Research Article

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Optimization and Characterization of Polyhydroxybutyrate (PHB) Production from *Bacillus thuringiensis*: A Sustainable Approach to Biodegradable Polymer Synthesis

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ABSTRACT

Polyhydroxybutyrate (PHB) is a naturally existing, biodegradable polymer accumulated as intracellular granules by various bacteria. The research centers around *Bacillus thuringiensis* SWPHB1, an isolated bacterial strain exhibiting notable ability in PHB synthesis. The investigation strategically optimizes multiple culture conditions, uncovering that the bacterial strain attains peak PHB production at 35°C and pH 7, with glucose and ammonium nitrate serving as optimal carbon and nitrogen sources, respectively. The thorough characterization of the PHB produced by the strain employs sophisticated techniques such as scanning electron microscopy (SEM) coupled with energy-dispersive X-ray spectroscopy (EDX), Fourier-transform infrared (FT-IR) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy. These analyses collectively provide crucial insights into the morphological properties, elemental composition, and chemical structure of PHB. The results not only emphasize the PHB-producing capabilities of *Bacillus thuringiensis* SWPHB1 but also highlight the significance of optimizing culture conditions for enhanced bioplastic yield.

Keywords: Polyhydroxybutyrate; *Bacillus thuringiensis*; Bioplastic; Optimization.

1. INTRODUCTION

In recent years, our planet has been facing serious environmental issues due to the growing problem of plastic waste. To tackle this challenge, scientists have been searching for materials that are both biodegradable and made from renewable resources since the 1970s (Yarsley and Couzens, 1945). Recently, there have been significant advancements in the development of bioplastics, driven by the demand for sustainable and carbon-neutral products. These bioplastics are essential to address global environmental concerns and restore the health of our planet (Rendón-Villalobos *et al.* 2016).

Bio-based plastics, specifically polyhydroxyalkanoates (PHAs), are anticipated to replace traditional fossil-based plastics because they are more environmentally friendly (Shen *et al*. 2020). Bioplastics come in both non-degradable and biodegradable forms, offering options depending on factors like manufacturing origin and post-use conditions (Mahitha and Madhuri, 2015). The high production rate of petroleum-based plastics has led to a major environmental challenge in managing solid wastes, mainly because these plastics resist biodegradation (Nishida *et al*. 2018). In contrast, bioplastics, being non-toxic, renewable, biocompatible, and biodegradable, are gaining global demand (Narayanan *et al*. 2020).

Polyhydroxyalkanoates are promising bioplastics (Quillaguaman *et al*. 2005). Among them, poly-3-hydroxybutyrate (PHB) stands out as it can replace conventional plastics. Polyhydroxyalkanoates are produced by bacteria as energy reserves in response to nutrient imbalance and stress (Halami 2008; Tindall *et al*. 2014). They have a wide range of applications due to their versatility in functional groups, such as methyl to tridecyl, unsaturated bonds, and chain length variations. Poly-3-hydroxybutyrate accumulates in various microorganisms and is expected to gradually replace polypropylene for multiple uses (Philip *et al*. 2007). It finds applications in diverse technologies, including automotive components, electronics, packaging, coatings, and disposable items (Khiyami *et al*. 2011). Additionally, researchers have explored the use of PHB in creating nanocomposites and silver nanoparticles for antimicrobial materials, beneficial in drug carriers for tissue engineering and wound treatment (Radu *et al*. 2019). Various analytical techniques are employed to characterize the structural makeup of PHB. Continuous efforts are made to develop economically viable and sustainable extraction techniques, microbial strains, and

industrial processes for PHB production (Juengert *et al*. 2018). Despite the environmental advantages, PHB currently has a higher production cost compared to petrochemical plastics, prompting ongoing research to find cost-effective solutions, improve fermentation techniques, and create efficient bacterial strains. To enable large-scale production, it is essential to optimize bioreactor configurations, substrate cost-efficiency, and downstream processing. Optimizing fermentation conditions, increasing yield by genetic engineering, and ensuring economical downstream processing are necessary to scale up PHB production from this strain for industrial uses. It is essential to address production limitations related to reactor design and substrate supply. Large-scale commercialization can be further supported by developments in process automation and bioreactor technology.

The current work aims to optimize PHB synthesis conditions, to investigate the PHB-based biofilm prepared by the produced PHB.

