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Optimisation of Polyhydroxy Butyrate Production by *Lysinibacillus fusiformis* and *Metabacillus indicus* isolated from Spent Engine-oil Contaminated Soil

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Abstract

This study isolated bacteria from spent engine oil-contaminated soil and optimized their production of polyhydroxybutyrate (PHB), a biodegradable polymer belonging to the polyesters classes that are of interest as bioderived and biodegradable plastics. Out of 12 bacterial isolates (species of Bacillus, Pseudomonas, Staphylococcus and Lactobacillus) recovered from the spent engine oil contaminated soils, and screened for their capacity to accumulate polyhydroxybutyrate (PHB), only two bacterial isolates (Lysinibacillus fusiformis and Metabacillus indicus), showed significant PHB production. L. fusiformis produced PHB at a concentration of 1.5 g/L, while M. indicus produced PHB at a concentration of 1.0 g/L. Optimal production conditions included a temperature of 35°C, agitation speed of 100 rpm, neutral pH of 7.0, glucose as the carbon source, and peptone as the nitrogen source. Gas chromatography-mass spectrometry confirmed the presence of PHB in the extracted samples, with hexadecanoic acid methyl ester identified as the predominant peak. These findings highlight the potential of bacteria from engine oil-contaminated soil as efficient PHB producers and contribute to the development of sustainable and biodegradable plastics.

Keywords: polyhydroxybutyrate (PHB), bacteria, biodegradable plastics, engine oil-contaminated soil.

INTRODUCTION

The proliferation of non-biodegradable synthetic plastics in our environment has ushered in a host of pressing environmental concerns, prompting the quest for sustainable and eco-friendly alternatives. Among the alternatives, biodegradable plastics produced by living organisms, including plants, fungi, and bacteria, emerge as promising solutions (Getachew and Woldesenbet, 2016). One such biodegradable polymer that has garnered substantial attention as a sustainable substitute is Polyhydroxybutyrate (PHB), a member of the Polyhydroxyalkanoate (PHA) family (Pei et al., 2011). Polyhydroxybutyrate, which can be synthesized by diverse organisms, holds tremendous potential across a spectrum of applications, ranging from packaging and cosmetics to agriculture, medicine, and pharmacology, presenting itself as a viable replacement for petrochemical-based polymers (Pei et al., 2011).

Optimizing these factors becomes paramount in the selection of microbial strains that can efficiently produce PHB (Shodhganga, 2011). Diverse techniques, such as Sudan-black and Nile

Within the realm of biodegradable plastics, bacteria have emerged as proficient producers of PHB, particularly under constrained growth conditions. Poly-3-hydroxybutyrate (PHB), composed of 3-hydroxybutyrate units, serves as an intracellular storage material synthesized by prokaryotic microorganisms to store energy and carbon during nutrient-depleted phases (Pei et al., 2011). Various bacterial species, such as *Ralstonia eutropha*, Azotobacter sp., Pseudomonas sp., and Bacillus sp., have exhibited remarkable prowess in PHB production and can be sourced from an array of environments, including oil-contaminated soils, activated sludge, rhizosphere, and hypersaline surroundings (Pei et al., 2011; Shodhganga, 2011; Getachew and Woldesenbet, 2016).

The production of PHB, however, is subject to an intricate interplay of factors encompassing incubation temperature of inoculants, pH, agitation rate, carbon source availability, and nitrogen source constraints (Shodhganga, 2011).

blue staining, in conjunction with extraction methodologies involving chloroform, sodium hydroxide, sodium hypochlorite, or their combinations, are harnessed for PHB determination and quantification (Saharan et al., 2014; Shodhganga, 2011).

Despite the promise of biodegradable polymers, their development as substitutes for traditional plastics encounters significant challenges, particularly the higher production costs compared to petrochemical-derived counterparts (Choi et al., 2013). The cost dynamics are influenced by factors encompassing the type of carbon source, the intricacies of the fermentation process, yields derived from chosen carbon sources, and downstream processing (Akaraonye et al., 2010; Maheshwari et al., 2018; Choi et al., 2013).

