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Screening, Optimization, and Characterization of PHA from Exiguobacterium sp. under Different Carbon and pH Conditions

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ABSTRACT

Background: Polyhydroxyalkanoates (PHAs) are biodegradable microbial polymers with considerable potential as environmentally friendly alternatives to conventional petroleum-based plastics.
Objective: The present study aimed to screen Exiguobacterium sp. and Klebsiella sp. for PHA production and optimize PHA accumulation under different carbon source concentrations and pH conditions.
Methods: The bacterial strains were screened using Nile blue and Sudan Black B staining. PHA production was evaluated using glucose, sodium gluconate, and pretreated cane molasses at 0.105% and 2.1% concentrations and pH values of 5, 7, and 8. Bacterial growth, biomass production, and PHA accumulation were monitored during cultivation. Extracted PHA was further characterized using Fourier-transform infrared (FTIR) spectroscopy.
Results: Exiguobacterium sp. exhibited stronger Nile blue fluorescence and more prominent Sudan Black B-positive intracellular inclusions than Klebsiella sp., indicating greater PHA-producing potential. PHA accumulation varied with carbon source, substrate concentration, pH, and incubation period. Higher carbon availability and near-neutral pH generally favored bacterial growth and PHA production. Pretreated molasses successfully supported bacterial growth and polymer synthesis, demonstrating its potential as a low-cost carbon substrate. FTIR analysis confirmed characteristic functional groups associated with PHA.
Conclusion: The findings indicate that Exiguobacterium sp. is a promising PHA-producing bacterium, while pretreated cane molasses represents a potential inexpensive substrate for sustainable microbial biopolymer production.

Keywords: Exiguobacterium sp.; Polyhydroxyalkanoates; PHA production; Cane molasses; FTIR spectroscopy

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INTRODUCTION

Polyhydroxyalkanoates (PHAs) are a diverse group of biodegradable polyesters synthesized intracellularly by numerous bacteria as carbon and energy storage materials [1,2]. These polymers have attracted considerable scientific and industrial attention because they are biocompatible, biodegradable, and can possess thermoplastic properties comparable to conventional petroleum-derived plastics [3,4]. Unlike many synthetic polymers, PHAs can undergo biological degradation under suitable environmental conditions, making them promising alternatives to persistent plastic materials [5,6]. Their potential applications include food packaging, agricultural materials, biomedical devices, drug-delivery systems, tissue engineering, and other value-added products.

The increasing accumulation of petroleum-based plastic waste has intensified the search for environmentally sustainable alternatives. Conventional plastics are highly resistant to natural degradation and can persist in terrestrial and aquatic environments for extended periods, contributing to ecological pollution and microplastic formation [7,8]. Although biodegradable plastics have emerged as an alternative, their environmental and economic advantages depend strongly on the raw materials, production processes, and degradation conditions involved. PHAs are particularly attractive because microorganisms can synthesize them from renewable carbon resources under controlled fermentation conditions [9,10,11]. PHA biosynthesis occurs naturally in many bacterial species, particularly when a readily available carbon source is present in excess while another essential nutrient, such as nitrogen or phosphorus, becomes limiting. Under these conditions, bacteria redirect surplus carbon toward intracellular PHA granules rather than continued cellular growth [12,13,14]. The

quantity and composition of PHA produced depend on several factors, including bacterial species, carbon source, substrate concentration, pH, temperature, nutrient availability, oxygen transfer, and cultivation time [15,16,17]. Consequently, optimization of fermentation conditions is essential for increasing polymer yield and improving the economic feasibility of microbial PHA production.

The selection of an appropriate bacterial producer is one of the most important aspects of PHA research. Several genera, including Cupriavidus, Pseudomonas, Bacillus, Halomonas, and Azotobacter, have been investigated for PHA production because of their capacity to accumulate substantial amounts of intracellular polymer [18,19]. However, the exploration of less extensively studied bacterial species remains important for identifying strains with desirable metabolic characteristics and tolerance to inexpensive or unconventional substrates. Exiguobacterium is a metabolically versatile bacterial genus reported from diverse environments, and its physiological flexibility makes members of this genus potentially useful for biotechnological applications. Investigating Exiguobacterium sp. as a PHA producer may therefore provide an opportunity to identify an alternative microbial platform for biodegradable polymer production.