2. MATERIALS AND METHODS

2.1 Isolation and Preservation of Bacterial Strains

Soil samples were collected at a depth of 10 cm and brought to the lab in sterile bags. These samples were processed by suspending 0.1 g of soil in 10 mL of distilled water and undergoing serial dilution. The resulting suspension was spread on nutrient agar plates using the spread plate technique and incubated for two days at 37 ℃ (Panigrahi and Badveli, 2013). Pure cultures were obtained through quadrant streaking, and slant cultures were preserved for future use.

2.2 Identification of Bacteria by Gram Staining

To determine the Gram type of bacterial isolates, Gram staining was employed. A tiny bacterial smear was heat-fixed on a glass slide, and a crystal violet stain was added. After rinsing, iodine was applied, followed by decolorization with acetone or ethanol and staining with safranin. The slides were then observed under a microscope.

2.3 Characterization of the PHB-producing Isolate

Identification of PHB-producing bacteria was done based on morphological, physiological, and 16s rRNA analysis. The morphological and physiological characteristics were done as described by "Bergey's Manual of Determinative Bacteriology" 8th ed., 1993. Based on the 16S rRNA sequencing, the isolate that produced PHB was further identified. The DNA was extracted utilizing standard Sambrook and Russell's (2001) protocol. As previously described (Weisburg *et al*. 1991), the PCR reaction mixture included 30 ng of genomic DNA, 1X buffer, 3 mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphates, 1U Taq DNA polymerase (Fermentas), and 2.5 µM of each Eubacterial universal primers (16s F(27F) 5'-AGAGTTTGATCMTGGCTCAG-3'; 16s R(1492R) 5'-TACGGYTACCTTGTTACGACTT-3'). The following thermal cycle program was employed: 5 minutes of denaturation at 94 ℃, 35 cycles of 1 minute at 94 ℃, 1 minute at 45 ℃, and 2 minutes at 72 ℃, followed by 5 minutes of extension at 72 ℃and 5 minutes of holding at 4 ℃ (Applied Biosystems Vertis thermocycler). Primer 27F was used to sequence the PCR result after it had been gel purified using the QIA gel extraction kit from Qiagen in Germany. The BLAST tool (National Centre for Biotechnology Information) was utilized to compare the sequence of the 16S rRNA gene, which was deposited in GenBank, using the GenBank database.

2.4 Screening of PHA-producing Bacteria

Bacterial isolates were recultured on nutrient agar+1% glucose medium using simple streaking on Petri plates. After 24 hours of incubation at 37 ℃, Sudan black stain was used to assay for PHB synthesis. Colonies were stained, washed, and observed (Quillaguaman *et al*. 2005).

2.5 Production of PHB by Selected Isolates

The isolate which exhibited the highest levels of PHB production was chosen for further analysis. The chosen isolate was used for PHB production in a specific medium. The selected isolate was cultured, inoculated into the production medium, and incubated for 48 hours at 37 ℃.

Table 1. Bacterial strains isolated from soil samples

Isolates	Cell Dry Weight (g/L)	PHB (g/L)	% PHB Yield
SA1	1.72	0.5	29.07
SA2	2.7	1.4	51.85
SA3	3.7	0.9	24.32
SA4	5.2	1.2	23.08
SA5	3.9	2.2	56.41
SA6	1.6	0.7	43.75
SA7	5.6	4.3	76.79
SA8	4.5	1.7	37.78
SA9	2.92	0.3	10.27
SA10	4.2	1.3	30.95

2.6 Optimization of Culture Conditions for PHB Production

Various factors influencing PHB production were studied, including different temperature, pH values, carbon sources (sucrose, glucose, starch), and nitrogen sources (ammonium sulfate, peptone, yeast extract) to

acquire the maximum production of PHB. The optimized conditions (35 °C, pH 7, glucose, and ammonium nitrate) are in line with those reported by (Shah, 2014) for *Bacillus subtilis* and (Wei *et al.* 2011) for *Cupriavidus taiwanensis*, who noted optimal PHB production at 30–37 °C and neutral pH.

