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Production and Characterization of Polyhydroxybutyrate (PHB) from an Agricultural By-Product Using *Bacillus thuringiensis* CICIM (B2031)

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ABSTRACT

The widespread overuse of petroleum plastics has led to serious environmental challenges. This has necessitated the pursuit of greener alternatives that would reduce or eliminate mankind's unsustainable and toxic dependence on the recalcitrant petroleum plastics. Polyhydroxybutyrate (PHB) is a potential substitute candidate for the production of biodegradable plastics. In this study, *Bacillus thuringiensis* CICIM (B2031) was utilized for the production of PHB using the agricultural by-product corn silage as a carbon source. The corn silage was pretreated with dilute sulfuric acid. The acid-pretreated corn silage hydrolysate was used at different loadings, producing a maximum biopolymer yield of 53.43% PHB. This was compared with the untreated corn silage which gave a maximum yield of 28.63% PHB, under the same conditions. The extracted PHB biopolymer was characterized using Fourier-Transform Infrared Spectroscopy (FT-IR), ultraviolet-visible (UV-Vis) spectroscopy, and gas chromatography-flame ionization detector (GC-FID). This study revealed that corn silage (CS) can be utilized as a renewable carbon source for PHB production.

1. INTRODUCTION

Petroleum refining and the various synthetic petrochemical materials derived from the process have emerged as both a necessary part of everyday life and an environmental problem of considerable proportions. These polymers are resistant to biodegradation, and it is these recalcitrant residues that pose the greatest environmental pollution threat (Charen et al. 2014, Tavella et al. 2025). These synthetic materials in the environment are not only harmful but also significantly affect the aesthetics of cities. Disposing of polyvinyl chloride (PVC) is costly and often involves incineration, which releases toxic emissions such as dioxins, making PVC a pollution problem (Gowda & Shivakumar 2014, Ghimire et al. 2025). The search for alternative plastic options is vital in reducing the current dependency on non-renewable resources (Getachew & Woldeesenbet 2016). This search should yield a suitable alternative that is biodegradable. Many solid waste disposal regulations have been instituted worldwide, and this has resulted in the limitation of plastic waste as well as the pursuit of novel ways to produce biodegradable plastic materials such as those derived from polyhydroxyalkanoate (PHA) (Alarfaj et al. 2015, Mallegni et al. 2025).

Microorganisms can make PHAs through utilization of various carbon sources that include lipids and sugars. Among the microbial PHAs, the most commonly produced is polyhydroxybutyrate (PHB). The crystalline structure of PHB contributes to its brittle properties. PHBs are natural biopolymers, which are synthesized and catabolized via microbial growth (Akar et al. 2006, Berlanga et al. 2006, de Roo et al. 2002). Bacteria have been reported to synthesize these biopolyesters, where they act as energy reserves that are deposited as insoluble cytoplasmic inclusions. The first bacterial PHB production was reported in *Bacillus megaterium*. Since then, the genus *Bacillus* has become a go-to source for PHBs, as they offer the advantage of growing fast, having genomic stability, and being able to adjust to a wide variety of carbon sources, producing PHBs which are, most importantly, endotoxin-free. *Bacillus thuringiensis* is a Gram-positive bacterium that can accumulate PHB and is an active PHB producer. However, the high production cost of the biopolymers and the availability of low-cost petrochemicals make the potential use of PHBs economically unattractive (Verlinden et al. 2011, Vigneswari et al. 2025).

The fact that PHBs are meant to be an alternative to petroleum plastics, which are abundant and low-cost, means that the feasibility of PHB production depends on cost-effective approaches. The cost of the carbon source substrate has been identified as the major economic impediment. It is therefore important to select carbon source feedstock that is cheap, readily available, and supports PHB production (Getachew & Woldesenbet 2016, Trapé et al. 2024).

Corn silage is produced through a microbially mediated process of preserving nutrients through moist anaerobic fermentation. Corn silage is therefore a highly nutritious substrate and is widely used as animal feed. Challenges during the feed-out stage, including substantial mold and yeast growth that can result in losses as high as 30%, motivate the repurposing of corn silage within the circular bioeconomy. Thus, the focus of this current study was to produce and characterize PHBs from agricultural corn silage (CS) using *Bacillus thuringiensis* CICIM (B2031).