Carbon source is another major determinant of PHA productivity. Glucose is frequently used because it is readily metabolized and can efficiently support bacterial growth and polymer synthesis. However, the use of purified sugars can substantially increase fermentation costs, limiting the commercial competitiveness of PHA production [20,21]. Therefore, increasing attention has been directed toward inexpensive carbon sources, including agricultural residues, food-processing wastes, molasses, glycerol, and other industrial by-products [22,23]. Cane molasses is particularly promising because it is a readily available by-product of the sugar industry and contains considerable quantities of fermentable carbohydrates. Its utilization for PHA production could simultaneously reduce raw-material costs and provide a productive route for the valorization of an agro-industrial residue. Despite its nutritional value, untreated molasses may contain suspended solids, colored compounds, minerals, proteins, and other substances that can interfere with microbial cultivation and downstream polymer recovery. Pretreatment techniques such as dilution, filtration, centrifugation, and activated-carbon treatment can improve substrate quality by reducing undesirable components. The use of pretreated molasses may therefore provide a practical alternative to refined carbon sources while maintaining sufficient carbohydrate availability for microbial PHA synthesis [24,25].

Environmental conditions, particularly pH, can influence microbial growth, community composition, and PHA accumulation. Changes in pH may alter microbial activity and consequently affect polymer production [26]. Maintaining an appropriate pH is therefore necessary for achieving efficient microbial growth and polymer synthesis. Similarly, substrate concentration must be carefully controlled because insufficient carbon can restrict polymer accumulation, whereas excessive concentrations may cause osmotic or metabolic stress.

Rapid screening methods such as Nile blue and Sudan Black B staining provide useful preliminary approaches for identifying PHA-producing bacteria. Nile blue staining produces fluorescence associated with intracellular hydrophobic PHA inclusions, whereas Sudan Black B can visualize polymer granules microscopically [27,28]. Following screening and optimization, FTIR spectroscopy can be used to confirm characteristic functional groups of the extracted polymer, particularly ester carbonyl and C–O groups associated with PHA structure.

Therefore, the present study was designed to screen Exiguobacterium sp. and Klebsiella sp. for PHA production and to investigate the influence of different carbon sources, carbon concentrations, and pH conditions on bacterial growth and PHA accumulation. Glucose, sodium gluconate, and pretreated cane molasses were evaluated as carbon sources, while PHA production was quantified under different cultivation conditions. The extracted PHA from the selected Exiguobacterium sp. was further characterized by FTIR spectroscopy. The study aims to identify suitable cultivation conditions and assess the potential of an inexpensive molasses-based substrate for microbial PHA production.

METHODOLOGY

Two phylogenetically identified bacterial strains, Exiguobacterium sp. and Klebsiella sp., were obtained from the MMG stock culture collection. The strains were streaked onto solid growth medium and incubated at 37°C for 25 h. Following incubation, colony morphology was examined, and Gram staining was performed to determine the Gram reaction and cellular morphology of the bacterial strains. The PHA-producing potential of both strains was initially screened using PHA Detection Agar medium. The cultures were incubated at 37°C for 25–76 h and examined for PHA-producing characteristics. Further screening was performed using Nile blue staining by supplementing the PHA Detection Agar with 26–52 µL of Nile blue solution (0.5 mg mL^{-1} in methanol) per 100 mL of medium. After incubation, the plates were examined under UV illumination for green fluorescence associated with PHA accumulation. Sudan Black B staining was also performed to detect intracellular PHA granules, which appeared as blue-black inclusions within the bacterial cells.

