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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 for greener alternatives that would reduce or eliminate mankind's unsustainable and toxic dependence on the recalcitrant petroleum plastic. 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 an agricultural by-product corn silage as a carbon source. The corn silage was pretreated with dilute sulphuric acid. The acid pretreated corn silage hydrolysate was used at different loadings producing a maximum biopolymer yield of 53.43 % PHB. This was in comparison to 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 pestilential proportion. These polymers are resistant to biodegradation, and it is these recalcitrant residue that poses the greatest environmental pollution threat (Charen et al., 2014; Tavella et al., 2025). These synthetic material in the surroundings are not only environmentally harmful but also significantly affects aesthetics of the cities. Costly disposal of poly-vinyl chloride (PVC) involves incineration which is accompanied by toxic emissions like dioxin, rendering PVC a pollution problem (Gowda and Shivakumar, 2014; Ghimire et al., 2025). The search for alternative plastic options is vital in reducing

the current dependency on non-renewable resources (Getachew and Woldesenbet, 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 poly-hydroxyalkanoate (PHA) (Alarfaj et al., 2015; Mallegni et al., 2025).

Microorganisms can make PHAs through utilization of various carbon sources that include lipids and sugars. Amongst the microbial PHAs, the most commonly produced is polyhydroxybutyrate (PHB). The crystalline structure of PHB made up of shorter chains gives it some 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 bio-polyesters where they act as energy reserves which are deposited as insoluble cytoplasmic inclusions. The first bacterial PHB production was reported on *Bacillus megaterium.* There the genus *Bacillus* has since then become a go to source for PHBs as they offer the advantage of growing fast, have genomic stability and are able to adjust to a wide variety of carbon sources producing PHB's which are most importantly, endotoxin-free. *Bacillus thuringiensis* is a gram-positive bacterium which has this capacity to accumulate PHB and it is an active PHB producer. However, the excessive production cost of the biopolymers and the availability of low-cost petrochemicals makes 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, mean that feasibility considerations for production of PHB have to zone in on cost effective means. The cost of the carbon source substrate has been identified as the major viability impediment. It becomes expedient to select carbon source feed stock that is cheap, readily available and supports PHB production.(Getachew and Woldesenbet, 2016; Trapé et al., 2024).

Corn silage is produced in microbiologically enabled process of preserving nutrients through moist anaerobic fermentation. Corn silage is therefore known to be highly nutritious substrate hence its wide application as animal feedstock e. It is the challenges encountered in the feedout stage of the silage where there is a massive breakout of molds and yeast, which can result in losses as high as 30% that leads to the current attempt at repurposing which is a fundamental pillar of the circular bioeconomy. Thus, the focus of this current study was to produce and characterize PHBs from agriculture corn silage (CS) using *Bacillus thuringiensis* CICIM (B2031).

2. MATERIALS AND METHODS

2.1. Compositional Analysis 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. Moisture content of the undried corn silage was determined following AOAC 7.003 method. The chemical composition of corn silage was carried out following AOAC 978.18 Compositional analysis was carried out in triplicate and the results are represented in Table 1.

Table 1: Chemical composition of the 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%

2.2. Corn Silage Hydrolysate

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

2.3. Cultivation of *Bacillus thuringiensis* CICI (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 yeast extract, 8 g/l tryptone and 2 g/l NaCl at 37°C for 48 h on

a shaker at 200 rpm. The bacterial cells were harvested at 6000 x g and the cells were 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 measurement 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 loop full 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 then fixed by immersing 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 sec as a counter stain. This was followed by washing using distilled water and then drying so as to observe using a light microscope (Selvakumar *et al*., 2011).

2.5. Biomass determination

A 50 ml sterilized Falcon tube was utilized to determine cell concentration which was defined as dry weight of cells per litre of culture broth. The cells solution was then centrifuged (4000 x 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 were used in the recovery of microbial PHB. A 1g 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 4000 x g for 10 min (Eppendorf, Centrifuge 5810R). A volume of 4ml methanol was added to the lower chloroform phase containing the solubilised polymer to precipitate the polymer (Rohini *et al*., 2006). Acetone was utilized to wash the precipitated polymer and air dried. In this study, all quantitative experiments were carried out in triplicate. The mean difference between different pretreatments was analyzed by ANOVA.

