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PHB(Polyhydroxybutyrate) Production by *Pseudomonas aeruginosa* and its Application in Dye Decolorization

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Abstract:
Polyhydroxyalkanoates(PHAs) are class of biodegradable,biocompactible plastics composed of polyester of R-hydroxyalkanoic acids. Polyhydroxybutyrate(PHB) is a short chain of PHA that is naturally produced by several microorganisms as a reserve material. Its accumulated in bacteria as intracellular storage granules in the presence of excess carbon source and limited nitrogen source.
The present work focused on the extraction of PHB from a gram negative bacteria *P.aeruginosa* and its application in dye decolorization. It showed significant results in the degradation of crystal violet dye. After the 64 hours of incubation, a combination of PHB and *Pseudomonas aeruginosa* culture gives 92% color removal efficiency that is highest as compare to individual treatment of PHB and *Pseudomonas aeruginosa* alone with dye.While individually treated dye with PHB and *Pseudomonas aeruginosa* alone gives 68% and 79.50% respectively. Thus, the combination of PHB and *Pseudomonas aeruginosa* culture is found to be significantly effective in decolorization of dye.

Keywords- Bioplastic, Polhydroxyalkanoate(PHA), Polyhdroxybutyrate(PHB), , *P.aeruginosa*, Dye degradation.

Introduction

Biodegredeble and enviromentally sustainable biopolymers such as PHA and its homopolymer. Polyhydroxybutyrate(PHB) hold the potential to replace plastics linear used and dispose partices with a fully circular life cycle for plastics (1). PHB is one of the most promissing material that are developed and evaluated as a biocompactible and non-toxic polymer which can be accumulated by a number of bacterial strains (2). It has high potential of replacement for petrochemical passed polymers such as ubiquitous high density polyethylene. To date high production costs minimal yeild, production technology complexities and difficulties relating to downstream processing and exapnsion in the marketplace(3).
PHB granules can be produced by many different bacterial strains out of which the extensively studied strains are: *Ralstonia eutropha* (also known as cupriavidus nector) Alcaligenes species, *Azatobacter* species (4), *Bacillis s*pecies, *Nocordia* species, *Pseudomonas* species and species (5). PHB are carbon and energy reserve polymers produced in bacteria, when carbon source is in plentiful and other nutrients such as nitrogen, phosphours, oxygen or sulpher are limited(1). *Pseudomonas aeruginosa* is a gram negative, rod shape, Asporogenous and monoflagellated bacterium. It has a pearlescent appearance and grape like or tortilla-like odour. *P.aeruginosa* grows well at 25°Cto 37°C. *P.aeruginosa* is a ubiquitous microorganisms which has the ability to survive under a varity of enviromental condition(6).
PHB supports the development of bacterial biofilm which is one of the natural physiological growth form for microorganism over these polymer structures. Different dyes used in textile industry usually have a synthetic origin and complex aromatic molecular structure which make them more stable and more difficult to be biodegrade(2). Bioremediation is a branch of biotechnology which deals with the use of living organism such as microbes and plant to remove contamination, Pollutant and toxins from soil and water. Application of bioremediation in waste water treatment is prominent technology which was succesfully used to treat various organic effluents and dye effluents.

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PHB is ecofriendly biocompatible and is accumulated up to 90% of cell dry weight. It finds applications in agriculture, packging and medical field including drug delivery and tissue engineering (7).

Pseudomonas aeruginosa is a gram negative, rod shape, asporogenous and monoflagallated bacteria in nature. It has pearlescent appearance and grape like or tortilla like odour. The optimum temperature growth range for *Pseudomonas aeruginosa* is 25 to 37°C. It is ubiquitus bacteria. The genome of *Pseudomonas aeruginosa* conatins single circular choromosome. It has large genome size of approximatelly 5.5 to 7.0 mb and high G+C contain about 65 to 67%. Because of this large size genome of *Pseudomonas aeruginosa* encodes most of the enzymes for various metabolic pathways and regulatory genes make this bacteria more compatible to the most growth condition.It has high antibiotic resistance, nutrional utility and abundant virulence factors.

Depending upon the various groups and their position on the main chain, PHA consists of different classes. PHAs can be found as homopolymers or as co-polymer and have approximately 150 different constituents (11). The current study involved screening of bacteria that produce PHB and the characterization of PHB which is produced for the quality (12)

Polyhydroxybutyrate (PHB) was first isolated and characterized through the PHAs (Polyhydroxyalkanoate) (2). PHB is crystalline in nature as it has linear chain structure, but it contains both crystalline as well as amorphous phases (8). PHB has some thermal properties includes two main temperatures, a glass transition temperature (Tg) for the amorphous phase and the melting temperature (Tm) for the crystalline phase given by Blaithin McAdam, Margaret Brennan Fournette and Marija Mojicevic of Multidisciplinary digital publishing Institute. (MOPI).