In light of these challenges, this research embarked on the endeavor of isolating, identifying, and optimizing the production of polyhydroxybutyrate (PHB) using bacteria sourced from soil contaminated with spent engine oil (SEO). The research encompassed the isolation of bacteria from SEO-polluted soils, screening for PHB-producing strains, the subsequent identification and characterization of PHB-producing bacteria through both cultural and molecular techniques, determination of optimal conditions for PHB synthesis, and comprehensive characterization of PHB.

The outcomes of this research promise to make substantial contributions to the quest for sustainable and environmentally-friendly alternatives to traditional plastics. By shedding light on the potential of bacteria derived from SEO-contaminated soil as efficient PHB producers, this research paves the way for innovative solutions in the realm of biodegradable plastics, fostering a greener and more sustainable future (Maheshwari et al., 2018).

MATERIALS AND METHODS

Sample Collection, Isolation & identification of Bacteria

Soil samples were collected from soil contaminated with spent engine oil (SEO) in Ikot Akpaden, Mkpat Enin Local Government Area, Akwa Ibom State using a soil auger and transported to the microbiology laboratory of Akwa Ibom State University for immediate processing. The samples were homogenized, and the total culturable heterotrophic bacterial loads were determined using pour and streak plate techniques, following the methods described by Olutiola et al. (2000). Pure colonies of the isolates were obtained through repeated sub-culturing on Nutrient Agar (NA) using the streak method (Cheesbrough, 2006). These pure colonies were stored in McCarthy bottles containing NA agar slants and incubated at 25 ± 2°C for 18 to 24 hours before storage at 4°C for future use. Identification of the isolates was conducted based on their growth characteristics on differential media and biochemical properties, following the standard protocols described by Cheesbrough (2006) and Holt (1994).

Screening for PHB Production

The screening of PHB-producing bacterial strains was performed using Sudan Black B stain (Burdon and Stokes, 1942). The PHB-positive strains were preserved in working and stock vials, containing NA agar slants with 2% glycerol for preservation.

Molecular Identification of PHB producers

DNA extraction utilized the Zymo Research Quick-DNA Fungal and Bacteria Kit (Zymo Research Corporation, USA). DNA quantification was achieved using a spectrophotometer (Gene Quant Pro) by measuring the optical density (OD) at 260 nm and 280 nm. Genomic DNA (gDNA) presence and quality were assessed via agarose gel electrophoresis. PCR amplification involved a master mix comprising PCR buffer, $MgCl_2$, DMSO, DNTPs, and Taq polymerase, alongside specific forward and reverse primers, template DNA, and ddH_2O. The final reaction volume was 20 µl, with the use of specific gene primers (16S F and 16S R). PCR conditions included an initial denaturation at 95 °C, followed by 30 cycles of denaturation, annealing, and elongation, and gel electrophoresis using a 100 bp DNA ladder. PCR products amplified with 16S primers underwent purification and bidirectional sequencing to determine the nucleotide sequence of the 16S RNA gene in each isolate. Sanger sequencing was carried out on an ABI Prism 3130X1 Genetic Analyzer (Applied Biosystems) using the BigDye terminator V3.1 kit (Applied Biosystems Inc., USA). Raw sequences obtained underwent editing by trimming both ends and removing bad chromatograms using Technelysium Pty Ltd (Australia). Nucleotide sequences were aligned using Clustal W (Thompson et al., 1994). Alignment is done after similarity searches using BLAST on the NCBI website. A phylogenetic tree was constructed using MEGA X, employing the maximum-likelihood method based on the Tamura-Nei model (TN+G+I) (Tamura et al., 2013). Branch support was assessed by bootstrap resampling with 1000 replications (Ali et al., 2018; Elshafey et al., 2022).

