INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print); 2222-5234 (Online)
Website: https://www.innspub.net
Email contact: info@innspub.net
Vol. 27, Issue: 4, p. 130-139, 2025

RESEARCH PAPER OPEN ACCESS

Production of bioplastics (PHB) using waste paper as feed stock by *Cupriavidus taiwanensis*

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Key words: Polyhydroxy butyrate, Waste paper, Rhizosphere soil, Biodegradablee

DOI: https://dx.doi.org/10.12692/ijb/27.4.130-139 Published: October 17, 2025

ABSTRACT

Plastics are widely used for in variety of applications in modern society. However, these synthetic polymers are not biodegradable and pose a global waste management challenge. Polyhydroxy butyrate (PHB) granule is a biodegradable and biocompatible plastic seen as intracellular reserve granules by many bacteria during adverse conditions. PHB has versatile properties like solubility in water, oxygen permeability, UV resistance, biocompatible etc. hence it is ideal for medical applications. Biopolymer producing bacteria was isolated from rhizosphere soil samples of leguminous plants. From the seven different bacterial were obtained from agricultural fields, the most efficient isolate was identified as *Cupriavidus taiwanensis* LMG 19424 by molecular characterisation. The target of the present work is the isolation and identification of microorganisms producing PHB from rhizosphere soil, chemical characterization of the PHB polymer and optimization of acid hydrolysis conditions for maximum production of PHB using waste paper as substrate. The high yield of bacterial cultures combined with its ability to grow in a range of environment make them the most versatile feedstock for plastic production. The bioplastics such as PHB can reduce the amount of plastic waste, and also helps in the efficient management of paper waste if they are used as a feedstock.

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INTRODUCTION

Plastics are an integral part of the society due to their dynamic properties such as durability and resistance to degradation. Plastics are generated from the polymersation of monomers from oil or gas. But due to the synthetic origin, they are not recognized by the microorganisms. Plastics take long periods to degradation. The degradation of a plastic bottle usually takes 450 years on an average (Christina *et al.*, 2018). The largest share of plastic production is contributed by packaging industry in which 50% comes from single-use applications such as disposable consumer items. Plastics were the second largest component in waste from electrical and electronic equipment (WEEE) (Nwachukwu *et al.*, 2013).

Bioplastics are biodegradable polymers among which the most prominent one is poly 3-hydroxy butyric acid (PHB). A Polyhydroxyalkanoic acid (PHAs) is seen as intracellular deposits in bacteria, archaea, and such as yeasts and fungi. These polymers display potential interest as substitutes of common plastics because they are completely degradable by the microoganisms present in the environment and they can be regenerated from carbon sources. Regardless of these dynamic properties, industrial production of PHAs is still not well established.

The large-scale production is cumbersome due to the production cost as compared with the oil-derived plastics. So diverse efficient fermentation studies are underway to reduce the production cost by employing bacterial strains. Various types of waste products have been used for PHB production because it provides dual benefits of utilizing the waste and also the production of biodegradable microbial bioplastic in a cost effective manner. (Bhuwal *et al.*, 2013)

The abundance of lignocellulosic biomasses makes them promising renewable and sustainable feedstock for production of polyhydroxyalkanoates which can be a substitute of synthetic plastics. Waste paper is one among the major components of municipal and industrial solid wastes which accounts more than 35% of total lignocellulosic wastes (Dubey *et al.*, 2012).

Annually only about 50–65% of waste paper is being recycled from 400 million tons of waste paper generated due to the constraints in recycling of paper fibers. Utilization of waste paper as feedstock for production of other value added products is considered as a much valuable and an alternative route for waste management (Neelamegam *et al.*, 2018).

The objectives of this study are as follows:

1. Isolation and purification of microorganisms from rhizosphere soil.
2. Screening of isolated cultures for the production of PHB.
3. Identification of potent strain producing PHB by biochemical, morphological and further by 16s rDNA analysis PCR and BLAST analysis.
4. Characterization of PHB by FTIR, Xray Diffraction and Differential scanning calorimetry.

