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Original Researcher Article

Screening, Optimization and Characterization of PHA- Producing Bacteria from Zuari Estuary Goa Using Agro-Waste Substrates

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Abstract: Polyhydroxyalkanoates (PHAs) are biodegradable bacterial polymers synthesized in bacteria under nutrient-limiting conditions. Some microorganisms are known to produce biocompatible and biodegradable polymers themselves, which are environment-friendly alternatives to conventional plastics. Rich in organic content and microbial diversity, the estuarine environment is a potential site of PHA producers. In this study PHA-producing bacteria from the Zuari River Estuary were isolated and characterized and agro-industrial wastes bagasse and cotton oil cake were investigated as low cost substrates. Two isolates IS-1 and IS-5 were strong PHA producers. IS-1 was identified as Micrococcus sp, while IS-5 was identified as Bacillus sp. The polymer was found to be PHA by FTIR and GC-MS analyses. Cotton oil cake gave the maximum yield (59%). This study brought a sustainable solution to the production of bioplastic from natural microbial sources.

Keywords: PHA, Biodegradable polymers, Agro-industrial waste substrates, Bagasse, Bioplastic production.

INTRODUCTION:

The significant environmental impact of traditional petroleum-based plastics has led to urgent research on sustainable and biodegradable alternatives sources. Polyhydroxyalkanoates (PHAs), a type of microbial polyester, have emerged as promising

bioplastics because of their compatibility with living organisms, full biodegradability, and have thermoplastic qualities [1,2,3]. Various bacteria produce these biopolymers as materials for storing carbon and energy, especially in nutrient-poor but carbon-rich environments [4,5,6]. Their wide range of applications includes eco-friendly packaging and biomedical implants, making them a viable option in the push for green technology [7,8].

However, one of the main challenges in adopting PHAs commercially is their high production cost. This is mainly due to the high cost of raw materials and processing [9]. To address this issue, using agro-industrial waste products such as sugarcane bagasse and cotton oil cake has gained interest. These materials offer a cost-effective and renewable carbon source while supporting waste recovery and a circular bioeconomy an approach that aligns with global sustainability targets [10].

Estuarine environments like the Zuari River Estuary in Goa provide a rich array of microbial life and organic matter, making them excellent places to find new PHA-producing bacteria. These dynamic habitats experience tidal changes and variable salinity, leading to the development of microbial communities that can withstand stress and possess unique metabolic abilities, including the ability to synthesize polymers.

Therefore, this study aims to screen, isolate, and characterize PHA-producing bacteria from the Zuari Estuary and assess the practicality of using locally available agro-industrial wastes for cost-effective PHA production. By combining local microbial diversity with renewable waste materials, this research seeks not only to improve bioplastic production efficiency but also to support sustainable environmental management.

MATERIALS AND METHODS

2.1 Sample Collection

Samples of water and sediment were aseptically taken from the Zuari River Estuary (Vasco Da Gama, Goa) and transported on ice to the laboratory for processing [11].

2.2 Isolation of Bacteria

The samples were cultivated on M1 Marine Agar and Zobell Marine Agar after being serially diluted. The streaking procedure was repeated to isolate and purify six distinct bacterial colonies.

2.3 Primary Screening with Sudan Black B

For primary screening, bacterial smears were stained with 0.3% Sudan Black B for 40–50 minutes, then decolorized with xylene, counterstained with safranin, and observed in oil immersion. PHA-positive cells appeared pink due to the presence of blue or black lipid granules [12].

2.4 Secondary Screening with Nile Blue A

In the secondary screening, isolates were incubated for 48 hours at 37°C after being inoculated into ZMA plates containing 0.5 to 1.0 µg/mL of Nile Blue A. Fluorescence observed under UV light was indicative of PHA accumulation [3].

2.5 Morphological and Biochemical Identification

The isolates were described using biochemical tests such as IMViC, oxidase, catalase, urease, starch hydrolysis, and gelatin liquefaction, as well as colony morphology, motility, and Gram reaction. [13] used Bergey's Manual of Determinative Bacteriology for identification.

