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Bioproduction of Poly-β-hydroxybutyrate (PHB) Using Dairy Wastewater: A Sustainable and Greener Approach

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ABSTRACT

Escalating usage of non-degradable plastics is raising significant concern. The search for bio-based degradable alternatives commenced far back, and the burgeoning progress in the development of bioplastics is featured as a critical solution to ongoing plastic pollution. Bioplastics are becoming a promising substitute for petroleum-based plastics, depending on the production source and post-use disposal management. Among all the promising materials, microbially produced polyester and polyhydroxybutyrate (PHB) belong to the polyhydroxyalkanoate (PHA) family and are biocompatible and non-toxic. PHB has remarkable thermal and mechanical properties, making it a potential replacement for ubiquitous plastics. In this study, PHB-producing bacteria were isolated from mangrove soil and checked for PHB accumulation using preliminary and confirmatory staining. Out of a total 25 isolates, 13 were found positive for PHB accumulation. Dairy wastewater was used as a cultivation medium for PHB production; the potential PHB-producing strain was selected for morphological and biochemical characterization up to the genus level and was found to be *Bacillus sp* (3.6±0.15g/L). Extracted PHB was characterized using FTIR, XRD, and TGA; in FTIR, the characteristic peak was recorded at 1724 cm^{-1}, and XRD showed the crystallinity of PHB. TGA curve displayed maximum mass loss between 254-290°C, indicating thermal stability. The outcome of the present study shows that dairy wastewater is an indispensable medium for PHB production in an eco-friendly way.

Keywords: *Bacillus*, Bioplastics, Dairy wastewater, FTIR, Polyhydroxybutyrate, Polyhydroxyalkanoates, TGA, XRD.

INTRODUCTION

Production of plastics has become an essential polymer across several aspects of today's life, including applications in the healthcare sector, construction, agriculture, clothing, cosmetics, technology and immeasurably more. Plastics have remarkable properties compared to other materials such as being lightweight, moldable, versatile, chemically resistible, and thermal-electric insulation. It has become ubiquitous owing to its widespread use, its inexpensive and frequent disposable nature makes it to be used and rejected easily and unnecessarily. Despite extensive awareness

that unrestricted usage and mismanagement of plastics are increasing contamination and environmental impairment worldwide, plastic production is continuously booming. Absolute degradation of plastics may take years to centuries in the natural environment, out of which hydrolysis does not account as plastic is hydrophobic in nature. In brief, plastic is resistant to degradation altogether making it persistent in the surroundings which typically triggers pollution.[1] Plastic pollution is now considered a paramount environmental problem, principally in aquatic ecosystems. Plastic takes a prolonged time for its biophysical breakdown and causes unpropitious effects on flora and fauna. Due to confined removal approaches, they are mainly landfilled along with other waste that can leach harmful chemicals and eventually lead to detrimental environmental and public health effects.[2] Due to various adverse effects on the environment, plastic agitates significant concern and requirement for substitute bio-based biodegradable polymers.

Polyhydroxyalkanoates (PHAs) are polyesters of biological origin, carried by microorganisms within the cellular structure as energy storage material in granular form. PHAs are natural polyester of 3-, 4-, 5-, and 6-hydroxyalknoic acids; more than 90 genera of altogether *Gram-positive* and *Gram-negative* bacteria are recorded positive for PHAs production under both aerobic and anaerobic conditions.[3] PHAs are stored in the cytoplasm of bacteria, where they exist as granules within size range of 0.2-0.5 µm. PHAs are water-insoluble, bio-compatible, thermoplastic and biodegradable in nature, which often make them as luring agents as bioplastics. The first discovery of PHA was made by French scientist Lemoigne in *Bacillus megaterium* in form of poly (3-hydroxybutyrate) (PHB) in 1925.[4] PHB is a class of PHAs, immensely like petroleum-based plastics. PHB has a characteristic methyl group (CH_3) and an ester linkage group (-COOR); these present groups are liable for the material being thermoplastic, hydrophobic and crystalline. It has the tendency to get mold, spun into fibres and, fabricated into films and utilized to make numerous commodities. However, the production of PHB is expensive due to the use of pure carbon sources, fermentation, and downstream processing. About half of the cost accounts only for pure sugars needed for production. These organisms utilize numerous substrates for the production of PHB; renewable feedstocks such as industrial byproducts, palm oil mill effluent, agricultural residues, paper mill wastewater, date syrup and dairy wastewater, are some of the examples of so far used substrates for PHB production. In past years, an approach of using "waste products" for the production of valuable products is rapidly growing. Dairy industrial wastewater possesses high BOD and COD, making it a primary pollutant. Cheese whey is the chief source of high organic load, also separation of this from dairy wastewater is often expensive and questions economic feasibility. Dairy wastewater earned attention due to numerous benefits such as being economical and cheap source of carbon for PHA production. In the present study, dairy wastewater was used as feedstock for the production of PHB to attempt sustainable and feasible biopolymer generation. By this approach, the circular economy can be enhanced as it is an attempt to recover resources from wastewater.