2. MATERIALS AND METHODS

2.1. Compositional Analysis of Corn Silage

Corn silage samples were dried overnight in an oven at 75°C to remove water. Samples were then milled using a Wiley laboratory mill to a particle size of 1 mm. The moisture content of undried corn silage was determined following AOAC Method 7.003. The chemical composition of corn silage was determined following AOAC Method 978.18.

Table 1: Chemical composition of corn silage.

Chemical	Composition
Hemicellulose	20.7%
Cellulose	27.0%
Lignin	6.54%
Ash	3.7%
Fat	1%
Protein	9.28%
Starch	26.89%
Mineral salts	4.01%

Compositional analysis was carried out in triplicate, and the results are represented in Table 1.

2.2. Corn Silage Hydrolysate

Corn Silage substrate was hydrolyzed with 2.5% v/v sulfuric acid (solid: liquid, 1:10). The mixture was autoclaved for 30 min at 121°C. The hydrolyzed mixture was cooled to room temperature and filtered through Whatman No. 1 filter paper. The supernatant was neutralized using 6 N NaOH. Reducing sugar content was quantified using the photometric method originally reported by Marais et al. (1966). The growth medium was prepared using these filtrates at concentrations of 6%, 12%, 24%, and 48% (v/v).

2.3. Cultivation of *Bacillus thuringiensis* CICIM (B2031) Strain

A *Bacillus thuringiensis* CICIM (B2031) culture was obtained from the Culture and Information Centre of Industrial Microorganisms of China Universities (CICIM). The fermentation was carried out in 250 mL flasks in a medium containing 4 g L⁻¹ yeast extract, 8 g L⁻¹ tryptone, and 2 g L⁻¹ NaCl at 37 °C for 48 h on a shaker at 200 rpm. The bacterial cells were harvested at 6,000 × g and washed with deionized water (Charen et al. 2014). The bacterial growth profile was monitored by sampling 2 mL aliquots of the culture at 6 h intervals. All cultures and measurements were performed in triplicate.

2.4. Screening of PHB-Producing Bacteria

Screening for PHB-producing *B. thuringiensis* cells was done using the lipophilic stain Sudan Black B method (0.3 g of Sudan Black B in 100 mL of ethanol). A loopful of culture was taken from the culture medium, mixed with 20% brine, and then smeared on a glass slide. The smear was allowed to dry and then fixed by immersing it in 2% acetic acid for 5 min. The slide was flooded with Sudan Black B solution for 15 min at room temperature. The slide was dried, and xylene was added to clear. A 0.5% aqueous Safranin aliquot

was added for 10 s as a counterstain. This was followed by washing using distilled water and then dried for observation using a light microscope (Selvakumar et al. 2011).

2.5. Biomass Determination

A 50 mL sterilized Falcon tube was utilized to determine cell concentration, defined as dry weight of cells per liter of culture broth. The cell suspension was then centrifuged ($4,000 \times g$ for 15 min) (Eppendorf, Centrifuge 5810R). The supernatant was discarded, and the collected cell pellet was washed twice with deionized water and freeze-dried (the ice condenser was set at -84°C and vacuum set at 1.0 mbar) (Rohini et al. 2006). The dried cells were used for PHB extraction.

2.6. PHB Extraction and Production

A mixture of sodium hypochlorite and chloroform was used in the recovery of microbial PHB. A 1 g sample of lyophilized cell pellet was mixed with chloroform and 15% sodium hypochlorite (1:1) and shaken at 100 rpm for 90 min at 37°C . Centrifugation followed at $4,000 \times g$ for 10 min (Eppendorf, Centrifuge 5810R). A volume of 4 mL of methanol was added to the lower chloroform phase containing the solubilized polymer to precipitate the polymer (Rohini et al. 2006). The precipitated polymer was washed with acetone and air-dried. In this study, all quantitative experiments were carried out in triplicate. The mean differences among the pretreatments were analyzed by ANOVA.