2.6 Preparation of Agro-Industrial Waste Hydrolysates

Locally sourced agro-wastes, like cotton oil cake and sugarcane bagasse, were dried and ground into a fine powder. 1% sulphuric acid (H_2SO_4) was used for hydrolysis, with a solid-to-liquid ratio of 1:10 (w/v) (Pandey et al., 2000).

Fermentable sugars were extracted from the mixture by autoclaving it for 30 minutes at121°C. Following hydrolysis, the slurry was allowed to cool to room temperature before being neutralized with 1 N NaOH to adjust the pH to 7. After removing any solid residues using Whatman No. 1 filter paper, the hydrolysate was added to Minimal Salt Medium (MSM) as a carbon source for bacterial fermentation. This strategy is based on research by [9,14], which demonstrates that lignocellulosic biomass can be efficiently converted into fermentable sugars through acid hydrolysis and neutralization.

2.7 PHA Production and Yield Estimation

When bacterial cultures were inoculated into Minimal Salt Medium (MSM) for fermentation, the only carbon source was either glucose (control) or agro-waste hydrolysates [15]. The flasks were incubated at 30°C and shaken at 150 rpm for 72 hours to encourage aerobic growth and PHA production.

Harvesting and Biomass Quantification

After fermentation, cells were collected by centrifugation at 8000 rpm for 15 minutes at 4°C.

Dry cell weight (DCW) was determined by rinsing the biomass with 70% ethanol and sterile distilled water, then drying it at 60°C until a constant weight was reached.
The DCW was recorded and used to calculate % PHA yield.
PHA was taken out and weighed.
% Yield = (PHA weight / DCW) × 100
This approach adheres to the established protocols for yield analysis and microbial PHA production as described by [16].

2.8 PHA Extraction

After a 72-hour incubation period, the bacterial cells were extracted by centrifugation (8000 rpm, 15 minutes, 4°C). The cell pellets were incubated for one hour at 30°C after being treated with 4% sodium hypochlorite at a biomass-to-lysing solution ratio of 1:10 in order to degrade non-PHA materials. The released intracellular PHA granules were collected by centrifugation and subsequently thoroughly cleaned with distilled water. The purified granules were dissolved in chloroform and then incubated at 50°C for four hours. PHA was precipitated by adding cold methanol (using a 1:3 chloroform: methanol ratio) after the chloroform extract had been filtered. The mixture was maintained at 4°C throughout the night to ensure complete precipitation. The polymer was centrifuged, dried at 40–50°C, and then weighed. The protocol was modified from [7]. To ensure high recovery and purity of PHA.

2.9 PHA Characterization

Fourier Transform Infrared Spectroscopy (FTIR):
The structure of polyhydroxyalkanoates (PHA) was investigated using FTIR analysis. To find functional group vibrations, dried PHA samples were examined between 4000 and 400 cm^{-1}. The presence of ester linkages characteristic of PHA was confirmed by key absorption peaks at about 1278 cm^{-1}, reflecting C–O–C stretching vibrations, and at about 1720 cm^{-1}, indicating ester carbonyl (C=O) stretching. Standard spectra and reference data served as the basis for identification [5].

Chromatography–Mass Spectrometry (GC-MS):
PHA samples were methanolyzed to change polymer monomers into their corresponding methyl esters for compositional analysis. GC-MS was used to analyze the resultant methyl esters. The presence of short- and medium-chain-length hydroxyalkanoate units was confirmed by the presence of major monomeric units such as dodecanoic acid and methyl-3-hydroxybutyrate. Standard spectra and reference information from [17,18]. were used for identification.

RESULTS AND DISCUSSION

3.1 Sample Collection

A variety of microorganisms for isolation were obtained from the Zuari River Estuary (Vasco Da Gama, Goa) through the effective collection and processing of water and sediment samples.

3.2 Isolation of Bacteria

With the use of M1 Marine Agar and Zobell Marine Agar, six different bacterial isolates (designated IS-1 through IS-6) were obtained. Colonies were purified through repeated streaking to ensure pure cultures for further screening.

3.3 Primary Screening with Sudan Black B

Under oil immersion microscopy, isolates IS-1 and IS-5 showed dark granules when stained with Sudan Black B in fig. 1 and fig. 2, indicating the presence of intracellular lipid (PHA) inclusions.