MATERIALS AND METHODS

Sample collection

Mangrove soil samples were collected from a coastal town of Bhavnagar (Gujarat), Ghogha (21.6767°N, 72.2845°E); consequently, samples were aseptically brought to the laboratory for analysis. Several parameters, such as pH, temperature, and salinity, were measured on-site using portable meters (OAKTON). Dairy wastewater was collected from the Vita milk plant, Rohtak (Haryana), India, and maintained at 4°C, and dairy wastewater was filtered using filter papers and muslin cloth. Chemicals used in the current study were purchased from Himedia (Maharashtra, India).

Isolation of PHB-producing bacteria

The classic serial dilution method was used to isolate predominant PHB-producing bacteria from samples. 50µL of dilutions was spread on LB agar plates supplemented with 1% glucose as carbon source and incubated at 37°C for 48 hours. The individual colonies that appeared were picked and purified by quadrant streaking. subsequently maintained on agar slants at 4°C for further experiments.

Screening of PHB-producing bacteria

Primary screening was performed

according to Singh and Parmar (2014)[5] with slight modifications. To screen the PHB-producing isolates, lipophilic Sudan black B stain was prepared as 0.3% solution (w/v) in 60% ethanol. For further staining, isolates were smeared on a glass slide and heat-fixed in the usual way. Smears were flooded with Sudan black B solution for 10 min and further rinsed with distilled water (DW). Then, counter-staining was done using 0.5% safranin for 10 sec and again rinsed with DW, subsequently observed under a light microscope (Stereo Olympus, America). After the primary screening, secondary screening was performed as per the method of Spiekermann *et al.,* (1999),[6] isolates were further analyzed using an *In vivo* Nile Red A stain. For this, stain was prepared by dissolving 0.25 mg of Nile Red A in 1 mL of dimethyl sulfoxide (DMSO). Added directly to the culture media with a concentration of 0.5ug/mL^{-1}, isolates were allowed to grow in the presence of stain. Finally, grown isolates were exposed to UV irradiation to check for fluorescence.

PHB extraction and quantification

PHB production was performed in a 250 mL Erlenmeyer flask containing 100 mL of dairy wastewater as feedstock under stationary growth conditions, incubated for 72 h at 37.5^{O}C. After this, PHB was extracted as per the method of Alafraj *et al.,* (2015),[7] grown biomass was centrifuged at 8,000–10000 rpm for 8–10 min, then acetone and ethanol was further added in the ratio of 1:1 to break the cell wall and to liberate the PHB. Again, spun at 8000 rpm for 5 min, and then the supernatant was disposed off. In the procured pellet, 4% of NaOCl was added and incubated for 30 min at room temperature, again centrifuged at 8000 rpm for 8 min and the supernatant was disposed off. The procured pellet was rinsed with acetone, ethanol, and chloroform, which were added to liquify the polymeric granules and further sieved using Whatmann No. 1 filter paper. To quantify PHB production, sulphuric acid was added to the filtrate it converted polymeric particles into crotonic acid. Readings were recorded using a spectrometer (Shimadzu UV 2450, Japan) at 235nm. Residual biomass was calculated, taking the difference between cell dry weight and dry weight of PHB.