MATERIALS AND METHODS

Isolation of bacterial cultures

About 20 soil samples were collected from the outskirts of Ernakulum and Thrissur districts of Kerala state, India. The samples were serially diluted up to 10^{-7} dilutions to isolate pure cultures of bacteria by pour plate technique. Around One mL of sample from dilutions such as 10^{-3}, 10^{-5} and 10^{-7} were pour plated in carbon rich nutrient agar (NA) medium with the following composition of glucose (1%), beef extract (0.3 %), peptone (0.5%), sodium chloride (0.8%) and agar (1.5%). The plates were incubated at 37°C for 72 hours. Individual colonies with different morphologies were picked from the master plate and purified by repeated streaking. The colonies were subsequently transferred to fresh agar slants and stored at 4°C for further processing.

Screening of PHB producers

PHB producers were screened from these isolates through primary and secondary screening. The

primary screening of PHB synthesizing organisms was carried out by staining the cells with lipophilic Sudan black-B dye followed by observation under oil immersion objective (100X, Labomed). Secondary screening was carried out on PHB selective media with the composition of chemicals such as 2.5 g, 25 g, 100 g, 20 g, 1 g, 100 g, 10 g, 1.2 g, 20 g/L of K_2HPO_4, Na_2HPO_4, Mannitol, NaCl, $MgSO_4$, $C_3H_3NaO_3$, Peptone, Bromothymol blue, and agar respectively (Narayanan *et al.*, 2020). The cultures were streaked on selective medium and incubated for three days at 37 °C. The growth of colonies on the medium confirmed that the isolates can synthesize PHB.

Production and extraction of PHB from selected isolates

The Mineral Salt Medium (MSM) was used for production of PHB. About 1% (OD - 600) of culture was inoculated into 100 mL MSM media in 250 mL Erlenmeyer flasks and incubated for 72 hours. The pellets obtained after centrifugation at 10,000 rpm for 5min were air dried and weighed, to obtain the cell dry weight (CDW). Sodium hypochlorite (4% v/v) solution was used to break the cell wall. The pellet suspension was then centrifuged at 10,000 rpm for 5min and washed with acetone and diethyl ether (1:1). The Pellet was suspended in hot chloroform. Excess chloroform was evaporated in water bath to obtain PHB crystals. The rate of PHB accumulation was calculated from the concentration of PHB to CDW (Sabarinathan *et al.,* 2018). All the experiments were performed in triplicates. PHB yield of the selected isolates were assessed to identify high yielding strains.

Molecular identification of PHB high yielding strains

Conventional morphological and biochemical approaches for identification of bacteria are time consuming and labor intensive. On the other hand molecular techniques like 16S rDNA analysis give faster and reliable results. The uniqueness of 16S to each bacterial species makes it an ideal target for identification (Franco-Duarte *et al.*, 2019). Genomic DNA was isolated from PHB high yielding strains by phenol-chloroform method. The universal primers 27F and 1492R were used to amplify 16S rDNA with Thermocycler (Wee 32, HiMedia) (Weisburg *et al.,* 1991) (Forward primer: 5'AGAGTTTGATCCGGCTCAG3''; Reverse primer: 5'ACGGCTACCTTGTTACGACTT-3''). PCR reaction was performed in 25µl reaction mixture containing the components 6.25µl of Taq buffer, 0.315µl dNTP mix, 0.25µl each of forward and reverse primer, 2.5µl of Taq polymerase, 2.5µl of template DNA and 12.935µl of HPLC grade water. The amplified PCR product was then sequenced using an automated sequencer (AgriGenome Labs Pvt Ltd. Kochi, India). Unknown organism was identified by performing a sequence similarity search for 16S rDNA sequence using NCBI BLAST and results were noted.

The Phylogenetic and molecular evolutionary analyses were conducted with the help of MEGA version X using neighbor joining method (Kumar *et al.*, 2018).

Preparation of raw material

The raw material used in the study (WOP) was collected from St. Peters College, Kolenchery, India. It was then shredded into 2-6 mm pieces and soaked in distilled water at 60°C. After soaking, shredded materials were washed by continuous stirring for ink removal. The water content was removed by repeated squeezing followed by overnight drying in oven at 50°C. Dry matter was then grinded using a mixer grinder (Al Azkawi Ahlam *et al.*, 2018). Pretreatment of WOP (5%) was carried out with 0.5 % hydrogen peroxide (H_2O_2) at 121°C and 15 psi for 30 minutes (Al Battashi *et al.*, 2018). The H_2O_2 treated WOP was again subjected to overnight drying in oven at 50°C followed by grinding.

