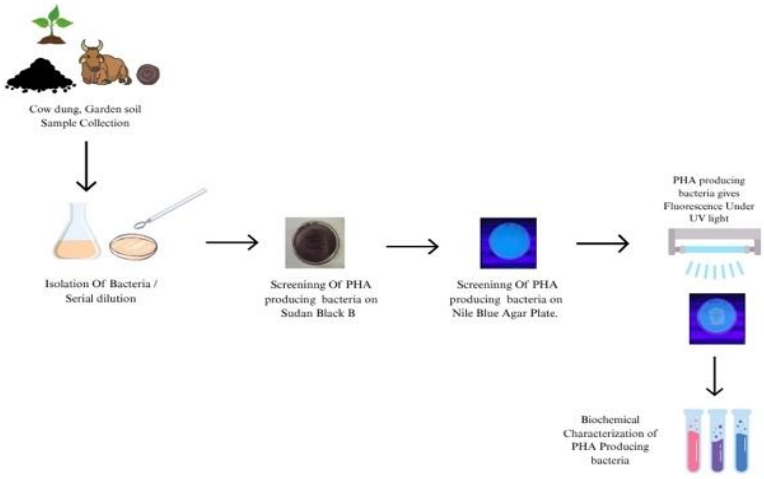
International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 21s, 2025
https://theaspd.com/index.php

Isolation, Screening And Production Of Polyhydroxyalkanoate (PHA) From Bacteria Isolated From Natural Habitats And Organic Waste Environments

Urjitsinh Zala[1], Abedhusain Seliya[2], Vijay Jagdish Upadhye[3], Anupama Shrivastav[2]
[1]Department of Medical Laboratory Technology, Faculty of Life Health and Allied Sciences, Institute of Technology and Management-ITM Vocational University, Vadodara, Gujarat, India.
[2]Department of Microbiology, Faculty of Life Health and Allied Sciences, Institute for Technology and Management-ITM Vocational University, Vadodara, Gujarat, India
[3]India Research and development Cell (RDC), Parul Institute of Applied Sciences (PIAS), Parul University (DSIR-SIRO Recognized), Vadodara, Gujarat, India.
Corresponding Author: Anupama Shrivastav, anupamashrivastav@gmail.com

Abstract
Polyhydroxyalkanoates (PHAs) are intracellular storage biopolymers synthesized by numerous microorganisms as polyesters under nutrient-limited conditions. These biopolymers are of considerable interest as sustainable alternatives to conventional petroleum-based plastics. This study aims to isolate and conduct preliminary screening of bacteria capable of PHA production from diverse environments, including soil, water bodies, and organic waste sites. Environmental samples were serially diluted and cultured on selective nutrient media to obtain pure bacterial colonies. Potential PHA-accumulating bacteria were identified through staining with Nile blue A and Sudan Black B, which revealed fluorescent or dark-stained intracellular granules under microscopy—confirming PHA synthesis potential upon nutrient depletion. Positive isolates from preliminary screenings were further subjected to PHA production studies under standard laboratory conditions. The extent of PHA accumulation was quantified by measuring cell dry weight and calculating PHA yield. Initial results indicated that several of the isolated bacterial strains demonstrated significant PHA accumulation capacity. Further research will aim to optimize growth conditions and maximize PHA production levels for these strains. Identification and characterization of effective PHA-producing bacteria support the development of environmentally friendly alternatives to synthetic plastics.
Keywords: *Polyhydroxyalkanoates (PHAs),Biodegradable plastics, PHA-producing bacteria, Biopolymer production, Sustainable alternatives, Microbial bioplastics*

Graphical abstract:



[Isolation and Screening Of PHAs Producing Bacteria from diverse environment sources]

1. INTRODUCTION

Because petroleum-based plastics are not biodegradable and disposal is difficult, their widespread use has become a major environmental concern, resulting in a significant accumulation of synthetic plastic waste. Polyhydroxyalkanoates (PHAs), which are biodegradable and biocompatible thermoplastics that are naturally produced by a variety of bacteria as intracellular energy and carbon reserves, have emerged as promising substitutes in response.[1] PHAs are appropriate for a variety of uses in coatings, packaging, and

biomedical devices because they share characteristics with traditional plastics like polypropylene, such as flexibility, elasticity, and adaptability[1.]

The need for sustainable and biodegradable substitutes has accelerated due to growing environmental concerns about synthetic, petroleum-based plastics. Polyhydroxyalkanoates (PHAs) are among the promising candidates that have attracted a lot of attention because of their mechanical qualities that are comparable to those of traditional plastics like polypropylene and polyethylene, as well as their biodegradability and biocompatibility.[1,2]

With over 150 different monomer structures and over 92 genera in both the Gram-positive and Gram-negative groups, PHA-producing bacteria are remarkably versatile. These bacteria can use a wide range of carbon sources, from simple sugars to complex industrial and agricultural wastes, and they live in a variety of ecological niches, such as soil, water, and harsh environments.[3] Notably, because of their high PHA productivity and substrate adaptability, genera like Alcaligenes, Ralstonia, Bacillus, and Pseudomonas have been the subject of much research.[4]

PHAs are important for the environment not only because of where they come from. In nature, many microorganisms can easily break them down, which means they have a small impact on the environment and help create a circular bio economy.[5] However, the high cost of making PHAs makes them less commercially viable right now. This is mostly due to the choice of substrate and process optimization. So, it is very important to find and describe efficient PHA-producing bacteria from a wide range of environmental sources in order to find new strains that have better yield, substrate versatility, and resistance to process stresses.[6]

The goal of this study is to find and describe PHA-producing bacteria from a variety of environmental sources. The goal is to find new strains that can efficiently store PHA and use cheap substrates. The results should help us learn more about the diversity, physiology, and biotechnological potential of PHA-producing bacteria. This will help us protect the environment and manage materials in a way that is good for the future.

2. MATERIALS AND METHOD

2.1 Sample Collection and Isolation of bacteria

Three different sites in the Akota area of Vadodara, Gujarat, were used to gather soil samples: garden soil, soil near a fuel pump, and soil enriched with cow dung. To avoid contamination, each sample was taken with sterile spatulas and kept in sterile zip-lock bags. After that, the samples were aseptically transported to the lab for additional microbiological analysis. The sampling strategy used here is in accordance with accepted procedures for environmental microbiology research.