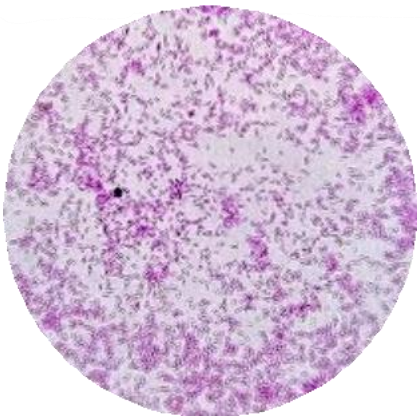


Fig.1: IS-1

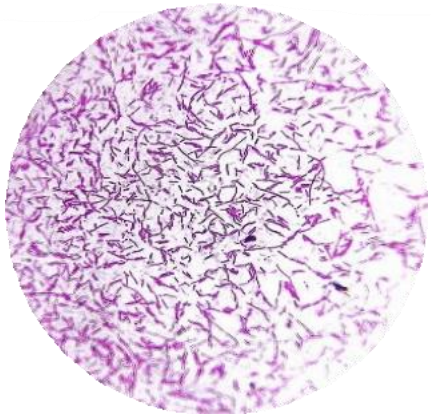


Fig.2: IS-5

3.4 Secondary Screening with Nile Blue A

A Nile Blue PHA accumulation was further supported by a staining that revealed fluorescence in IS-1 in fig.3 and IS-5 in fig. 4. when exposed to UV light. These isolates were selected for identification and characterization.

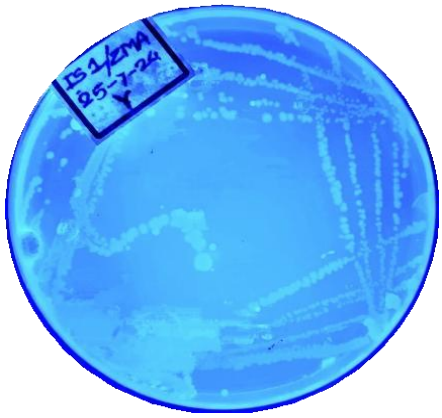


Fig.3: IS-1

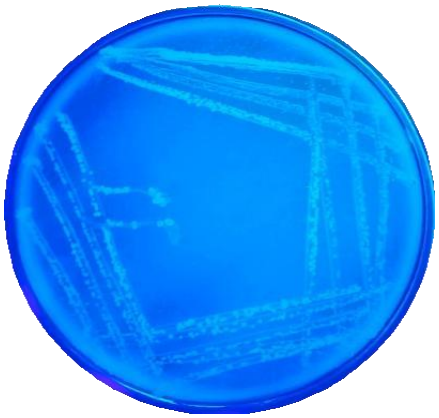


Fig.4: IS-5

3.5 Morphological and Biochemical Identification

Based on morphological characteristics and biochemical test profiles, isolate IS-1 was identified as *Micrococcus* sp., while isolate IS-5 was identified as *Bacillus* sp., following the diagnostic criteria outlined in Bergey's Manual of Systematic Bacteriology [13].

Table. 1: Morphological and Biochemical characteristics of IS1 and IS5

Morphological / Biochemical Test	IS-1 (*Micrococcus sp.)*	IS-5 (*Bacillus sp.*)
Gram Reaction	Gram-positive cocci	Gram-positive rods
Motility	Non-motile	Motile
Catalase	+	+
Oxidase	+	+
Urease	+	–
Starch Hydrolysis	+	+
Gelatin Liquefaction	–	+

Indole	–	–
Methyl Red (MR)	–	–
Voges–Proskauer (VP)	–	+
Citrate Utilization	–	+
Nitrate Reduction	–	+

3.6 Agro-Waste Hydrolysate Preparation

Bagasse and cotton oil cake acid hydrolysates were made and effectively neutralized. Both served as alternative carbon sources in minimal salt medium (MSM) for PHA production.

