JOURNAL OF PURE AND APPLIED MICROBIOLOGY, Dec. 2014. Vol. **8**(6), p. 4817-4821

Accumulation Pattern of Polyhydroxyalkonoates by *Agrobacterium tumefaciens* SU-11 in Glucose Containing Medium

S. Alexandria Jennifer[1], Faizal Ali[1], Jyotsana[1], P. Senthilkumar[2] and Antony V. Samrot[1#]

[1]Department of Biotechnology, Sathyabama University, Jeppiaar Nagar, Sholinganallur, Chennai – 600 119, Tamil Nadu, India.
[2]Department of Chemical Engineering, Sathyabama University, Jeppiaar Nagar, Sholinganallur, Chennai – 600 119, Tamil Nadu, India.

(Received: 10 September 2014; accepted: 25 Novembe 2014)

Polyhydroxyalkonoates (PHA) are intracellular energy storage granules of most bacteria and these PHA has got potential use in industries, pharmaceuticals and nanotechnology. In this study, PHA accumulation by a soil isolate, *Agrobacterium tumefaciens* SU-11 was analysed. Highest accumulation of PHA by *A.tumefaciens* SU-11 was found in glucose (6g/l) containing medium. FTIR and GC analysis confirmed the accumulation of polyhydroxydeconoate (PHD) by *A.tumefaciens* SU-11.

Key words: PHA – polyhydroxyalkonoates; PHD - polyhydroxydecanoic acid; FTIR – Fourier transform infrared spectroscopy; GC- Gas chromatography.

Polyhydroxyalkanoate (PHA) are biopolymers accumulated when bacterial cells are grown in excess carbon and limited other nutrients[1]. These PHAs are utilized as intracellular carbon and energy source at late stationary phase and reduces mortality rate[2]. PHA granules accumulated in cytoplasm are mostly amorphous and spherical in shape; these are also appeared to be electron transparent with clear boundaries under Transmission Electron Microscope[3]. The number and size of these PHA granules varies among PHA accumulating microorganisms[4], which depends on the PHA granule associated proteins and enzymes such as PHA synthase, PHA depolymerase, structural proteins (Phasins) and regulatory proteins[5]. PHA granules can be stained with Sudan Black B[6] or Nile Blue A[7]. There are two groups among the PHAs producing bacteria on the basis of number of carbon atoms present in monomer units[8] i.e. Short –chain length PHAs (scl-PHA) (3-5 carbon atoms) and medium chain length PHAs (mcl-PHA) (6-14 carbon atoms)[9].

The biosynthesis of different types of PHAs significantly depends on the chosen microorganism, fermentation condition and different carbon source. The vast majority of microbes synthesize either scl-PHAs containing primarily 3-hydroxybutyrate units or Mcl-PHAs containing 3-hydroxyoctanoate (3HO) and 3 hydroxydecanoate (3HD) as the major monomers[1]. The co-polymers of PHA are also produced which varies according to substrates used. These co-polymer of scl-PHA and mcl-PHA are as it got more tensile strength and other mechanical properties[10]. Kluttermann[11] found *Agrobacterium radiobacter*, to accumulate PHB while grown on minimal medium containing different trimethylammonium and also found highest PHB formation (71%) on 1% *D*(+)-carnitine containing medium.

* To whom all correspondence should be addressed.

In this study, *Agrobacterium tumefaciens* SU-11 was isolated from soil and optimization of PHA production was done. PHA produced was characterized by GC-MS and FTIR.

MATERIALS AND METHODS

Isolation and identification of bacteria

Microorganism was isolated from garden soil collected from Sathyabama University, Chennai, Tamil Nadu – 600119, India following conventional microbiological methods. Isolated microorganism was identified based on their biochemical reaction and 16SrRNA sequencing. Thus obtained organism was preserved in nutrient agar at 4^0C in refrigerator.

Production and optimization of PHA

The following concentration of minimal media was used for PHA production. Glucose (5g/l), Potassium dihydrogen phosphate (3g/l), disodium hydrogen phosphate (17.8g/l), ammonium chloride (2g/l), sodium chloride (0.5g/l) and magnesium sulphate (2.4g/l) as earlier[12]. In order to optimize the production of PHA the concentration of the carbon source, temperature, pH, incubation time and $MgSO_4$ were varied.