Enrichment & Optimization of PHB producing Bacteria

Bacterial isolates capable of producing PHB were subjected to selective enrichment following the method described by Madhumathi et al. (2016). The selected bacterial isolates capable of producing PHB were used for inoculum preparation. To optimize PHB production, the effect of media components and various process parameters such as temperature, carbon source, nitrogen source, and incubation time were studied using the one-factor-at-a-time method. The optimized pH and carbon source for each bacterium were used for PHB extraction using the solvent extraction method (Chang et al., 1994) with slight modifications. Firstly, 1% of PHB positive strain was inoculated in PHB media with optimized pH and carbon source and it was incubated at 37°C. After each 4-hour, 1 ml of media was centrifuged at 11,800 rpm for 20 minutes and Sudan staining was done to confirm PHB production. When the PHB production was confirmed, which mostly ensued after 48 hours, 50 ml of bacterial cell culture growth was taken and pelleted at 5000 rpm for 25 minutes. The dry weight of the pellet was taken and then it was washed with acetone and ethanol successively. For the recovery of PHB, equal volume of 6% sodium hypochlorite was used to re-suspend the pellet and it was incubated at 37°C for 10 minutes. This was followed by centrifugation at 5000 rpm for 30 minutes to sediment the lipid granules. The pellet obtained was washed with acetone and ethanol followed by hot chloroform treatment. After the pellet dissolved in chloroform, Whatman filter paper was used to filter out the cell residues so that only PHB is present in the chloroform solution. Finally, the filtrate was evaporated in hot air oven at 40°C and dry weight of extracted PHB was measured. The percentage of PHB accumulation was calculated using the formula described by Thapa et al. (2018):

$$\frac{Dry\ weight\ of\ extracted\ PHB\ \left(\frac{g}{ml}\right) \times 100}{Dry\ weight\ of\ biomass}$$

Methanolysis of the PHB was determined following the method described by Mostafa et al. (2020), and the fragmentation pattern of the obtained mass spectra was analyzed using NIST 98 mass library software, Gaithersburg, MD, USA.

RESULTS

The total bacterial load of soil samples varied among different sample locations (Table 1). Soil sample from location A exhibited the highest total bacterial count of 2.4×10^6 $CFUg^{-1}$, followed by soil from location B with a count of 5.0×10^5 $CFUg^{-1}$. The soil sample from location C had the lowest bacterial load of 9.7×10^4 $CFUg^{-1}$.

Table 1: Total bacterial load of engine oil contaminated soil samples

S/No	Sampling Location code	Bacterial count (CFU g^{-1})
1	A	2.4×10^6
2	B	5.0×10^5
3	C	9.7×10^4

A = Ikot Abasi; B= Ikot Akpaden; C= Ndon Obolom

A total of 12 bacterial isolates were obtained from the spent engine oil-contaminated soils. Based on morphological, physiological, and biochemical characteristics. The isolates were tentatively identified as species of *Bacillus*, *Pseudomonas*, *Staphylococcus* and *Lactobacillus*. Among the 12 bacterial isolates, only two displayed the potential to accumulate polyhydroxybutyrate (PHB) as revealed by the Sudan Black B stain. However, the quantity of PHB produced varied significantly among the two isolates. Isolate 1, tentatively identified as *Bacillus* sp showed the highest PHB production, with a mean value of 1.5 g/L, followed by Isolate 2 (*Pseudomonas* sp) with a mean value of 1.0 g/L (Figure 4.1). Statistical analysis conducted using XLSTAT PREMIUM Software (Evaluation 2023.1.5.1409 Version) revealed significant differences ($p<0.05$) in PHB production among the bacterial isolates, with *Bacillus* sp. showing significantly higher production (a mean value of 1.5 g/L) compared to the other isolate mean value of 1.0 g/L.

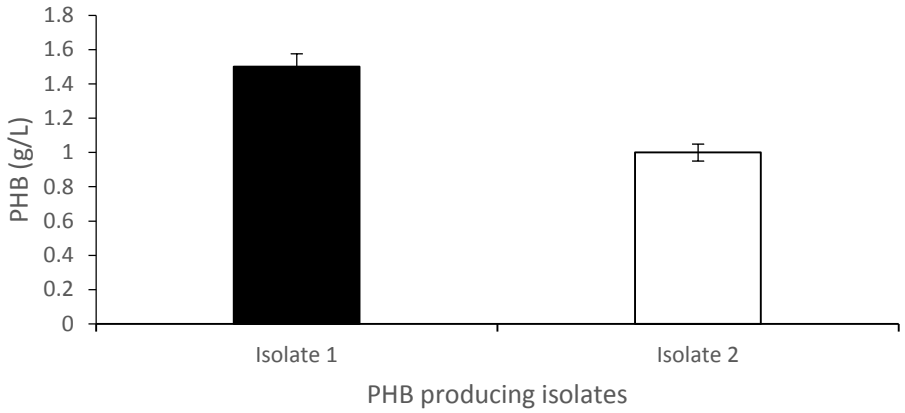

Figure 1: PHB production by bacteria isolated from engine oil contaminated soil

The PHB-producing isolates were further identified through 16S rRNA gene sequencing. Alignment results indicated high sequence homology and similarity to *Metabacillus* and *Lysinibacillus* (Figure 2). Accession numbers for the sequences are provided in Table 2.