2.7. UV-Vis Characterization

A 10 mg sample of the extracted polymer was dissolved in 2 mL of chloroform and scanned over a UV-Vis range of 200–800 nm (Thermo Scientific, GENESYS 150, UV-Vis Spectrophotometer) against a chloroform blank, as reported by Alarfaj et al. (2015).

2.8. FT-IR Spectroscopy

The structural analysis was performed using a PerkinElmer 537 FT-IR spectrophotometer. The spectrum of the extracted polymer from *B. thuringiensis* CICIM (B2031) [poly(3HB)] was compared with a pure poly(3HB) standard obtained from Sigma-Aldrich. Structural analysis of the functional groups was performed utilizing a Fourier-transform infrared Perkin Elmer 537 spectrophotometer. The sample was placed on the sample holder and scanned from $4,000\text{--}400\text{ cm}^{-1}$. An attenuated total reflectance (ATR) was used to record the spectra, and absorption frequencies were expressed as ν_{max} (cm^{-1}).

2.9. GC-FID Characterization

The monomer composition of the polymer was determined

using a Varian 2100T gas chromatograph with flame ionization detection (GC-FID). The Varian 2100T gas chromatograph was equipped with a ZB-5MS (Phenomenex) column (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness) and a flame ionization detector. A 5 mg sample of the polymer was subjected to transesterification using 1 mL chloroform and 0.85 mL of methanol with 0.15 mL of sulfuric acid as the catalyst. This reaction was carried out in a small screw-cap test tube for 60 min at 100°C . The mixture was allowed to cool down to room temperature. After cooling, 0.5 mL of distilled water was added to the test tube and mixed for 1 min using a vortex. The upper phase was removed using a plastic pipette, and the lower chloroform phase containing the methyl ester of β -hydroxy acid was recovered and transferred to a small screw-cap vial. A 2 μL sample was injected into the injector port, and the inlet pressure of the carrier gas (nitrogen) was set at 109.2 kPa. For effective separation of the esters, the following temperature program was used (60°C for 1 min, temperature increase $25^\circ\text{C min}^{-1}$ to 230°C (230°C for 1 min); flow controller: split, total flow 301.3 mL min^{-1} , column flow 1.48 mL min^{-1} ; the temperature of the injector and detector were 200 and 230°C , respectively (Pantazaki et al. 2009). Different β -hydroxyalkanoic acid methyl ester standards were used as references.

3. RESULTS AND DISCUSSION

It has been reported that both Gram-positive and Gram-negative bacterial species produce PHBs (Gomaa 2014). This vast microbial diversity calls for accurate identification and characterization of the producing strains and their ability to grow on cheaper substrates in the production of PHBs. In this study, PHB was produced in broth supplemented with corn silage (CS) as a cheap carbon source collected from the Department of Agriculture, KwaZulu-Natal. *B. thuringiensis* is known for both the production of an insecticidal δ -endotoxin and accumulation of PHB. It was also noted that maximum biomass production at stationary phase (maximum OD) coincided with the accumulation of the polymer after 48 h (Rohini et al. 2006). However, accumulated PHB may gradually decline after the stationary phase if the cell mass is not harvested because of PHB metabolism (Rohini et al. 2006).

3.1. PHB Production

The *B. thuringiensis* CICIM (B2031) was screened for PHB accumulation in the cytoplasm using Sudan Black B stain (Selvakumar et al. 2011). Sudan Black B has high lipophilic staining sensitivity in PHB screening. *B. thuringiensis* CICIM (B2031) showed PHB accumulation,

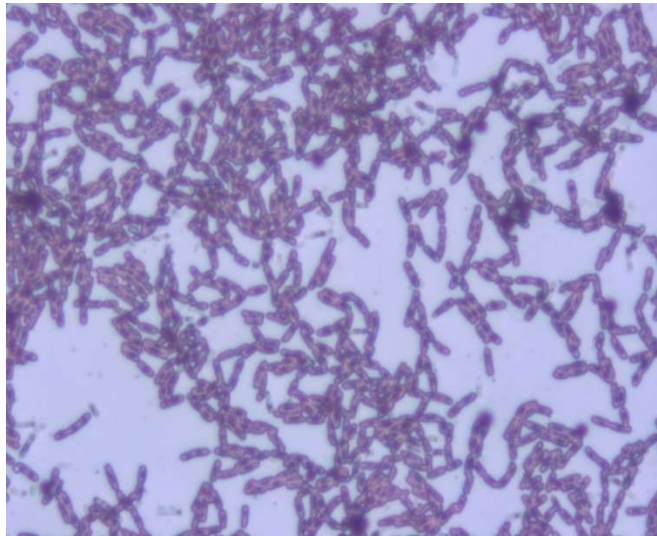


Fig. 1: Micrograph of *B. thuringiensis* CICIM (B2031) showing PHB accumulation; magnification 1000 $\times$.

