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Screening and Isolation of Bacterial Isolates for Polyhydroxyalkanoates Production from Oil-contaminated Sites

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Abstract

Synthetic polymers are generally manufactured from petroleum and chemical-based sources. These polymers are employed in day-to-day life basic needs which leads to the production of huge number of synthetic polymers that accumulate in nature for thousands of years. There are limitations associated with synthetic polymers apart from their non-biodegradability, non-compatibility their mechanical and physical properties that cannot be easily altered narrows their few applications. PHAs (polyhydroxyalkanoates) are biopolymers that can be a promising solution to this problem, and it is one of the major candidates in reducing the dependency on synthetic polymers. In this study, oil-contaminated soil samples were collected from 2 cm depth from different locations. Soil samples were serially diluted and were used for the isolation of bacterial colonies on nutrient agar enriched with 2% glucose. Seven isolates were obtained after incubation and were screened for PHA production potential using Sudan black B and Nile blue sulfate. Results showed that out of seven isolated colonies, only three were found positive for primary Sudan black B screening, and out of 3 only 2 were found positive for confirmatory Nile blue screening. There are only two isolates that were positive for both screening dyes and were selected for further studies on PHA production. Furthermore, morphological characterization and biochemical characterization of these two isolates are done for the identification of bacterial isolates.

Keywords: PHA, Sudan black B, Nile blue, morphological, biochemical characterization

INTRODUCTION

Increased awareness of environmental sustainability in recent years has raised concerns regarding the use of synthetic polymers made from non-renewable petroleum-based resources because of their poor degradability and persistence in nature. This has led to an increase in the demand for biopolymers [1, 2]. The worldwide biopolymers market was USD 17.54 billion in 2023, with a compound annual growth rate (CAGR) of 10.4% between 2024 and 2030 [3]. Biopolymers are used in various industrial and biomedical applications, including biomedical, pharmaceutical, and food applications. Owing to their biocompatibility, they are in significant demand for biomedical applications [4].

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Polyhydroxyalkanoates (PHAs) are naturally occurring biocompatible and biodegradable polymers that belong to a versatile group of polyesters comprising R-hydroxyalkanoic acids. They are produced as intracellular polymeric granules by bacteria and archaebacteria, where they function as energy and carbon reserves [5]. PHA production is enhanced by unbalanced microbial growth during the fermentation process, either

under nutrient limitation or in excess of a carbon source. They are produced as insoluble granules in the cytoplasm and help protect microorganisms from unfavorable environmental conditions, such as UV exposure, osmotic shock, and pressure [4]. PHA are generally classified based on the number of carbon atoms in their monomers produced by them (a) Short short-chain PHAs consist of 3-5 carbon monomeric units, and common examples of short-chain PHAs are Poly-3-hydroxy butyrate (PHB). (b) Medium-chain PHAs have 6–14 carbon atoms in hydroxyalkanoate units. Poly(3-hydroxynonanoate) (PHN) and poly(3-hydroxyoctanoate) (PHO) are examples of medium-chain PHAs. (c) Long-chain PHAs have ≥15 carbon atoms. Examples include poly (3-hydroxypentadecanoate) and Poly (3-3-hydroxhexadecanoate) [5]. More than 150 distinct PHA monomers have been reported for the biosynthesis of different PHAs, making them the largest category of natural polyesters. Short-chain-length PHAs possess physiochemical characteristics similar to those of conventional plastics, making them a promising alternative to fossil-fuel-based polymers [5].

The global PHA market demand of PHA was valued at USD 57.8 million in 2023, which is expected to reach USD 98.5 million by 2028. Market growth is driven by the increased demand for sustainable, eco-friendly, and biodegradable raw materials to replace synthetic plastics, government initiatives to eliminate single-use plastics, and growing demand for PHA-based environmental plastics [6].

Major concerns that restrict PHA utilization as an industrial plastic material are the high cost of production PHAs production, difficulty in processing PHA homopolymers, poor mechanical strength, and thermal stability [7]. Important factors affecting the successful large-scale production of PHA include the cost of raw material, downstream processing cost, choice of PHA-producing microorganisms, and type of fermentation process [7]. Research efforts have been made to utilize agroindustry residues as inexpensive carbon sources to reduce substrate costs [8, 9]. The development of modified PHA as composites or blends of PHA has been shown to possess improved physicochemical properties and widen its applicability [10, 11]. Bioprospecting approaches based on the screening and isolation of new PHA producers are important for identifying a potential PHA producer with a new polymer. Therefore, this study aimed to screen and isolate new PHA-producing bacteria from hydrocarbon-contaminated soil. The strains were screened for their PHA-producing abilities and characterized using morphological and biochemical methods [12–14].