2.7. UV-Vis characterization

A 10 mg sample of the extracted polymer was dissolved in 2 ml of chloroform and subjected to scanning in UV-Vis range of between 200-800 nm (thermoscientific, GENESYS 150, UV–Vis Spectrophotometer) against chloroform blank as reported by Alarfaj *et al*. (2015).

2.8. FT-IR spectroscopy

The structural analysis was performed using a FTIR Perkin Elmer 537 spectrophotometer. The spectrum of the extracted polymer from *B. thuringiensis* CICIM (B2031) [poly (3HB)] was compared with [poly (3HB)] pure standard obtained from Sigma-Aldrich. Structural analysis of the functional groups was performed utilizing Fourier-transform infrared Perkin Elmer 537 spectrophotometer. The

sample was placed on the sample holder and scanned from 4000 – 400 cm^{-1}. An attenuated total reflectance (ATR) was used to record the spectra and absorption frequencies were expressed as $vmax.cm^{-1}$.

2.9. GC-FID characterization

The monomer composition of the polymer was determined by gas chromatography (GC) Varian 2100T GC–FID (Thermo). 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) using a flame ionization detector. This was utilized to characterize the monomer composition of the extracted polymer. A 5 mg sample of the polymer was subjected to transesterification using 1 ml chloroform and 0.85 ml methanol mixture with a 0.15 ml sulphuric acid 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 into the test tube and mixed for 1 min using a vortex. The upper phase was removed using plastic pipettes, then the lower chloroform phase containing the methyl ester of β-hydroxyl acid was recovered and transferred to a small screw cap vial. A 2μl sample was injected into the injector pot and the inlet pressure of the carrier gas (nitrogen) was set at 109.2 kPa. For effective separation of esters, the temperature program was used (60°C for 1 min, temperature increase 25 °C/min to 230°C (230°C for 1 min); Flow controller: split, total flow 301.3 ml/min, column flow 1.48 ml/min; the temperature of the injector and detector were 200 and 230°C, respectively (Pantazaki *et al*., 2009). Different β-hydroxyalkanoatic 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 characterisation 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 (max ODs) coincided with the accumulation of the polymer after 48 hrs (Rohini *et al*., 2006). However, there is the risk of the amount amount of accumulated PHB gradually declining after stationary phase if cell mass is not harvested, due to 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 accumulated PHBs evidenced by blue-black inclusions in cytoplasm. The existence of blue-black granules showed the produced PHB and capability 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 counter stained with safranin was used to show positive accumulation of PHBs in *B. thuringiensis* under a light microscope

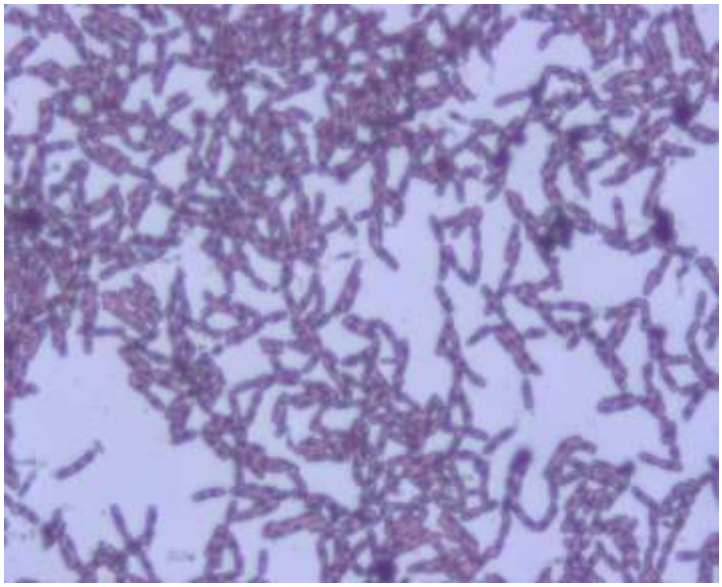

Figure 1 Micrograph of *B. thuringiensis* CICIM (B2031) showing PHB accumulation; magnification 1000×.

The cultures were harvested when the cells reached the early stationary phase and followed by determination of polymer composition. During bacterial growth, the composition of the substrate changes with subsequent build up and utilisation of metabolic intermediates., hence changes in the PHB monomer composition proportional to the age of the culture are expected (Brandl *et al*., 1988).

