JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

A STUDY ON EFFECT OF MUTAGENIC AGENTS ON POLYHYDROXYALKANOATES (PHA) PRODUCTION

Jayaseelan Aravind *, *Hubbathalai Sunderraj Sangeetha*

Address(es): Dr. J. Aravind,
Faculty of Biotechnology, Kumaraguru College of Technology, Coimbatore - 641049, Tamilnadu, India. Ph: +91422 2669401.

*Corresponding author: dr.j.aravind@gmail.com

ARTICLE INFO

Received 25. 5. 2013
Revised 5. 1. 2014
Accepted 24. 2. 2014
Published 1. 4. 2014

Regular article

OPEN ACCESS

ABSTRACT

The effect of mutagenic agents on Polyhydroxyalkanoates (PHA) accumulation was investigated in two micro-organisms *Cupriavidus nectar* and *Kluyvera intermedia*. Three mutagenic agents- ultraviolet light, heat, chemical mutagens (acriflavin and 5 bromouracil) were selected for the study. The cultures were treated at various time intervals and chemical at varying concentration and cultured using hydrolyzed grass (*cyanodon dactylon*) as a substrate. It was found that higher accumulation was obtained in *C. nectar* when treated at a concentration of 50µg/ ml acriflavin and 5 bromouracil (25µg/ ml). *K. intermedia* showed a higher accumulation at acriflavin concentration of just 25µg/ ml and 5- bromouracil at 50µg/ ml concentrations. It was observed that % PHA accumulation significantly decreased with increase in exposure to UV in both *C. nectar* (17 % - 1.18%) and *K. intermedia* (15 % - 7%). Exposure of culture to heat resulted in less PHA accumulation in *C. nectar* (16 % - 11%), *K. intermedia* (17 % - 19 %) compared to their parent strain *C. nectar* (17 %) and *K. intermedia* (25 %). FTIR spectra revealed the presence of characteristic medium chain length (mcl) PHA in the obtained sample.

Keywords: PHA - mutation - mutagenic agents - FTIR

INTRODUCTION

Polyhydroxyalkanoates (PHAs) are natural biopolymers, excess carbon accumulated by bacteria under nutrient limiting conditions (N or P). They occur as an intracellular product either as inclusion bodies or as complexes with calcium and polyphosphates in the cytoplasmic membranes and constitute for about 80% of the dry cell weight and can be identified by staining with Sudan black B or using Nile blue A under fluorescent microscopy **(Ostle *et al.*, 1982)**. PHA posses physical and mechanical properties similar to synthetic plastics **(Poirier *et al.*, 1995)** and are widely used in manufacturing of packaging films, sutures, cardiovascular stents, scaffold and fixation rods etc. **(Scholz, 2000)**. *Ralstonia eutropha*, a facultative autotroph accumulate about 80 % of its cell dry weight as PHA from simple carbon sources like glucose, fructose and acetic acid and is known to be widely exploited for large scale production **(Anderson *et al.*, 1990)**. The major concern related to PHA production is its high cost of raw materials and lower yield restricting its large scale production in industries **(Ojumu *et al.*, 2004)**. Several alternative strategies have been exercised to overcome these problems by using low cost renewable substrates and strain improvement strategies via mutation and genetic engineering making alternation in genes has also been investigated recently. In cyanobacteria it has resulted in increased accumulation of about 52% of its cell dry weight as PHA using naturally available sources **(Akiyama *et al.*, 2011)**. The biosynthetic pathway for PHB production in microorganism is regulated by three genes phb A, B and C and synthesize three enzymes namely β-ketoacyl-CoA thiolase, acetoacetyl CoA reductase and PHB polymerase respectively. PhaA gene catalyses the synthesis of acetoacetyl–CoA from acetyl–CoA, phaB stereo specifically reduces acetoacetyl–CoA to (R)-3-hydroxybutyryl– CoA and phaC promotes the incorporation of (R)-3-hydroxybutyryl–CoA **(Lee, 1995).**

Metabolic engineering of genes by mutation has been investigated for higher accumulation of PHA in microorganisms. Mutation can be brought about by exposure to physical and chemical mutagens. Mutation in *Bacillus sp.,* induced by UV exposure and chemical mutagens like acriflavin and 5-bromouracil resulted in higher production in mutant strains compared parent strains and also showed different protein profiles **(Katircioglu *et al.*, 2003)**.