Optimization of acid hydrolysis

Pretreated WOP material was then hydrolyzed by the addition of sulfuric acid. The various parameters influencing the hydrolysis step are the

concentration of sulphuric acid (1–3% v/v), temperature (60–100°C) and time of hydrolysis (20–120 min). The response surface methodology with central composite design was used to optimize the significant and contributing factors of hydrolysis with Design Expert software version 8.0.7.1 (Stat-Ease, Inc. USA). The factors chosen were concentration of acid, temperature and time of hydrolysis. A set of seventeen experiments were generated by the software. All the runs were performed in triplicate. The validation of the experiment was done by the predicted model and experimental conditions derived from RSM. The experimental results were fitted with the response surface regression using second order polynomial equation. The polynomial equation was then expressed as three dimensional surface plots to demonstrate the relationship between the effects of the variables and the responses.

Estimation of reducing sugars

Reducing sugars released during substrate hydrolysis were estimated by 3, 5-dinitrosalicylic acid (DNS) method (Ghose, 1987). The hydrolysate obtained was neutralized to pH 7.0 before estimation of reducing sugars. Concentration of sugar was determined by taking average value of three measurements in each run. Optimized conditions with maximum release of sugars were further used to carry out fermentation for production of PHB. The hydrolysate obtained from RSM optimized conditions was then used as the carbon source for production of PHB.

Optimization of fermentation conditions

Effect of nitrogen sources on growth and PHB production

These experiments were done in MSM medium containing WOP hydrolysate (5%) as carbon source and nitrogen sources such as ammonium chloride, ammonium sulfate, yeast extract, and urea (2 g/L). The flasks were incubated at 30°C and 150 rpm. The biomass and PHB were then estimated in cell broth after 48 h (Aramvash *et al.*, 2015).

Characterization of PHB

Fourier transforms infrared spectroscopy

The side chains as well as the functional groups were analyzed by Fourier transform infrared spectroscopy. Infrared spectral data were collected on Thermo Nicolet iS50 FTIR spectrometer equipped with attenuated total reflectance (ATR). Spectra were collected over a range of 4000–100 cm^{-1} at 0.2 cm^{-1} resolution with an interferogram of 64 scans.

Differential scanning calorimetry

The most widely used thermal analysis technique is the Differential Scanning Calorimetry (DSC). The heating or cooling process at a constant rate or during isothermal steps, generates characteristic changes in sample properties, with regard to glass transition, polymorphic transition and melting or crystallization conditions. The melting enthalpy and crystallinity of PHB was accurately measured using the differential scanning calorimeter (Netzsch DSC 204 F1 Heat flux DSC).

X-Ray diffraction

X-ray diffraction measurement was carried out with Bruker D8 Advance diffractometer operated at 40 kV and 35 mA. XRD patterns were then recorded in the 2θ range 10° - 80°.

MALDI-TOF- mass spectrometry

The MALDI-TOF mass spectra were recorded using Bruker Autoflex max LRF. Spectra were taken in Positive Reflector mode and analyzed using polymer spectral analysis software, Polymerix.

RESULTS AND DISCUSSION

Isolation of pure cultures

Soil samples were collected aseptically from various agricultural fields in Kerala (Table 1). The isolation of endospore forming bacteria was carried out by dissolving one gram of the sample in 10ml of sterile distilled water and thereafter by heating at 80°C for 10 minutes. The cultures were serially diluted and then spread plated on nutrient agar plates. The plates were then incubated at 30°C for 48 hours. The well grown bacterial cultures were then used for further screening techniques and stored at 4°C.

Table 1. Site of collection of samples

#	Labelled as	Type of rhizosphere soil	Site of collection
1.	PHB1	*Pisum sativum*	Muriyad, Thrissur (District), Kerala
2.	PHB2	*Pisum sativum*	Vadavucode, Ernakulam (District), Kerala
3.	PHB3	*Mimosa pudica*	Kolenchery, Ernakulam (District), Kerala
4.	PHB4	*Pisum sativum*	Kunnathunadu, Ernakulam (District), Kerala
5.	PHB5	*Pisum sativum*	Pullur, Thrissur (District), Kerala
6.	PHB6	*Pisum sativum*	Muriyad, Thrissur (District), Kerala
7.	PHB7	*Pisum sativum*	Muriyad, Thrissur