Polyhydroxybutyrate (PHB) are energy groups synthesized by different microorganisms such as Pseudomonas, Staphylococcus etc. in excess of carbon and limitation of nutrients like nitrogen. These biopolymers are suitable alternate to synthetic polymers. The microorganisms for this purpose were isolated and the culture conditions were optimize by changing the parameters (9). The extracted compound on FTIR analysis gave characteristics C=O peak of PHB at optimized conditions (7). Charles *et al* (2015) isolated a bacterial strain from tannery effluent which can tolerate high concentrations of potassium dichromate.

The isolated microorganisms were identified as *Pseudomonas aeruginosa*. PHB effect on spent combination of PHB and the *Pseudomonas aeruginosa* culture was added to spent wash, a maximum colour removal was found, rather than when treated individually with the PHB and *Pseudomonas aeruginosa*. PHB act as support material for the bacteria to bind it and thus develops biofilm (10). The bacterial growth in the biofilm and the polymer together acted in synergy, adsorbing and coagulating the pollutants in the form of colour pigments

Materials and Methods

Collection of Sample:

- **Bacterial Culture:-** Bacterial Culture that is *Pseudomonas aeruginosa* was taken from laborotary at government institute of science Aurangabad.

- **Inoculum Preparation, Media and Culture Conditions: -**

Yeast Extract Mannitol (YEM) medium is use for Polyhydroxy butyrate (PHB) production. YEM consists of following ingredients in (g/L) as: - Mannitol ;10g, K_2HPO_4 ;0.5 g, $MgSO_4.7H_2O$; 0.2g, NaCl; 0.1g, Tryptone; 0.2g, Peptone; 2.5 g, Yeast Extract; 2.5 g. The pH of the medium was adjusted to 7. 1oo mL of YEM broth was inoculated with loopful of *Pseudomonas aeruginosa* culture and incubated at 30°C for 48 hours. The inoculated culture medium was agitated continuously at 110 rpm. After 48 hours the production medium was subjected to PHB extraction (2).

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➢ **Gram's Staining: -**

Small drop of bacterial sample was taken on glass slide and heat fixed. By using dropper primary stain i.e. crystal violet was applied to it and kept for 1 min. Then the slide was washed with water to remove excess stain.Gram's iodine was added to it for 1 min. It was then rinsed with alcohol or acetone. Then the slide was immediately washed with water. Safranin was added to it and kept for 1 min. Then the slide was washed with water and observed under microscope.

➢ **Sudan Staining: -**

Method: -

Smear of bacterial culture was prepared on grease free glass slide and heat fixed. Prepared Sudan black stain was added on it and kept for 15 min. It was then immersed in xylene and counterstaining with safranin was performed. The slide was then observed under microscope for blue black appearance of cells which will be the positive PHB strain (1).

Extraction of PHB Granules: -

➢ **Method I**

Inoculated production medium after 48 hours was subjected to centrifugation for 45 min at 6000×g. The cell pellets were suspended in 5 mL of deionized water and homogenized for 2 min in sonicator. The 2 mL of cell suspension mixed with 2 mL of 2N HCl and incubated in boiling water bath for 120 min. Centrifuge at 6000×g for 2 min.The precipitate was obtained by adding 5 mL of chloroform to it in the test tubes and were left overnight at 28°C on Shaker at 150 rpm. After overnight incubation sample was centrifuge at 6000×g for 20 min and 0.1 mL of chloroform extract was dried at 50°C. About 5 mL of concentrated H_2SO_4 was added and heated at 100°C in water bath for 20 min. Then sample was cooled at room temperature and subjected to FTIR analysis.

Method II

PHB production was confirmed by Sudan staining and after confirmation the production medium was subjected to centrifugation at 5000 rpm for 25 min. The pellet was collected and supernatant was discarded. The dry weight of cell pellet was taken and washed with acetone and ethanol successively. Then to recover the PHB equal volume of 6% sodium hypochlorite was added to resuspended the pellet and it was incubated at 37°C for 10 min. After that it was centrifuged at 5000 rpm for 30 min. The pellet was collected and washed with acetone and ethanol followed by hot chloroform treatment. The extracted sample then anlysed by FTIR analysis (1).