3.7 PHA Production and Yield Estimation

Table.2: Fermentation and Yield Data: IS-1 (*Micrococcus sp*)

Substrate	Dry Cell Weight (DCW)	PHA Yield (g)	% Yield
Zobell Marine Agar (ZMA)	16.0 g	6.08 g	38.00%
Bagasse Hydrolysate	28.0 g	11.48 g	40.90%
Cotton Oil Cake Hydrolysate	34.0 g	20.06 g	**59.00%**

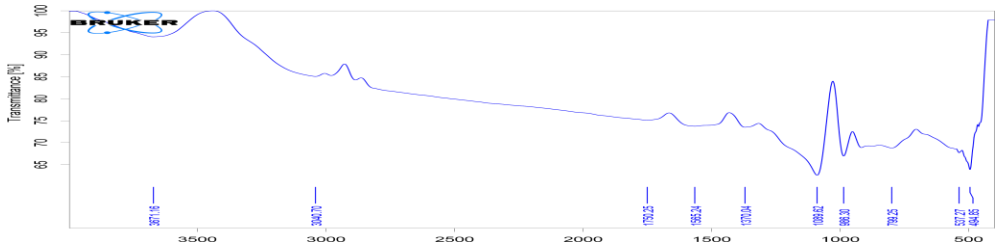
According to the results, the most efficient carbon source was cotton oil cake hydrolysate, which produced the most PHA (59% of DCW). This suggests its strong potential as a cost-effective agro-industrial substrate for large-scale PHA production [19]. Compared to conventional media (ZMB) and other waste substrates (bagasse), cotton oil cake significantly improved both biomass and PHA yield, confirming its suitability in sustainable bioplastic development.

3.8 PHA Extraction

Following the incubation period, bacterial biomass was collected and chemically lysed using 4% sodium hypochlorite, a common method for breaking down non-PHA cellular material. The oxidative action of hypochlorite specifically breaks down proteins, nucleic acids, and other cytoplasmic constituents while preserving intracellular PHA granules. Chloroform, a non-polar solvent that dissolves PHA polymers, was then used to extract the crude PHA. PHA is separated from insoluble cell debris with the help of this extraction procedure. When cold methanol was added to the chloroform phase to purify the extracted polymer, PHA precipitated because it is poorly soluble in alcohols. This process concentrates the polymer while also eliminating low-molecular-weight impurities. The final product was a high-purity polymeric powder that ranged from white to off-white

3.9 PHA Characterization

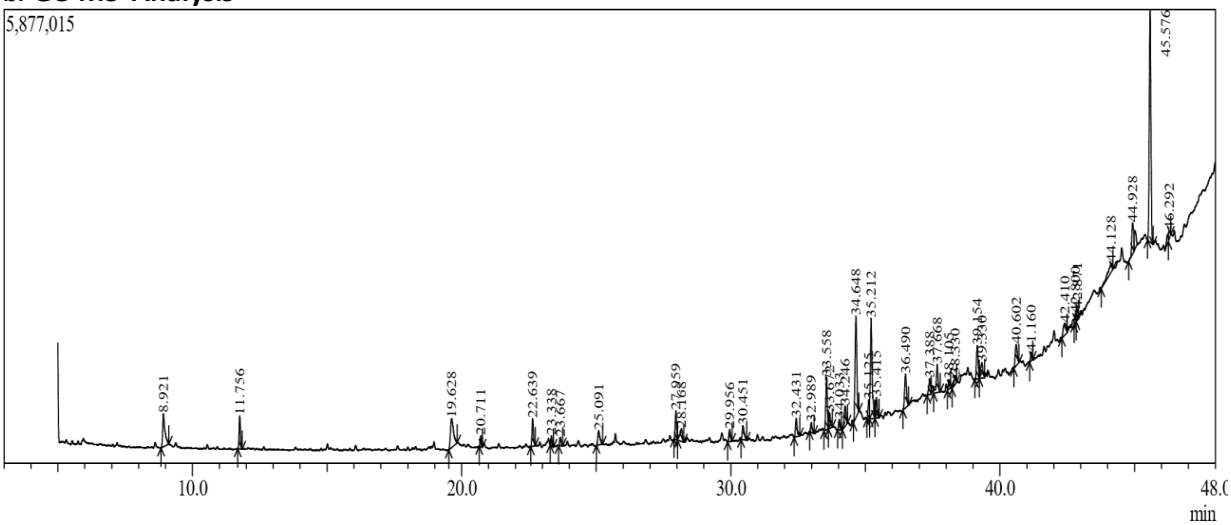
a. FTIR Analysis



The extracted polymer's functional groups and chemical structure as polyhydroxyalkanoate were verified using FTIR. The FTIR spectrum of the purified PHA showed absorption bands that corresponded to known PHA profiles. There was a noticeable peak at 1720 cm^{-1}, which is indicative of ester carbonyl (C=O) stretching. At 1278 cm^{-1}, another peak emerged, signifying C–O–C stretching. According to [5], these findings are in line with previously published FTIR spectra of poly(3-hydroxybutyrate) and its copolymers. This suggests that bioplastics made by different bacterial strains are similar to the isolated material. The success of the extraction and