In a sterile test tube, a 1.0 g aliquot of each environmental sample was suspended in 10 mL of sterile distilled water. To guarantee homogeneity, the suspension was vortexed for one minute. In accordance with accepted microbiological procedures, sterile distilled water was used as the diluent in serial ten-fold dilutions up to 10^{-8} . 0.1 mL aliquots were aseptically transferred onto sterile Petri dishes containing carbon-enriched Nutrient Agar (NA) medium from the 10^{-2}, 10^{-4}, 10^{-6}, and 10^{-8} serial dilutions. The carbon-enriched NA medium contained the following ingredients (per liter): 6.0 g of Na_2HPO_4, 3.0 g of KH_2PO_4, 1.0 g of NH_4Cl, 0.5 g of NaCl, 0.05 g of yeast extract, 17.0 g of agar, and 10.0 g of glucose. Before sterilization, the medium's pH was brought to 7.0 [7,8,11]

This medium was created especially to encourage the growth of microorganisms that produce polyhydroxyalkanoate (PHA) in a targeted manner.

Under strict aseptic conditions, each dilution was plated twice using the spread plate method. The plates that had been inoculated were kept at 37 °C for 48 hours.After incubation, separate, well-isolated colonies were chosen and grown on new NA plates to get pure isolates.

2.2 Screening Of PHAs Producing bacteria

Sudan Black B staining was used to do a first test on the bacterial colonies after they had been incubated for 24 hours to see if they could make polyhydroxyalkanoate (PHA). A 0.02% (w/v) alcoholic solution of Sudan Black B was poured over the agar plates that had the bacterial colonies on them. The plates were left alone at room temperature for 20 minutes so that the staining could happen properly. After that, the extra dye was carefully poured out without disturbing the colonies.[12] After that, the plates were gently rinsed with pure ethanol to get rid of any dye that wasn't bound to them. Colonies that could make PHAs kept the dye and looked bluish-black, which showed that PHA granules were building up inside the cells. On the other hand, colonies that didn't make PHAs didn't keep the stain and looked white.[1]

As per the protocol described by[14] , bacterial isolates that tested positive after the initial screening with Sudan Black B were then confirmed to accumulate polyhydroxyalkanoate (PHA) using the viable colony staining method with Nile Blue A. In order to create a carbon-rich environment that is conducive to the production of PHA, each of the Sudan Black B-positive isolates was aseptically streaked onto a separate sterile Petri plate that contained nutrient agar enriched with 1% (w/v) glucose. Furthermore, 0.5 µg/mL of Nile Blue A was added to the medium. Viable colonies were able to form because the plates were incubated under the right growth conditions. Under UV light, the existence of intracellular PHA granules was later verified because Nile Blue A selectively binds to PHAs and fluoresces when excited, making it possible to visually identify and distinguish PHA-producing strains. The ability of bacterial isolates to accumulate polyhydroxyalkanoates (PHAs) was assessed by additional analysis after they tested positive for Sudan Black B staining. These isolates were streaked onto nutrient agar plates supplemented with 1% glucose, a carbon-rich substrate, and 0.5 µg/mL of Nile blue A stain. Because of its capacity to bind selectively to intracellular PHA granules, nile blue A has been proven to be a sensitive and selective fluorescent dye for the identification and differentiation of PHA-producing bacteria.[1] The plates were exposed to ultraviolet (UV) light after incubation, which made it easier to see the PHA granules inside the bacterial cells. Under UV light, colonies that were accumulating PHAs showed a pronounced bright orange fluorescence, which was explained by the interaction of intracellular PHA inclusions and Nile blue A. Semi-quantitative differentiation between PHA-positive and PHA-negative bacterial colonies was made possible by the positive correlation between the fluorescence intensity and the intracellular PHA content.[1,15] Isolates demonstrating intense bright orange fluorescence were therefore identified as potential PHA-accumulating strains. These selected colonies were subsequently sub-cultured by streaking onto fresh nutrient agar plates to obtain pure cultures for further characterization and study. This fluorescence-based screening method provided a rapid, reliable, and non-destructive approach for identifying and isolating bacteria capable of synthesizing and storing PHAs intracellularly, thereby facilitating subsequent biochemical and molecular analyses.[1]

2.3 Biochemical Analysis of Polyhydroxyalkanoate-Producing bacteria.

Based on the properties of their cell walls, bacterial isolates were distinguished using Gram staining. After being heat-fixed on sanitized glass slides, the smears were successively stained with crystal violet for one minute and then treated with iodine for an additional minute. After a brief ethanol decolorization, the smears were counterstained for 30 seconds with safranin. The 100× oil immersion objective was used to view the stained preparations under a light microscope. Gram-negative bacteria absorbed the safranin counterstain and turned pink or red, while Gram-positive bacteria kept the crystal violet-iodine complex and looked blue or violet[16] . To perform the catalase test, a drop of 3% hydrogen peroxide was added to a tiny piece of the bacterial colony that had been placed onto a sterile glass slide. The organism's capacity to break down hydrogen peroxide into water and oxygen was confirmed by the instantaneous bubble formation, which signified a positive catalase reaction [16]. Bacterial isolates were added to MR-VP broth and incubated for 48 hours at 35 °C in order to perform the Methyl Red (MR) test. Each tube received five drops of methyl red indicator following incubation. A positive MR reaction, indicating mixed acid fermentation, was indicated by the appearance of a red color, whereas a negative result was indicated by the appearance of a yellow color .[17]After streaking the isolates onto starch agar plates and incubating them for 48 hours at 37 °C, the starch hydrolysis was evaluated. The plates were flooded with iodine solution following the incubation period. Extracellular amylase activity caused starch to hydrolyze, as evidenced by the clear halo that surrounded the bacterial colonies. [17] Isolates were streaked onto cetrimide agar slants and incubated for up to seven days at 35–37 °C in order to evaluate cetrimide resistance. Every day, the plates were checked for pigment production and growth. Pseudomonas aeruginosa is known to be resistant to cetrimide, as evidenced by bacterial growth on this selective medium.[16,17] Peptone water containing 1% glucose, sucrose, or mannitol was used in the tests for carbohydrate fermentation. A Durham tube was added for each test to monitor the production of gas. For 24 to 48 hours, inoculated tubes were incubated at 37 °C. The broth turned yellow, indicating the production of acid, and a bubble appeared in the Durham tube, indicating the formation of gas.[18] Nutrient gelatin tubes inoculated by stabbing with the test organism were used to assess gelatin hydrolysis. For a maximum of seven days, the tubes were incubated at temperatures between 25°C and 37°C. The tubes were cooled in an ice bath after incubation. Even after chilling, the medium's liquefaction verified the organism's capacity to hydrolyze gelatin.[18] The soft agar stab method was used to assess the bacterial isolates' motility. A sterile needle

containing the bacterial culture was used to inoculate semisolid agar tubes, which were then incubated for 24 to 48 hours at 37 °C. The organism's ability to move actively was demonstrated by the diffuse and hazy growth that was seen radiating outward from the stab line, confirming motility.[16,18]