2.7 Extraction and Quantification of PHB

The biomass-containing medium was subjected to centrifugation at 8,000–10,000 rpm for 8–10 minutes in a cooling centrifuge, followed by the addition of a 1:1 mixture of acetone and ethanol to the resulting pellet. This combination facilitated the disruption of the cell wall, releasing PHB granules. After centrifuging the mixture at 8,000 rpm for 5 minutes, the supernatant was discarded. The pellet obtained was then dissolved in 4% sodium hypochlorite (NaOCl), incubated at room temperature for 30 minutes, and subjected to centrifugation at 8,000 rpm for 8 minutes, with subsequent removal of the supernatant. The pellet, post-NaOCl treatment, underwent washing with a 2:1 mixture of acetone and ethanol. The final polymeric particles were dissolved in chloroform, filtered through Whatman No. 1 filter paper, and treated with 10 mL of concentrated H_2SO_4 for 1 minute, converting the polymeric granules into crotonic acid. The spectrophotometer reading at 235 nm was used for PHB quantification, with concentrated H_2SO_4 serving as the standard. The entire process allowed for the efficient extraction and quantification of PHB from the biomass (Alarfaj *et al*. 2015).

2.8 Polymer Characterization

Polyhydroxybutyrate granules were analyzed using an electron microscope (SEM) with EDAX, and the findings were documented. The JEOL SEM JSM-7900F instrument was utilized to analyze energy dispersive X-ray spectroscopy data to obtain a basic understanding. Using a Perkin Elmer 1600 FTIR spectrometer and potassium bromide pellets between 4000 and 400 cm^{-1}, the FTIR spectrum of the polymer sample was acquired while taking care to prevent moisture uptake (Hong *et al*. 1999). Additionally, it was subjected to ^{1}H NMR and ^{13}C NMR investigations utilizing Varian NMR spectrometers operating at 600 MHz and 150 MHz, respectively. The solvent used was $CDCl_3$.

3. RESULTS AND DISCUSSION

3.1 Isolation and Preservation of Bacterial Strains

A total of ten bacterial strains were isolated from the soil samples. A screening process was conducted to assess the PHB production capability of these isolates, as outlined in Table 1. All isolated strains underwent screening for PHB production using Sudan Black staining. Among these 10 isolates, the SA7 isolate produced significant amount of PHB, which was confirmed through multiple stages of the screening process. This particular isolate was subsequently cultivated in a production medium for more in-depth examination. During the primary screening using Sudan Black B staining on the isolate grown on a Petri dish, the color of the colonies changed to a dark greenish-blue (Fig. 1). This change is indicative of PHB accumulations in the test isolates, as noted by (Mostafa *et al*. 2020). (Mahitha and Madhuri, 2015) have also considered colonies displaying a bluish color on PHB selective media to be indicative of a strain capable of synthesizing and accumulating PHB.



Fig. 1: Screening of strains by Sudan Black staining method (strain SA7)

The isolate demonstrating PHB production in the test was identified as *Bacillus thuringiensis* through morphological and biochemical characterization and the 16S rRNA sequence analysis was done and submitted to NCBI GenBank (Accession No: OR807719). A phylogenetic tree analysis revealed genetic similarity within the same genus and species, as depicted in Fig. 2. The evolutionary history was inferred using the neighbour joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown below the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths (next to the branches) in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al*. 2004) and are in the units of the number of base substitutions per site. This analysis involved 8 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1364 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura *et al*. 2021). The phylogenetic analysis confirmed the hereditary origin of the test isolates as *Bacillus thuringiensis* SWPHB1. Similarly, (Mahitha and Madhuri, 2015) employed molecular tools such as 16S

rRNA analysis to identify novel bacterial species exhibiting PHB-producing capabilities from polluted sites.

Table 2. Interpretation of morphological and biochemical characteristics

Culture Morphology and Characterization	
Colony morphology	Elevated, slimy, white round colonies
Shape	Rod
Gram staining	Gram Positive
Motility	Motile
Capsule staining	Positive
Spore staining	Positive
Catalase	Positive
Oxidase	Negative
Urease	Positive
Nitrate reduction	Positive
Methyl red	Negative
H_2S production	Negative
Vogues- Proskauer	Positive
Indole	Negative
Citrate Utilization	Negative
Mannitol fermentation	Positive
Sucrose fermentation	Positive
Glucose fermentation	Positive
Maltose fermentation	Positive
Lactose fermentation	Negative
Gelatin Hydrolysis	Positive