PHA isolation

The culture was centrifuged and the pellet was washed with distilled water and then freeze dried. PHA was isolated by following the method of Law *et al*[13]. Percentage of PHA accumulation was determined by following calculation. % PHA = amount of PHA produced / cell dry weight x 100

FTIR analysis

Infrared spectra (IR) were recorded on polymer films cast from chloroform solution onto KBr plates using FTIR (Shimadzu, DR-800) at 27°C.

Gas chromatography analysis

About 2mg sample was subjected to methanolysis following modified method of Bhuwal[14] with a solution consisting of 1ml chloroform, 2ml methanol and 0.15ml H_2SO_4 at 100°C for 140 min. The methanolysed esters were analyzed by GC Clarus 500 Perkin Elmer. Helium (1 ml/min) was used as carrier gas. The injector and detector are at 250° C and 200°C respectively. The program was used for 36 min; ramp of 2 min up to 110°C; 10°C per min rise up to 200° C and hold at 280°C for 5 min.

RESULTS AND DISCUSSION

Agrobacterium tumefaciens SU-11 isolated from garden soil sample which was identified by biochemical parameters and confirmed by 16s rRNA sequence. The sequence is being deposited in GENBANK and the accession number is JQ746125. *Agrobacterium tumefaciens* SU-11 was found to accumulate more amount of PHA (i.e.43%) in glucose containing medium (6g/l) than in lactose containing medium. The highest PHB accumulation (60% PHB/cell dry weight) was reported in *Agrobacterium radiobacter*[11].

In this present study, pH, temperature and incubation time were found to influence the PHA accumulation and highest accumulation of PHA was seen in glucose containing medium (6g/l) at pH 7 with incubation time of 48h at 30°C (Figure 1 and 2). Limitation of nitrogen source usually increases the production of PHA as reported[15]. Contrasting to this finding, nitrogen source limitation was found to decrease PHA

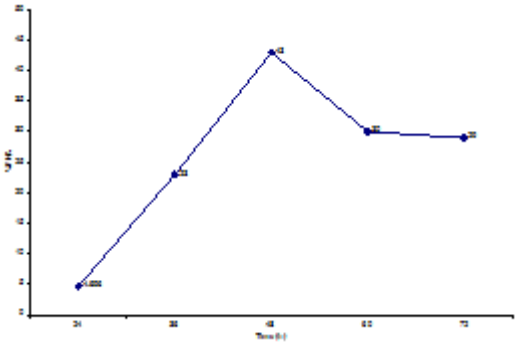

Fig. 1. PHA accumulation by *Agrobacterium tumefaciens* SU-12 at various incubation time in glucose containing medium

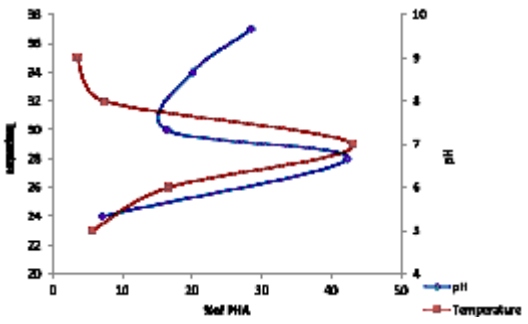

Fig. 2. PHA accumulation by *Agrobacterium tumefaciens* SU-12 at various temperature and pH in glucose containing medium

accumulation and limitation of $MgSO_4$ increased PHA accumulation upto 52% in glucose containing medium (Figure 3).

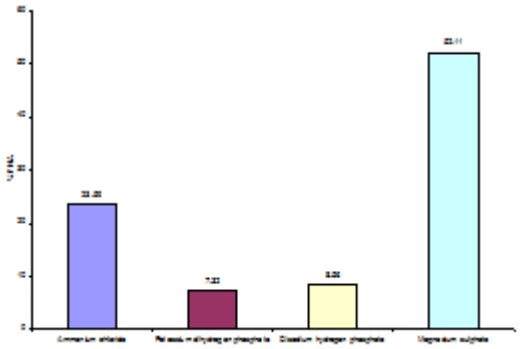

Fig. 3. PHA accumulation by *Agrobacterium tumefaciens* SU-12 at limitation of any nutrition in glucose containing medium

The FTIR spectrum indicated peaks at 1736 and 1730 attributing to the stretching vibration of the CO group (ethyl carbonyl) in the polyester. Bands at 1156, 1190, 1213 and 1261 cm^{-1} indicated presence of COC group. Transmission bands at 2851, 2923, 2958, and 2969 were attributed to stretching vibration of CH bonds of methyl (CH_3) and methylene (CH_2) group. Other characteristic bands for mcl-PHA were noticed at 3409, 1097, 1025, 974, 860, 802 and 486cm^{-1} (Figure 4).