Table 2.1 Common Microbiological Tests

Test	Media/Reagents Used	Incubation Time	Positive Result Indicator
Gram Staining	Crystal violet, iodine, safranin	—	Blue/violet (G+), pink/red (G–)
Catalase Test	3% H_2O_2	—	Bubbling
MR Test	MR-VP broth, methyl red	48h	Red color
Starch Hydrolysis	Starch agar, iodine	48h	Clear zone
Cetrimide Agar	Cetrimide agar	Up to 7 days	Growth, pigment
Sugar Fermentation	Peptone water + sugar, Durham tube	24–48h	Yellow (acid), gas (bubble)
Gelatin Hydrolysis	Nutrient gelatin	Up to 7 days	Liquefaction
Motility Test	Semisolid agar	24–48h	Diffuse growth

2.4 Fermentation Procedure for the PHA Production

Polyhydroxyalkanoates (PHA) were produced by bacterial isolates using a submerged fermentation approach in basal mineral salt medium (MSM). The production medium was formulated with the following components (per liter): urea, 1.0 g; yeast extract, 0.16 g; KH_2PO_4, 1.52 g; Na_2HPO_4, 4.0 g; $MgSO_4 \cdot 7H_2O$, 0.52 g; $CaCl_2$, 0.02 g; and glucose, 10 g. Additionally, 0.1 ml/l of a trace element solution was incorporated, consisting of (in g/l): $ZnSO_4 \cdot 7H_2O$, 0.13; $FeSO_4 \cdot 7H_2O$, 0.02; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.06; and H_3BO_3, 0.06. The pH of the production medium was adjusted to 7.0 prior to sterilization. All media were sterilized using autoclaving at 121°C and 15 psi for 15 minutes.

Seed cultures were prepared by inoculating a loopful of each bacterial isolate into sterile nutrient broth containing 1% glucose. The seed medium composition per liter was: glucose, 10 g; peptone, 3 g; beef extract, 5 g; and sodium chloride, 5 g, with pH adjusted to 7.0. Seed flasks were incubated at 37°C with shaking at 120 rpm until logarithmic phase growth was achieved.

Fermentation was initiated by inoculating the production medium with 2% (v/v) of the prepared seed culture under aseptic conditions using a laminar airflow cabinet. Inoculated flasks were incubated at 37°C in a rotary shaker set at 120 rpm for a total of 120 hours. All conditions were maintained consistently for each bacterial isolate tested. Samples were withdrawn at the end of fermentation for further analyses of biomass and PHA accumulation.[1,18]

2.5 PHA Extraction and Quantification Method

Extraction and measurement of polyhydroxyalkanoates (PHAs) from bacterial cultures involved a carefully structured protocol, designed for both efficiency and selectivity. Starting with a 5ml sample of the production broth, bacterial cells were separated from the liquid by spinning the mixture at high speed (8,500×g, 15 minutes). The leftover liquid was removed, and the dense cell layer was gently rinsed with a buffered saline solution (pH 7.4) to remove any traces of the culture medium.The cleaned cells were then left to air-dry for about two hours, which allowed for precise measurement of their dry mass—a key reference used in later calculations. To specifically isolate PHAs from the dried cells, a combination of chloroform and 4% sodium hypochlorite, each added at 12.5µL per milligram of cell mass, was used. This step involved incubating the mixture at 37°C with gentle shaking for 90 minutes. This process digests almost all cell components except for the PHAs, which are naturally resistant to the chemicals used.[1,19]

After incubation, the mixture was spun again at 6,500×g for 10 minutes at room temperature, causing the formation of different liquid layers. The bottom layer rich in chloroform contained the dissolved PHAs. This layer was carefully separated and transferred to a clean tube. To further purify and collect the PHAs, a mixture of methanol and water (in a 7:3 ratio) was added at five times the volume of the chloroform extract. After mixing and an additional centrifugation at 8,500rpm for 15 minutes, solid PHAs formed as a visible precipitate.

This precipitate was transferred onto a petri plate and left to air-dry overnight, resulting in a pure, dry PHA sample. By weighing this final dried material, researchers could accurately determine the amount of PHAs extracted.[9]

For quantitative analysis, three main calculations were performed:

- **Cell Dry Weight (g/ml):** Total dry mass of all cells divided by the initial sample volume.
- **PHA Concentration (g/ml):** Mass of extracted PHAs divided by the sample volume.
- **PHA Yield (%):** Percentage of cell mass converted to PHA, calculated as:

$$\text{PHA Yield (\%)} = \left(\frac{\text{PHA Concentration (g/ml)}}{\text{Cell Dry Weight (g/ml)}}\right) \times 100$$

This procedure not only ensured the selective extraction and purification of PHAs from bacterial cells, but also provided reliable quantitative data for evaluating production efficiency.

3. RESULT & DISCUSSION

3.2 Isolation of PHAs Producing Bacteria

Three distinct sources of soil samples were gathered: a garden, a fuel pump, and cow dung in the Akota area of Vadodara, Gujarat, India. Using conventional serial dilution and plating methods, thirteen different bacterial isolates were extracted from these samples. (Table 3.1.) Sudan Black B and Nile Blue A staining techniques were then used to perform a preliminaryscreening for the production of polyhydroxyalkanoate (PHA) in these isolates.