Residual Biomass = DCW(g/L)-Dry weight of PHB (g/L), PHB accumulation (%) = Dry weight of PHB (g/L)*100% /DCW(g/L)

Morphological and Biochemical Identification

Morphological characteristics such as shape, color, structure, elevation, margin, and arrangement were observed to characterize the isolate. A Stereo Olympus microscope was used in the study to observe and characterize the isolate. Carbohydrate utilization and biochemical tests were also performed using a Himedia identification kit (KB013), which included Sucrose, Mannitol, Glucose, Arabinose, Trehalose utilization reactions and Malonate, Voges Proskauer's, Citrate, ONPG, Nitrate reduction, Catalase, and Arginine tests.

PHB Characterization

X-ray Diffraction (XRD)

The extracted PHB sample was analyzed using XRD spectroscopy to elucidate the crystalline nature. The examination was performed with a a Rigaku Miniflex600 diffractometer, 2-theta range within 10^{o}-50^{o} and scan speed of 10 min^{-1}.

Fourier Transform Infrared Spectroscopy (FTIR)

PHB sample was characterized with FTIR to analyze the functional groups. Analysis was performed on Nicolet iS50 FT-IR recorded in the KBr pellet. The wavelength range used for observations was between 400 to 4000 cm^{-1}.

Thermogravimetric Analysis (TGA)

Thermal decomposition of extracted PHB was measured on TGA HiRes1000. Extracted PHB was vacuum-dried first at 35^{o}C for 24 h, and then analysis was carried out at a temperature range from 35^{o}C to 600^{o}C, with a heating rate of 20^{o}C/minute. Simultaneously, a graph was plotted between relative mass change against temperature, and the acquired curve was analyzed.

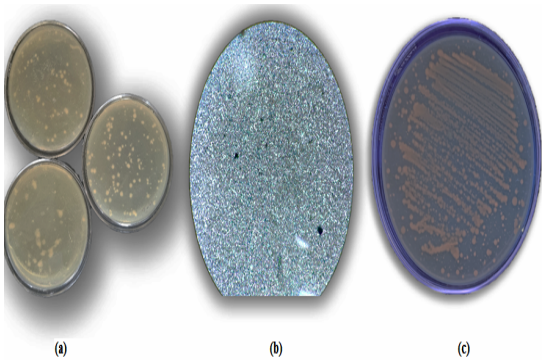

Fig. 1(a). Isolated bacterial colonies from mangrove soil (b) Sudan Black B stained cells of SN043 under microscope imparting blue colour (c) Nile Red A stained cells of SN043 showing florescence under UV light irradiance

RESULTS AND DISCUSSION

Isolation and screening of PHB-producing bacteria

A wide variety of bacteria are reported for the presence of PHB inclusions from various environments.[8] The presence of isolates in several habitats might depend on the predominant environment and culture conditions. A total of 25 isolates were procured from mangrove soil samples, and preliminary screening of obtained isolates was performed using Sudan Black-B stain to distinguish the presence of PHB. Out of the total 25 isolates, 19 were reported positive for PHB accumulation. Positive isolates appeared bluish in color, while non-accumulating isolates were not able to incorporate the stain. Sudan Black B is a lipophilic stain and has excellent sensitivity for PHB screening; it clearly elucidates the presence of intracellular lipid inclusions in the cytoplasm.[9]

Further, Secondary screening was performed using a highly sensitive stain for PHB, Nile Red A. Isolates were allowed to grow in the presence of Nile Red A acting as an *In vivo* stain and detection of PHB was performed using UV light irradiation. PHB accumulating isolates showed bright orange fluorescence while non accumulating one did not show any fluorescence.