After 48hrs it was noticed that pH 7.0 yielded the highest biomass as compared to 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 play the role of being an energy reserve which mean a high biomass would mean a high accumulation of PHB. *B. thuringiensis* has been reported to show maximum biomass peaking at around 48 hours which signals the early stages of the stationery phase.

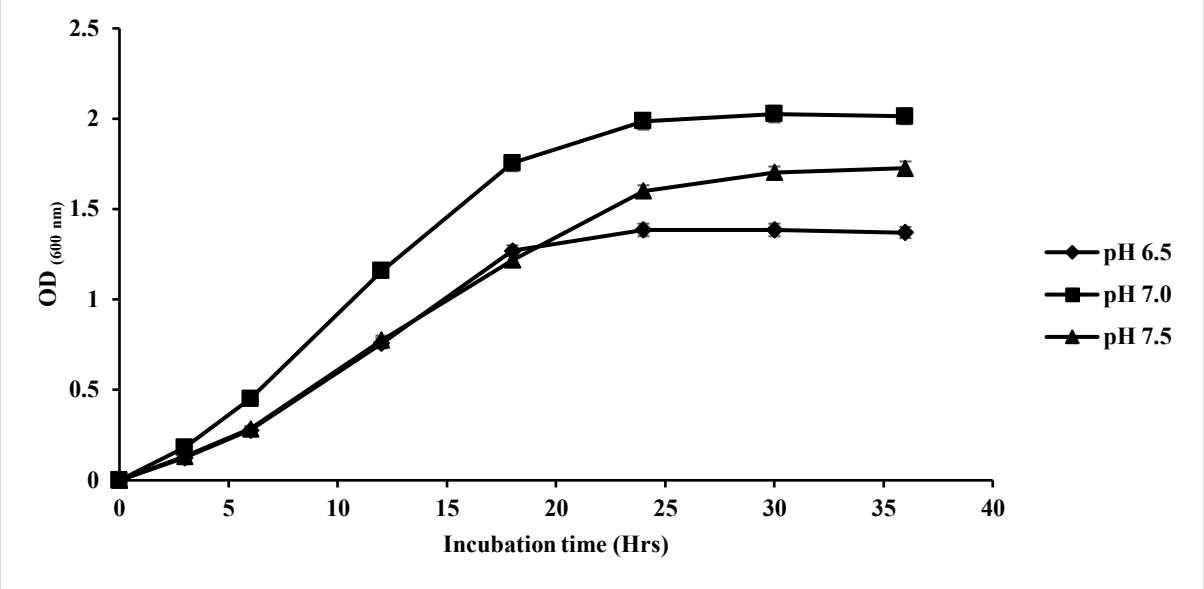

Figure 2 Growth of *B. thuringiensis* at different pH 6.5, 7.0, and 7.5.

The corn silage supported PHB production even in its unhydrolysed form. There was about 27% accumulation of PHB after 24% loading, however, a doubling of the substrate loading did not show a commensurate increase in biomass and PHB accumulation (Table 2). This could be due to the fact that a further increase in unhydrolysed substrate is an increase in un-pretreated substrate which is not easily accessible to *B. thuringiensis.*

Table 2 Effect of unhydrolysed corn silage supernatant concentration on biomass and polymer production after 48 hrs of cultivation.

% cs supernatant in media	Biomass (g/L)	Polymer (g/L)	% 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

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 4.99g/L was also achieved (Table 3). This can be attributed to the hydrolysed substrate being richer in simple sugars that are easily accessible to the bacterium

Table 3 Effect of hydrolysed corn silage supernatant concentration on biomass and polymer production after 48 hrs of cultivation.

% cs supernatant in media	Biomass (g/L)	Polymer (g/L)	% 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 3 different readings ± standard deviation

B. thuringiensis ability to accumulate PHB has been reported. The focus has been on efficiency in terms of production durations. Most reports have placed optimum durations of between 24 and 96 hours for Bacillus strains. What has informed the focus on conditions for the production of PHB is the link between the 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 the PHB concluding that the characteristics of the PHB are dependent on the growth conditions. It is the characteristics of the PHB that would determine applicability and in fact there is a possibility that 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 carbohydrates content, that includes starch content (>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). In view of all these compounds and minerals, corn silage as an agricultural residue was used in this present study as a less expensive substrate to produce PHBs.