evidenced by blue-black inclusions in the cytoplasm. The presence of blue-black granules indicated PHB production and the ability of *B. thuringiensis* to produce these polymers (Fig. 1). This supported the findings of Charen et al. (2014) and Sawant et al. (2014), where Sudan Black B counterstained with safranin was used to show positive accumulation of PHBs in *B. thuringiensis* using a light microscope

The cultures were harvested when the cells reached the early stationary phase, followed by determination of the polymer composition. During bacterial growth, the composition of the substrate changes with subsequent buildup and utilization of metabolic intermediates; hence,

changes in PHB monomer composition proportional to culture age are expected (Brandl et al. 1988).

After 48 h, it was observed that pH 7.0 yielded the highest biomass compared with pH 6.5 and pH 7.5 (Fig. 2). *B. thuringiensis* and some other bacteria are known to produce PHB naturally (Charen et al. 2014). PHB serves as an energy reserve, so greater biomass may result in greater accumulation of PHB. *B. thuringiensis* has been reported to show maximum biomass peaking at around 48 h, which signals the early stages of the stationary phase.

The corn silage supported PHB production even in its unhydrolyzed form. There was approximately 27% PHB

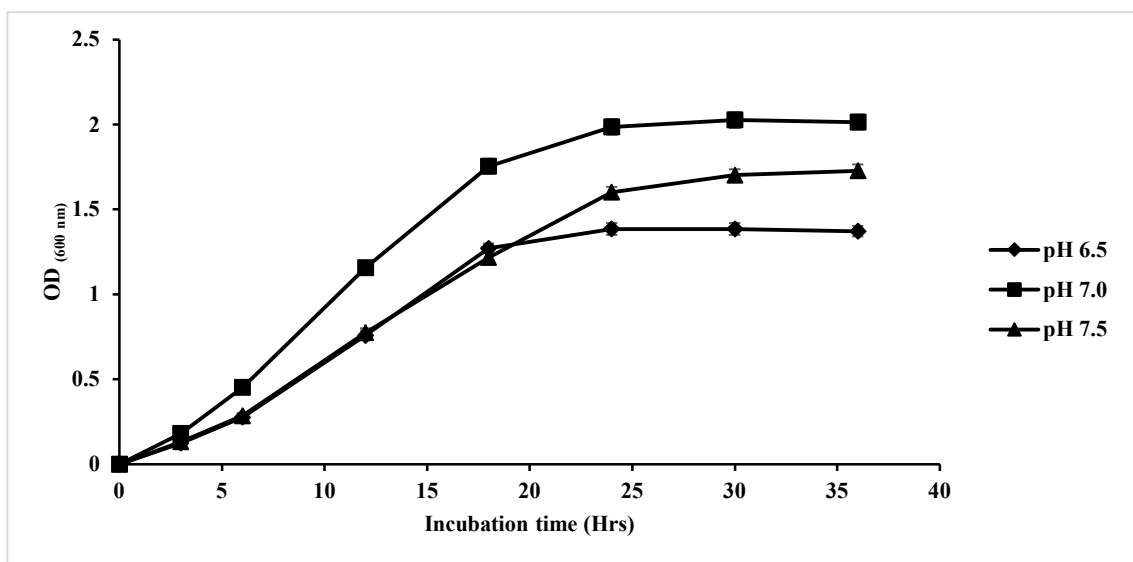


Fig. 2: Growth of *B. thuringiensis* at pH 6.5, 7.0, and 7.5.

Table 2: Effect of unhydrolyzed corn silage supernatant concentration on biomass and polymer production after 48 h of cultivation.