The main objective of our research is to increase the accumulation of PHA by studying the effect of mutagenic agents on two micro-organisms *Cupriavidus nectar* and *Kluyvera intermedia*. This work was carried out in Kumaraguru College of Technology, Coimbatore.

MATERIAL AND METHODS

Media composition

Minimal salt media was used for the growth conditions. Media composition: Glucose (10g/l), sodium chloride (5g/l), di- potassium hydrogen phosphate (5g/l), magnesium sulphate (1g/l), ammonium chloride (1g/l). The culture was incubated for 48h at room temperature in orbital shaker using hydrolyzed grass as a substrate.

Substrate hydrolysis

The hydrolysis of the substrate was carried out according to the procedure followed by **Cao *et al.*, (1995)** were cellulose is converted in to glucose using zinc chloride method. The finally obtained white mass was used as a substrate.

Glucose estimation

The amount of glucose in hydrolyzed grass was estimated using 3, 5 Di nitro salicylic acid method and absorbance value was measured at 540 nm **Miller (1959)**.

Extraction of PHA

Extraction of PHA was done using sodium hypochlorite digestion followed by extraction with hot chloroform according to procedure **Sasidharan and Santhanum (2010).**

Determination of % PHA accumulation and PHA concentration

The intracellular PHA accumulation was estimated as ratio of dry weight of PHA extracted to cell dry weight **(Zakaria *et al.*, 2010).**

$$\text{PHA accumulation} = \frac{\text{Dry weight of PHA (mg/ ml)}}{\text{Dry cell weight (mg/ml)}} \text{X } 100$$

Effect of UV exposure on PHA production

The effect of UV light on PHA production was studied by exposing the culture to UV at various time intervals. Fresh 24 h inoculated culture was spread plated on a sterile petri plate and incubated at 37 ˚C for 24 h in an incubator. Later the plates were transferred to UV chamber and placed at a height of 40 cm from the light source and exposed to UV at different time interval ranging from 5 min to 30 min. The plates after irradiation were immediately transferred to a black box to avoid photo - reactivation by light. The plates were kept inside for a period of 2 h and then the culture was inoculated in production medium containing hydrolyzed grass as a substrate.

Heat induced mutation

One ml of the culture was taken in a sterile test tube and incubated at 60 ˚C for various time interval ranging from 1 min to 4 min and then was allowed to cool at room temperature **(Sideropolous *et al*., 1968)**. The cultures were then inoculated in mineral salt media containing hydrolyzed grass as a carbon source and incubated for 48 h and the % PHA accumulation was determined for each time of exposure.

Effect of chemicals mutagens on PHA production

Chemical mutagens like acriflavin and 5 bromouracil were used to study their effect on PHA accumulation. One ml of 24 h culture was collected in an Eppendorf tube and centrifuged at 10000 rpm for 5 min. To the pellet obtained, mutagens were added at various concentrations ranging from 25µg/ml to 100 µg/ml and left for about one hour. Later the exposed culture was centrifuged at 5000 rpm for 10 min to remove the traces of mutagen and suspended in sterile saline and was inoculated in minimal salt media for 48 h.

FTIR

The obtained PHA granule was processed in to KBr pellets and infrared spectroscopy was recorded in the spectrum range 400-4000 cm^{-1}. The spectrum obtained was compared with standard PHB.

RESULTS AND DISCUSSION

Determination of glucose by DNS method

The amount of glucose in the hydrolyzed sample was estimated from the standard glucose curve and the concentration was found to be 3700 µg/ml.