Bioremediation {Dye Decolorization}: -

Preparation of Dye: - 50 ppm dye was prepared by adding 0.005 g crystal violet in 100 mL of distilled water. 100mL dye solution of 100 ppm concentration was taken in 250mL of Erlenmeyer flask. 4 such flask containing dye solution were used in assay. Assay for dye decolorization was performed in set as follows: 2 mL of 48 hours old culture of *Pseudomonas aeruginosa* was added in one of the 4 flasks. 2 mL of extracted PHB sample was added separately in another flask. 2 mL of PHB and 48 hours old culture of *Pseudomonas aeruginosa* in 1:1 ratio was added in remaining one flask. As mentioned above 4 flask containing 100 mL of dye solution and respective components were kept on incubator shaker for 5 days at 50 rpm. One flask was kept as control having only 100 mL of dye solution. Color removal was measured at a characteristic wavelength of 475 nm using UV- Visible Spectrophotometer. The color removal efficiency was calculated by: -

Color removal (%) = $C_0 – C_t / C_0 * 100$

Where, C_0 = Initial Absorbance

C_t = Absorbance at time t

Results and discussions:

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Grams Staining:

Pseudomonas aeruginosa Obtained from laboratory was used for PHB production. Gram staining was performed for the identification of *Pseudomonas aeruginosa* which shows gram negative small rods of pink color, under microscope (Fig.1).

Sudan Staining:

The strain *Pseudomonas aeruginosa* was analyzed for PHB production ability. The blue black dots within the *Pseudomonas aeruginosa* visualization of rods indicates that the strain is capable to production of PHB under excess carbon source and limiting nitrogen source.

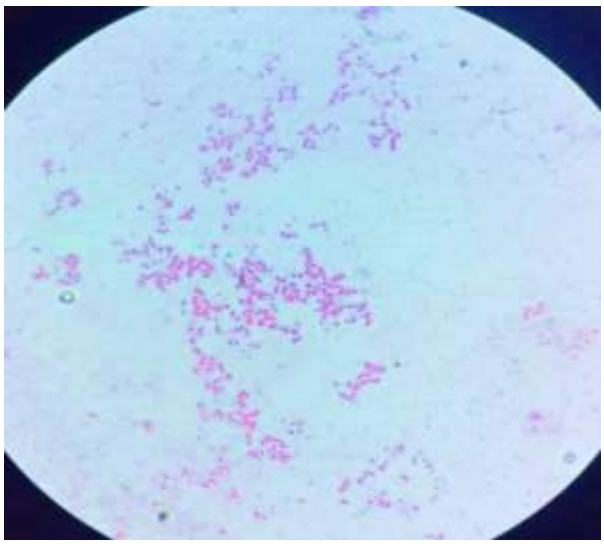

Figure 1: Gram's staining showing Gram-negative rods of isolated *Pseudomonas aeruginosa*.

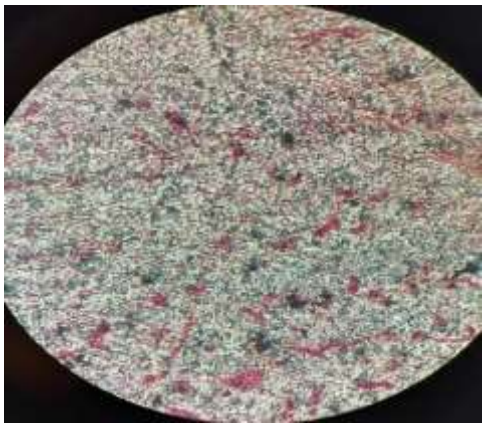

Figure 2: Sudan Staining for PHB production ability. The blue-black dots within the *Pseudomonas aeruginosa* indicates that the strain is capable to production of PHB under excess carbon source and limiting nitrogen source.

FTIR Analysis

FTIR is most useful for identifying types of chemical bonds (functional groups) and therefore can be used to elucidate the presence of PHB mixture. Hence, we tried to expose the different functional group present in our product by FTIR spectroscopy, carried out in Dept of Chemistry, Institute of Forensic Science, Aurangabad. 5 µI of the sample was loaded in FTIR BRUKER system having wavelength range from 400-4000cm^{-1} and consist of GOLAY detector. Instrument was running on

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OPUS software of 7.0.129 version. Infrared absorption spectra were recorded in the above range with a spectral resolution and wavenumber accuracy of 4 and 0.01 cm−1, respectively.