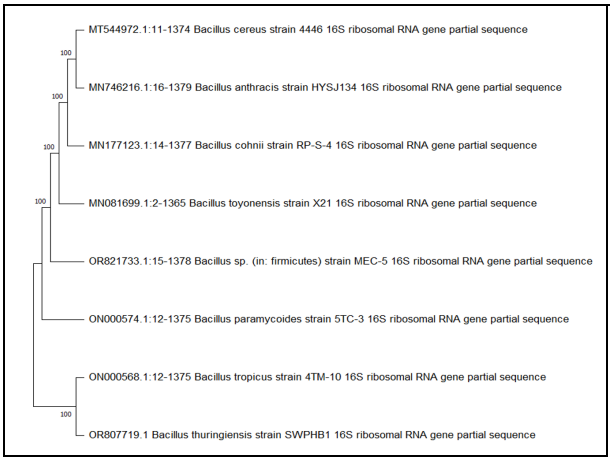


Fig. 2: Phylogenetic tree of *Bacillus thuringiensis* SWPHB1

3.2 Optimization of Culture Conditions for PHB Production

Variations in temperature, pH, carbon, and nitrogen sources have been carefully considered when optimizing the culture conditions for strain SA7 (Tables 2, 3, 4, 5 and 6).

The bacterial isolate SA7 demonstrated optimal PHB production at 35 ℃ with 66.7%, with a decline in bacterial activity observed as temperatures rose at 35 ℃ with 22.22%. Higher temperatures were associated with reduced bacterial performance. This finding aligns with the research results of (Wei *et al*. 2011). At pH 7, PHB production peaked to 63.6%. Rising pH values result in a decrease in PHB production. The optimized conditions (35 ℃, pH 7, glucose, and ammonium nitrate) are in line with those reported by (Shah, 2014) for *Bacillus subtilis* and (Wei *et al*. 2011) for *Cupriavidus taiwanensis*, who noted optimal PHB production at 30–37 ℃ and neutral pH.

Table 3. Effect of temperature on PHB production

Temperature (℃)	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
25	1.2± 0.3	0.4± 0.31	33.3
35	5.4± 0.12	3.6± 0.13	66.7
45	0.9±0.11	0.2±0.13	22.22

Table 4. Effect of pH on PHB production

pH	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
5.5	1.66 ± 0.24	0.5± 0.16	30.12
7	5.11± 0.24	3.25± 0.11	63.6
8.5	1.66± 0.18	0.7 ± 0.21	42.16

Table 5. Effect of different carbon sources on PHB production

Carbon sources	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
Sucrose	1.3± 0.11	0.5± 0.01	38.5
Glucose	3.1± 0.03	1.5± 0.03	48.38
Starch	1.8 ± 0.03	0.4 ± 0.11	16.7

Table 6: Effect of different nitrogen sources on PHB production

Nitrogen sources	Cell dry weight (g/L)	PHB (g/L)	% PHB yield
Ammonium nitrate	2.8± 0.03	1.2± 0.03	43.38
Yeast extract	1.3± 0.11	0.4± 0.01	30.76
Peptone	1.2± 0.2	0.2± 0.01	23.6

To evaluate the bacterial activity involved in PHB production, the medium was supplemented with multiple carbon sources. Glucose was the most effective carbon source for PHB creation which accounted for 48.3%, while starch has the lowest PHB generation (16.7%). This finding agrees with (Shah, 2014) and (Joraleerut *et al.* 2014). According to them, they

confirmed that when glucose is the only carbon source in the medium, *Bacillus sp.* can create PHB.

When sucrose was used as the carbon source, the production of PHB was moderate (38.5%). The production was maximum (48.38%) with ammonium nitrate as the nitrogen source. A low (23.6%) PHB of cell dry weight was obtained in a medium containing peptone, but yeast extract had a moderate (30.76%) cell dry weight. The optimal nitrogen source, according to (Lakhawat *et al.* 2012) is ammonium acetate, which promotes bacterial growth and PHB synthesis.

3.3 Extraction of PHB

It was effectively possible to extract the PHB from the *Bacillus thuringiensis* SWPHB1 (Fig. 3). The NaOCl-$CHCl_3$ method was selected due to its high PHB recovery and purity. Alternatives such as enzymatic digestion or solvent extraction using less toxic agents like DMSO were considered, but were found to be less efficient or more costly for small-scale lab use (Alarfaj *et al*. 2015; Juengert *et al*. 2018).