Table 3.1 Samples & No. Of Isolates

No.	Samples	Total No. Of Isolates
1	Cow Dung Sample	06
2	Petrol Pump Soil	03
3	Garden Soil	04

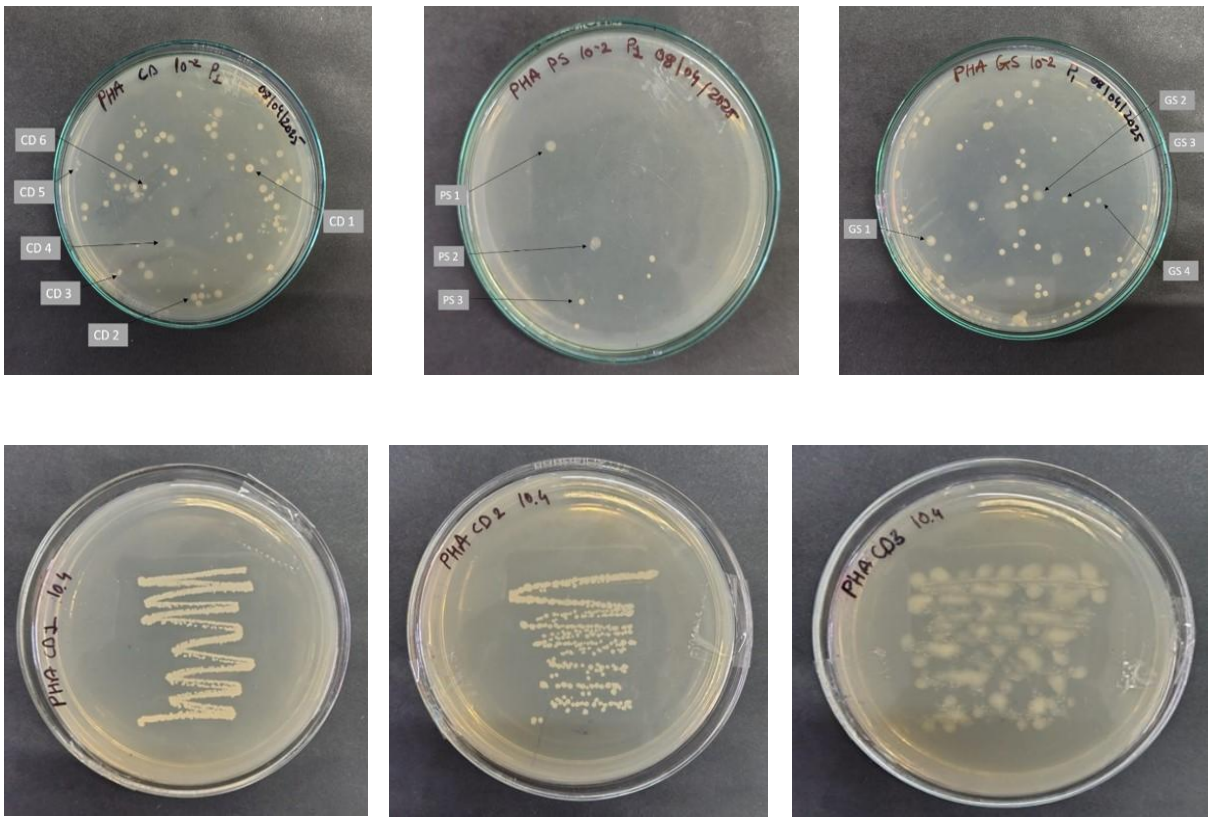


Fig. 3.1.1 Isolation of bacteria from different environmental sample.

3.3 Pure Culture of Isolates

Pure cultures were developed from all the isolates obtained during the study.

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 21s, 2025
https://theaspd.com/index.php

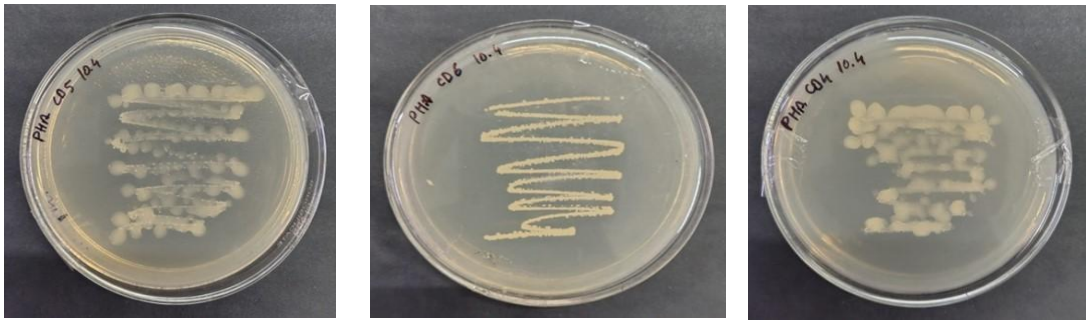


Fig 3.2.1 Pure Culture Of Cow Dung Isolates.

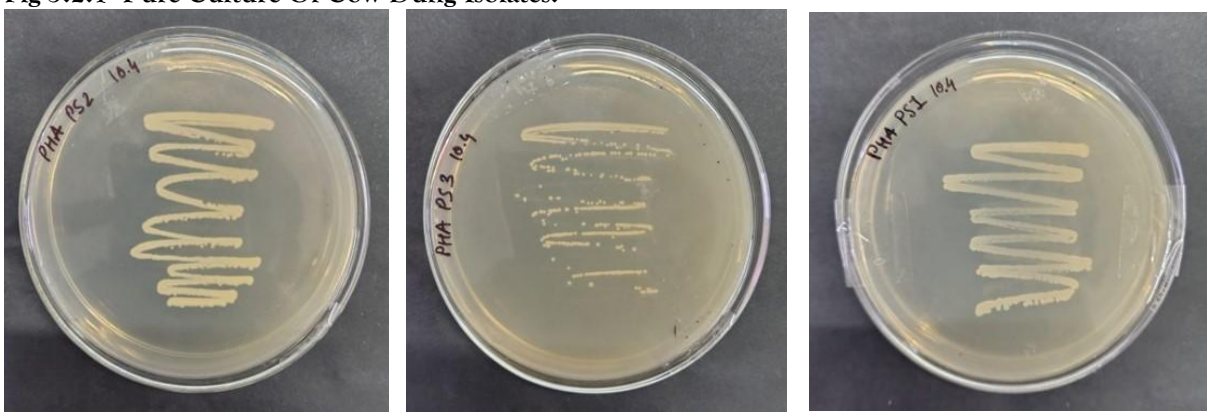


Fig 3.2.2 Pure Culture Of Petrol Pump Soil Isolates.