GC analysis of the PHA produced in glucose containing medium was confirmed as Polyhydroxydecanoate (PHD) (Figure 5). Mcl polymers such as HD, HTD, HDD are usually accumulated only when abnormal carbon sources are used[16]. Mcl polymers are usually products of intermediates of fatty acid de novo biosynthesis

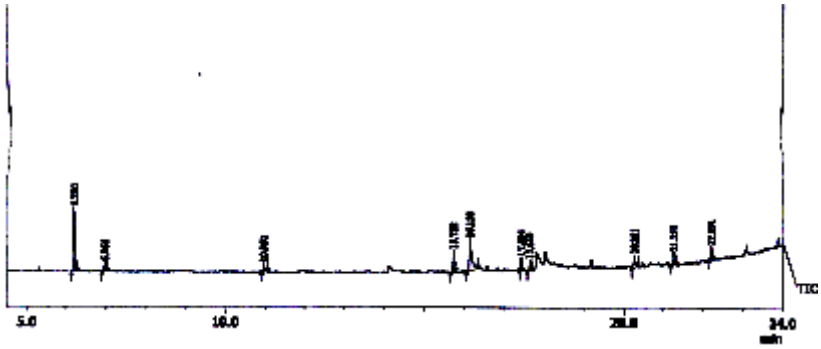

Fig. 5. GC spectrum of PHA accumulated by *Agrobacterium tumefaciens* SU-12 grown in glucose containing medium

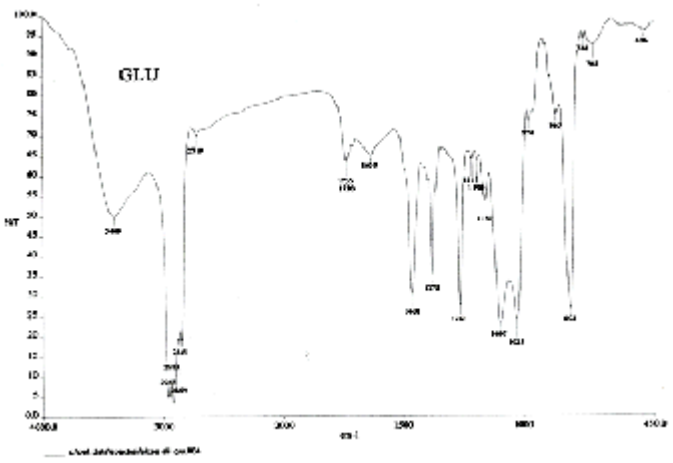

Fig. 4. FTIR spectrum of PHA accumulated by *Agrobacterium tumefaciens* SU-12 grown in glucose containing medium

and β-oxidation fatty acid cycles and also depends on the metabolic routes involved[17,18,19,20,21].

CONCLUSION

The isolated organism was identified as *Agrobacterium tumefaciens* SU-11 and it was found to accumulate more amount of PHA at Glucose (5g/l), Potassium dihydrogen phosphate (3g/l), disodium hydrogen phosphate (17.8g/l), ammonium chloride (2g/l), sodium chloride (0.5g/l) and magnesium sulphate (2.4g/l) pH 7 and 30ºC with 48 hrs of incubation time. The accumulation of PHA was decreased when nitrogen source and other nutrients such as disodium hydrogen phosphate and potassium dihydrogen phosphate were limited but limitation of $MgSO_4$ resulted in increased production of PHA. FTIR and GC analysis confirmed the presence of PHD accumulation.