pu[illegible] supported by the spectral data, [illegible] the existence of functional group[illegible] necessary for PHA identification.

b. GC-MS Analysis



Following methanolysis of the extracted PHA, additional structural confirmation was acquired via GC-MS analysis. By breaking down the polymer into methyl ester derivatives, which are subsequently separated and examined using gas chromatography and mass spectrometry, this method finds individual hydroxyalkanoate monomers. The methanol-treated product's GC-MS chromatogram displayed a number of noteworthy peaks that corresponded toM3HB, or methyl-3-hydroxybutyrate: The fact that poly(3-hydroxybutyrate) (PHB) contains this common short-chain-length (scl) monomer suggests that PHB is the main constituent of the generated PHA, which is distinguished by its crystallinity and biodegradability. Lauric acid, another name for dodecanoic acid (C12), is a medium-chain-length (mcl) fatty acid that indicates the presence of mcl-PHA segments in the polymer. This illustrates how the bacterium can add medium-chain monomers to the polymer chain to produce a less brittle and more elastic copolymer. Bis(2-ethylhexyl) phthalate: Detected as a minor component, this compound is not a typical monomer of microbial PHA. Its presence may signal trace environmental contamination, residue from solvents, or possible metabolism of phthalates by the microorganism. However, since it occurred in small amounts, its effect on the overall polymer structure is minimal. The bacterium's broad substrate specificity and metabolic flexibility, which most likely involve both β-oxidation and de novo fatty acid synthesis pathways, are indicated by the presence of both scl and mcl monomers. This metabolic ability is desirable, as it allows the production of customized PHA copolymers with improved mechanical and thermal properties. Standard spectra and reference information from [20]. who noted the diversity of PHA monomer composition depending on the bacterial strain and carbon source used.

CONCLUSION

The current study effectively illustrates the potential for sustainable polyhydroxyalkanoate (PHA) production using native bacterial isolates from the Zuari River Estuary in Goa. The most effective PHA producer among the six isolates, as determined by morphological, biochemical, and Sudan Black B and Nile Blue A staining methods, was Micrococcus spp. (IS-1).

Making innovative use of agro-industrial waste hydrolysates, particularly cotton oil cake, significantly increased the yield, reaching up to 59% PHA of the dry cell weight (DCW), surpassing the yields from traditional carbon sources like glucose or bagasse. This supports the circular bioeconomy and waste valorization by reaffirming the feasibility of agro-waste as an inexpensive, renewable substrate.

While GC-MS analysis identified both short-chain (Methyl-3-hydroxybutyrate) and medium-chain monomers (Dodecanoic acid), FTIR comprehensive characterization of the extracted polymer confirmed the presence of signature ester functional groups (C=O and C–O–C). This suggests that the isolate can produce copolymeric PHAs with enhanced biodegradability and mechanical

qualities. The purity of the polymer was not considerably impacted by the trace amount of bis (2-ethylhexyl) phthalate, which is probably a contaminant.

All things considered, combining estuarine microbial resources with agro-industrial waste substrates offers a viable approach to producing bioplastics that is both economical and environmentally benign. This strategy promotes the use of underutilized agricultural residues and lessens reliance on petroleum-based plastics.

Authors' Contributions

"All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Swapnil Patil, Abhijeet Bambare, Prasad Kamble. The final draft of the manuscript was written by **Jadhav S.B.**, **Pravin Bambare.** and all authors commented on previous versions. All authors read and approved the final manuscript.

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Declaration of competing interest

All authors declare that no financial/personal interest or belief could affect their objectivity, and there is no potential competing interest to declare.

Data availability

Data will be made available on request.

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