Table 2. Description of BLAST hits on the query sequence PHB 3

Description	Scientific name	Max score	Total score	Query cover	E value	Per. Ident	Len	Accession
Cupriavidus taiwanensis LMG 19424 16S ribosomal RNA, partial sequence	*Cupriavidus taiwanensis* LMG 19424	309	309	100%	3e-84	99.41%	1535	NR_074823.1
Cupriavidus taiwanensis LMG 19424 16S ribosomal RNA, partial sequence	*Cupriavidus taiwanensis* LMG 19424	305	305	100%	4e-83	98.84%	1514	NR_028800.2
Cupriavidus nantongensis strain X1 16S ribosomal RNA, partial sequence	*Cupriavidus nantongensis*	303	303	100%	1e-82	98.82%	1405	NR_149305.1
Cupriavidus alkaliphilus strain ASC-732 16S ribosomal RNA, partial sequence	*Cupriavidus alkaliphilus*	303	303	100%	1e-82	98.82%	1520	NR_109152.1
Cupriavidus numazuensis NBRC 100056 16S ribosomal RNA, partial sequence	*Cupriavidus numazuensis* NBRC 100056	298	298	100%	7e-81	98.24%	1457	NR_113871.1
Cupriavidus pinatubonensis strain 1245 16S ribosomal RNA, partial sequence	*Cupriavidus pinatubonensis*	294	294	100%	9e-80	97.67%	1542	NR_040987.1
Cupriavidus oxalaticus strain CCUG 2086 16S ribosomal RNA, partial sequence	*Cupriavidus oxalaticus*	289	289	100%	4e-78	97.09%	1539	NR_117108.1

Table 3. Levels of independent variables for the experimental design

Symbol	Independent variables	Low level	Mid-level	High level
A	Concentration of acid (%)	1	2	4
B	Temperature (°C)	46	80	114
C	Hydrolysis time (minutes)	20	72	154

Screening for PHB producing bacteria

Primary screening- Sudan black staining

The detection of PHB producing colonies was done using lipophilic stain Sudan Black B. The stain was prepared by dissolving 0.3 gm powdered stain in 100 ml of 70% ethanol. The smears of colonies were heat-fixed on clean, grease-free glass slides, followed by staining with 0.3% solution of the Sudan Black B. The slides were then kept undisturbed for 15 minutes, immersed in xylene and counterstained with safranin (5% w/v in sterile distilled water). Sudan black stained the PHB granules as black spots, while the cells appeared dark blue in colour (Fig. 1). The cells appearing blue-black under microscope were accredited as PHB positive strains and preserved on agar slants with 2% glycerol.

Secondary screening

PHB selective media: The PHB selective media was used in this study for secondary screening. The growth of colonies on PHB selective media confirms the ability of strains to produce PHB (Fig. 2). The constituents of Modified agar plates are: Beef extract (0.3%), Peptone (0.5%), Sodium Chloride (0.8%), Glucose (1%), and Agar (1.5%).

Extraction of PHB

The extraction of PHA from the positive isolates was carried out (Sabarinathan *et al.*, 2018) to determine PHB yield of each isolate. Seven bacterial strains with high PHB content were used for comparison of cell growth and PHB (Table 1). Based on the dry weight of

the extracted PHB, positive colonies of PHB producing bacteria were identified. The yield of PHB was found to be 1.98, 2.18, 1.78, mg/dl in the colonies labelled as PHB1, PHB3 and PHB 4.

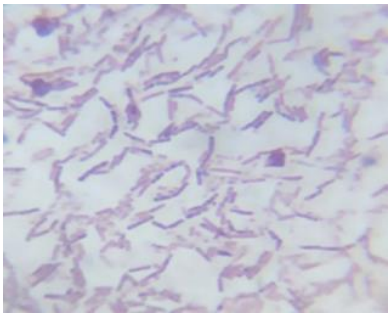

Fig. 1. Sudan black B staining

Fig. 2. PHB selective media

Polymerase chain reaction

The DNA samples from the cultures PHB 1, PHB 3 and PHB 4 were amplified with forward and reverse primers specific for 16S rDNA which generated specific amplicons of 1500bp. The samples were then loaded in 1.5% agarose gel and visualized in UV light (Fig. 3).