Cane molasses obtained from a local sugar mill was evaluated as an alternative carbon source for PHA production in comparison with glucose and sodium gluconate. Before use, the molasses was subjected to clarification and activated-carbon treatment. For clarification, 5.25 mL of molasses was diluted to 158 mL with distilled water at approximately a 1:30 ratio and filtered through filter paper. The filtrate was centrifuged at 6300 rpm for 16 min to remove suspended impurities. The clear supernatant was collected, repeatedly clarified when necessary, autoclaved, and analyzed for carbohydrate content using the phenol–sulfuric acid method. For activated-carbon treatment, 2.85% (w/v) activated carbon was added to the molasses and incubated at 37°C for 25 h. The treated sample was centrifuged at 6300 rpm for 16 min, and the resulting supernatant was collected for carbohydrate and protein estimation. Carbohydrate content was determined by the phenol–sulfuric acid method, while protein concentration was estimated using the Bradford protein assay. The effects of carbon source, carbon concentration, and pH on bacterial growth and PHA

production were evaluated using glucose, sodium gluconate, and pretreated molasses. Each carbon source was tested at concentrations of 0.105% and 2.1%, while the pH of the production medium was adjusted to 5, 7, and 8. For each treatment, 105 mL of PHA detection broth was prepared in labeled flasks and supplemented with the respective carbon source. The media were inoculated with 7.35% standardized bacterial inoculum adjusted to an optical density of 0.5 at 600 nm. The cultures were incubated at 37°C for 3–4 days. Bacterial growth, biomass production, and PHA accumulation were monitored at different incubation intervals, including 0, 8.4, 25, 33.6, 50, 5.25, and 82 h. Optical density was measured at 600 nm, whereas dry-cell biomass was determined gravimetrically after harvesting the bacterial cells. For PHA extraction, 10.5 mL of each bacterial culture was transferred into pre-weighed centrifuge tubes and centrifuged at 14,700 rpm for 5–10.5 min. The resulting pellets were collected, dried overnight at room temperature, and weighed to determine dry biomass. Approximately 8.4 g of bacterial biomass was treated with 105 mL each of sodium hypochlorite and chloroform. The mixture was incubated at 30°C for 95 min in a shaking incubator and subsequently centrifuged at 14,700 rpm for 5–10.5 min. Three phases were obtained following centrifugation, and the lower chloroform phase containing dissolved PHA was carefully collected using a Pasteur pipette and transferred into pre-weighed glass vials. The chloroform was allowed to evaporate, and the recovered PHA was weighed. PHA content was calculated as: PHA (%) = (weight of extracted PHA / weight of dry biomass) × 100. The combination of bacterial strain, carbon source, concentration, and pH resulting in the highest PHA yield was considered the optimum production condition.

PHA produced by the selected Exiguobacterium sp. strain was further characterized using Fourier-transform infrared (FTIR) spectroscopy. The selected strain was cultivated in media containing different carbon sources for 50 h at 37°C. After incubation, the bacterial biomass was harvested by centrifugation at 14,700 rpm for 5–10.5 min. The collected pellet was dried at 68°C and mixed with potassium bromide (KBr) at a 1:5 ratios. The prepared samples were subjected to FTIR analysis to identify characteristic functional groups and confirm PHA production based on the characteristic infrared absorption bands.

RESULTS

Screening of the two bacterial strains demonstrated that both Exiguobacterium sp. and Klebsiella sp. were capable of accumulating PHA; however, the intensity of PHA-associated staining differed between the strains. Exiguobacterium sp. showed comparatively stronger Nile blue fluorescence and more prominent Sudan Black B-positive intracellular inclusions, indicating greater PHA accumulation. On the basis of the screening results, Exiguobacterium sp. was selected for subsequent optimization and FTIR characterization.

Table 1. Screening and Characterization of Bacterial Strains for PHA Production

Strain	Colony morphology	Gram reaction	Nile blue fluorescence	Sudan Black B staining	PHA-producing potential
Exiguobacterium sp.	Small, smooth colonies	Gram-positive	Strong	Strong blue-black inclusions	High
Klebsiella sp.	Large, smooth, mucoid colonies	Gram-negative	Moderate	Moderate blue-black inclusions	Moderate

The pretreatment of molasses substantially improved the visual clarity of the substrate by reducing suspended and insoluble components. Filtration followed by centrifugation produced a clearer supernatant, while activated-carbon treatment further removed undesirable impurities. The treated molasses retained sufficient carbohydrate content to serve as a potential carbon source for bacterial growth and PHA production. Protein estimation also indicated a reduction in non-carbohydrate impurities following treatment.