Effect of UV exposure on PHA production

The effect of UV on PHA accumulation was studied by exposing culture to UV at various time intervals. It was found that the PHA accumulation decreased with increase in exposure time for *C. nectar* and *K. intermedia* when compared to their unexposed culture as shown in figure 1. The decrease in PHA accumulation on UV exposure was similar to the result obtained by **(Adwitya *et al*., 2008).** The decrease in accumulation may be due to mutation in the gene involved in PHA biosynthetic machinery resulting in decreased catalytic efficiency of the enzyme to produce polymers of PHA.

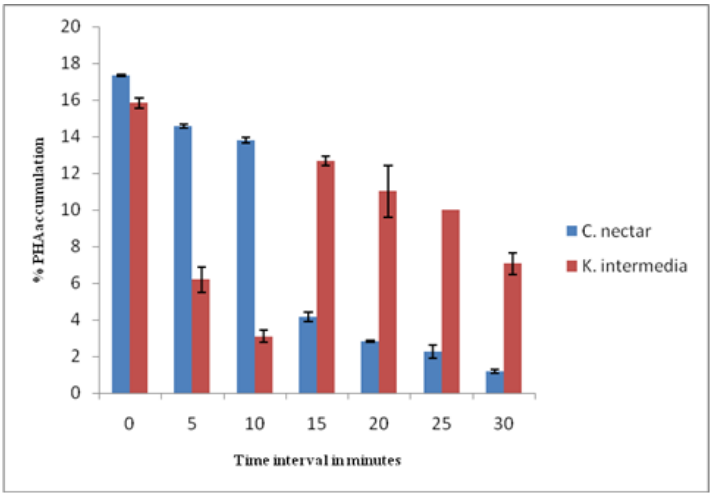

Figure 1 PHA accumulation (%) by *C. nectar* and *K. intermedia* on exposure to UV

Effect of heat on PHA production

On exposure of culture to heat it was found that PHA accumulation in *C. nectar* and *K. intermedia* decreased at various intervals of time as shown in figure 2. The exposure of culture to higher temperature for a long period of time would have been resulted in DNA damage resulting in decreased PHA accumulation.

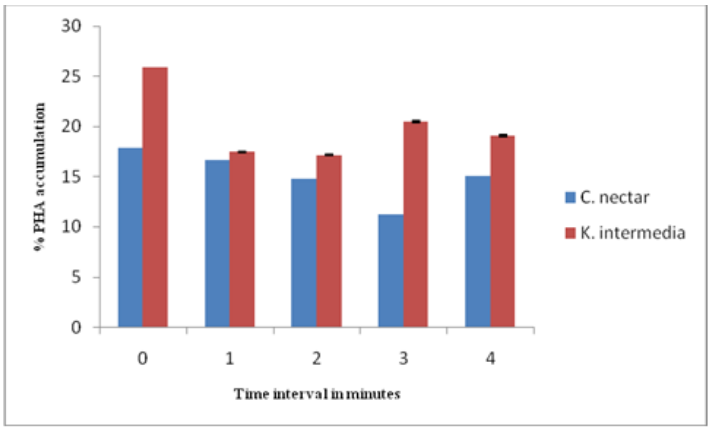

Figure 2 PHA accumulation (%) by *C. nectar* and *K. intermedia* on exposure to temperature

Effect of chemical mutagens on PHA production

Higher PHA accumulation was found in *C. nectar* when chemicals were treated at a concentration of acriflavin (50µg/ ml) and 5 bromouracil (25µg/ ml). *K. intermedia* showed a higher accumulation at acriflavin (25µg/ ml) and 5-bromouracil (50µg/ ml) (Figure 3 and 4). Increase in chemical concentration after the optimum resulted in decrease in PHA accumulation. The increase in PHA accumulation on treatment with various chemical mutagens is similar to the result obtained by **Katirciaglou *et al*., (2003)** in which mutant strains of *Bacillus sp*. resulted in higher PHB accumulation compared to their parent strain.