Fig. 3. Polymerase chain reaction of the three isolates

Identification of bacteria

The identification of isolated bacteria was done by both conventional biochemical tests and staining procedures. Molecular characterization was done by 16S rDNA sequence homology study.

In silico analysis

The BLAST results suggested 100% identity of PHB 1 sample with *Bacillus cereus* strain IAM 12605, 99.41 % identity of PHB 3 sample with *Cupriavidus taiwanensis* LMG 19424 and 100% identity of PHB 4 sample with *Bacillus cereus* strain IAM 12605. Among the three the potent strain, *Cupriavidus taiwanensis* was selected for further analysis. The description of BLAST hits on the Query sequence PHB 3 sample is shown in Table 2.

Phylogenetic tree

The phylogenetic tree obtained for PHB 3 sample using neighbor joining method by comparing the blast sequence of various *Cupriavidus* species is shown in the Fig. 4. The tree was constructed using MEGA-X software.

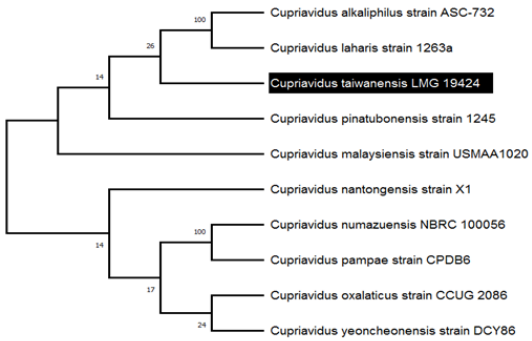

Fig. 4. Phylogenetic tree of PHB 3

Optimization of waste office paper hydrolysis by response surface methodology

Pretreated raw material (WOP) was hydrolyzed in acidic conditions for the release of simple sugars like glucose from cellulose that can be directly utilized by bacteria (Lenihan *et al.*, 2010). The influence of various factors on acid hydrolysis of WOP was optimized through central composite design (CCD) of RSM. Effects of the most important variables for acid hydrolysis including, acid concentration, time and

temperature on paper hydrolysis were studied as mentioned earlier. The maximum and minimum ranges of independent variables are given in Table 3. The concentrations of reducing sugar released by acid hydrolysis at different combinations of the independent parameters were determined by DNS method.

In the experimental design, ANOVA was performed. In the present study, the model F value of 4.83 suggested that the model is significant. Results of ANOVA are represented in Table 4. Here the values of "Prob > F" indicates that model terms are significant whereas values greater than 0.1000 indicate the model terms are not significant. It was found that in this case temperature (B) was significant model term (Table 3).

The comparison of curvature variance with residual variance is done by the F-value. It was found that if the variances observed are close to the same value, the significance of the curvature is likely less. If the null hypothesis is true, Probability >F (*p*-value) is the probability of seeing the observed F-value. If the probability value is small, it indicates the rejection of the null hypothesis and also that the curvature is not significant. The results of the present linear model exhibit small probability value ($p< 0.05$) indicating that there is a model effect. The adequate precision indicates the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 7.577 indicates an adequate signal. Therefore, this model can be conveniently used to navigate the design space. The final equation for linear model suggested in terms of coded factors for concentration of sugar (Y) is given below, Y= +121.67+ 55.34 A + 78.19 B + 67.39 C

Here the coded equation explains the effect of independent variables on the dependent variable, response (Y). The response changes of each factor is given by the perturbation plot as it moves from the chosen reference point, keeping all other factors constant at the reference value (Fig. 5). The single most significant variable for increasing concentration of sugar was fond to be Temperature (B) followed by time (C) and concentration of acid (A).

Table 4. ANOVA table for calculating the of reducing sugar on acid hydrolysis of WOP as affected by the variables such as H_2SO_4 concentration, temperature and time

Source	Sum of squares	d.f.	Mean square	F value	*p*-value
Model	1.730E+005	3	57674.50	4.83	0.0180*
Concentration of acid (A)	36119.64	1	36119.64	3.02	0.1058
Temperature (B)	83511.74	1	83511.74	6.99	0.0203*
Time (C)	53595.06	1	53595.06	4.48	0.0541
Residual	1.554E+005	13	11952.51	-	-
Lack of Fit	1.549E+005	11	14081.33	57.71	0.0172
Pure error	488.00	2	244.00	-	-
Cor total	3.284E+005	16	-	-	-

Bold values indicate the significant parameter. * Values less than 0.05 indicate significance at 95% confidence interval.