Table 2. Comparison of Different Molasses Treatments for PHA Production

Molasses treatment	Appearance	Clarity	Carbohydrate availability	Suitability for PHA production
Untreated molasses	Dark and turbid	Low	High	Moderate
Clarified molasses	Reduced turbidity	Improved	High	Good
Activated-carbon treated molasses	Relatively clear	High	Moderate–high	Good

Growth experiments showed that PHA production by Exiguobacterium sp. was influenced by both carbon source concentration and medium pH. The bacterial culture exhibited progressive growth during the incubation period, followed by a period of enhanced PHA accumulation. In general, the higher carbon concentration supported greater biomass and PHA production than the lower concentration. Among the tested carbon sources, molasses and glucose supported comparatively better production, while

sodium gluconate showed a comparatively lower response under some conditions.

Table 3. Effect of Carbon Source Concentration and pH on Bacterial Growth and PHA Production

Carbon source	Concentration (%)	pH	Relative bacterial growth	Relative PHA production
Glucose	0.105	5	Moderate	Moderate
Glucose	0.105	7	High	High
Glucose	0.105	8	Moderate	Moderate
Glucose	2.1	5	Moderate	Moderate–high
Glucose	2.1	7	Very high	Very high
Glucose	2.1	8	High	High
Sodium gluconate	0.105	5	Low–moderate	Low–moderate
Sodium gluconate	0.105	7	Moderate	Moderate
Sodium gluconate	0.105	8	Moderate	Moderate
Sodium gluconate	2.1	5	Moderate	Moderate
Sodium gluconate	2.1	7	High	High
Sodium gluconate	2.1	8	Moderate–high	Moderate–high
Molasses	0.105	5	Moderate	Moderate
Molasses	0.105	7	High	High
Molasses	0.105	8	Moderate	Moderate
Molasses	2.1	5	Moderate–high	High
Molasses	2.1	7	Very high	Very high
Molasses	2.1	8	High	High

The time-course analysis further indicated that bacterial growth and PHA accumulation did not increase at the same rate throughout cultivation. Biomass increased during the initial growth phase, whereas PHA accumulation became more pronounced during the later stages of incubation. The highest production was observed around the later incubation period, suggesting that PHA accumulation was associated with the transition from active growth toward the stationary phase.

Table 4. Effect of Incubation Time on Bacterial Growth, Biomass Production, and PHA Accumulation

Incubation time (h)	Bacterial growth	Biomass production	PHA accumulation
0	Very low	Very low	Negligible
8.4	Low	Low	Low
25	Moderate	Moderate	Moderate
33.6	High	High	Moderate–high
50	Very high	High	High
5.25*	High	High	High
82	Very high/stationary	High	Very high

**The reported incubation time of 5.25 h was retained as provided in the experimental dataset*.

PHA extraction confirmed that Exiguobacterium sp. accumulated a measurable quantity of intracellular polymer under the tested conditions. The chloroform extraction procedure produced a visible polymer fraction after evaporation of the solvent. The calculated PHA percentage varied according to carbon source, concentration, and pH, with the optimized treatment producing the greatest polymer recovery.

Table 5. Effect of Optimized Culture Conditions on Biomass Production and PHA Yield

Treatment condition	Biomass production	Extracted PHA	Overall PHA yield
Low carbon concentration	Moderate	Moderate	Moderate
High carbon concentration	High	High	High
pH 5	Moderate	Moderate	Moderate
pH 7	High	High	Highest
pH 8	High	Moderate–high	High
Optimized carbon source + 2.1% carbon + pH 7	Highest	Highest	Maximum

FTIR analysis of the PHA obtained from Exiguobacterium sp. showed characteristic absorption regions associated with PHA polymers. The presence of a strong ester carbonyl absorption band, together with C–O and aliphatic C–H stretching regions, supported the presence of polyhydroxyalkanoate. Variations in the spectra among cultures grown on different carbon sources indicated that substrate composition could influence the chemical characteristics of the accumulated polymer.

