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PRODUCTION AND ISOLATION OF POLYHYDROXYALKANOATES FROM *Pseudomonas sp* USING WASTE COOKING OIL AS A SOLE CARBON SOURCE

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ABSTRACT

Polyhydroxyalkanoates (PHA) are a class of polymers which are produced by plants and microbes. In this study, PHA producing bacteria were isolated from soil samples contaminated with waste cooking oil. The strains were then screened for PHA production by the Nile Blue A and Sudan Black staining. Two isolates display a bacteria's ability to accumulate PHA granules. Both positive strains PS1 and PS2 were identified to be as *Pseudomonas sp* by biochemical identification as per Bergey's Manual of Determinative Bacteriology. On Optimization studies both the strains have showed the maximum production at pH -7, Temperature 30 ºC, 2% carbon source and 0.5% Nitrogen source. Mass production was carried out for 120 hours with optimized media parameters. Quantification by crotonic acid method has showed a higher yield 2.7 µg/ml of PHA for PS2 as compared to PS1 with 2.3 µg/ml. Thus, this work has shown that waste cooking oil is an ideal, cheap and easily available source for the production of PHA.

Keywords : Polyhydroxyalkanoates, Optimization, *Pseudomonas sp*, Nile blue, Sudan black

INTRODUCTION

Polyhydroxyalkanoates (PHA), a family of biopolyesters [1] with diverse structures, are the only bioplastics completely synthesized by microorganisms. PHA can be synthesized by over 30% of soil-inhabiting bacteria [2]. Microorganisms are capable of producing PHA from various carbon sources ranging from inexpensive, complex waste effluents to plant oils, fatty acids, alkanes and as well as simple carbohydrates [3]. Each year, a large amount of waste materials are discharged from agricultural and food processing industries and these wastes represent a potential renewable feedstock for PHA production. Utilization of waste materials as carbon source for PHA production not only reduces the substrate cost, but also saves the cost of waste disposal [4].

Poly-hydroxyalkanoates (PHA) is one of the biodegradable plastics produced mainly by bacteria. In the last three decades, PHA have attracted industrial interest as biodegradable plastics not only because of their compatible material properties like synthetic thermo-plastics but also could PHA be synthesized from

renewable carbon resources, based on agriculture or even on industrial wastes [5]. Due to these unique characteristics of PHA, various kinds of bacterial strains have been tested for their PHA production capability. To date, there are more than 300 different microorganisms, which can synthesize PHA [6].

Most PHA have been produced by prokaryotic microorganisms, including bacteria and archaea, although transgenic plants were reported to produce PHA [7]. The functions of prokaryotic PHA were found to be related to carbon and energy storage as well as enhanced survival. Under environmental stress conditions it was shown that the bacteria containing PHA storage materials would be able to survive during starvation period compared to those without PHA as this energy-reserve material slows down the cell autolysis and subsequently its mortality [8, 9]. This study exploits the fast growth of prokaryotes for our benefit to mass-produce PHA for its applications as bio-plastics and biofuels.

MATERIALS AND METHOD

Sample collection and Isolation

Oil contaminated soil samples were collected in sterile plastic bags from different cooking oil vending outlets at depth of 3-15 cm from the surface using a sterile spatula. The isolation was carried out for the all soil samples by standard isolation procedure by serial dilution methods and plating on nutrient agar media followed by incubation at 37ºC for 24-48 hours.

Screening of PHA producing Microorganisms

Nile Blue staining:

Bacterial isolates were characterized morphologically, purified, subcultured and maintained as the test organisms on nutrient agar slants. Screening of PHA producing microorganisms using Nile Blue Staining was carried out according to the method given by Ostle [10]. The stained plates were visualized under 230 nm UV transilluminator [11].

Detection of PHA granules by Sudan black staining:

Secondary screening for the identification of PHA by Sudan black staining was done for a loopful of 24-48hours old culture. The staining was done according to the procedure given by Burdon K.L [12]. The sudan black stained slide was then counter stained with 0.5% malachite green for 10 seconds and examined under light microscope.

Biochemical Identification

The pure cultures of the PHA positive organisms was selected and identified by morphological and various biochemical characterization as per *Bergey's Manual of Determinative Bacteriology* [13].