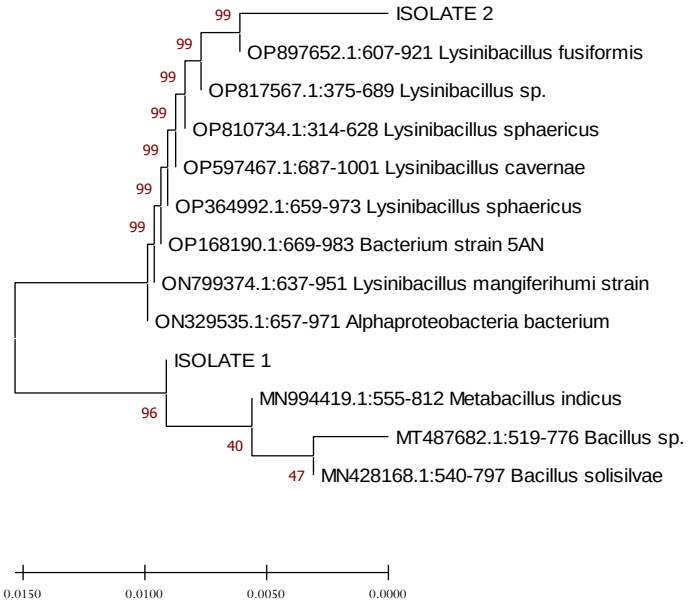

Figure 2: The phylogenetic tree based on the patterns and the evolutionary genetic relationship of PHB producing isolates by 16S rRNA nucleotide sequence. The scale bar corresponds to a 0.01 nucleotide substitution per sequence position. The numbers at the nodes indicate the levels of bootstrap support (%) based on 1000 data sets. The number that begins with alphabets represents the accession number

Table 2: Molecular identification, based on 16S rDNA BLAST sequencing data of the isolated PHB producers

Samples	Description	Query coverage (%)	Similarity (%)	NCBI Accession ID
Isolate 1	*Metabacillus indicus*	100	89	gi|1802471198|MN994419.1
Isolate 2	*Lysinibacillus fusiformis*	100	98	gi|2358615728|OP897652.1

Production of PHB by the isolates was influenced by various parameters. The highest PHB content (1900.05 ± 174 mg/L and 1500.50 ± 4.5 mg/L) for *Metabacillus indicus* and *Lysinibacillus fusiformis*, respectively, was observed at 35°C (Figure 3). Lower levels of PHB production (400 and 500 mg/L) were obtained at 25°C, while a further increase to 45°C led to a significant reduction in PHB production by the isolates. Agitation speed also affected PHB production. Maximum production (1850 and 2100 mg/L) was achieved at an agitation speed of 100 rpm for *Lysinibacillus fusiformis* and *Metabacillus indicus*, respectively (Figure 4). However, increasing the agitation speed to 200 rpm resulted in a significant decrease in PHB production (440.3 to 693.5 mg/L) for both isolates. Further reduction (79.8 to 100.5 mg/L) was observed at an agitation speed of 300 rpm. The pH of the production media had an impact on PHB production. Neutral pH (7) was found to be the most favorable for maximum PHB production (2515 ± 62.5 mg/L *by Lysinibacillus fusiformis* and 2085.5 ± 1.5 mg/L by *Metabacillus indicus*) (Figure 5). pH values slightly below 7.0 also supported PHB production, although at significantly lower levels. Glucose was observed as the most suitable carbon source for PHB production by both *Lysinibacillus fusiformis* (2450.5 ± 174 mg/L) and *Metabacillus indicus* (2210.2 ± 14 mg/L) (Figure 6). Sucrose was the second-best carbon source, *with Lysinibacillus fusiformis* producing 630.4 ± 121 mg/L and *Metabacillus indicus* producing 520.2 ± 17 mg/L. Among the nitrogen sources tested, peptone resulted in the highest PHB production for both isolates (2205.1 ± 121 mg/L for *Lysinibacillus fusiformis* and 2213.0 ± 134 mg/L for *Metabacillus indicus*), significantly outperforming yeast extract and urea (Figure 7).