3.2. PHB characterization with UV-Visible spectroscopy.

UV-Vis scanning of synthesized PHB revealed a similar spectrum with commercial PHB **(Fig. 3).** This scan range indicates the occurrence of the PHBs and confirms corn silage as potential PHB substrate. UV-Vis spectra of PHB isolated from *B. thuringiensis* was measured between 200 to 800 nm against the commercial standard using $CHCl_3$ as blank. Both spectra of the commercial PHB standard and extracted PHB indicated a sharp peak of maximum absorbance of 240 nm. These results are consistent with the findings of Shah, (2014) which recorded maximum absorbance of PHB between the range 230-240 nm

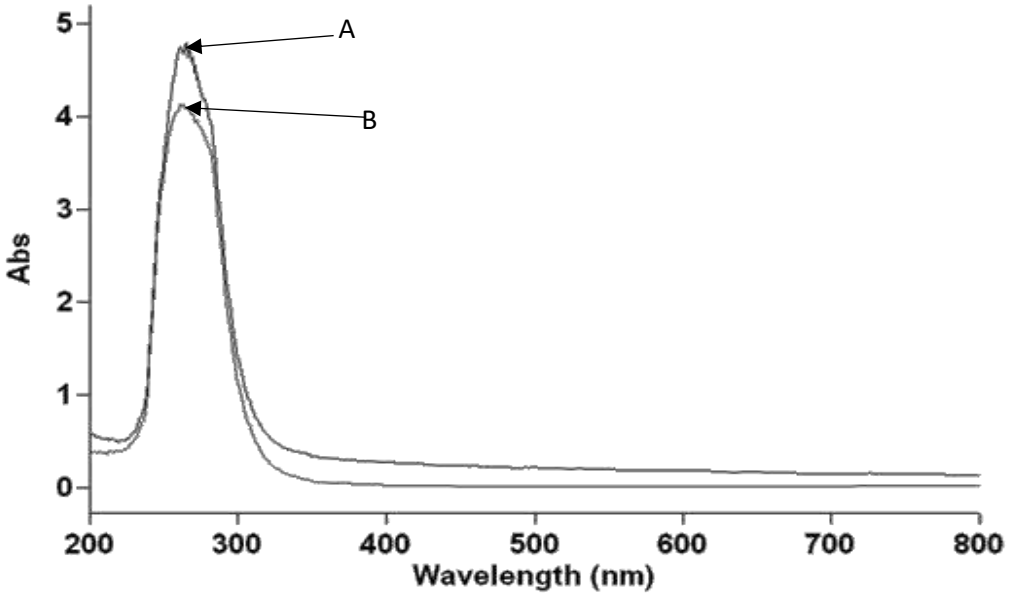

Figure 3 UV-Vis absorption spectra of standard PHB (Sigma-Aldrich) (A) and the PHB polymer obtained from *B. thuringiensis* CICIM (B2031) (B).

3.3. FT-IR spectroscopy analysis

From the FT-IR spectrum obtained **(Fig. 4)** it can be concluded that the 3375 cm^{-1} band vibrations is evidence of the strong hydrogen bond formed by the terminal OH groups. Comparable results have been reported in other studies (Getachew and Woldesenbet, 2016, Gumel *et al*., 2012). The region of 4000 – 300 cm^{-1} is commonly shared in commercial polymer and bacterial polymers absorption bands. According to Misra *et al*. (2000), Getachew and Woldesenbet (2016), the absorption band at 1719 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 1060 – 1281 cm^{-1} characterizes the valence vibration of the carboxyl (-C-O-) polymeric group (Getachew and Woldesenbet, 2016). These are characteristics of polyhydroxybutyrate. The band at 1456 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 1380 cm^{-1} band (Alarfaj *et al*., 2015). This spectrum confirms the report by Alarfaj *et al*., (2015), verifying that PHB was the isolated compound.