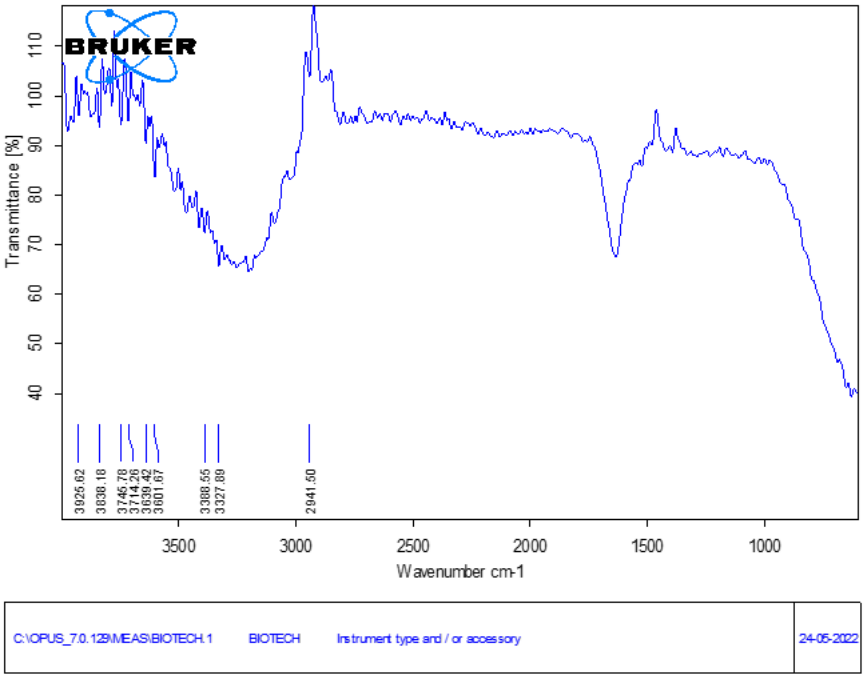

Figure 3: PHB+ Sample I

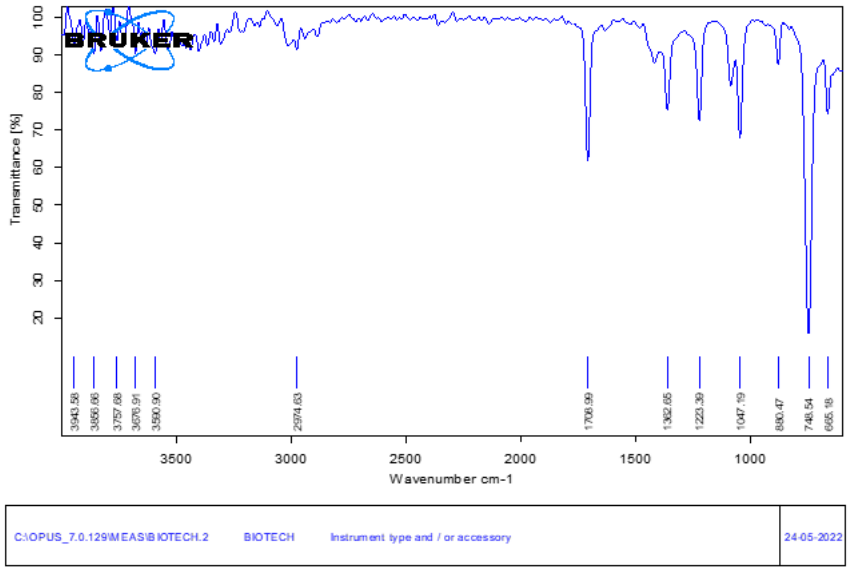

Figure 4: PHB+Sample II

The FTIR analysis of SAMPLE 1 revealed that wavenumbers 2,941, cm−1 resulting from the C–H stretching mode suggested the presence of aliphatic chain. The presence of peaks from 3327 to 3925 cm^{-1} indicated the presence of –OH stretching. The C–O bonds were observed at 1,658 cm−1. The obtained wavenumbers are consistent with the functional groups present in PHB.The FTIR analysis of sample 2 revealed that wavenumbers from 600 to 1700 cm-1 resulting from the C–H, C-C and O-H stretching mode suggested the presence of aliphatic chain. The peak at 1706cm-1 again indicated the presence of keto or aldehyde group in the sample. Beside these the presence of –OH stretching was also reflected through peaks arrived in the region of 3500 to 3900 cm^{-1}.

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Dye decolorization

The Extracted PHB was applied for the bioremediation purpose as dye decolorization. The Crystal violet stain was used for the same. The crystal violet concentration taken as 100 ppm.Three sets were analysed for the dye decolourization assay.