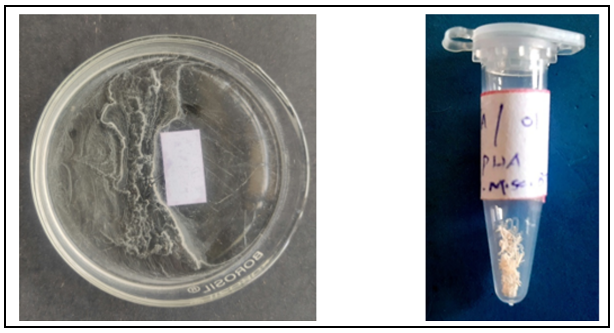


Fig. 3: Extracted PHB from *Bacillus thuringiensis* SWPHB1

3.4 SEM with EDAX Analysis

The produced PHB powder was subjected to SEM examination along with EDAX (Fig. 4 and 5). After 48 hours of incubation, the PHB produced by SEM analysis revealed a surface texture that was uneven with scaly and cavity morphologies. Table 7 lists the elemental composition of PHB. This outcome is in line with Hernández *et al.* (2019) findings.

The results of the FT-IR analysis (Fig. 6) show that the polyhydroxybutyrate has peaks around 3400-3600 cm^{-1}, which are connected to a terminal –OH group but are not very significant. The peak at 2078 cm^{-1} is associated with silicon compounds, while the peaks at 3,307 cm^{-1} and 3,587 cm^{-1} show stretching of amines and amides. A sharp peak at 1,737 cm^{-1}indicates the presence of aliphatic ester carbonyl C=O, which is characteristic of PHB. Shi *et al.* (2017) and Hong *et al.* (2019) reported peaks at 1724 cm^{-1} and 1728 cm^{-1}, respectively, for PHB isolated from *Alteromonas lipolytica* and *Vibrio proteolyticus*. On the other hand, alkyl halides are present in the bands detected at 668 cm^{-1} (Castro-Mayorga *et al*. 2018).

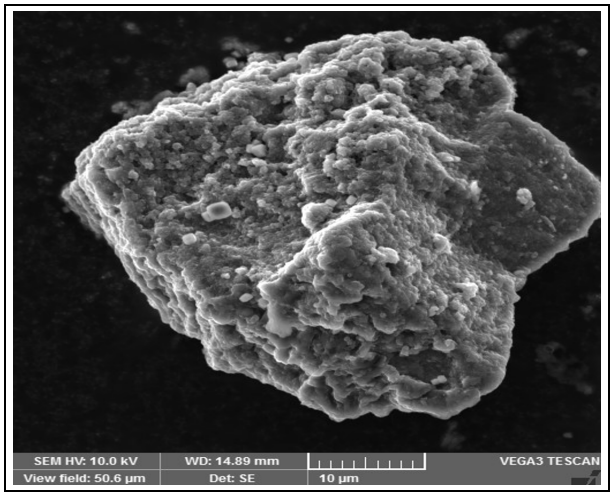


Fig. 4: Observations of PHB granules under the scanning electron microscope

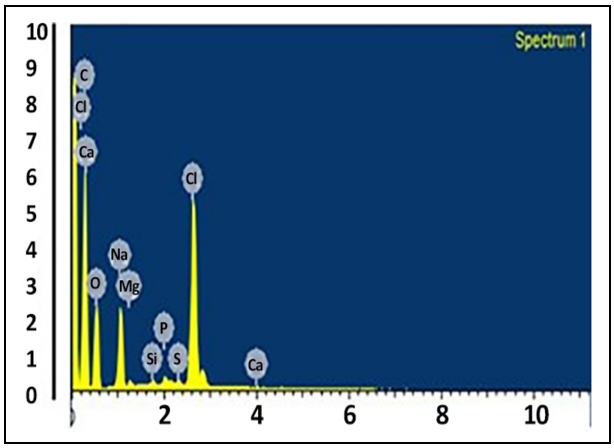


Fig. 5: EDX analysis of PHB

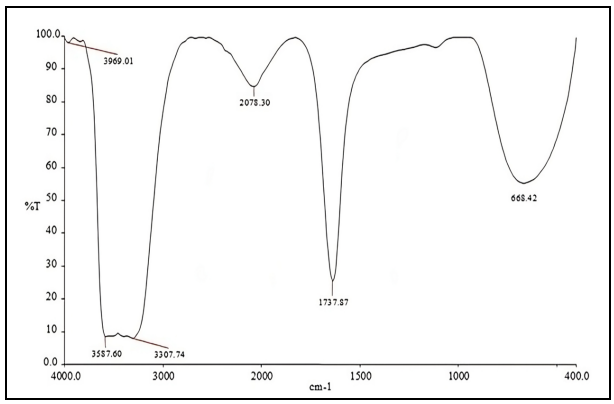


Fig. 6: FT-IR spectrum of PHB polymer produced by *Bacillus thuringiensis*

The chemical structure of PHB produced by the strain *Bacillus thuringiensis* SWPHB1 has been determined using ^1H NMR (Fig. 7 (b)). The ^1H NMR

spectrum containing multiple signals at 0.9, 1.3, 1.67, 2.05, 2.34, 5.28, and 5.4 ppm (Fig. 5). These peaks corresponded to various protons of PHB. The present study on *Bacillus thuringiensis* SWPHB1, which can produce PHB polymers, was supported by these reports.