PHB content increased over time for *both Lysinibacillus fusiformis* and *Metabacillus indicus*. The content was 300 and 400 mg/L for *Lysinibacillus fusiformis* and *Metabacillus indicus*, respectively, on day 1 of incubation and gradually increased to 1900 ± 100 and 2100 ± 120 mg/L on day 7 (Figure 8). The polymer extracted from *Metabacillus indicus* and *Lysinibacillus fusiformis* was characterized using GC-MS (Figures 9 and 10). Hexadecanoic acid methyl ester was identified as the predominant peak at 17.756 and 17.744 mins, respectively.

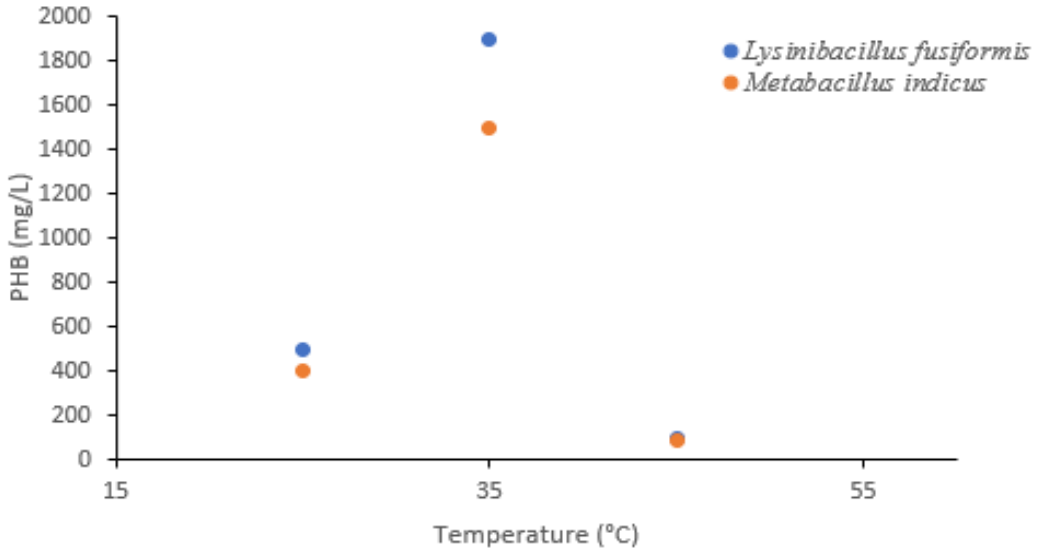

Figure 3: Effect of temperature on PHB production by *Metabacillus indicus* and *Lysinibacillus fusiformis*

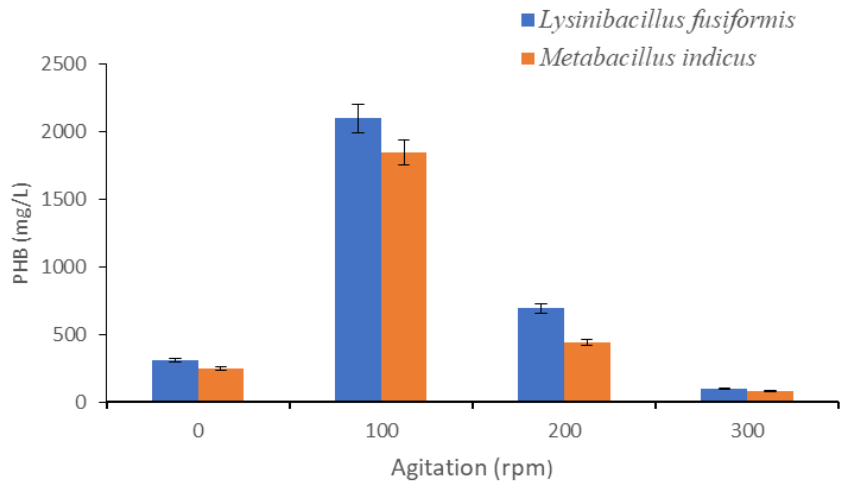

Figure 4: Effect of agitation on PHB production by *Metabacillus indicus* and *Lysinibacillus fusiformis*