The statistical model was validated using point prediction tool of RSM wherein an optimum value of all the three variables (concentration of acid (3%), temperature (100°C) and time (120 minutes)) were evaluated. The validity of the model is confirmed by the observed concentration of sugar (323 mg/dL) which was so close to the predicated one (322.6mg/dL).

Optimization of nitrogen sources

Optimization studies are carried out by the potential isolate PHB 3. The effect of different nitrogen sources on growth and PHB production of culture was carried out using MSM media containing WOP hydrolysate as carbon source, supplemented with different nitrogen sources.

The Cell Dry Weight (CDW) and yield of PHB were analyzed after the fermentation reactions.

Ammonium chloride, ammonium sulfate, yeast extract and urea at a concentration of 2 g/L were used nitrogen sources. The highest rate of PHB

accumulation was shown by ammonium chloride followed by urea (Fig. 6). Earlier reports (Aramvash *et al.*, 2015; Yu Hong Wei *et al.*, 2011) also confirmed ammonium chloride as the best nitrogen source for PHB production by *Cupriavidus* species.

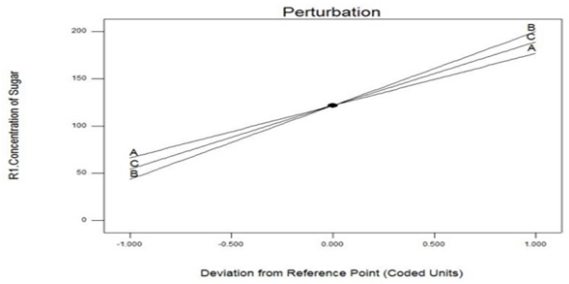

Fig. 5. Perturbation plot for three variables

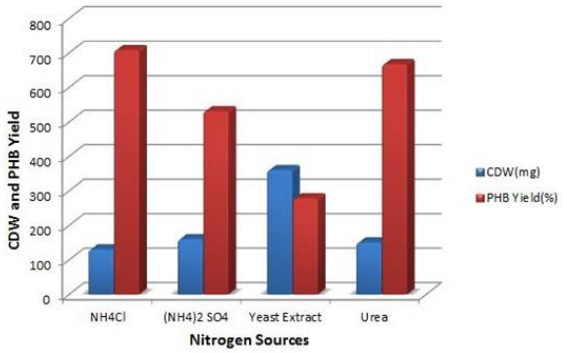

Fig. 6. CDW and PHB Yield of *C. taiwanensis* using different Nitrogen sources

Characterization of PHB

FT-IR analysis

Qualitative study of molecular structures was done by FTIR spectroscopy. The main functional groups of the PHB polymer could be verified (Fig. 7) by this method. The absence of specific bands in single bond and triple bond regions indicates the absence of such bonds. Numerous absorbance bands were observed in the double bond region between 2000-1500 cm^{-1}. The specific absorbance bands of the sample corresponds to wavenumbers 1737 cm^{-1}, 1639 cm^{-1}, 1553 cm^{-1}. This suggests some carbonyl double bond, which can be from ketones, aldehydes, esters, or carboxyl groups. The fingerprint region has many bands between 1500-500 cm^{-1}. The presence of diverse groups at the following regions indicates the presence of groups such as 1720 (ester C=O valence); 1639 (thioester C=O valence); 1380; 1302; (CH2-S); 1162 (ester C-O).

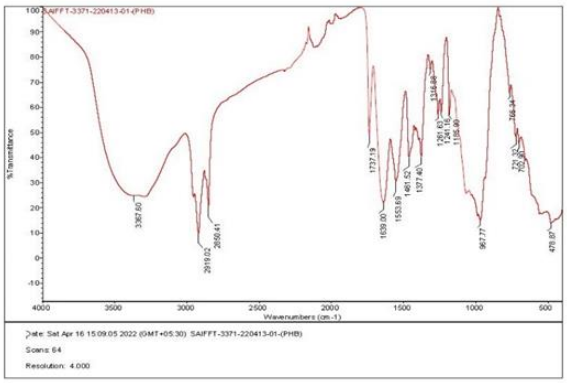

Fig. 7. FTIR analysis

Other bands located near 1261 and 1185 cm^{-1} were attributed to C-O-C groups. The presence of C-O bonds was confirmed by the bands located at 1050 and 979 cm^{-1}. The bands located in the spectral region around 2900 cm^{-1} was indicated by the C-H stretch bonds. Further the the terminal O-H bonding or water adsorption on the PHB was indicated by the specific peak at wave numbers 3367 cm^{-1}. Based on the above results, it was assumed that the extracted compound from *C. taiwanensis* LMG 19424 can be PHB.