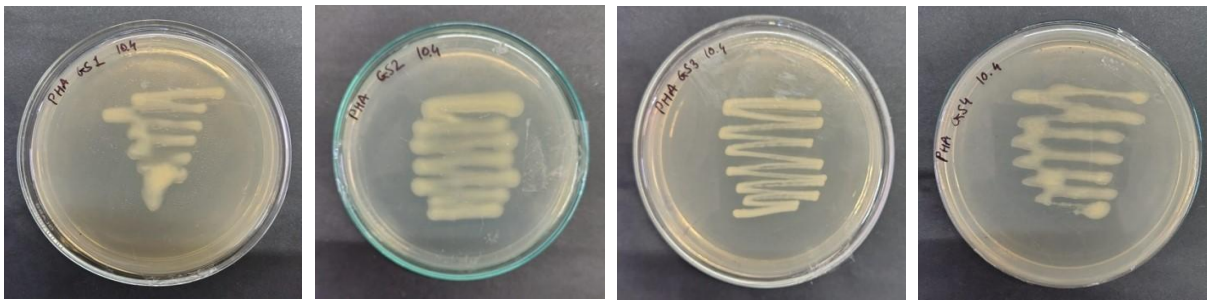


Fig 3.2.3 Pure Culture Of Garden Soil Isolates

3.4 Screening Of PHA Producing bacteria.

Sudan Black B dye staining was used to screen all of the obtained bacterial isolates for the production of polyhydroxyalkanoate (PHA).

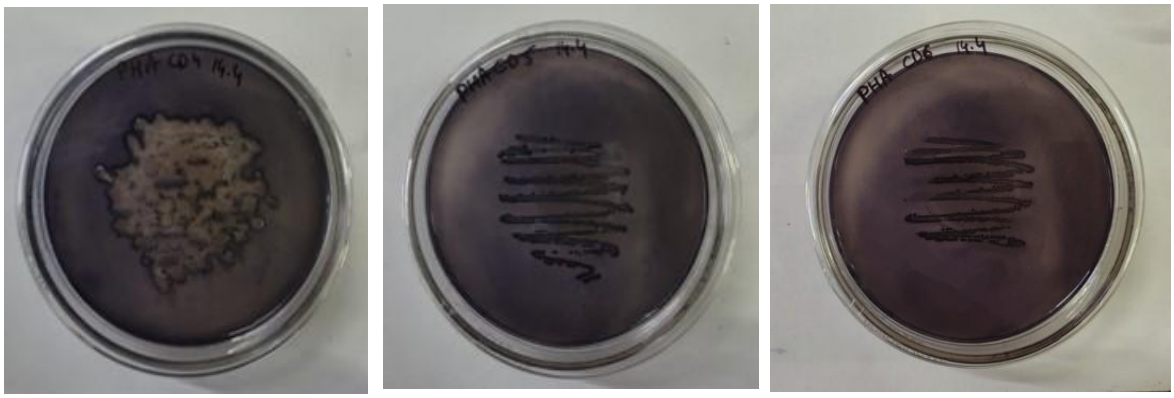


Fig. 3.3.1 Sudan Black Screening of Cow Dung Isolates

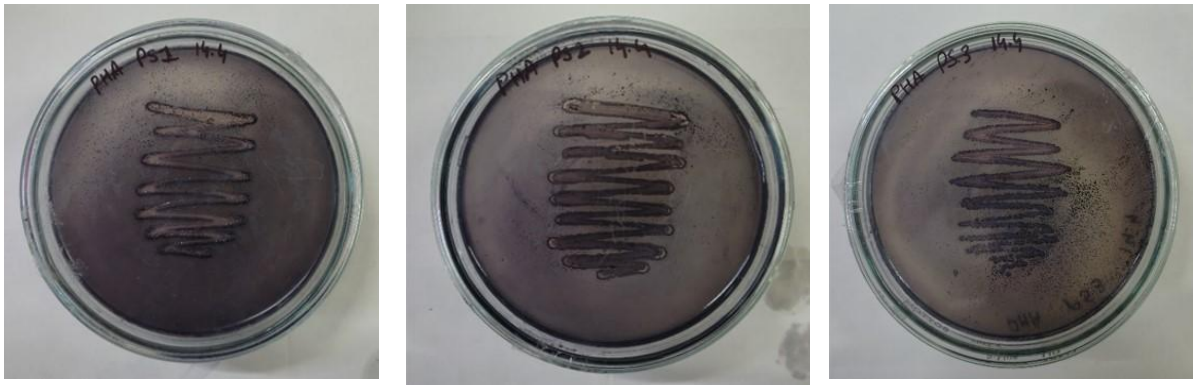


Fig. 3.3.2 Sudan Black Screening of Petrol Pump Isolates

Fig. 3.3.3 Sudan Black Screening of Garden soil Isolates

Table No. 3.2 Sudan Black Screening Result

Isolate	Result
CD1	Positive
CD2	Positive
CD3	Positive
CD4	Negative
CD5	Positive
CD6	Positive
PS1	Positive
PS2	Positive
PS3	Positive
GS1	Negative
GS2	Negative
GS3	Negative
GS4	Negative

International Journal of Environmental Sciences
ISSN: 2229-7359
Vol. 11 No. 21s, 2025
https://theaspd.com/index.php

To further screen for PHA isolates, Sudan Black Screening positive isolates were streaked on carbon-enriched nutrient agar plates with Nile blue stain.