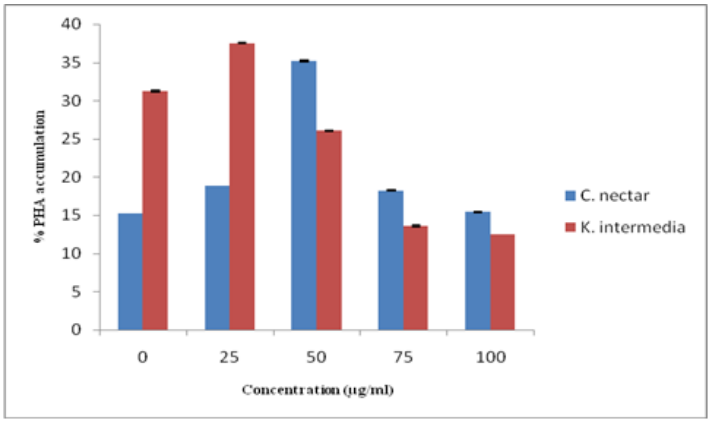

Figure 3 PHA accumulation (%) by *C. nectar* and *K. intermedia* on exposure to acriflavin

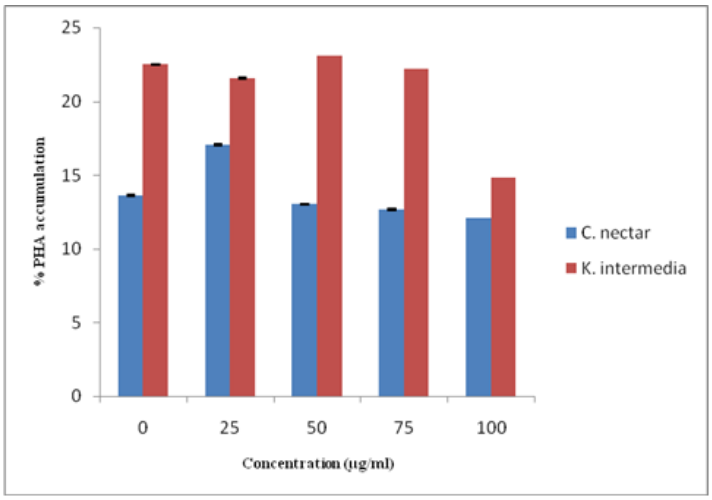

Figure 4 PHA accumulation (%) by *C. nectar* and *K. intermedia* on exposure to 5 bromouracil

FTIR

FTIR spectra were recorded in range from 400-4000 cm^{-1}. Figure 5 shows the spectra of standard PHB molecule with a strong signal at 1728 cm^{-1} representing the characteristic peak of PHB. The intense bands at 2980-2850 cm^{-1} correspond to the aliphatic C-H group, medium signals at 1000-1500 cm^{-1} represent the bending due to CH_2 and CH_3, 1280-1050 cm^{-1} due to valence symmetric and asymmetric stretch vibration of C-O-C **(Olivera *et al*., 2007; Sandhya and Aravind 2013).**

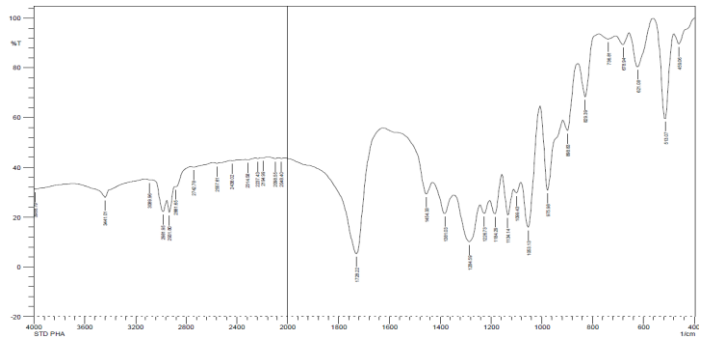

Figure 5 FTIR spectra for standard PHA

Figure 6 and Figure 7 shows the spectra obtained for PHA extracted from *C. nectar* and *K. intermedia.* The absorption bands at 3600-3100 cm^{-1} represent the OH stretch and aliphatic C-H group at 2954 and 2854 cm^{-1} **(Sanchez *et al*., 2003; Aravind *et al*., 2012-13)** similar to standard PHA. The presence of weak symmetric peaks near 1447- 1380 cm^{-1} suggest the presence of carboxyl group (C=O) in COOH in the polymer representing alkanoic acids in the sample **(Noghabi *et al*., 2007).** Intense peaks at 1500-1000 cm^{-1} represent the alkene and alkane bending of C-O stretch.

