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BIOSYNTHESIS AND CHARACTERIZATION OF BIODEGRADABLE PLASTICS FROM P*SEUDOMONAS OLEOVORANS* AND *ALCALIGENS EUTROPHUS*

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ABSTRACT:

There is a worldwide concern regarding the development of biodegradable plastic materials as a remedy towards harmful effects caused by plastic wastes on the environment. Biodegradable plastics are easily disposable and degradable. Bacteria synthesis and accumulate PolyHydroxyAlkanoates (PHA) as carbon source under limiting condition of nutrients. *Pseudomonas oleovorans* and *Alcaligens eutrophus* were isolated from sewage water, in an Industrial area, which produce PHA. PHA extraction was done by solvent chloroform method, subsequently. Extracted PHA was analyzed by Gas chromatography and Gas chromatography- Mass Spectrometer for specific polymer composition. The structure and its functional group were determined by NMR and Fourier Transform Infrared spectroscopy.

Keywords: *P. oleovorans, A. eutrophus*, PHA, Polymer, Biosynthesis, Metabolism

[I] INTRODUCTION:

Nowadays there has been considerable interest in the development and production of biodegradable plastics in response to problems associated with plastic waste and its effect on environment. PolyHydroxyAlkanoates is naturally synthesized biodegradable polyester of hydroxyacid stored as carbon reserve [1]. They have properties ranging from stiff and brittle plastics to rubber like materials [2]. PolyHydroxyAlkanoates in particular are attractive substitutes for conventional petrochemical plastics because of their similar material properties to various thermoplastics and elastomers and their complete degradability upon disposal in various environments [3]. The presence of intracellular granules consisting of poly(3-hydroxyoctanoate) (PHO) has been observed in *P. oleovorans* grown in two-phase medium containing 50% (vol./.vol.) octane [4]. When the organism was supplied with l-octene, 3-hydroxy-7-actenoate was incorporated as a major monomer, although a substantial amount of 3-hydroxyoctanoate was also present in the PHA. This strain of P. *oleovorans* also produces substantial amounts of PHA from n-alkanoic acids [5]. By changing the carbon source and bacterial strains used in the fermentation process, it is possible to produce the different range of biopolymers [6]. The bacterium *Alcaligens eutrophus* [7] and P*seudomonas oleovorans* [8] which accumulate PHA only when carbohydrates and fatty acids are provided as carbon source. PHA extracted was separated from lipids by precipitating with diethyl ether, hexane, methanol, or ethanol. Finally, PHA was redissolved in chloroform and further purified by precipitation with hexane [9]. PHA is produced by a variety of bacterial species *Pseudomonas oleovorans*, *P. Putida*, *Alcaligens eutrophus* and *A.latus* [10]. Jian yu [7] produced PHA from starch waste water via organic acids. Biodegradation of PHA under aerobic conditions results in CO_2 and H_2O, whereas in anaerobic conditions, the degradation products are CO_2 and CH_4. PHA is compostable over a wide range of temperatures, even at a maximum of around 600^0C with moisture levels at 55 percent. Studies have shown that 85 percent of PHA was degraded

in seven weeks [11]. Biodegradation is dependent on a number of factors such as microbial activity of the environment and the exposed surface area, moisture, temperature and pH [12]. PHA can be used as biodegradable carriers for long-term dosage of insecticides, herbicides and fertilizers, seedling containers and plastic sheaths protecting saplings, biodegradable matrix for drug release in veterinary medicine, and tubing for crop irrigation [13]. Recently, much effort has been given to produce PHA in pilot scale of continuous mode from waste water treatment to make the production feasible [14]. In this study, it was investigated that the production of PHA from *P.oleovorans* and *A.eutrophus* was isolated and screened from sewage water near industrial area and increased its production utilizing different carbon sources such as of glucose, fructose, oleic acid and stearic acid.