Table 7. Elements (% wt.) by EDX analysis of the PHB

Sample	Elements (%wt.) Normalised at 100							
	C	Ca	Na	O	Mg	Si	P	S
PHB	8.90	6.57	3.99	2.86	2.79	0.12	0.23	0.10

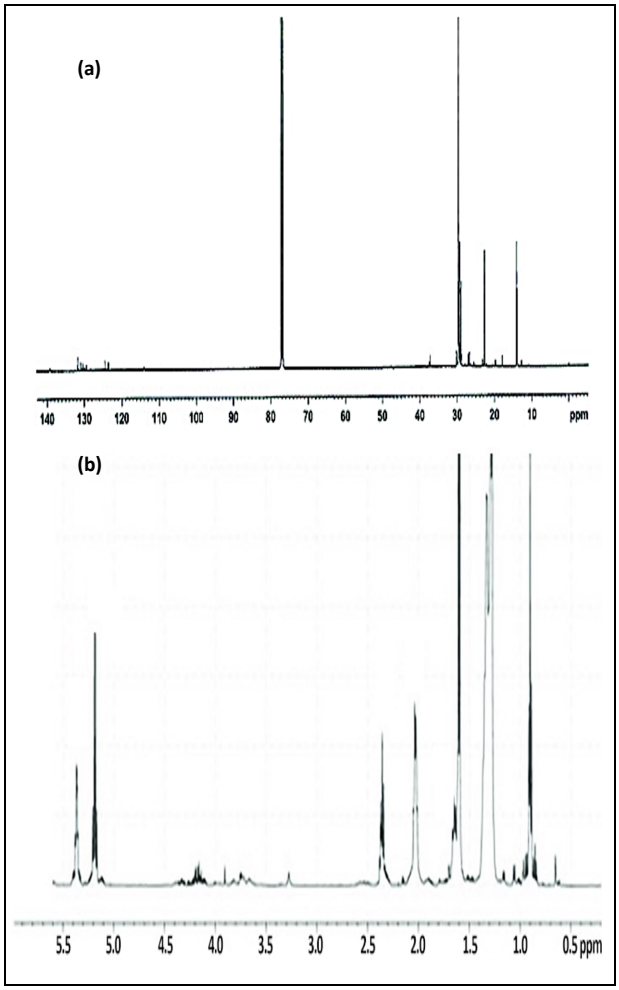


Fig. 7: (a) 13C-NMR spectra and (b) 1 H-NMR spectra of PHB polymer produced by *Bacillus thuringiensis*

Further, the carbon moieties of the produced PHB were determined by C NMR analysis, which identified four significant peaks at 12.04, 23.6, 29.75, and 77.08 ppm (Fig. 7 (a)). The identified peaks in the NMR spectra agree with the previously mentioned studies, confirming the presence of poly (3-hydroxybutyrate) PHB obtained from the *B. thuringiensis* species. Alarfaj *et al*. (2015) also reported similar signals that corresponded to the carbon moieties of PHB produced by *B. thuringiensis* KSADL127 in ^{13}C NMR analysis utilizing $CDCl_3$.

4. CONCLUSION

In conclusion, this study optimized the production, extraction, and characterization of polyhydroxybutyrate from *Bacillus thuringiensis* SWPHB1, isolated from soil. Under optimized conditions (35 °C, pH 7, with glucose and ammonium nitrate as carbon and nitrogen sources), the strain yielded the highest PHB production. The polymer was extracted through a multi-step process and characterized using SEM-EDX, FTIR, and NMR, confirming its morphology, elemental composition, and chemical structure. The findings show that *B. thuringiensis* SWPHB1 produces PHB consistent with previously reported properties and supports its potential use in biofilm formation. However, the study lacks PHB degradation and toxicity assessments, as well as economic and long-term stability analyses. Future work may explore fed-batch or continuous fermentation systems, strain improvement through mutagenesis or genetic engineering, and cost-effective carbon substrates such as agro-industrial waste to scale PHB yield for commercial production.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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