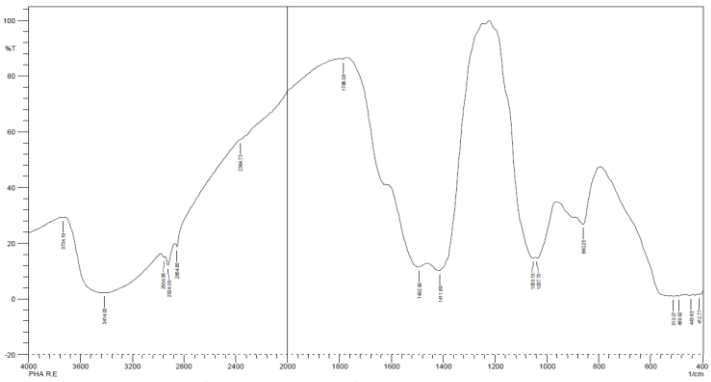

Figure 6 FTIR spectra for PHA isolated from *C. nectar*

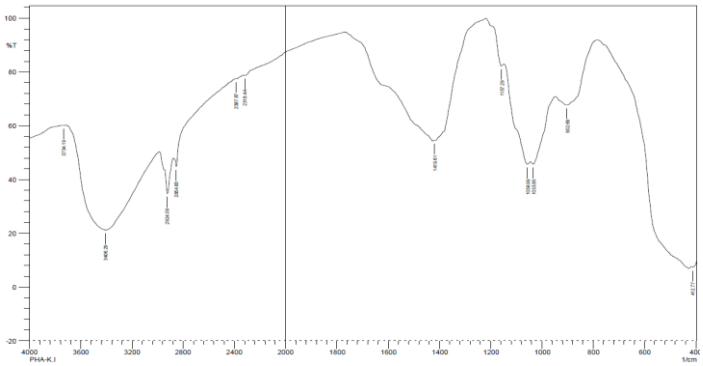

Figure 7 FTIR spectra for PHA isolated from *K. intermedia*

CONCLUSION

Effect of mutagenic agents (UV, temperature, chemicals) on PHA accumulation was studied in two microorganism namely *C. nectar* and *K. intermedia* to increase the yield of PHA. Chemical induced mutation was studied using two chemical mutagens, acriflavin and 5 bromouracil. It was found that *C. nectar* (35. 2 %, 17 %) and *K. intermedia* (37.5%, 23.12%) treated with acriflavin at 50µg/ ml and bromouracil (25 µg/ ml, 50 µg/ml) resulted in accumulation of higher amount of PHA compared to their parent strains *C. nectar* (15. 25 %, 13. 63 %) and *K. intermedia* (31. 25%, 22. 5%) respectively. Exposure of culture to UV and temperature resulted in decrease in accumulation ratio when compared to their parent strain. FTIR spectrum revealed the presence of mcl-PHA molecule in sample. Thus treatment of cultures with chemical mutagens provides a new strategy for high yield of PHA production.

REFERENCES

ADWITIYA, P., ASHWINI, P., AVINASH, A., BADRI, R., KAJAL, D., VOMSI, P. and SRIVIDHYA, S. 2008. Mutagenesis of *bacillus thuringensis* IAM 12077 for increasing polyhydroxybutyrate production. *Turkish Journal of Biology*, 33, 225-230.

ANDERSON, A.J. and DAWES, E.A. 1990. Occurrence, metabolism, metabolic rate, and industrial uses of bacterial polyhydroxyalkonate. *Microbiological Reviews*. 54, 450-472.

ARAVIND, J., SASIKALA, P. and PREETHI, R. 2012-13. Production of polyhydroxyalkanoate (pha) using hydrolyzed grass and *Syzygium cumini* seed as low cost substrates. Journal of Microbiology Biotechnology and Food Sciences, 2 (3), 967- 982.