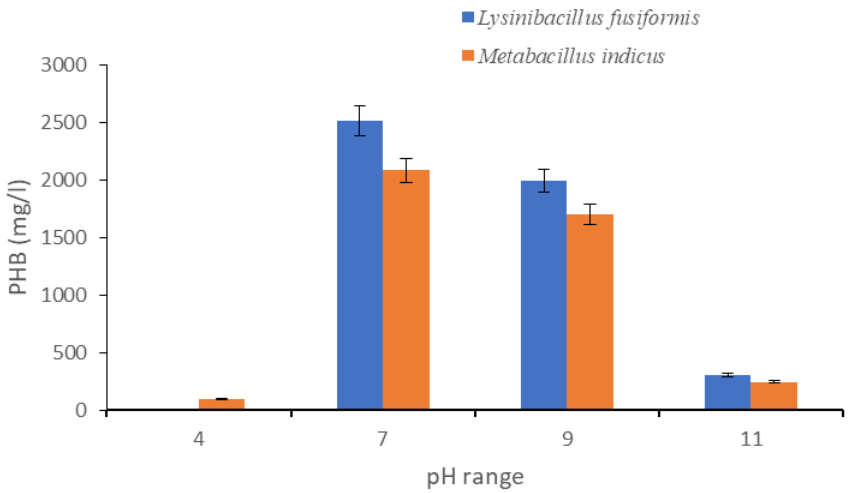

Figure 5: Effect of pH on PHB production by *Metabacillus indicus* and *Lysinibacillus fusiformis*

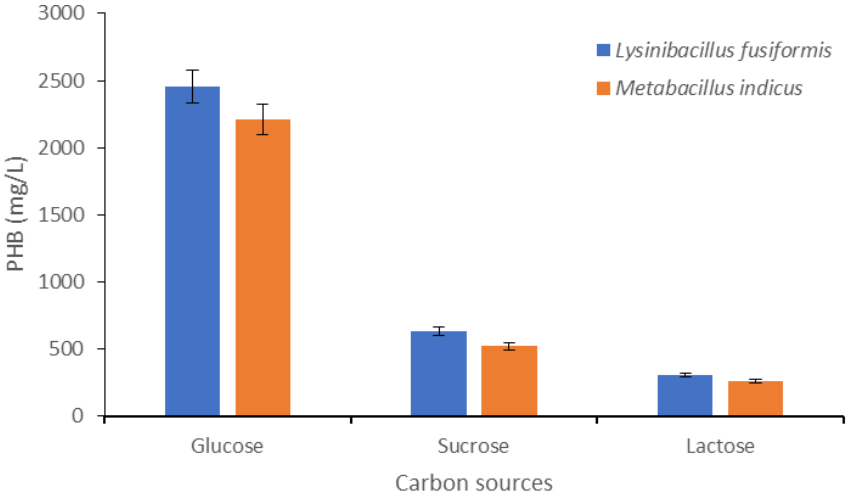

Figure 6:Effect of carbon sources on PHB production by *Metabacillus indicus* and *Lysinibacillus fusiformis*