MATERIAL AND METHODS

Collection of Soil Samples

PHA contains fatty groups; therefore, for isolating PHA-producing bacteria, soil samples were collected from two different sites: (a) soil samples were collected from an automobile repair shop at Indirapuram Ghaziabad, and this soil was contaminated with Mobil oil. (b) Soil samples were collected from Jaypee Institute of Information Technology, Noida, Sector 62, under a peepal tree where mustard oil-contaminated soil was present. These sites are richer in carbon percentage and have a lower nitrogen content. The probability of getting PHA producer microorganisms was higher.

Isolation of Pure Bacterial Colonies from Soil Samples

For isolating bacteria, serial dilution of soil samples was performed in which 1 g of soil was diluted in 9 mL of 0.9% saline water and serially diluted up to six dilutions. Each dilution was spread on nutrient agar plates enriched with 2% glucose and incubated at 37°C for 24 h [15–17]. These plates were observed for the isolation of bacteria, and streaking was performed to obtain pure colonies of isolates from mixed communities of bacteria [18]. Seven colonies were isolated from both samples; colonies that were isolated from automobile repair shops are represented as AR and colonies obtained from the Jaypee Institute of Information Technology are represented as JR.

Screening of PHA-producing Bacterial Isolates by Sudan Black B and Nile Blue Staining

The obtained pure colonies were screened by Sudan black B staining to identify polyhydroxyalkanoate-producing bacteria. Bacterial colonies were flooded with an alcoholic solution of Sudan black and kept undisturbed for 30 min, after which the solution was removed. Wash plates

with alcohol and observe petri dishes for colonies that retain a bluish-black color. Isolates that were found to be positive in the primary screening were further screened for confirmation of PHA-producing isolates. Nile blue agar plates (0.05 µg/ml) were prepared, and the bacterial culture was streaked on plates and kept in an incubator at 37°C for 24 h. The plates were then observed under a UV transilluminator for fluorescent colonies. Isolates that are found to be positive for both screening dyes are considered PHA-producing [18–20].

Morphological and Biochemical Characterization

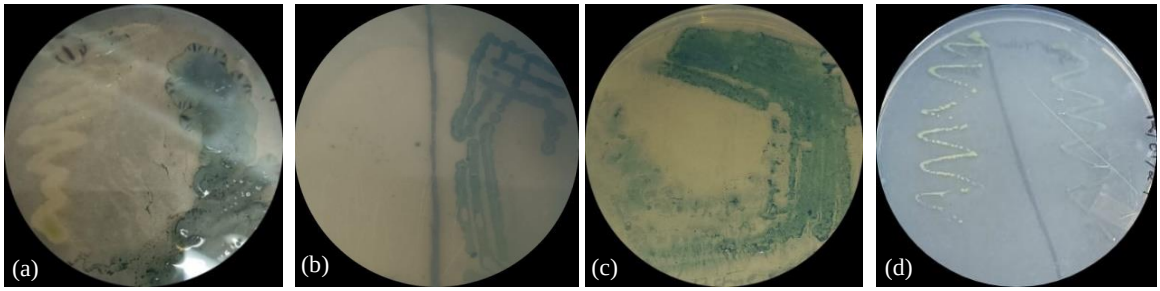
Morphological identification of bacterial colonies was performed based on their shape. After the selection of PHA-producing isolates, they were examined by Gram staining for morphological characterization [21–23]. Motility testing was performed to determine whether the isolates were motile or non-motile. The spores were also examined for spore formation. Biochemical characterization was performed based on Bergey's manual [24], and multiple tests were performed for identification [25, 26, 24].

RESULT AND DISCUSSION

Screening and isolation of PHA-producing bacteria from soil samples from two different sites: Four colonies were isolated from automobile repair shops and three colonies from the Jaypee Institute of Information Technology campus. The cells were cultured to obtain pure colonies. Subsequently, they were screened by Sudan black B for the selection of PHA-producing isolates. The results of Sudan black B are shown in Figure 1, of 7 colonies only three were found to be positive for the primary screening dye. Confirmation of PHA-producing isolates was performed using Nile blue staining plates. After incubation for 24 h, Nile blue agar plates were examined under a UV transilluminator for the fluorescence of PHA-producing colonies. In Figure 2, Nile blue plate results are shown in which we can see fluorescence in the colonies. Of the three colonies, two showed fluorescence under a UV transilluminator [27, 28]. Only two isolates were positive for both screening dyes. Table 1 shows the screening results for all the isolated colonies.