CS supernatant in medium (%)	Biomass (g L ⁻¹)	Polymer (g L ⁻¹)	PHB content (% w/w)
6	1.91 ± 0.16	0.22 ± 0.01	11.91 ± 1.65
12	2.64 ± 0.21	0.51 ± 0.02	19.42 ± 2.26
24	3.22 ± 0.17	0.88 ± 0.06	27.39 ± 2.65
48	3.82 ± 0.22	0.67 ± 0.02	28.63 ± 1.51

Table 3: Effect of hydrolyzed corn silage supernatant concentration on biomass and polymer production after 48 h of cultivation.

CS supernatant in medium (%)	Biomass (g L ⁻¹)	Polymer (g L ⁻¹)	PHB content (% w/w)
6	3.15 ± 0.24	1.32 ± 0.142	42.34 ± 3.25
12	3.26 ± 0.21	1.52 ± 0.123	46.42 ± 3.28
24	4.47 ± 0.36	2.25 ± 0.145	50.52 ± 2.29
48	4.99 ± 0.28	2.66 ± 0.232	53.43 ± 3.08

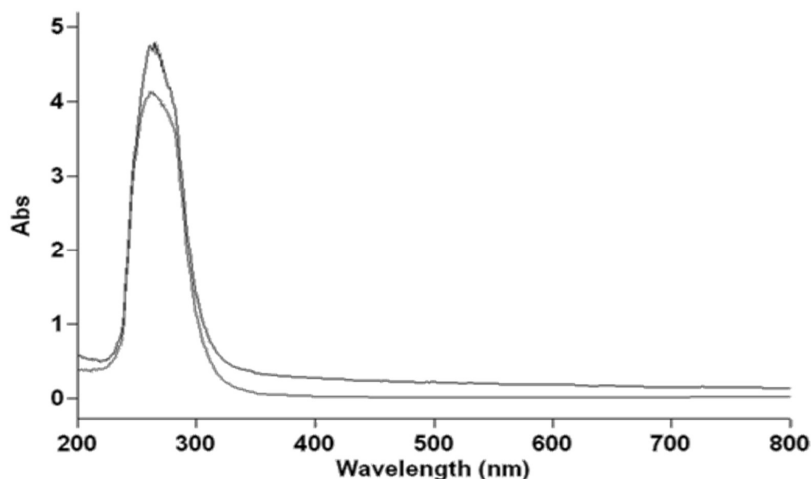
*Data represent the mean of three readings ± standard deviation.

accumulation at 24% loading; however, a doubling of the substrate loading did not show a commensurate increase in biomass and PHB accumulation (Table 2). This may be because a further increase in unhydrolyzed substrate increases the amount of untreated material that is not readily accessible to *B. thuringiensis*.

The acid-hydrolyzed substrate gave better biomass and PHB accumulation yields. A 53.43% PHB accumulation was recorded from the highest loading. The highest biomass of 4.99 g L⁻¹ was also achieved (Table 3). This can be attributed to the hydrolyzed substrate being richer in simple sugars that are readily accessible to the bacterium.

The ability of *B. thuringiensis* to accumulate PHB has been reported. The focus has been on efficiency in terms of production duration. Most reports have indicated optimum durations between 24 and 96 h for *Bacillus* strains. The focus on PHB production conditions is informed by the

relationship between growth conditions and the properties of the PHB produced. Ray and Kalia (2017) monitored the link between the different growth conditions, bacterial strains, and the degradation of PHB and concluded that PHB characteristics depend on the growth conditions. PHB characteristics determine its applicability, and the various intermediates produced from the degradation can be used for PHB modifications for industry-specific applications. As an agricultural by-product, corn silage is a cost-effective agro-based substrate that may help address the feasibility concerns in PHB production. Corn silage has a very high fermentable carbohydrate content, including starch (>28%, w/w) and reducing sugars (8%) (Chahine 2009). It also contains trace elements and vitamins (0.09%) (Erdman et al. 2011, Jiao et al. 2025). Given these compounds and minerals, corn silage as an agricultural residue was used in this study as a less expensive substrate to produce PHBs.

Fig. 3: UV-Vis absorption spectra of standard PHB (Sigma-Aldrich) (a) and PHB polymer obtained from *B. thuringiensis* CICIM (B2031) (b).