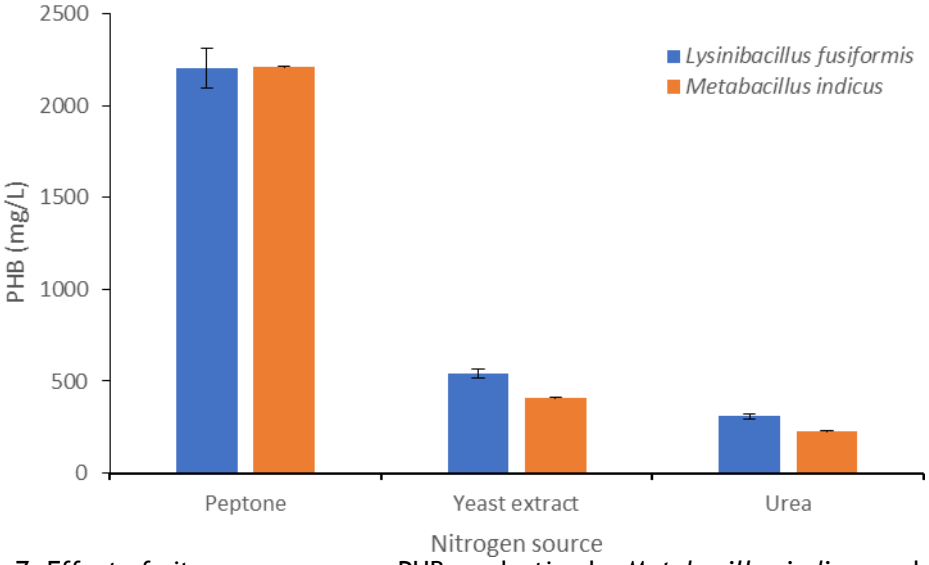

Figure 7: Effect of nitrogen sources on PHB production by *Metabacillus indicus* and *Lysinibacillus fusiformis*

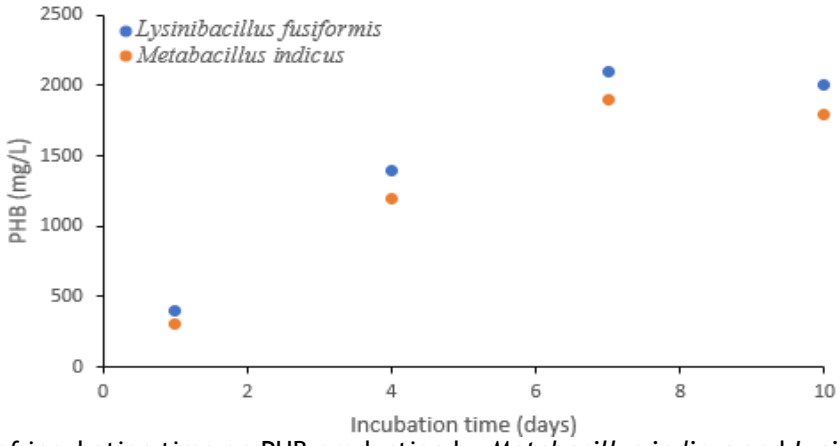

Figure 8: Effect of incubation time on PHB production by *Metabacillus indicus* and *Lysinibacillus*

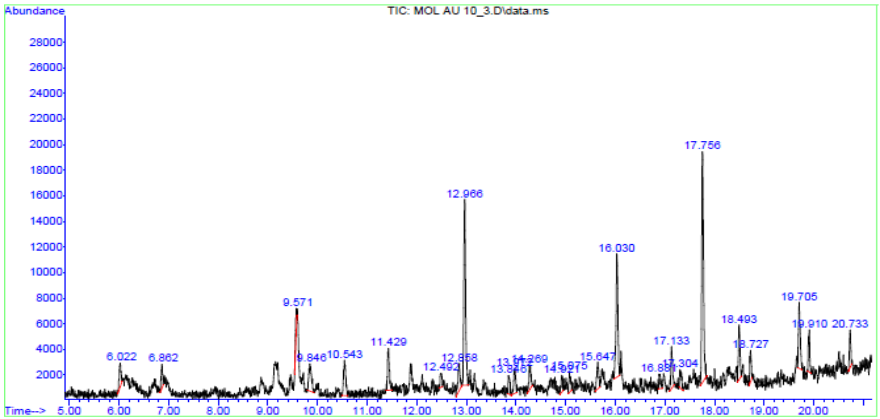

Figure 9: Chromatogram of PHB extracted from *Metabacillus indicus* in submerged formation with glucose as the carbon source

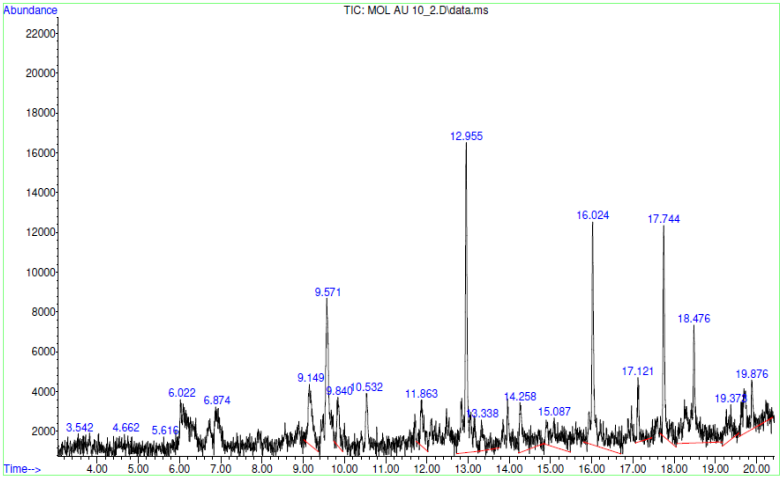

Figure 10: Chromatogram of of PHB extracted from *Lysinibacillus fusiformis* with glucose as the carbon source

