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PRODUCTION AND CHARACTERIZATION OF BIOPOLYMER (PHB) USING BACTERIAL STRAINS ISOLATED FROM ALLUVIAL SOIL

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ABSTRACT: Biopolymers are gaining much interest and are considered appropriate substitutes for petroleum-based plastics. It is used for various applications in the medical, agriculture, and packaging fields. The bacterial strains from river soil samples were isolated and screened for biopolymer production. The objective of the present study was to produce polyhydroxybutyrate (PHB) from the isolated strains under optimized conditions in a shake flask. The optimization was done with the software tool Design expert – Response surface methodology (RSM). The optimum environmental conditions were: initial pH 7.0, Carbon Source 35g/l, and Nitrogen source 1g/l. Under these controlled conditions, biopolymer yield was obtained 2.25g/L. The FT-IR spectra of extracted biopolymer indicate symmetric (O-C-O) presence at 1600cm^{-1} and asymmetric absorption peak at 1400 cm^{-1}. The TGA result indicates the thermal degradation of the obtained biopolymer was at 106 °C. The obtained results suggest that these biopolymers may be similar to PHB.

INTRODUCTION: Biopolymer is a bio-degradable polymeric material obtained from various biological sources such as plants, microorganisms, *etc*. It is the fastest and most efficient developing method to provide alternatives to fossil-fuel-based polymers and the best way to reduce global warming and pollution caused by conventional plastics [1]. Various kinds of biopolymers are available based on their source, degradation type, structural and functional backbone.

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The present studies focus on the microorganism based production of biopolymer, an easy and efficient method to produce large quantity of biopolymer from suitable microorganisms under controlled culture conditions irrespective of environmental factors like climatic conditions [2]. Apart from availability, easy handling, and production process, bacteria offers additional design space for *in-vitro* modification of the desired polymer than other microorganisms [3].

The biopolymers are majorly applied in food Packaging, medical sectors as scaffolds in tissue engineering, Implants, sensors, and drug delivery and enzyme immobilization techniques [18]. Currently, polyhydroxybutyrate holds high demand, and in 2030, the expected market size of PHB is USD 121 Million [25]. Increase in demand for biopolymers; even plants was genetically modified now a day, to produce biopolymers in large scales [27]. The PHBs are interesting biopolymers. These are the polyesters from the PHA family, produced from microorganisms in the presence of excess carbon source and lack of other essential nutrients in the culture conditions [4]. The PHA family has good mechanical and thermal stability, biodegradability, and biocompatibility properties. PHB shows optical activity, Piezoelectricity, and good barrier properties [5]. PHBs are natural biopolymers 100% resistant to water and moisture, thermoplastic, and 100% biodegradable. However, they have some specific properties, such as low permeability to gases [14].

The chemical configuration of PHB

- {O-CH (CH3)-CH2-(C=O)} n [20]

The molecular structure of PHB is not based on the features of the strain and the carbon-nitrogen composition given for its production [24]. Biopolymers of this kind are made from various microorganisms, including *Bacillus spp, Pseudomonas spp, Azotobacter spp,* using various low-cost substrates like organic wastes, industrial waste by-products, and other carbon-rich soils [17]. Some environmental factors influencing PHB production include pH, Temperature, size of the inoculum, *etc*. These factors can be controlled by means of optimization methods [30]. The optimization of the media conditions was done using the optimization tool response surface methodology (RSM). This tool helps widely to identify suitable media composition and culture conditions [6]. Our research aims to optimize and characterize the PHB produced from bacteria isolated from the alluvial soil sample.

MATERIALS AND METHODS:

Isolation and Screening of Bacteria: The soil sample was collected from the Kodiveri River bank. The bacteria were isolated using the serial dilution and grown in Nutrient broth [24]. The isolates were tested for PHB production with the help of the Sudan black B staining method. The morphology of the bacteria was identified by the Gram's Staining method and biochemical tests like the Catalase test, Indole test, MR& VP test [13, 16].

Production of Biopolymer: The isolated bacteria were grown in Nutrient broth. Then the Mineral Salt Media (MSM) was prepared, pH was adjusted to 7 and sterilized. The isolated bacteria were inoculated to sterilized media and the 250ml shake flask culture was kept at 37℃ in the shaker for 24-48 hours at 150 rpm [12].

Optimization of Biopolymer Production: To optimize the media components for the production of biopolymer, the Response Surface Methodology (RSM) tool from Design Expert Software was used. It provides experimental designs for the given factors influencing the process and its variables to optimize, improve and enhance the process. The Central Composite Design (CCD) was applied to optimize the PHB production from the isolated bacteria by varying pH factors. These carbon and nitrogen source concentrations influence biopolymer production. The design was analyzed by ANOVA and provided the actual and predicted values of the given variables for the factors chosen [7].