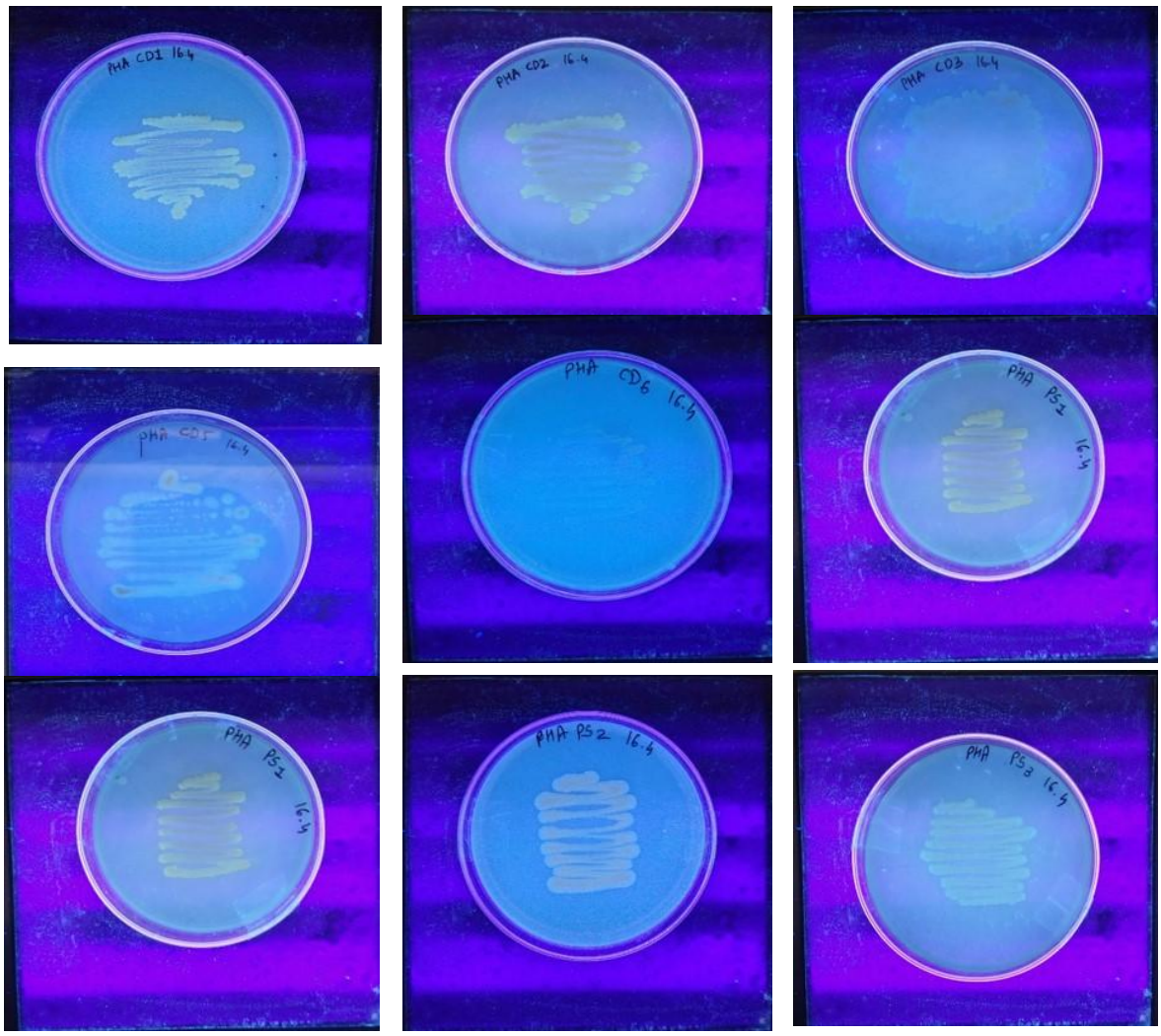


Fig. 3.3.4 Nile Blue Screening of bacterial isolated giving positive results with Sudan Black Screening.

Table No. 3.3 Nile Blue Screening Result

Isolate	Result
PS1	Positive
PS2	Positive
PS3	Positive
CD1	Positive
CD2	Positive
CD3	Negative
CD5	Positive
CD6	Negative

Two isolates (CD3 and CD6) tested positive with Sudan Black B staining but negative with Nile Blue A staining. Since Sudan Black B stains all types of lipids non-specifically, while Nile Blue A exhibits fluorescence specifically in the presence of polyhydroxyalkanoates (PHAs), the positive result observed with Sudan Black B may be attributed to the presence of other intracellular lipids rather than PHAs.

3.5 Biochemical Characterization Of PHA producing bacteria.

The biochemical characterization data of all the isolates that tested positive for PHA production are presented below.

Table No. 3.4. Biochemical Characterization Of PHA producing Isolates.

Test	CD1	CD2	CD5	PS1	PS2	PS3
Gram stain	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Catalse	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve
Methyle Red	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve
Starch Hydrolysis	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Cetrimide	-Ve	-Ve	-Ve	-Ve	-Ve	-Ve
Sugar Fermnentation (Glucose)	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Sugar Fermnentation (Sucrose)	+Ve	+Ve	+Ve	+Ve	+Ve	+Ve
Sugar Fermnentation (Mannitol)	+Ve	+Ve	+Ve	-Ve	+Ve	-Ve
Gelatin Hydolysis	+Ve	+Ve	-Ve	+Ve	+Ve	+Ve
Casein Hydrolysis	+Ve	-Ve	-Ve	+Ve	-Ve	-Ve
Motility	Non-Motile	Motile	Non-Motile	Non-Motile	Non-Motile	Non-Motile

3.6 Quantitative Analysis of PHA Production from bacteria.

Table No.3.5 Cell dry weight, PHA Concentration and PHA Yield

Cell Dry Weight

Strain	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.
PS 1	0.053	0.08	0.065	0.023	0.066
PS 2	0.043	0.05	0.035	0.026	0.043
PS 3	0.063	0.055	0.051	0.033	0.036
CD 1	0.036	0.053	0.086	0.065	0.046
CD 2	0.055	0.076	0.06	0.03	0.06
CD 5	0.018	0.063	0.035	0.025	0.038

PHA Concentration

Strain	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.
PS 1	0.006	0.01	0.013	0.013	0.028
PS 2	0.005	0.01	0.013	0.013	0.016

PS 3	0.005	0.008	0.01	0.008	0.016
CD 1	0.005	0.008	0.01	0.011	0.02
CD 2	0.003	0.01	0.015	0.013	0.018
CD 5	0.001	0.008	0.011	0.015	0.021

PHA Yield

Strain	24 Hrs.	48 Hrs.	72 Hrs.	96 Hrs.	120 Hrs.
PS 1	11.32	12.5	20	56.52	42.42
PS 2	11.62	20	37.14	50	37.2
PS 3	7.93	14.54	19.6	24.24	44.44
CD 1	13.88	15.09	11.62	16.92	43.47
CD 2	5.45	13.15	25	43.33	30
CD 5	5.55	12.6	31.42	60	55.26