Variation of Physical and Chemical Parameters for maximum production of PHA

Optimization studies was carried for maximum production of PHA by varying two physical and chemical parameters of Mineral Salt Media [In g/l, Na_2HPO_4 (3.6), $(NH_4)_2SO_4$(1.0), KH_2PO_4(1.0), $MgSO_4$(1.0), Fe(NH_4) citrate (0.01), $CaCl_2$ (0.10), containing 10 ml/L of the trace element solution consisting (mg/L) of $ZnSO_4.7H_2O$ (10.0), $MnCl_2.4H_2O$ (3.0), $CoCl_2.6H_2O$ (1.0), $NiCl_2.6H_2O$ (2.0), $Na_2MoO_4.2H_2O$ (3.0), H_3BO_3 (30.0), $CuCl_2.2H_2O$ (1.0). The carbon source (5.0) and yeast extract (0.1) were sterilized separately and added to MSM. The final pH of the MSM solution was adjusted to 7.0] supplemented with different percentage of Carbon and Nitrogen source, pH, and Incubation Temperature.

Effect of pH for enzyme production

The effect of pH on PHA production was determined by preparing MSM broth with varying the pH from 5 to 9. pH was adjusted with 1N NaCl or 1N HCl. The broth was then inoculated with the isolated *Pseudomonas sp*. The amount of PHA produced after five days of incubation at 37°C was quantified by by crotonic acid assay.

Effect of Temperature for enzyme production

The effect of incubation temperature on PHA production was determined by preparing broth with pH 7. The media was then inoculated with *Pseudomonas sp* and incubated at different temperature such as 20°C, 25°C, 30°C, 37°C and 40°C. PHA quantification was performed after five days of incubation.

Effect of Carbon source concentration on enzyme production

Optimization of carbon source was done by preparing the MSM with different concentration of waste cooking ranging from 0.5% to 2.5% keeping the other constituents constant. PHA quantification was performed after five days of inoculation with the identified *Pseudomonas sp*.

Effect of Nitrogen source concentration on enzyme production

Optimization of nitrogen source was done by preparing the MSM media with different concentration of ammonium sulphate ranging from 0.5 % to 2.5% keeping the other constituents constant. PHA quantification was performed after five days of inoculation with the identified *Pseudomonas sp*.

Mass production

PHA production was carried out in 1.5 liter Sartorius B-Lite fermentor for the *Pseudomonas sp* using the optimized media parameters (w/v: MSM with Oil- 2.0 %, Ammonium sulphate 0.5%, with pH-7) and incubated at 37°C for five days. After completion of fermentation the product was drawn out in a sterile conical flask and subjected to centrifugation at 10,000 rpm for 10 minutes. The pellet obtained was air dried and weighed.

Extraction of PHA

The PHA extraction was carried out with the procedure given by Santhanam and Sasidharan **[**14**]** with modification**.** The PHA was extraction was done by using the solvent chloroform. The cell pellet was suspended in sodium hypochlorite solution and incubated at 37°C for 1-2 hours for complete digestion of cell components except PHA. The mixture was centrifuged to collect PHA granules and the supernatant was discarded. The sediment was washed twice with distilled water and centrifuged again. Finally PHA granules in the sediment were washed twice with acetone and diethyl ether (1:1 ratio). The resultant polymer granule was dissolved in boiling chloroform and air dried to obtain powdered form of PHA.

Estimation of PHA by crotonic acid assay

Estimation was done according to the method given by Chen [15]. The Extracted polymer granule was dissolved in concentrated sulphuric acid (1mg/ml) and heated at 100˚C for 10 minutes to convert PHA into Crotonic acid and the absorbance was read at 235 nm against a concentrated sulphuric acid as blank. A standard curve was prepared with Pure PHA powder (PHA (poly-[(R)-3-hydroxybutyric acid] obtained from Sigma-Aldrich, Bangalore) concentrations ranging from 1- 6µg/ml.