Extraction and Quantification of Biopolymer: After Incubation time, the media containing the inoculated colonies were subjected to the sodium hypochlorite extraction method to extract the intracellular polymer produced under given stress conditions. The cultures were taken in 50 mL centrifuge tubes and centrifuged at 8000rpm for 30 minutes. The supernatant was removed. Pellets were treated with 1M Sodium hypochlorite and kept for 1-2 hours of incubation at 37℃. Sodium hypochlorite is the disinfectant that helps in cell disruption and digestion of cellular lipids and proteins. The sample was centrifuged at 6000 rpm for 15 minutes. Then the pellets were washed with distilled water, absolute ethanol, and Acetone. After washing, the pellet was dissolved in 10 mL of chloroform and incubated overnight at 50°C. After drying, the biopolymer was stored at 4°C until further use [15, 22, 28].

Characterization of Biopolymer:

Fourier Transform Infrared Spectroscopy (FTIR): FTIR is an analytical method to study functional groups. It can quantify some components of an unknown mixture and analyze solids, liquids and gases. The obtained biopolymer was characterized by FTIR in the range of 4000 – 400cm^{-1} to study the nature of functional groups present in it [12].

Thermogravimetry/ Differential Thermal Analysis (TG/DTA): TG-DTA can simultaneously analyze the multiple thermal properties of a single sample. It measures the sample's thermal stability and determines the oxidative or reductive effect of the given sample. The sample analysis was carried out in a nitrogen atmosphere by heating of about 5mg of sample from 33℃ to 550℃ at 10℃/min [8].

X-Ray Diffraction (XRD): The crystal structure of extracted PHB formed by powder XRD patterns was analysed using an X-Ray diffractometer using Cu K-beta radiation source. XRD patterns were recorded in the 2Θ range 20° - 70° at a scan speed of 1.0° min^{-1} at room temperature [11].

RESULTS AND DISCUSSION:

Screening of Bacteria for PHB Production: The bacteria isolated were stained with Sudan black B and Gram's staining methods for preliminary screening of PHB production. Bacteria were found to be gram-positive and capable of producing PHB since it shows bluish-black colour [9].

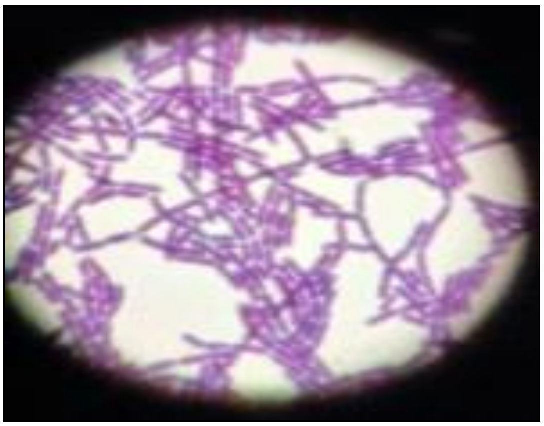

FIG. 1: GRAM'S STAINING –GRAM POSITIVE BACTERIA

FIG. 2: SUDAN BLACK B STAINING - PHB POSITIVE

FIG. 3: INDOLE TEST - NEGATIVE

FIG. 4: MRVP TEST -POSITIVE; VP NEGATIVE

FIG. 5: CATALASE TEST – POSITIVE

Biochemical tests like the Catalase test, Indole test, MR test, and VP tests were done to identify the bacterial species [29]. **Fig. 3, 4, 5** depicts that the bacteria isolated was Indole negative, MR Positive, VP Negative, and Catalase positive, respectively.

The results from these tests indicate that the isolated bacteria have similar characteristics to *Bacillus spp* [10, 13, 16]. *Bacillus* species were found to produce 11-69% of PHAs, more than any other bacterial strains [19].

Optimization of PHB Production using Response Surface Methodology (RSM): RSM provides the experimental design, which was analyzed by ANOVA and provided the actual and predicted values of the given variables for the factors chosen.

The factors were optimized by CCD with five central points. The optimal levels of each variable for maximal yield of PHB and their 3D plots of interaction were made [11, 21].

RSM is the best tool to minimize the trials and increase outcomes at a time. The maximum yield of obtained PHB was found to be at 35g/l of fructose and at pH of 9.

The model designed was significant, and the predicted R-Square value agrees reasonably with the adjusted value of R-Square.