In first set 2ml of 48 hours old culture of *Pseudomonas aeruginosa* was added in 100 ml of 100 ppm concentration of crystal violet. The second set that is, 2ml of PHB sample was added in 100 ml of 100 ppm concentration of crystal violet. The third set is prepared by adding the 2ml of PHB sample and 48 hours old culture of *Pseudomonas Aeruginosa* in 1:1 ratio to 100 ml of 100 ppm concentration of crystal violet. These three sets were monitored for several hours and maintained on orbital shaker. Any change in dye concentration was measured after each 8 hours. At the end of 3 days batch study the third set containing PHB sample along with *Pseudomonas aeruginosa* culture showed highest percent removal of dye that is of 90.21 %. While individually treated dye with PHB and *Pseudomonas aeruginosa* alone gives68% and 79.50% respectively(fig..

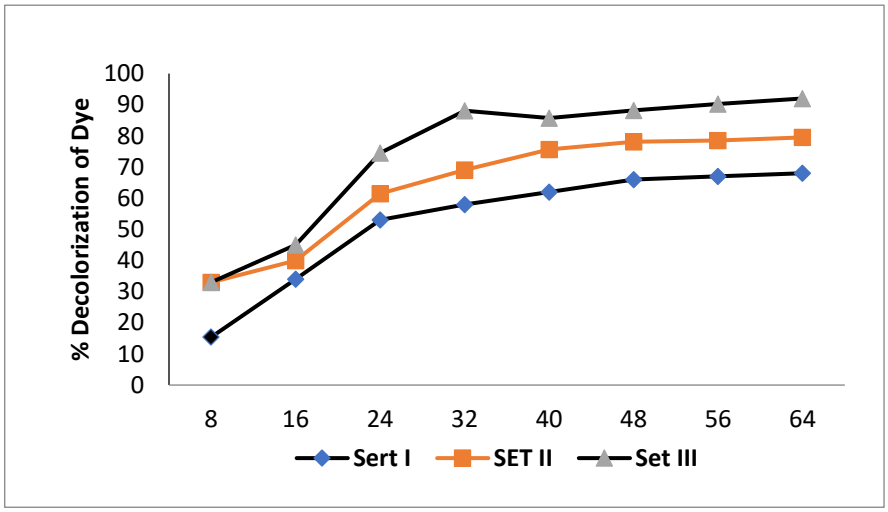

Figure 5: Percentage removal of dye

Discussion and Conclusion:

In the present work and attempt was made to use *Pseudomonas aeruginosa* gram-negative bacteria for characteristics PHB production when grown in excess carbon source and limiting nitrogen source. The YEM medium use for production of PHB contains mannitol(10g/L) as carbon source and yeasts extract (2.5g/L) as a nitrogen source after 48 h of incubation of inoculated production media significant amount of PHB was produced and it was detected using Sudan Black staining. The blue black appearance of PHB crystals inside the *Pseudomonas aeruginosa* rods confirm that the used strain was able to produced PHB in significant amount.

The PHB was extracted by using two different methods. The first method includes the use of sonicator, HCl along with chloroform for the extraction of PHB. While the second method uses acetone, ethanol, sodium hypochlorite along with hot chloroform.

The extracted PHB then applied for the study of dye decolorization activity. The extracted PHB shows positive effect and significant results in the degradation of crystal violet dye. After the 64 hours of incubation, a combination of PHB and *Pseudomonas aeruginosa* culture gives 92% color removal efficiency that is highest as compare to individual treatment of PHB and *Pseudomonas aeruginosa* alone with dye.While individually treated dye with PHB and *Pseudomonas aeruginosa* alone gives 68% and 79.50% respectively. The color removal was carried out due to the binding of bacteria to PHB forms a biofilm. Together it acts as ion exchange so the other suspended organic particles attract towards the biofilm. This biofilm act as a support medium for the growth of bacteria. Thus, the combination of PHB and *Pseudomonas aeruginosa* culture is most effective in bioremediation.

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➢ Conclusion:

Plastic waste pollution is a global environmental challenge which affect land, oceans and many creatures. Non degradable nature of petrochemical based plastics cause hazards to the environment. And here PHB comes to the rescue as it generates ecofriendly, biodegradable and biocompatible plastics.

The present study reveals the use of *Pseudomonas aeruginosa* as a efficient PHB producer that gives considerable amount of PHB granules. Extracted PHB shows effective dye decolorization activity and in turn act as a tool for bioremediation.

PHB can be used for manufacture of pots, spoons and plastics bags providing a alternative to the synthetic plastics. High production cost is the most important drawback which limit the use of PHB for main stream application. If the PHB production market grows many new and emerging processes will be implemented commercially.

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