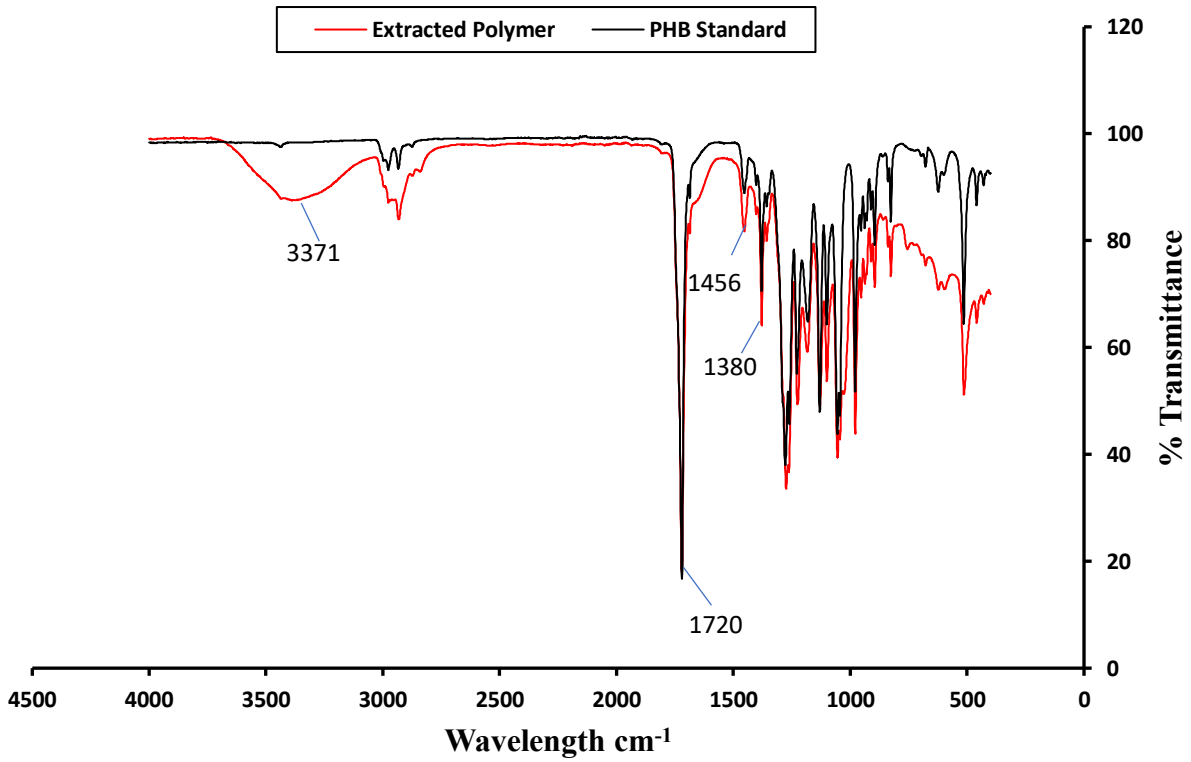

Figure 4 - FTIR analysis of polyhydroxybutyrate polymer commercial standard (Sigma -Aldrich) and polymer isolated from *B. thuringiensis* CICIM (B2031).

3.4. GC-FID chromatography analysis

GC qualification of PHB is a commonly applied method in PHA research as it involves the decomposition of the molecule by methanolysis (Alves *et al*., 2017). The key compounds of concern in this study were identified based on their retention peak. GC-FID analysis of the commercial PHB and extracted polymer eluted a similar peak at 5.7 min **(Fig. 5).** This peak signifies that the compound extracted was of a biodegradable polyester family and therefore GC confirmed that the polymer produced in this study is a short-chain length PHB consisting of 4 carbons (Chee *et al*., 2010;).