The graph represents that the pH has a major influence over the yield of PHB. Among the Carbon (Fructose) and Nitrogen (Ammonium chloride) sources, fructose has more effects on yield since the PHB is the carbon and energy-storing site of bacteria in nutrient stress conditions.

With RSM, a maximum of about 0.5 g/l of PHB were extracted from the isolated strain of bacteria [26].

TABLE 1: EXPERIMENTAL DESIGN

Std	Run	Factor 1 A:pH	Factor 2 B: Fructose g/l	Factor 3 C: NH4Cl g/l	Response1 PHB g/l (Actual Value)	Predicted Value
1	13	5.00	20.00	0.50	4.32	4.04
2	1	9.00	20.00	0.50	8.16	8.38
3	3	5.00	35.00	0.50	5.12	4.49
4	18	9.00	35.00	0.50	10.5	10.27
5	5	5.00	20.00	1.00	4.35	4.45
6	14	9.00	20.00	1.00	7.67	8.16
7	10	5.00	35.00	1.00	5.24	4.89
8	8	9.00	35.00	1.00	9.89	10.04
9	16	3.64	27.50	0.75	0.71	1.34
10	6	10.36	27.50	0.75	9.75	9.32
11	2	7.00	14.89	0.75	7.22	6.84
12	7	7.00	40.11	0.75	8.23	8.80
13	4	7.00	27.50	0.33	6.93	7.42
14	15	7.00	27.50	1.17	7.86	7.57
15	9	7.00	27.50	0.75	7.62	7.57
16	11	7.00	27.50	0.75	7.62	7.57
17	12	7.00	27.50	0.75	7.58	7.57
18	19	7.00	27.50	0.75	7.56	7.57
19	17	7.00	27.50	0.75	7.49	7.57

TABLE 2: ANOVA (ANALYSIS OF VARIANCE)

Source	Sum of Squares	Degree of Freedom	Mean Square	F Value	p-Value Prob>F
Model	91.93	9	10.21	39.01	<0.0001 (significant)
A-pH	76.84	1	76.84	293.43	<0.0001
B- Fructose	4.63	1	4.63	17.67	0.0023
C - NH_4Cl	0.028		0.028	0.11	0.7528
AB	1.03	1	1.03	3.93	0.0787
AC	0.20	1	0.20	0.75	0.4102
BC	1.125E-004	1	1.125E-004	4.296E-004	0.9839
A^2	8.57	1	8.57	32.72	0.0003
B^2	0.11	1	0.11	0.42	0.5328
C^2	9.841E-003	1	9.841E-003	0.038	0.8506
Lack of Fit	2.814E-003	3	9.379E-004	1.34	0.4543 (not significant)

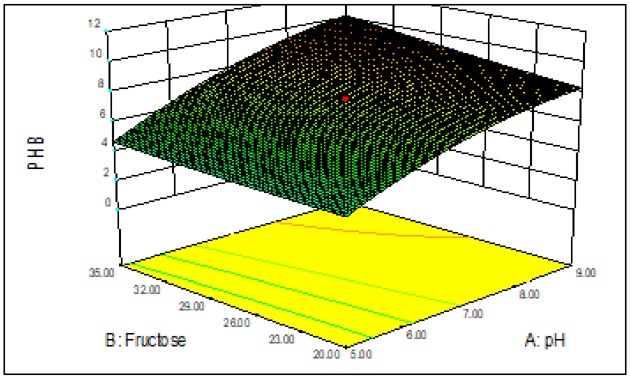

FIG. 6: 3D PLOT OF THE EFFECT OF PH AND CARBON SOURCE TO THE PHB OBTAINED

FIG. 7: OBTAINED PHB

Characterization of PHB:

Fourier Transform Infrared Spectroscopy (FT-IR) Analysis: The FT-IR spectrum of obtained PHB shows the absorbance peak at 3730.33 cm^{-1} and 3263.56 cm^{-1} representing the terminal hydroxyl (OH) group. The absorbance band at 2927.94 cm^{-1} represents C-H Stretching. The band at 1728.22 cm^{-1} denotes the C=O group. The peak at 1641.42 cm^{-1} indicates the C=C group.

The absorbance bands at 1259.52 cm^{-1}, 1228.66 cm^{-1}, 1182.36 cm^{-1} and 1051.20 cm^{-1} denote the C-O group. The peaks at 500-800 cm^{-1} represent the C=C group. The FT-IR results of the obtained polymer agree with the FT-IR of PHB [12, 21, 28].