3.2. PHB Characterization by UV-Visible Spectroscopy

UV-Vis scanning of synthesized PHB revealed a spectrum similar to that of commercial PHB (Fig. 3). This spectral similarity indicates the presence of PHB and supports the use of corn silage as a potential PHB substrate. UV-Vis spectra of PHB isolated from *B. thuringiensis* were measured from 200 to 800 nm against the commercial standard using CHCl_3 as a blank. Both spectra of the commercial PHB standard and extracted PHB indicated a sharp maximum absorbance peak at 240 nm. These results are consistent with the findings of Shah (2014), which reported maximum absorbance of PHB within the range 230–240 nm.

3.3. FT-IR Spectroscopy Analysis

From the FT-IR spectrum obtained (Fig. 4), it can be concluded that the $3,375\text{ cm}^{-1}$ band is evidence of the strong hydrogen bond formed by the terminal OH groups. Comparable results have been reported in other studies (Getachew & Woldeesenbet 2016, Gumel et al. 2012). The region of $4,000\text{--}300\text{ cm}^{-1}$ contains absorption bands common to the commercial and bacterial polymers. According to Misra et al. (2000) and Getachew and Woldeesenbet (2016), the absorption band at $1,719\text{ cm}^{-1}$ is a PHB marker band due to C=O (carbonyl) stretches in the (COO) ester group located in the chain. The bands ranging from $1,060\text{--}1,281\text{ cm}^{-1}$ characterize the valence vibrations of the carboxyl (-C-O-) polymeric group (Getachew & Woldeesenbet 2016). These are characteristic of polyhydroxybutyrate. The band at $1,456\text{ cm}^{-1}$ is a result of CH vibrations, due to the asymmetric stretching and CH_3 group bending vibrations, with the COH bond being represented by the $1,380\text{ cm}^{-1}$ band (Alarfaj et

al. 2015). This spectrum confirms the report by Alarfaj et al. (2015), verifying that PHB was the isolated compound.

3.4. GC-FID Chromatography Analysis

GC identification of PHB is a commonly applied method in PHA research as it involves the decomposition of the molecule by methanolysis (Alves et al. 2023). The key compounds of concern in this study were identified based on their retention time. GC-FID analysis of the commercial PHB and extracted polymer showed a similar peak at 5.7 min (Fig. 5). This peak indicates that the extracted compound belongs to the biodegradable polyester family; therefore, GC confirmed that the polymer produced in this study is a short-chain-length PHB consisting of four carbon atoms (Chee et al. 2010).

The extracted polymer was analyzed by gas chromatography (GC) after methanolysis. The hydrolysis and derivatization reactions took place in a single vessel. The resulting methyl esters were separated by GC for qualitative analysis. A poly(3HB-co-3HV) copolymer reference standard was used (Fig. 5(a)). The peaks at 5.7653 min and 6.802 min represent PHB and PHV, respectively. These results indicate that the extracted polymer is poly(3HB-co-3HB) compared with the pure standard. The peak area of the extracted polymer showed additional peaks (Fig. 5(b)) compared with the reference standard, which may result from by-product formation during transesterification with sulfuric acid and methanol (Elhami et al. 2022). Derivatization of 3-hydroxyalkanoic acid with HCl and propanol to form a propyl ester may be preferred because the propyl ester is more lipophilic than the methyl ester, which improves the partitioning coefficient (Sharma et al. 2021).