[II] MATERIALS & METHODS

2.1 Isolation of Targeted Bacterial Strain from Sewage Water:

The sewage samples were serially diluted and by spread plate pure cultures were isolated. The isolated bacterial colonies were re-streaked on nutrient agar plates and strains were subsequently analyzed for Gram character. *Pseudomonas olevorans* and *Alcaligens eutrophus* were confirmed by specific biochemical tests. For PHA production, the stock cultures were initially inoculated in a nutrient rich growth medium. After growth for 24 h in the above medium, the cells were harvested and again re-inoculated into nitrogen free medium containing the carbon source. 1% to 5% of inoculums and 10g/L of carbon source were used throughout the study. The organisms were allowed to grow for 48 h under aeration at 30°C. Polymer production was carried out at 30°C for 48 h, and then, the samples were withdrawn and analyzed for PHA production. [15].

2.2 PHA Extraction and Characterization:

The PHA was directly extracted using the solvent chloroform from the organisms in triplicate. First, the bacterial cultures were harvested by centrifugation at 5000 rpm for 10 min. The lipids were removed from the cell pellet-using methanol (40 times the volume of cell pellets) and the cells were incubated at 95°C for 1 h. Then it was filtered to remove the methanol completely and the sediment granules were incubated in an oven at 65°C till dry. Chloroform was added to the dried granules and was incubated at 95°C for 10 min and after cooling, the mixture was gently mixed overnight. The solution was then filtered to get the debris. Finally, the PHA was precipitated from the debris with 7:3 (v/v) mixtures of methanol and water. The precipitated PHA was then washed with acetone and dried [16]. The presence of PHA as intracellular granules was confirmed by staining the cells with Sudan black-B [17]. To assess the degrading capacity of the extracted PHA obtained from *P. oleovorans* and *A .Eutrophus* the chloroform solvent method was used [18]. The Extracted PHA was determined by gas liquid chromatography to find the monomeric composition of extracted PHA. The gas liquid chromatography-mass spectrometer (GLC/MS) is used to analyze the molecular and ionic composition of PHA. Analysis of a NMR spectrum provides information on the number and type of chemical entities in PHA. Fourier Transform Infrared Spectroscopy (FTIR) identifies chemical bonds in a PHA by producing an infrared absorption spectrum. Estimation of PHA was done by Law and Slepecky [19].

2.3 Statistical Analysis:

Results were analyzed by using one way analysis of variance (ANOVA). P values of <0.05 were considered significantly. Statistical calculations were performed using statistical package for social sciences. (SPSS)

[III] RESULTS:

P.oleovorans and *A.eutrophus* were grown under nitrogen limiting medium. *A.eutrophus* produced a higher amount of PHA compared to *P. oleovorans*. The concentration of PHA polymer produced in different ranges of initial inoculums in *A.eutrophus* and *P.oleovorans* are as summarized in [Table 1].

Microorganism	PolyHydroxyAlkanoate (g/L) with different inoculums				
	1%	2%	3%	4%	5%
A.eutrophus	1.79	3.58	4.01	3.82	2.96
P.oleovorans	0.35	0.72	0.64	0.60	0.50

Table: 1 Comparison of PHA by two different types of microorganisms with different concentration of initial inoculums.

The production of PHA was higher in 3%(v/v) innoculum of *A.Eutrophus* and 2%(v/v) innoculum of *P.oleovorans* respectively. From the experiment it was noticed that the synthesis of polymer was decreased further on increase in size of the innoculum. This shows that the higher inoculum of bacterial cells rapidly utilizes the accumulated intracellular PHA granule as an energy and carbon source [20]. The PHA production of *A.eutrophus* from different carbon sources showed significant difference. [Fig 1].

A.eutrophus produced the maximum concentration of 4.8 g/L of PHA using glucose as carbon substrate. Production of PHA from different sugars via condensation of acetyl-CoA units are stemming from hexose catabolism [21]. In the present study *P.oleovorans* produced low concentration of PHA with glucose. This was proved by Haywood *et al*. *P.oleovorans* does not synthesize PHA granules in a medium containing glucose. This organism yields higher amount of PHA 2.4g/l with oleic acid and 5.6 g/l with stearic acid. (Fig: 1).