Production and Extraction of PHB using Dairy wastewater

PHA accumulating isolates after confirmatory screening were grown in 100 mL of dairy wastewater, and PHB was extracted after 72 h of incubation under stationary growth conditions. The isolate SN043 showed the highest PHB accumulation (3.6g/L), using dairy wastewater as feedstock, and it was further identified using morphological and biochemical characterization. Dairy wastewater contains high BOD and COD; hence, microbes have the potential to use organic material present in water for their growth. Baei *et al.,* (2010) utilized dairy wastewater (Cheese Whey) using Azohydromonas lata DSMZ 1123 and optimized PHB production.[10] Similarly, Pagliano *et al.,* (2020) used volatile fatty acids from biodigestion of dairy wastewater, which accounts for a new and economical substitute for PHB production using *Cupriavidus necator* DSM 13513.[11] Maximum accumulation of PHB was achieved after 48 h and extracted PHB showed similar properties compared to petroleum-based plastics such as thermal stability and poor affinity to water. Jurado *et al.,* (2019) optimized fermentation conditions for efficient PHB production using *Bacillus subtilis* EPAH18 and also evaluated the economics of the process by simulation and concluded that dairy wastewater can be utilized as a production medium for PHB biorefinery.[12]

Table 1: List of PHB-accumulating isolates screened using dairy wastewater

Isolate	Biomass (g/L)	PHB (g/L)
SN041	6.03 ±0.15	2.31 ±0.1
SN042	6.36 ±0.15	1.26 ±0.15
SN043	8.23 ±0.20	3.6 ±0.15
SNO44	7.31 ± 0.11	1.13 ±0.05
SN045	6.16 ±0.05	2.4 ±0.30
SN046	5.56 ±0.20	2.06 ±0.15
SN047	5.36 ±0.11	1.16 ±0.11
SN048	5.12 ±0.13	2.4 ±0.15
SN049	6.42 ±0.11	1.33 ±0.11
SN050	4.80 ±0.11	1.33 ±0.11
SN051	4.3 ±0.15	1.13 ±0.30
SN052	4.43 ±0.20	1.4 ±0.25
SN053	6.33 ±0.11	1.5 ±0.15

Table 2: Morphological Characters of isolate SN043

Morphological Characters	Results
Color	Creamish and opaque
Texture	Smooth
Gram-reaction	*Gram-positive*
Shape	Rod-shaped
Arrangement	Isolated Colonies

Table 3: Biochemical and Carbohydrate utilization tests results of SN043

Biochemical/Carbohydrate test	Results
Malonate (Well no. 1)	-ve
Voges Proskauer's (2)	+ve
Citrate (3)	-ve
ONPG(4)	+ve
Nitrate Reduction (5)	+ve
Catalase (6)	+ve
Arginine (7)	-ve
Sucrose (8)	-ve
Mannitol (9)	ve
Glucose (10)	+ve
Arabinose (11)	+ve
Trehalose (12)	+ve

Structural Characterization

FTIR

FTIR was carried out to investigate the vibrational frequencies of the anticipated functional groups. In the analysis, frequency bands were noted at 3437 cm^{-1} and 2977 cm^{-1} corresponding to terminal -OH group and C=H stretches of methyl and methylene group, these were in concordance with results of Mostafa *et al.,* (2020).[13] The carbonyl group (C=O) is a common attribute of PHB structure. FTIR spectra for extracted PHB showed a significant peak at 1724 cm^{-1}, which corresponds to C=O stretch of the ester group. A similar peak was observed in the study of Babruwad *et al.,* (2014) while characterizing biosynthesized PHB from *B.cereus*.[14] In the spectrum, peak at 1291 cm^{-1} was also observed corresponding to C-O group which was adeptly comparable with the recorded results of Narayanan *et al.,* (2020).[15] Briefly, the above major peaks resemble to the other studies carried out to characterize PHB, hence the presence of PHB can be concluded.

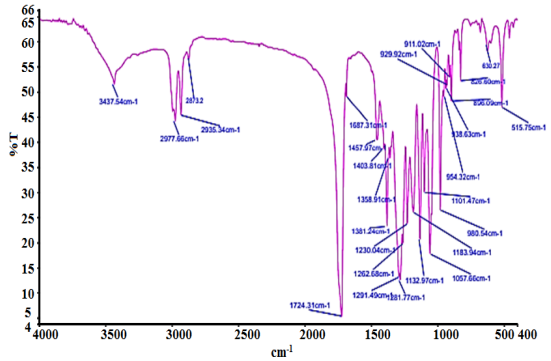

Fig. 2. FTIR spectra of PHB extracted from SN043

XRD

XRD was carried out to analyze the crystallinity of extracted PHB; XRD pattern exhibited intense peaks at 2-theta values of 13.28°, 16.72°, 22.36°, and 25.24° All the recorded peaks have narrow FWHM values, principally revealing crystalline nature of PHB. The recorded values are analogous to results acquired in the study of Rathna and Kulandhaivel (2024) while studying PHB synthesis from agro-residue by *Brevibacterium casei*.[16] Recorded XRD pattern was also comparable with the results of Uzun and Aydemir (2017).[17]