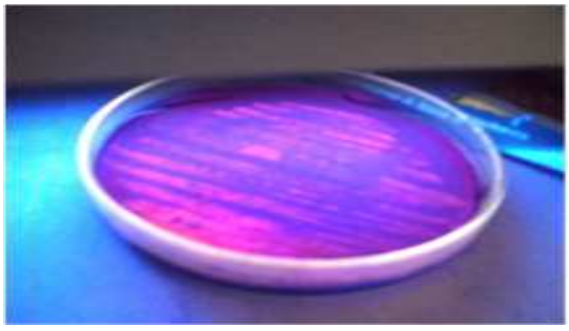
RESULTS

Isolation of Micro-organisms

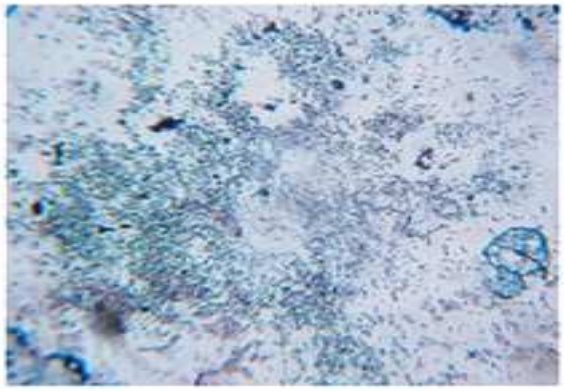
The soil samples yielded different types of colonies on nutrient agar after incubation after 24 hours at 37ºC. Bacterial isolates from the four different soil samples were characterized into 26 types based on its morphological characters and gram staining. All the 26 bacterial strains were screened for PHA by Nile blue and Sudan black staining dyes. Sample (S-18) showed positive for PHA production and was maximum compared to other isolates (Figure-1 a & b).

Figure 1- Staining by Nile blue and Sudan black

a. Nile blue staining



b. Sudan black staining



Biochemical Identification

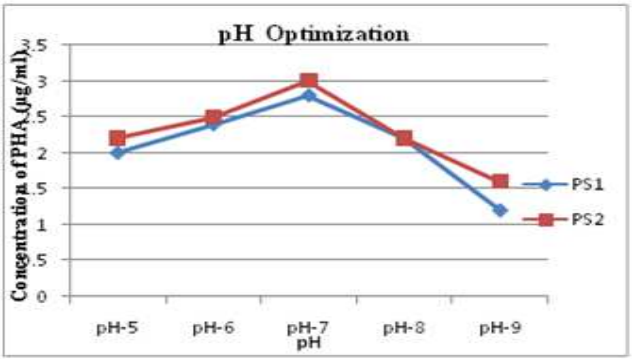
According to the Bergey's manual of determinative bacteriology the test organisms which showed positive was identified to be as *Pseudomonas sp.* based on its biochemical characteristics.

Optimization studies

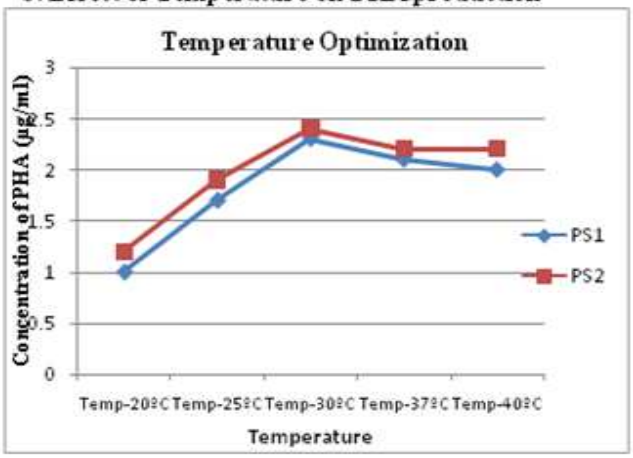
Media optimization studies were carried out for pH, Temperature, Carbon source and Nitrogen source. The PHA quantification was done by Crotonic acid method. The pH and temperature studies of have showed the maximum production at pH -7 (Figure-2a) and Temperature 30 ºC (Figure-2b). Upon optimization of carbon and nitrogen source with different percentage, 2.0 % cooking oil (Figure-2c).and 0.5% ammonium sulphate (Figure-2d) has given maximum production.

