biosystems Engineering 25(6):377-385.

Santhanam, A and Sasidharan, S. 2010. Microbial production of polyhydroxyalkanote (PHA) from *Alcaligens* spp. And *Pseudomonas oleovorans* using different carbon sources. Afri. J.Biotechnol., 9: 3144-3150.

Slepecky, R. A and J. H. Law. 1960. A rapid spectrophotometric assay of alpha, beta-unsaturated acids and B-hydroxy acids. Anal. Chem. 32: 1697-1699.

Steinbüchel, A and Valentin, H,E. 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol Lett. 128:219–228.

Yamanaka, K., Kimura, Y., Aoki, T and Kudo, T. 2010. Effect of ethylene glycol on the end group structure of poly (3-hydroxybutyrate). Polym.Degrad. Stab., 95: 1284-1292.

Yan, S., Tyagi, R. D and Surampalli, R. Y. 2006. Polyhydroxyalkanoates (PHA) production using wastewater as carbon source and activated sludge as microorganisms, Water Science & Technology 53 (6):175–180. 429.

Yang, Y. H., Brigham, C. J., Budde, C. F., Boccazzi, P., Willia, L. B., Hassan, M.A., Yusof, Z. A.M., Rha, C and Sinskey, A. J. 2010.Optimization of growth media components forpolyhydroxyalkonoate (PHA) production from organic acids by *Ralstonia eutropha*. ApplMicrolBiotechnol., 87: 237-2045.

Zakaria, M. R., Ariffin, H., Johar, N. A. M., Aziz, S. A., Nishida, H., Shirai, Y and Hassan, M. A. 2010. Biosynthesis and characterization of poly (3-hydroxybutyrate-co-3-hydroxybutyrate) Co-polymer from wild type *Comamonas* sp. EB172. Polym.Degrad. Stab., 95: 1382-1386.

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