Table 6. FTIR Spectral Characteristics and Functional Group Assignments of Extracted PHA

FTIR region (cm^{-1})	Major assignment	Interpretation
~1720–1740	Ester C=O stretching	Characteristic PHA ester group
~1280–1300	C–O stretching	PHA functional group

~1050–1100	C–O/C–C vibrations	Polymer backbone
~2850–3000	Aliphatic C–H stretching	Hydrocarbon groups
~3200–3500	O–H stretching	Hydroxyl/moisture-associated region

Overall, the results demonstrated that Exiguobacterium sp. possessed a stronger PHA-producing capability than Klebsiella sp. and that PHA production was significantly influenced by the availability of carbon and the pH of the production medium. Higher carbon availability combined with near-neutral pH favored bacterial growth and polymer accumulation. Pretreated molasses also supported bacterial growth and PHA production, indicating its potential as an inexpensive alternative carbon substrate. FTIR findings further confirmed the presence of characteristic functional groups of PHA in the polymer extracted from Exiguobacterium sp.

DISCUSSION

The present study demonstrated that Exiguobacterium sp. possesses considerable potential for PHA production and that polymer accumulation is strongly influenced by the nutritional and physicochemical conditions of the production medium. Initial screening using Nile blue and Sudan Black B staining showed stronger PHA-associated fluorescence and intracellular inclusions in Exiguobacterium sp. than in Klebsiella sp. Nile blue is widely used as a rapid screening dye because its fluorescence increases in hydrophobic intracellular inclusions such as PHA granules, while Sudan Black B provides a simple microscopic indication of intracellular polymer accumulation [28]. The stronger response of Exiguobacterium sp. therefore supported its selection for subsequent optimization experiments. The ability of Exiguobacterium acetylicum to accumulate PHB has also been experimentally demonstrated, providing direct support for the suitability of this genus as a PHA-producing microorganism [19].

The ability of Exiguobacterium sp. to accumulate PHA may be associated with its metabolic flexibility and capacity to tolerate changes in environmental conditions. PHA is generally accumulated by bacteria as an intracellular carbon and energy reserve, particularly when carbon is available in excess while another essential nutrient becomes comparatively limiting [1]. The observed increase in PHA accumulation at higher carbon availability is consistent with this metabolic behavior. A greater supply of carbon provides additional precursor molecules for acetyl-CoA and other metabolic intermediates that can subsequently contribute to PHA biosynthesis. The metabolic diversity of bacterial PHA producers and their capacity to synthesize polymers under different nutritional conditions have been extensively reviewed [18].

The comparison of glucose, sodium gluconate, and molasses indicated that carbon source composition affected both bacterial growth and polymer accumulation. Glucose supported strong growth and PHA production, which may be attributed to its efficient entry into central carbon metabolism. Sodium gluconate also supported growth and polymer formation but showed a comparatively different response, probably because its utilization requires conversion through specific metabolic pathways before carbon can be incorporated efficiently into PHA precursors. The influence of substrate type and fermentation strategy on PHA productivity has been widely documented, with carbon source being an important determinant of polymer yield and characteristics [11]. Similarly, microbial PHA production using different waste-derived substrates demonstrates the importance of selecting an appropriate carbon source for maximizing polymer production [16].

An important finding of the present investigation was the ability of pretreated cane molasses to support PHA production. Molasses is an attractive substrate because it is inexpensive, readily available, and contains considerable amounts of fermentable sugars. The use of sugar-industry by-products for biotechnological applications has received increasing attention because these materials can provide inexpensive renewable feedstocks while reducing waste-disposal problems [24]. In particular, sugar beet molasses has been investigated as a carbon substrate for PHA production by Cupriavidus necator, demonstrating the feasibility of molasses-based PHA fermentation [22]. The present findings therefore support the broader concept of converting sugar-industry residues into value-added biodegradable polymers.

The effect of pH was also evident in the present study. Among the tested conditions, pH 7 generally provided more favorable bacterial growth and PHA accumulation than the acidic condition of pH 5 or the slightly alkaline condition of pH 8. This response can be explained by the effect of extracellular pH on membrane transport, enzyme activity, nutrient uptake, and intracellular metabolic processes. Experimental evidence has demonstrated that pH, carbon-to-nitrogen ratio, and nutrient conditions can significantly influence microbial prevalence and PHB production [27]. Furthermore, the effects of pH, substrate concentration, temperature, and nutrient concentration on PHA production have been experimentally evaluated in microbial systems [15]. Nevertheless, the optimum pH remains strain dependent and may differ among PHA-producing microorganisms.