TGA

TGA was carried out to check the thermal stability of extracted PHB, it gives information related to the thermal decomposition of a material. The acquired TGA curve showed the highest mass decomposition (92.82%) of PHB occurred up to 290°C, maximal mass decomposition of 92.82% of PHB occurred up to 290°C with initiation prevailing at around 254°C; it may be because of β-elimination as it is responsible for ester cleavage reactions in PHB molecule. These results were comparable with the results demonstrated by Sirohi *et al.,* (2021).[18] Bhuwal *et al.,* (2013) observed a similar pattern in a study carried out for PHB production using cardboard industry wastewater.[19]

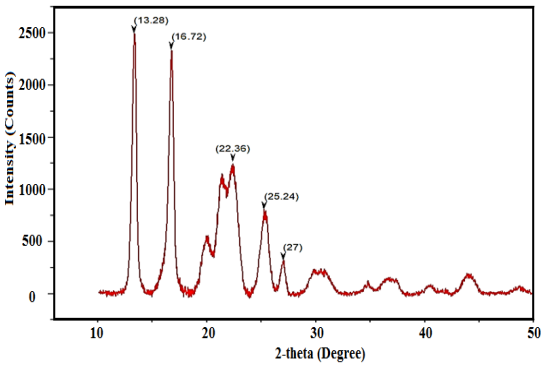

Fig. 3. XRD pattern of extracted PHB from SN043

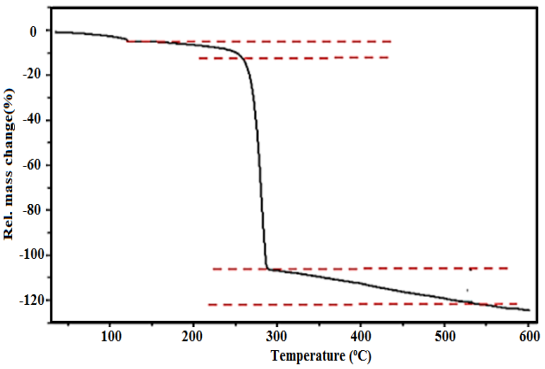

Fig. 4. TGA curve of extracted PHB from SN043

CONCLUSION

Millions of tons of petroleum-based plastic have accumulated in the environment over the last few years, attempted awareness has made some prompt progress in disposal methods of plastics but this is not the virtuous solution to this plight. Production of substitute biomaterials using renewable feedstocks is only underway, PHAs have the potential to surmount petroleum-based plastics which enables sustainability and circular economy. However, the application of PHAs is limited due to a lack of inexpensive carbon sources. This conducted study is an attempt to use cheap

feedstock for the economical production of PHB. The results strongly anticipated that the expensive cost of carbon sources can be curtailed by using renewable sources such as dairy wastewater. In the conducted study, bacterial strain isolated from mangrove soil has the ability to accumulate PHB, and it was established by Sudan Black B and Nile Red A staining. Morphological and biochemical characterization was carried out for the isolate, and it was identified up to the genus level as *Bacillus sp*. PHB production was carried out using inexpensive dairy wastewater as a carbon source and the highest recorded PHB accumulation was 3.6±0.15g/L of cell dry mass. Structural characterization showed characteristic groups of PHB and also depicted its thermal stability. The outcome of the present study can be concluded that *Bacillus sp*. has the capability to fabricate PHB by utilising cheap nutritional sources. This bacterial strain will be identified, and further cultural parameters will be optimized for maximum PHB production to make PHB production economical.

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Conflict of interest

The authors declare that they have no financial interests (e.g. owning stocks of a related company, having received honoraria, consultancy fees), research interests (research support by grants or otherwise), organizational interests and others (e.g. trips, travel, or gifts).

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