REFERENCES

1. Anderson, A.J., Daves, E.A. Occurence, metabolic role and industrial uses of bacterial polyhydroxyalkonate. *Microbiological Reviews*, 1990; **54**(4): 450-472.
2. Macrae, R. M., Wilkinson, J. F. Poly-²-hyroxybutyrate metabolism in washed suspensions of *Bacillus cereus* and *Bacillus megaterium*. *Journal of general microbiology*, 1958;**19**(1), 210-222.
3. Madison, L. L., Huisman, G. W. Metabolic engineering of poly (3-hydroxyalkanoates): from DNA to plastic. *Microbiology and molecular biology reviews*, 1999; **63**(1), 21-53.
4. Chicago Byrom, D.: Polyhydroxyalkanoates, In: *Plastic from microbes: microbial synthesis of polymers and polymer precursors* (Mobley DP, ed). Hanser Munich, 1994; pp 5-33.
5. Griebel, R. J., Merrick, J. M. Metabolism of poly-²-hydroxybutyrate: effect of mild alkaline extraction on native poly-²-hydroxybutyrate granules. *Journal of bacteriology*, 1971; **108**(2), 782-789.
6. Burdon, K. L. Fatty material in bacteria and fungi revealed by staining dried, fixed slide preparations. *Journal of bacteriology*, 1946; **52**(6), 665-678.
7. Ostle, A. G., Holt, J. G. Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. *Applied and Environmental Microbiology*, 1982; **44**(1), 238-241
8. Steinbüchel, A., Valentin, H. E. Diversity of bacterial polyhydroxyalkanoic acids. *FEMS Microbiology Letters*, 1995; **128**(3), 219-228.
9. Lee, S. Y. Bacterial polyhydroxyalkanoates. *Biotechnology and bioengineering*, 1996; **49**(1), 1-14.
10. Noda, I., Lindsey, S. B., Caraway, D. (2010).: Nodax™ class PHA copolymers: their properties and applications. In: *Plastics from Bacteria*. Springer Berlin Heidelberg, 2010; pp 237-255.
11. Klüttermann, K., Tauchert, H., Kleber, H. P. Synthesis of Poly ² hydroxybutyrate by Agrobacterium radiobacter after Growth on D Carnitine. *Acta biotechnologica*, 2002; **22**(34), 261-269.
12. 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), 195-203.
13. Law, K. H., Leung Y.C., Lawford. H., Chau, H., Lo, W.H., Yu, P. H. Production of polyhydroxybutyrate by Bacillus species isolated from municipal activated sludge. *Appl. Biochem. Biotechnol*., 2001; **91**: 515–524
14. 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.
15. Din, M. M., Ujang, Z., Loosdrecht, M. V., Ahmad, A., Sairan, M. F. Optimization of nitrogen and phosphorus limitation for better biodegradable plastic production and organic removal using single fed-batch mixed cultures and renewable resources. *Water science and technology*, 2006; **53**(6), 15-20.
16. Xi, J., Wu, Q., Yan, Y., Zhang, Z., Peter, H. F., Cheung, M. K., Chen, G. Q. Hyperproduction of polyesters consisting of medium-chain-length hydroxyalkanoate monomers by strain Pseudomonas stutzeri 1317. *Antonie van Leeuwenhoek*, 2000; **78**(1), 43-49.
17. Tsuge, T. Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates. *Journal of bioscience and bioengineering*, 2002; **94**(6), 579-584.
18. Poirier, Y., Nawrath, C., Somerville, C. Production of polyhydroxyalkanoates, a family of biodegradable plastics and elastomers, in bacteria and plants. *Nature Biotechnology*, 1995; **13**(2), 142-150.

19. Liu, Q., Ouyang, S. P., Chung, A., Wu, Q., Chen, G. Q. Microbial production of R-3-hydroxybutyric acid by recombinant E. coli harboring genes of phbA, phbB, and tesB. *Applied microbiology and biotechnology*, 2007; **76**(4), 811-818.
20. De Smet, M. J., Eggink, G., Witholt, B., Kingma, J., Wynberg, H. Characterization of intracellular inclusions formed by Pseudomonas oleovorans during growth on octane. *Journal of bacteriology*, 1983; **154**(2), 870-878.
21. He, W., Tian, W., Zhang, G., Chen, G. Q., Zhang, Z. Production of novel polyhydroxyalkanoates by Pseudomonas stutzeri 1317 from glucose and soybean oil. *FEMS microbiology letters*, 1998; **169**(1), 45-49.