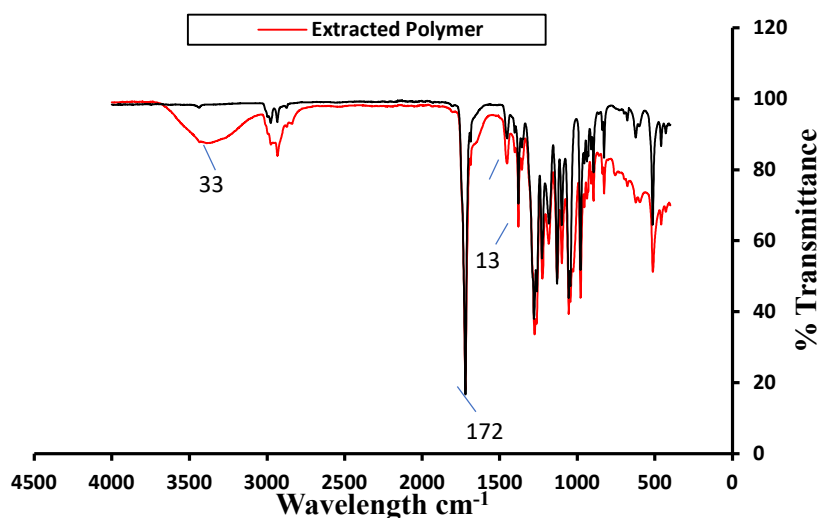


Fig. 4: FT-IR analysis of the commercial polyhydroxybutyrate standard (Sigma-Aldrich) and polymer isolated from *B. thuringiensis* CICIM (B2031).

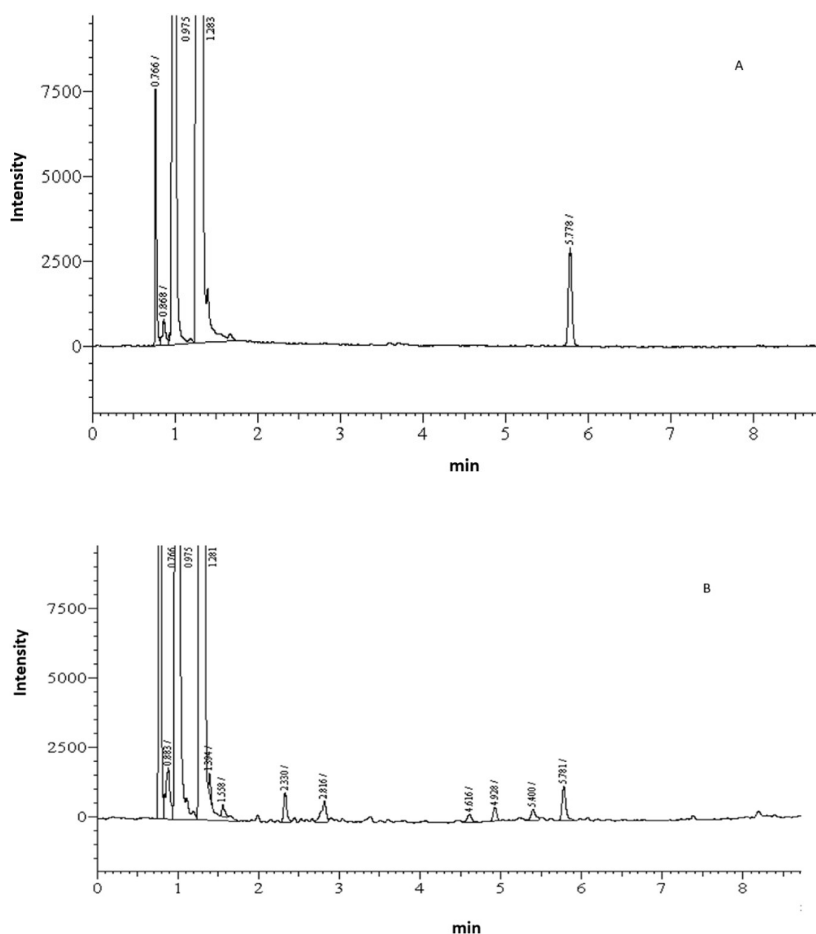


Fig. 5: GC-FID analysis of PHB produced by *B. thuringiensis*: chromatogram of the standard (a) and sample (b).

4. CONCLUSIONS

This study demonstrates PHB production using *Bacillus thuringiensis* CICIM (B2031) with the agricultural by-product corn silage. The bacterially derived PHB was confirmed by UV-Vis, FT-IR and GC-FID. This study provides an alternative approach to PHB production by repurposing an animal-feed carbon source such as corn silage. This strain shows potential for producing biopolymers from numerous agricultural by-products. However, this study revealed the need for novel derivatization methods for the analysis of scl-PHB to avoid excessive formation of by-products. PHB yields could also be further increased beyond the achieved 53.43% by media optimization.

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