Differential scanning calorimetry

For the commercial applications the thermal stability of PHB is a potent factor. The important industrial properties of the polymer are the thermal properties and chemical characteristics. DSC study of PHB gives an understanding of the crystallinity of PHB (Fig. 8). The thermal properties of PHB were determined using the DSC method. Differential scanning calorimetry (DSC) can calculate the heat flow into or out of a material as a function of time or temperature.

The key parameters to polymer processing and applications are the melting temperature (Tm), glass transition temperature (Tg) and crystallinity (XC). The curve obtained from the second heating from which Tm was found at 124.1°C and glass transition temperature (Tg) was determined at 16°C.

The area under the melting curve is calculated by the melting enthalpy which gives a measure of the

crystallinity of PHB (Xc). The percentage of crystallinity is given by the ratio of the PHB melting enthalpy to the melting enthalpy of 100% crystalline PHB (ΔHm100) (ΔHm/ΔHm100 × 100) (Ertan *et al.*, 2021). The melting enthalpy of PHB 3 was determined to be 83.08 J/g, while the melting enthalpy of the 100% crystalline PHB was taken as 146 J/g (Barham *et al.*, 1984). The crystallinity of PHB 3 was calculated as 56.9 %, which is within the range of 50–70% previously found for PHB homopolymers.

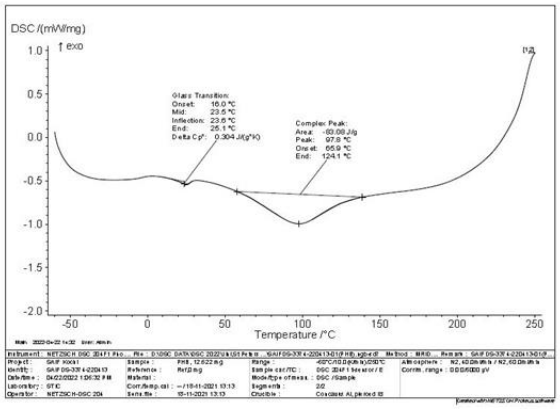

Fig. 8. DSC thermogram

X-ray diffraction

The characterization of unknown natural or synthetic solid materials can be done by X-ray diffraction (XRD) analysis of PHB. The phase and crystallinity of PHB crystals can be determined by this method. It was found that XRD (powder) analysis of PHB crystals can give valuable information regarding its structure which may be useful for target specific applications (Fig. 9).

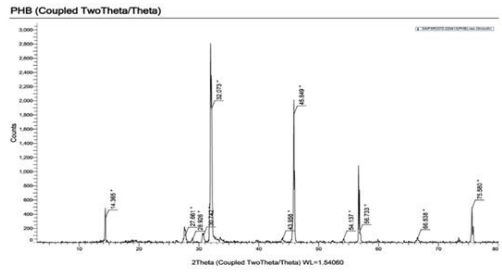

Fig. 9. X-ray diffractogram

The XRD data are normally presented as peak positions at 2θ and X-ray counts (intensity) in the form of a table or a xy plot. An X-ray diffractogram of PHB (Fig. 9) illustrated diffraction peaks in 2θ = 32.0◦, 45.8◦, 56.7◦, 75.5◦, 14.3◦ and 27.6◦ regions. The higher crystalline property of the crystal is confirmed by the sharp and slender peak at 32.0◦with relative intensity of 100% and peak at 45.8◦ with relative intensity of 96.2%. The crystallinity of the polymer is confirmed by the XRD data . The first two peaks reported, i.e., 14.3◦ and 27.6◦ are typical of orthorhombic structures, and have almost similar pattern when compared with previous crystallographic data for this material.

ACKNOWLEDEMENTS

The authors are grateful to Kerala State Council for Science, Technology and Environment (KSCSTE) for their financial aid and support.

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