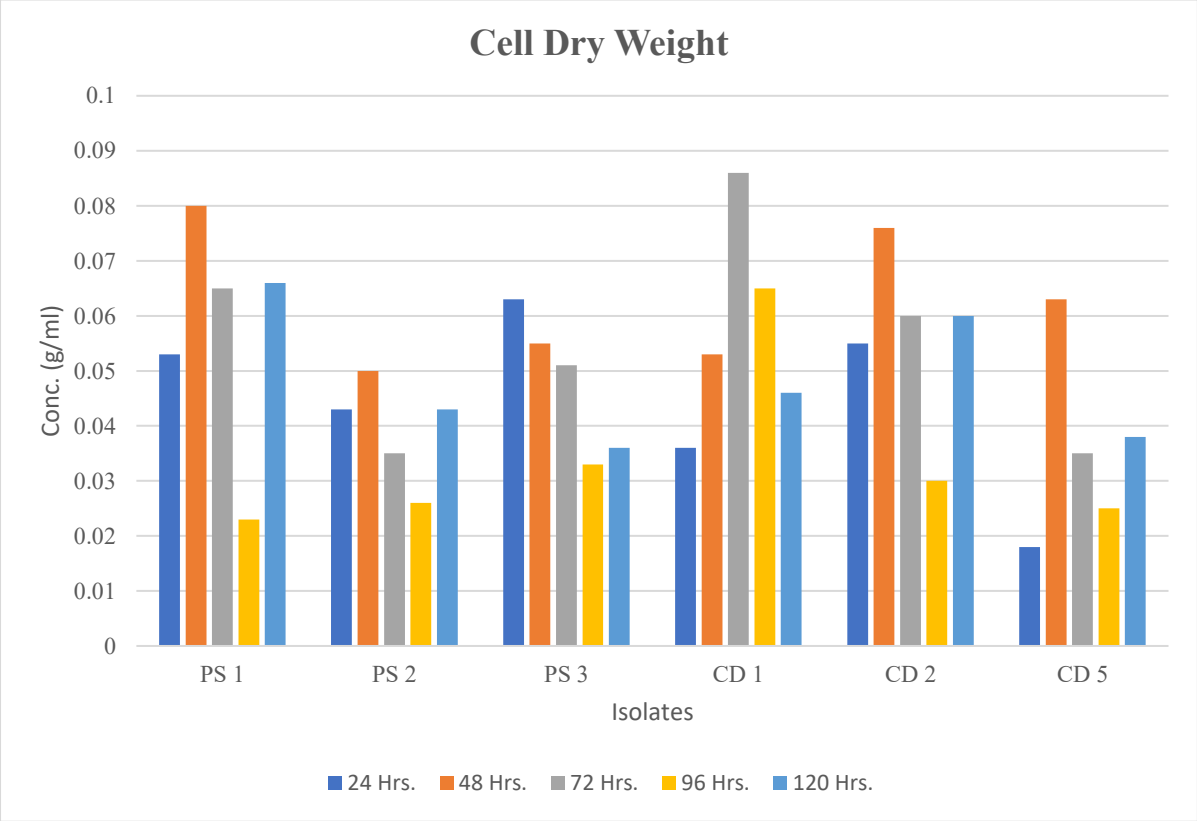


Figure No.3.5.1 Cell Dry Mass Variation During PHA Production

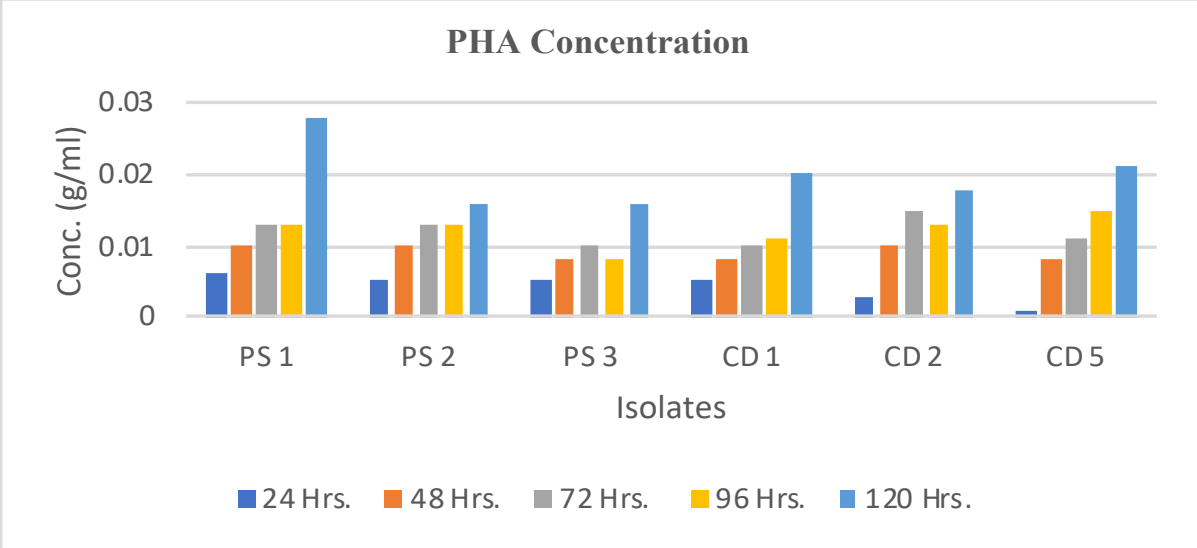


Figure No.3.5.2 Cell Dry Mass Variation During PHA Production

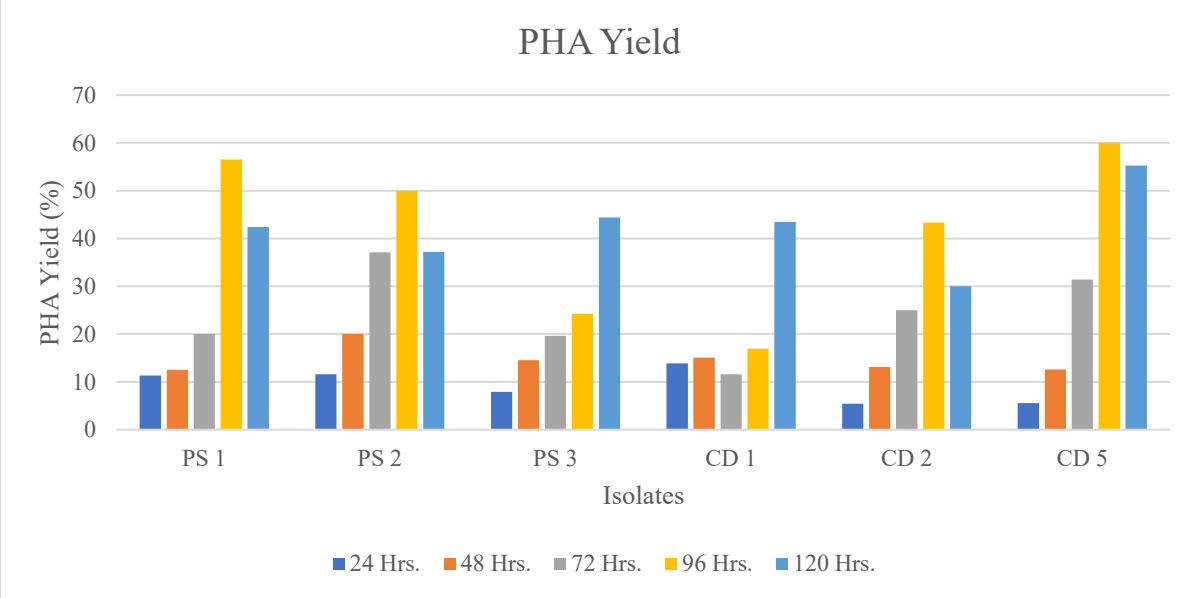


Figure No.3.5.3 PHA Yield During PHA Production

4. DISCUSSION AND COMPARISON WITH SIMILAR STUDIES

The present investigation successfully isolated thirteen morphologically distinct bacterial strains from three environmental matrices: cow dung-enriched soil, garden soil, and petrol pump soil, all sourced from the Akota region of Vadodara, Gujarat. Primary screening for polyhydroxyalkanoate (PHA) biosynthesis was conducted using Sudan Black B staining, a non-specific lipid stain, followed by definitive confirmation with Nile Blue A staining, which selectively binds to PHA granules. Of the thirteen isolates, six were validated as true PHA producers based on fluorescence under Nile Blue A, underscoring the necessity of implementing specific confirmatory methodologies for accurate identification of PHA-accumulating bacteria. This observation aligns with previous reports; Sudan Black B is prone to false-positive results due to the indiscriminate staining of intracellular lipids, whereas Nile Blue A affords superior specificity for PHA inclusions.

The phenotypic and biochemical heterogeneity observed among the Nile Blue A-positive isolates—including variable motility and metabolic traits—substantiates the broad physiological diversity characteristic of PHA-producing taxa, as documented in extant literature. Both Gram-positive and Gram-negative organisms, particularly those affiliated with Bacillus, Pseudomonas, and Alcaligenes genera, are widely recognized as efficient PHA producers inhabiting diverse ecological niches such as soil, aquatic systems, and waste environments. The successful recovery of PHA producers from both nutrient-rich and

stressed ecosystems in this study corroborates their ecological versatility and adaptability, consistent with prior findings by Bhuwal et al. (2013) and Naseem et al. (2024) who reported analogous outcomes from industrial and agricultural substrates.

The combinatorial staining strategy employed here—initial screening with Sudan Black B followed by Nile Blue A confirmation—is extensively validated in the scientific corpus. Studies by Mesquita et al. (2015) and Kansagara & Kothari (2015) have established the heightened reliability of Nile Blue A staining for PHA detection. Congruently, in the present study, two isolates displaying Sudan Black B positivity were negative by Nile Blue A staining, plausibly due to the accrual of non-PHA hydrophobic storage compounds. The employment of glucose-enriched media for preliminary screening is in accordance with standardized protocols; nevertheless, several reports indicate that agro-industrial waste substrates can also support robust PHA biosynthesis, highlighting future opportunities for substrate diversification and process optimization to enhance industrial feasibility.