Thermogravimetry / Differential Thermal Analysis (TG/DTA): Fig. 9 depicts the degradation of PHB in a nitrogen atmosphere by heating about 5mg of sample from 33℃ to 550℃ at 10℃/min. The thermal degradation occurs at 106℃, and residual mass at 549.6 ℃ was about 42.32% [8, 12, 23].

X-Ray Diffraction (XRD): Fig. 10 depicts that the XRD pattern is recorded revealed peak values of 2ϴ at 11.3, 19.28, 23.42, and 26.30, which are characteristic of PHB molecules. The increased intensity of peaks at 70 and 110 showed that the polymer may have a more organized/packaged crystalline structure [23].

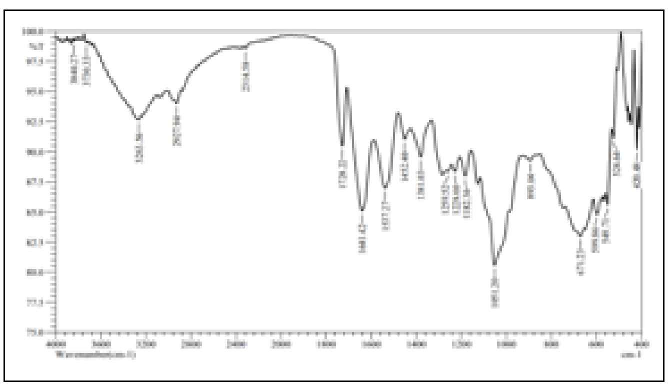

FIG. 8: FT-IR OF OBTAINED PHB

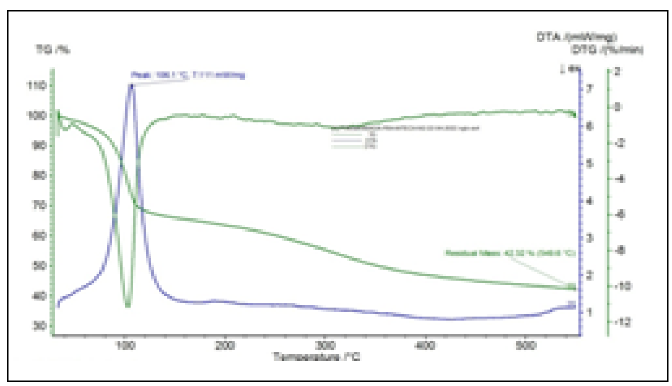

FIG. 9: TG/DTA OF OBTAINED PHB

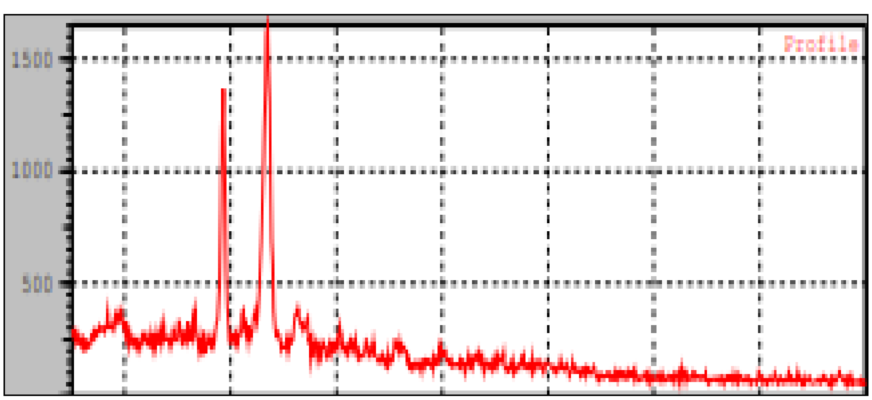

FIG. 10: XRD OF OBTAINED PHB

CONCLUSION: This study aimed to isolate the bacteria from the river bank soil sample from Sathyamangalam to produce polyhydroxy-butyrate. The bacteria's morphological characters were analyzed using the results of the biochemical tests, including Indole test, Catalase test, MR and VP tests. The production process was carried out in the shake flask. Mineral Salt media was used as the production media, and media optimization was done with the help of the Design Expert software tool – Response surface methodology (RSM). This tool analyses the effect of pH, Carbon source, and Nitrogen source on the PHB yield. The obtained PHB was characterized using FTIR, TG/DTA. The results depict that the functional groups obtained were similar to that of the standard PHB, and the thermal degradation was attained at 106°C. Hence, this work focused on the production, extraction, and characterization of the PHB from the isolated bacterial strain with a favourably high yield by optimized media conditions.

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CONFLICTS OF INTEREST: We declare no conflict of interest to disclose.

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