Figure 2- Effect of pH, Temperature, Carbon source and Nitrogen source on Enyme production

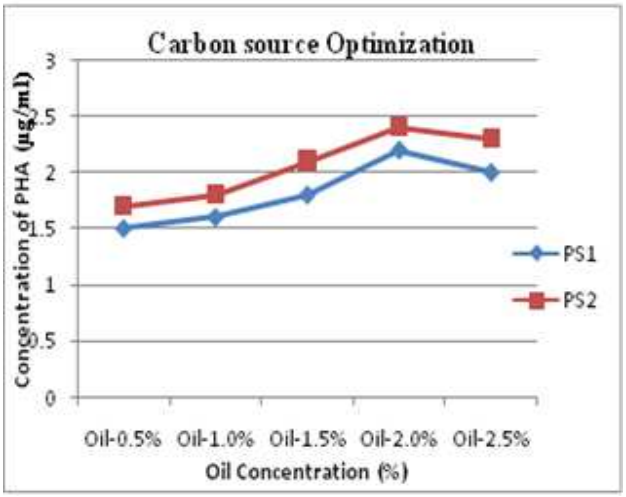
a. Effect of pH on PHA production



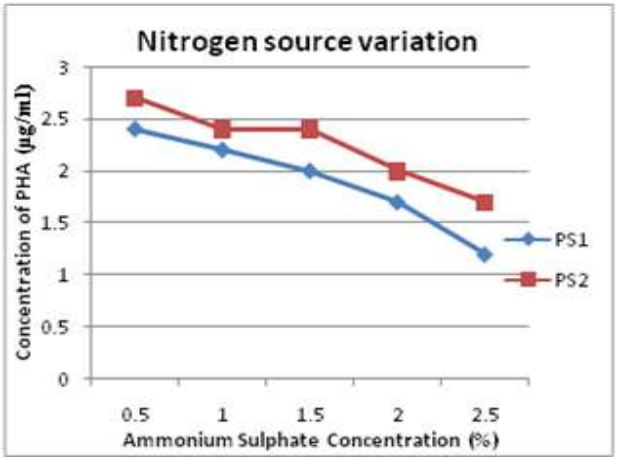
b. Effect of Temperature on PHA production



c. Effect of Carbon source on PHA production



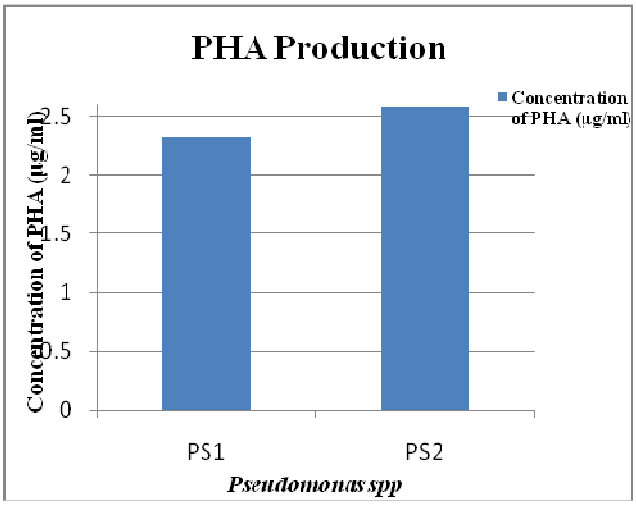
d. Effect of Nitrogen source on PHA production



Production and Estimation of PHA

Mass production of PHA was carried out with the Optimized media parameters and for both the *Pseudomonas spp* and extraction was done by hot chloroform method. The PHA granules was dissolved in concentrated sulphuric acid to convert into crotonic acid which is light brown in colour and absorbance was read at 235nm against sulphuric acid as blank. The amount of PHA produced was estimated to be 2.32 and 2.38 µg/ml (Figure-3) for the test organism 1 & 2.