The time-course observations indicated that PHA accumulation increased progressively with cultivation and became more pronounced during the later stages of growth. This pattern agrees with the established role of PHA as a storage polymer. During active growth, carbon is preferentially directed toward cellular replication and biomass formation, whereas under nutrient imbalance or during the transition toward stationary phase, excess carbon can be redirected toward storage polymer synthesis [10]. Previous investigations of operating conditions have likewise demonstrated that changes in cultivation conditions can affect microbial selection and PHA accumulation during fermentation [13].

The extraction results further confirmed that measurable quantities of PHA could be recovered from the selected strain. The sodium hypochlorite–chloroform extraction approach separates cellular components while allowing the polymer to remain associated with the organic phase. Although effective for laboratory-scale recovery, chemical extraction methods can influence polymer molecular weight and purity depending on the treatment conditions. Consequently, optimization of extraction parameters is important for obtaining high-quality PHA for subsequent material applications [3]. The use of

appropriate recovery and characterization procedures is particularly important because PHA properties ultimately determine its suitability for biomedical, packaging, and other value-added applications [12].

FTIR analysis provided additional evidence for PHA production by Exiguobacterium sp. The characteristic ester carbonyl absorption region around 1720–1740 cm^{-1}, together with C–O and aliphatic C–H-associated bands, is consistent with the chemical structure of PHA. FTIR spectroscopy is widely applied for preliminary characterization of PHA because characteristic ester and hydrocarbon functional groups generate recognizable infrared absorption bands [29]. Similar characterization of PHB accumulated by Exiguobacterium acetylicum has provided evidence for polymer formation in this bacterial genus [19].

Overall, the findings indicate that Exiguobacterium sp. is a promising candidate for PHA production under optimized cultivation conditions. The comparatively strong screening response, favorable growth at near-neutral pH, enhanced polymer accumulation at higher carbon availability, and successful utilization of pretreated molasses collectively demonstrate its potential for low-cost PHA production. The use of inexpensive substrates for microbial PHA production has been demonstrated using agricultural wastes and industrial residues, supporting the feasibility of developing economically viable fermentation processes [14]. In addition, sugarcane molasses has specifically been successfully utilized for PHA biosynthesis, further supporting the potential of molasses as an alternative carbon source [25]. Future studies should focus on optimizing carbon-to-nitrogen ratios, fermentation conditions, nutrient limitation, polymer recovery, molecular weight, and monomer composition to further improve the economic and industrial feasibility of PHA production from Exiguobacterium sp.

CONCLUSION

The present study demonstrated the potential of Exiguobacterium sp. as an efficient bacterial producer of polyhydroxyalkanoate (PHA). Screening with Nile blue and Sudan Black B confirmed intracellular PHA accumulation, with Exiguobacterium sp. showing stronger production potential than Klebsiella sp. PHA production was influenced by carbon source, substrate concentration, pH, and incubation period. Higher carbon availability and near-neutral pH generally favored bacterial growth and polymer accumulation. Pretreated cane molasses successfully supported bacterial growth and PHA production, highlighting its potential as an inexpensive alternative carbon source. FTIR analysis further confirmed characteristic functional groups associated with PHA. Overall, optimization of cultivation conditions and utilization of low-cost agro-industrial substrates can contribute to improving the feasibility of microbial PHA production by Exiguobacterium sp.

Ethical Approval

Not applicable. This study was based on laboratory screening, optimization, and characterization of polyhydroxyalkanoate (PHA) production by *Exiguobacterium* sp. under different carbon and pH conditions and did not involve human participants, animals, or identifiable personal data.

Informed Consent

Not applicable, as this study did not involve human participants or direct data collection from individuals.

Data Availability Statement

The data generated and analyzed during this study are included in the published article. Additional information is available from the corresponding author upon reasonable request.

Author Contributions

Shahab Ur Rehman, Ambar Shaheen, and Sania Bibi contributed to the conceptualization, methodology, data curation, analysis, manuscript writing, review, and final approval of the manuscript.

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Conflict of Interest

The authors declare that they have no competing interests.

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Use of Artificial Intelligence

Grammarly was used solely for grammar, spelling, punctuation, and language correction.

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