Table 1. Screening results of both dyes for isolated colonies.

Colonies	Sudan black B	Nile blue
AR1	Negative	Negative
AR2	Positive	Negative
AR3	Negative	Negative
JR1	Negative	Negative
JR2	Negative	Negative
JR3	Positive	Positive
JR4	Positive	Positive



JR1 JR3 JR2 JR4 AR2 AR1 AR3

Figure 1. Sudan black B staining results of 7 isolates, (a) JR1 is negative as it retained its original color while JR3 is positive as bluish color is retained, (b) JR2 retained its original color and JR4 retained bluish color, (c) AR2 retained bluish color, (d) AR1 and AR3 retained original color so negative for PHA production.

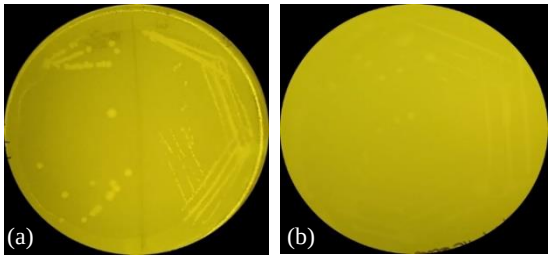


Figure 2. Nile blue screening results of Sudan black B positive isolates, (a) JR3 and JR4 both isolates showed fluorescence under UV transilluminator after growth in media containing Nile red dye, and (b) AR2 isolate did not show fluorescence.

Only two isolates were positive for both screening dyes. After screening, the morphological identification of the positive isolates was performed. The isolates were identified based on their morphology, motility, and spore formation. Both isolates were gram-negative rod-shaped bacteria. In Figure 3, the Gram staining results of unknown bacteria are shown. From microscopic examination, it can be seen that both bacteria have rod-shaped chains.

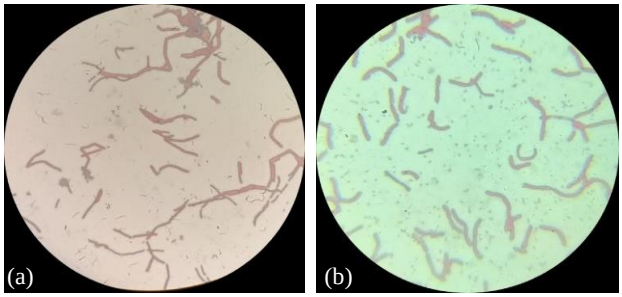


Figure 3. Gram staining and microscopic examination of unknown isolates; (a) staining result of JR3, and (b) staining result of JR4.

For further identification, biochemical tests were performed according to Bergey's manual [29] has been performed. These tests include indole, MR, VP, citrate utilization, and glucose fermentation tests (Table 2) [24].

Table 2. Morphological features and biochemical characteristics of positive PHA-producing bacterial isolates.

Test	JR 3	JR4
Gram stain	Gram-negative	Gram-negative
Cell shape	Rod-shaped	Rod-shaped
Colony color	White	Cream
Colony shape	Irregular	Circular
Cell arrangement	Chain	Chain
Spore formation	Negative	Negative
Oxidase	Negative	Negative
Catalase	Negative	Negative
Glucose fermentation	Acid produced but no production of gas	Acid produced but no production of gas
Motility	Non-motile	Non-motile
Methyl red	Negative	Negative
Voges Proskauer	Negative	Negative
Indole	Negative	Negative
Citrate utilization	Positive	Positive

CONCLUSION

The present study focused on the screening and isolation of potential PHA-producing bacteria from oil-contaminated soil. Of the seven isolates obtained, two potent PHA-accumulating bacteria were identified from soil samples collected from an automobile repair shop near Noida, India. Furthermore, morphological and biochemical characterizations were performed to identify the isolated bacterial colonies. From the characterization results based on Bergey's manual, we can assume that the unknown bacteria belong to *Klebsiella* genus. Therefore, molecular characterization must be performed for further confirmation.

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