Figure 3- PHA estimation by Crotonic acid



DISCUSSIONS

The PHA degradation capacity of microbes was reported to be due to the presence of intracellular depolymerases. This enzyme converts the polymer to water and carbon dioxide aerobically and methane under anaerobic condition. Four soil samples were collected from near roadside eateries where the soil was regularly contaminated by waste cooking oil from different locations of Bangalore. On screening of two bacterial isolates has showed positive for PHA production by Nile Blue A and Sudan Black tests. The PHA positive test organisms were identified to be as *Pseudomonas sp.* based on the Bergey's manual of determinative Bacteriology. To determine the ideal growth conditions for highest PHA yield, the most important physical and chemical parameters were varied. The parameters varied were pH, temperature, Carbon source concentration and Nitrogen source concentration. From the estimated PHA yield under these varying conditions, it was determined that the highest yield by both *Pseudomonoas sp* was at pH 7, temperature 30˚C, Carbon source (waste cooking oil) concentration 2% and nitrogen source concentration 0.5%.

CONCLUSION

The results of this study has led to the preliminary finding that the bacterial strains of *Pseudomonas sp* is capable of producing PHA while using waste cooking oil, which can further be used in commercial production of PHA as a biological source bring down the use of chemicals and also reducing the load on pollution.

REFERENCE

1. Hazer, B and Steinbüchel, A (2007), Increased diversification of polyhydroxyalkanoates by modification reactions for industrial and medical applications, *Appl. Microbiol. Biotechnol*.74, 1-12.
2. Wu Q, Sun, S.Q, Yu, P.H.F, Chen, A.X.Z and Chen, G.Q (2000), Environmental dependence of microbial synthesis of polyhydroxyalkanoates, Acta Polym Sin. 6,751–756.
3. Choi, J.I and Lee, S.Y (1997), Process analysis and economic evaluation for poly(3-hydroxybutyrate) production by fermentation, *Bioprocess Engineering*. 17:335-342
4. Yu, J (2007), Microbial production of bioplastics from renewable resources, S. T. Yang editor. *Bioprocessing for value-added products from renewable resources*.
5. Braunegg, G, Lefebvre, G, and Genser, K.F (1998), Polyhydroxyalk-anoates, biopolyesters from renewable resources: physiological and engineering aspects, J Biotechnol. 65,127–61.
6. Lee, S.Y (1996), Plastic bacteria? Progress and prospects for polyhydroxyalkanoates production in bacteria, TIBTECH. 14, 431–8.

7. Poirier, Y and Brumbley, S.M (2009), Metabolic engineering of plants for the synthesis of polyhydroxy-alkanaotes, Microbiol Monogr. doi:10.1007/978-3-642-03287-5_8.
8. Macrae, R.M, Wilkinson, J.F (1958), Poly-beta-hyroxybutyrate metabolism in washed suspensions of Bacillus cereus and Bacillus megaterium, Journal of General Microbiology.19, 210-222.
9. Castro-Sowinski, S, Burdman, S, Matan, O and Okon, Y (2009), Natural functions of bacterial poly-hydroxyalkanoates, Microbiol Monogr. doi:10.1007/978-3-642-03287-5_3.
10. Ostle, A.G and Holt, J.G (1982), Nile Blue A as a fluorescent stain for poly-β-hydroxybutyrate, Appl Environ Microbiol. 44, 238–241.
11. Spiekermann, P, Rehm, B. H, et al., (1959), The role of organic substrates in bacterial photosynthesis, *Proc. Natl. Acad. Sci.* U. S. 45, 1246-1260.
12. Burdon, K. L (1946), Disparity in Appearance of True Hansen's Bacilli and Cultured "Leprosy Bacilli" when stained for Fat, J. Bact. 52, 665–678
13. Holt, J.G, Krieg, N. R, Sneath, P.H.A, Staley, J.T and Williams, S.T (1994), Bergey's Manual of Determinative Bacteriology, 9th Edition, Williams and Wilkins. USA, 559-565.
14. Amutha Santhanam and Sreenivasan Sasidharan (2010), Microbial production of polyhydroxy alkanotes (PHA) from Alcaligens spp. and Pseudomonas oleovorans using different carbon sources, African Journal of Biotechnology. 9, 21, 3144-3150.
15. Chen, G.Q (2009), A polyhydroxyalkanoates based bio- and materials industry, *Chem Soc Rev*. 38,2434–2446.