DISCUSSION

In environmentally stressful conditions characterized by limited resources and fluctuating nutrient availability, microorganisms like *Lysinibacillus fusiformis* and *Metabacillus indicus* employ the production of polyhydroxybutyrate (PHB) as a survival strategy (Juengert et al., 2018). This study, isolating these PHB-producing bacteria from engine oil-contaminated soil in Mkpat Enin, adds significant depth to our understanding of their adaptive mechanisms. Genetic and phylogenetic analyses not only confirmed the isolates as *Lysinibacillus fusiformis* and *Metabacillus indicus* but also shed light on their evolutionary relationships within the bacterial classification, establishing a robust foundation for future research into their genetic makeup and adaptation strategies. The optimization of PHB production conditions reveals crucial insights. The preference for a mesophilic temperature of 35°C showcases the adaptability of these bacteria to moderate thermal conditions, aligning with previous findings (Narayanan et al., 2021). This optimal temperature likely enhances enzymatic activities vital for PHB synthesis, reinforcing the adaptive advantage of these bacteria in diverse environments. Moreover, the pH of 7.0 providing the highest PHB yield underscores the importance of maintaining a neutral pH to ensure metabolic efficiency. The sensitivity of metabolic processes to pH changes emphasizes the significance of this parameter in the context of PHB production. The choice of glucose as the preferred carbon source and peptone as the ideal nitrogen source reflects the metabolic versatility of *Lysinibacillus fusiformis* and *Metabacillus indicus*. Glucose, being readily available, serves as an efficient substrate for PHB production, emphasizing the adaptability of these bacteria to utilize common carbon sources effectively. Peptone, a complex organic nitrogen source, showcases the ability of these bacteria to efficiently metabolize diverse nitrogen compounds, highlighting their adaptability to varied nitrogen availability in their ecological niches. This adaptability is pivotal for their survival and dominance in challenging environments where nutrient availability fluctuates unpredictably.

The observed peak in PHB content on day 7 of incubation signifies the temporal dynamics of PHB accumulation within these bacterial cells. This finding aligns with previous studies on *Bacillus* species, suggesting a conserved pattern of PHB synthesis kinetics (Singh et al., 2011; Hamdy et al., 2022). Understanding the temporal aspects of PHB production provides valuable insights for bioprocess optimization and industrial-scale production, potentially leading to enhanced yields and cost-effectiveness. The validation of PHB through GC-MS analysis, identifying characteristic PHB monomeric groups, solidifies the credibility of these findings. This analytical rigor not only corroborates the PHB production but also paves the way for advanced applications. The biocompatibility, low inflammatory response, and biodegradability of microbially synthesized PHB, as indicated in previous studies (Mostafa et al., 2020; Mwamburi et al., 2019), make these findings significant for various practical applications. From environmental remediation to medical fields, these PHB-producing bacteria hold promising potential, providing sustainable solutions in the face of environmental challenges.

CONCLUSION

This study successfully isolated and identified two strains of bacteria, *Lysinibacillus fusiformis* and *Metabacillus indicus*, capable of producing polyhydroxybutyrate (PHB) from engine oil-contaminated soils. These strains exhibited significant PHB production under optimized environmental conditions, including a mesophilic temperature of 35°C, a lower agitation speed of 100 rpm, a neutral pH of 7.0, and the utilization of glucose as a carbon source and peptone as a nitrogen source. The 16S rRNA analysis and phylogenetic tree confirmed the genetic relatedness of these isolates to known strains within the genera *Lysinibacillus* and *Metabacillus*. The GC-MS analysis further supported the presence of PHB through the detection of characteristic monomeric groups. These findings contribute to our understanding of PHB production by spore-forming bacteria and highlight their potential as valuable resources for PHB production. Future applications of these PHB-producing bacteria can be explored in various fields, capitalizing on their unique properties and adaptability to challenging environments.

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