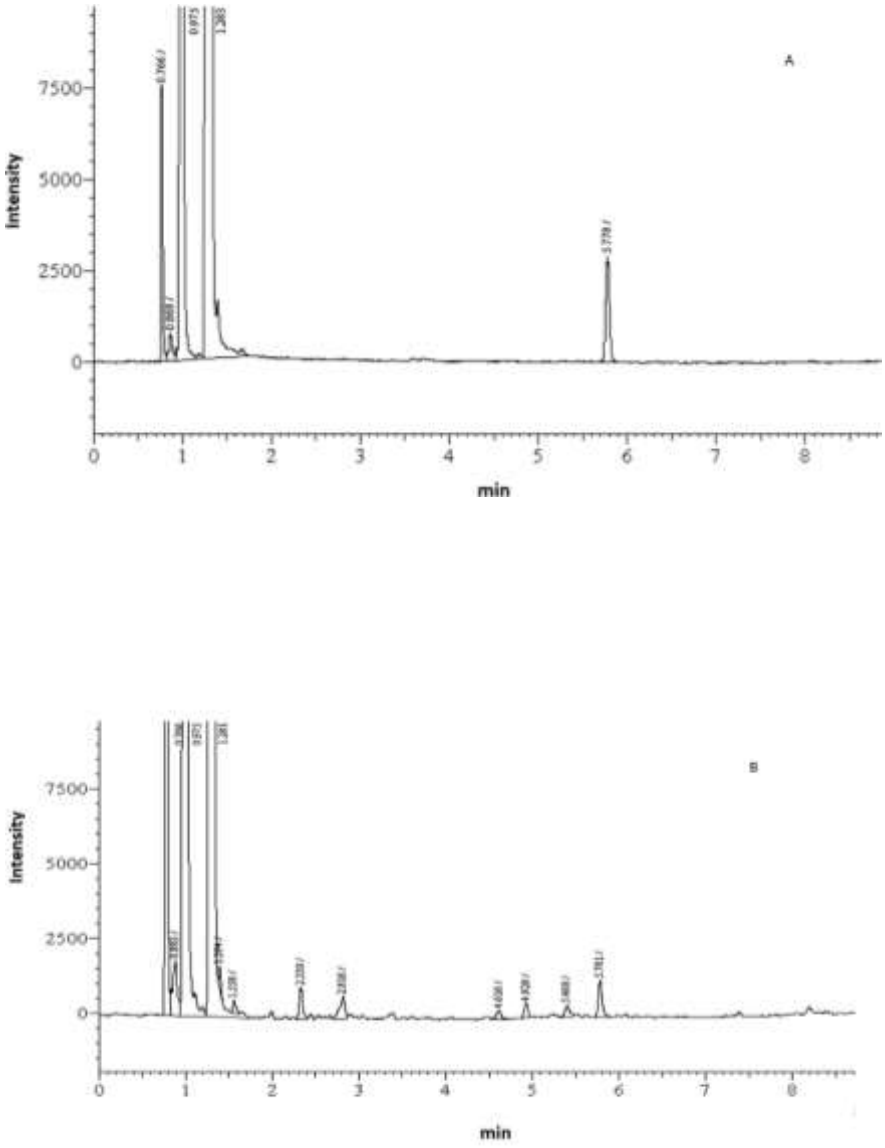

Figure 5. GC-FID analysis of PHB produced by *B. thuringiensis*: chromatography of standard (A) and chromatography of sample (B).

The extracted polymer was determined by gas chromatography (GC) after the methanolysis. The hydrolysis, and derivatization reactions took place in a single vessel. The resulted methyl esters were ran through separating technique of GC, as a qualitative analysis. A reference standard of a copolymer poly (3HB-co-3HV) was used (Fig 5a). The peaks at 5.7653 min represents PHB and 6.802 min represents PHV, respectively. These indicate that the extracted polymer is poly (3HB-co-3HV) in comparison to the pure standard. The peak area of the extracted polymer appeared to have other peaks (Figure 5b) compared to the reference

standard, this is due to the fact that transesterification using sulphuric acid and methanol degrade the purity of the compound causing by-products during the reaction (Godbole 2016;Elhami et al., 2022). Derivatization method of 3-hydroxyakanoic acid with HCl and propanol to a propyl ester may be preferred as this is more lipophilic when compared to methyl ester which improves the partitioning coefficient (Sharma et al., 2021)

4. CONCLUSIONS

This study offers PHB production using *Bacillus thuringiensis* CICIM (B2031) growing on agricultural by-product corn silage. The bacterial derived PHB was confirmed by UV-VIS, FTIR and GC-FID .This study opens an alternative way to achieve production of PHB by repurposing animal feed carbon source such as corn silage. This strain promises to produce biopolymers from numerous agricultural by-products. This study however, revealed the need for novel derivatization methods for the analysis of scl-PHB to avoid excessive formation of side-products. A common transesterification method using suphuric acid-methanol was shown to be inadequate for the quantitative analysis of scl-PHB due to a serious underestimation of the PHB content. PHB yields could also be further increased from the achieved 53.43% by media optimization.

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