PHA produced by *A.eutrophus* was found to be easily degraded than *P.oleovorans* by a number of soil microbes showing its utility as a biodegradable agent. The PHA was determined by Gas Chromatography. Two different peakswere found with retention times of 4.6 and 8.9 min, which correspond to those found for the chemically synthesized (S)-3-hydroxyhexanoate methylester and (S)-3-hydroxyoctanoate methylester, respectively [Fig: 2]. The molecular weights [Fig.3(A & B)] of the two compounds were confirmed by GC-MS analysis. The major peak in panels B (m/e = 147) and C (m/e = 175) are due to protonated forms of the 3-hydroxyalkanoic acid methylesters, while the heavier peaks are due to the corresponding ammonium derivates. The peaks at m/e 129 in panel B and m/e 157 in panel C are consistent with the dehydrated form of 3-hydroxyhexanoic acid methylester and 3-hydroxyoctanoic acid methylester, respectively. The ratio of the monomers 3-hydroxyhexanoate and 3-hydroxyoctanoate in PHA was found to be 1:9 [Fig: 3].The functional group of PHA was confirmed as C=0 group by FTIR spectroscopy. [Fig: 4].The structure of PHA was confirmed by NMR spectroscopy [Fig 5].

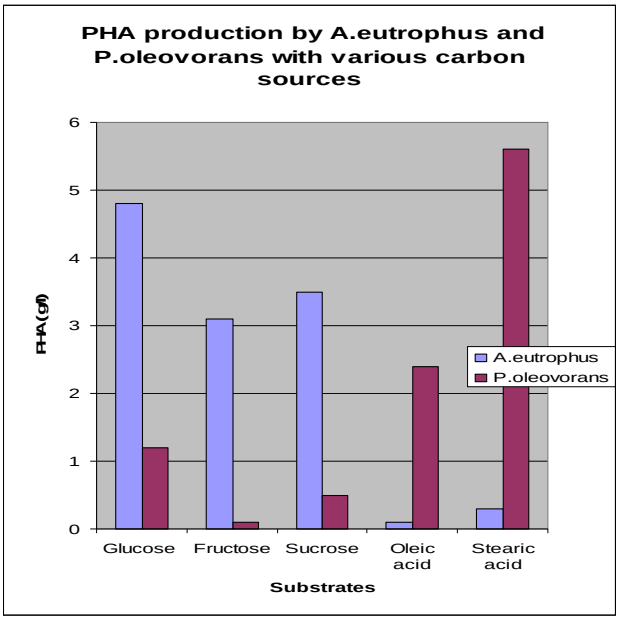

Fig: 1. Production of PHA using *A.eutrophus* and *P.oleovorans* with different carbon sources

Fig: 2.GC analysis of the methanol treated monomers of PHA produced by p.oleovorans

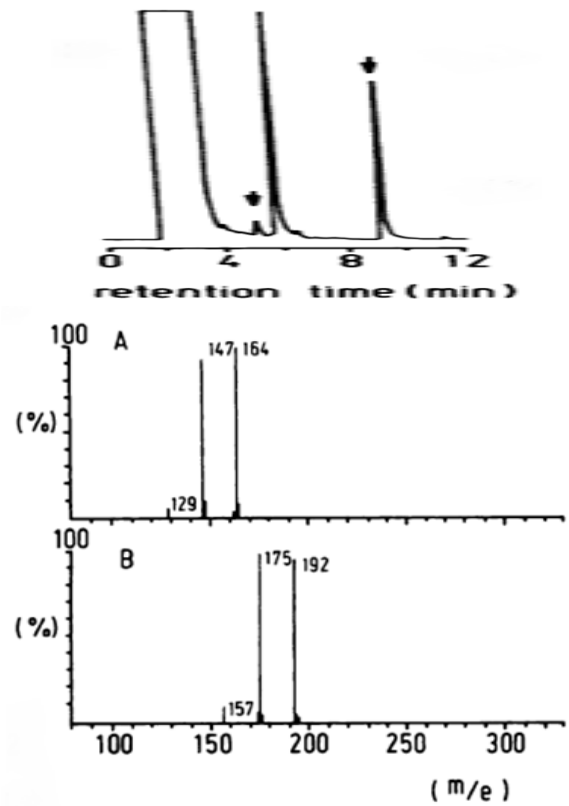

Fig: 3. GC-MS spectra of PHA produced by *p.oleovorans*

FT-IR Analysis:

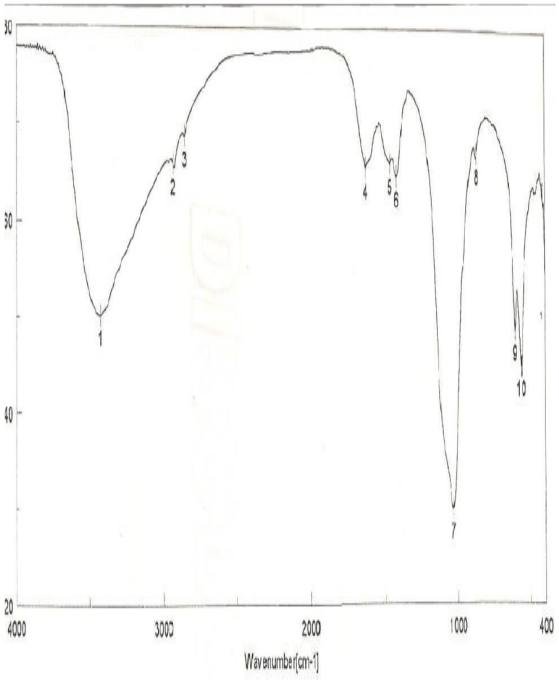

Fig: 4. FT-IR Spectrum of PHA produced by *A. eutrophus***.** Accumulation =16; Resolution = 4cm-1 Zerofilling = Off; Apodization = Cosine Gain = 2; Scanning speed = 2 mm/s; 1. 3426.89, 44.9160; 2. 2927.4, 59.9219; 3. 2854.13, 65.0217; 4. 1598.70, 58.1044; 5.1421.28, 55.0307; 6.1039.44, 35.6863; 7. 875.52, 65, 4236; 8. 565.04, 46.8555.

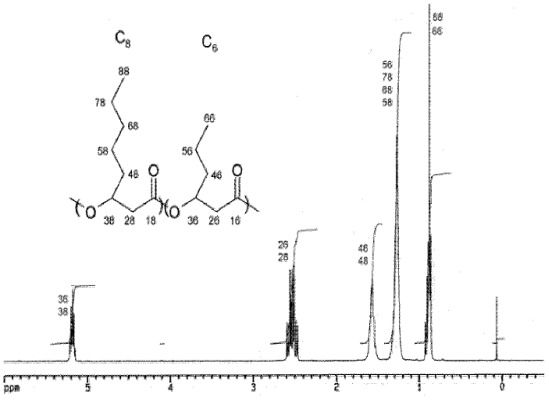

Fig: 5. H-NMR spectrum of PHA isolated from cells of *p.oleovorans*

[IV] DISCUSSION:

The rate of PHA synthesis was higher in nitrogen limiting conditions with moderate inoculum concentrations (2% to 3%). This is predominantly done by maintaining the strains under metabolic burden conditions and also most of the carbon sources are utilized for PHA synthesis rather than for making higher biomass. It was also noted from the literature that the produced PHA will be utilized for maintaining the cells – central metabolism when there is a scarcity of the carbon sources. The variation in the synthesis of PHA is due to the specificity in both strains and nutrients. The PHA production in Alcaligens eutrophus was higher in because it is capable of flow of metabolism through glycolysis and fatty acid synthesis pathway, but lesser PHA was produced, when fatty acid was used as a carbon source. At the same time pseudomonas strain are meant for early utilization of fatty acids through ß-oxidation pathway, it produced more PHA, but not in carbohydrates.

[V] CONCLUSION:

This study has led to the preliminary finding of bacterial sp. *P.oleovorans* and *A.eutrophus* from sewage water capable of producing PHA from carbohydrates and fatty acids as a sole carbon sources. It was also observed that various range of PHA produced in different inoculum sizes and different carbon sources. This is mainly due to their own expedient pathways. Eventually the higher PHA production is possible through the study of their central carbon metabolism, applying suitable process conditions such as batch or fed batch methods.

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