CAO, N.J., XU, Q., CHEN, L.F. 1995. Acid hydrolysis of cellulose in zinc chloride solution. *Applied Biochemistry and biotechnology*, 51, 1995.

KATIRCIOGLU, H., ASLIM, B., YUKSEKDAO, Z.A., MERCAN, N., and BEYATLI, Y. 2003. Production of poly-β-hydroxybutyrate (PHB) and differentiation of putative *Bacillus* mutant strains by SDS-PAGE of total cell protein. *African Journal of Biotechnology*. 2, 147-149.

LEE, E.Y., RANG, S.H. AND CHOI, C.Y. 1995. Biosynthesis of pol (3-hydroxybutyrate-co-3-hydroxyvalerate) newly isolated *Agrobacterium sp*. SH-1 and GW-014 from structurally unrelated single carbon substrate. *Journal of fermentation and bioengineering*, 79, 328–334.

MILLER, G.L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical. Chemistry, 31**,** 426, 1959.

NOGABI, A.K., ZAHIRI, S.H., YOON, S.C. 2007. The production of cold induced extracellular polymer by *Pseudomonas fluorescens* BM07 under various growth conditions and its role in heavy metals absorption. *Process Biochemistry*, 42, 847-855.

OJUMU, T.V., YU, J. and SOLOMON, B.O. 2004. Production of polyhydroxyalkonate, a bacterial biodegradable polymer. *African Journal of Biotechnology*, 43, 18-24.

OLIVERIA, F.C., DIAS, M.L., CASTILHO, L.R., and FREIRE, D.M.G. 2007. Characterization of poly (3-hydroxybutyrate) produced by *Cupriavidus nector* in solid state fermentation. *Bioresource Technology*, 98, 663-668.

OSTLE, A.G. and HOLT, J.G. 1982. Nile Blue A as a Fluorescent Stain for Poly-3- hydroxybutyrate. *Applied Environmental Microbiology*. 44, 238-241.

POIRIER, Y., DENNIS, D.E., KLOMPARENS, K. and SOMERVILLE, C. 1995. Polyhydroxybutyrate, a biodegradable thermoplastic produced in transgenic plants. *Science*. 256, 520-523.

SANCHEZ, R., SCHRIPSEMA, J., SILVA, F.L., TACIRO, K.M., PRADELLA, G.C.J. and GOMEZ, G. 2003. Medium chain length polyhydroxyalkonoic acids produced by P.putita IPT 046 from renewable resource. *European Polymer Journal*, 39, 1385–1394.

SANDHYA, M. and ARAVIND, J. 2013. Production of polyhydroxyalkanoates from *Ralstonia eutropha* using paddy straw as cheap substrate. International Journal of Environmental Science and Technology, 10(1), 47-54.

SANTHANAM, A. and SASIDHARAN, S. 2010. Microbial production of polyhydroxyalkonate (PHA) from *Alcaligens spp*. and *Pseudomonas oleovorans* using different carbon sources. *African Journal of Biotechnology*, 9, 3144-3150.

SIDEROPOULOS, S.A., JOHNSON, C.R. and SHANKEL, M.D. 1968. Mutational synergism between heat and sub lethal dosage of ultraviolet light in *Escherichia coli I* strains", *Journal of bacteriology*, 95, 1486-1488.

SCHOLZ, C., GROSS, R.A., 2000. Poly (β-hydroxyalkanoates) as potential biochemical material polymers: an overview from renewable resources – biopolymers and biocatalysis", *ACS Series*, 764, 328-334.

ZAKARIA, M.R., ARIFFIN, H., JOHAR, N.A.M., AZIZ, S.A., NISHIDA, H., SHIRAI and HASSAN, M.A. 2010. Biosynthesis and characterization of (Polyhydroxybutyrate–co-3-hydroxyvalerate) copolymer from wild type Comamonas sp. EB172', *Polymer Degradation and Stability*, 95, 1382-1386.