Quantitative assessment of five Nile Blue A-confirmed PHA-producing isolates was conducted to evaluate biopolymer productivity. Analytical parameters included cell dry mass (CDM), PHA concentration, and PHA yield, the latter quantified as the percentage of PHA relative to total CDM. Strain CD5 exhibited the highest biopolymer productivity, achieving a PHA yield of approximately 55% CDM post 120 hours of incubation. These yield values are commensurate with, or surpass, typical outputs reported for environmental PHA producers, where values in the range of 50–60% are frequently observed; albeit, select optimized strains may reach yields up to 90% under strictly controlled conditions. All of these findings support the value of focused environmental screening in identifying new PHA-producing microbes with beneficial characteristics like substrate flexibility and resistance to process-related stressors, which are critical for the development of sustainable and commercially viable bioplastic production technologies.

5. CONCLUSION

In this study, thirteen bacterial strains were successfully isolated from diverse environmental sources, with the objective of identifying robust polyhydroxyalkanoate (PHA) producers for sustainable bioplastic applications. A two-tier screening approach—employing both Sudan Black B and Nile Blue A staining—was utilized to enhance the specificity of PHA detection. While Sudan Black B staining indicated the presence of lipid inclusions in multiple isolates, only six strains were conclusively confirmed as PHA producers through the selective fluorescence exhibited with Nile Blue A. This discrepancy highlights the necessity of refining classical screening methodologies to improve accuracy in the identification of PHA-accumulating bacteria. Subsequent biochemical characterization of these six PHA-positive strains revealed notable phenotypic diversity, particularly with respect to motility; five isolates were classified as non-motile, whereas one displayed motility. This observed variability underscores the adaptive potential and broad physiological range of environmental PHA producers. Among the confirmed isolates, strain CD5 demonstrated superior PHA accumulation and exhibited favorable production parameters, emphasizing its potential as a model organism for future optimization studies. Collectively, these results contribute to the expanding body of knowledge regarding the diversity of PHA-producing bacteria in natural habitats and establish a solid framework for subsequent research focused on optimizing microbial bioplastic production for both industrial and environmental applications.

Funding/Conflicts of interests if any: The authors declare that there is no conflict of interest regarding the publication of this paper.

REFERENCES

[1.] Shah, S., & Kumar, A. (2021). Production and characterization of polyhydroxyalkanoates from industrial waste using soil bacterial isolates. Brazilian Journal of Microbiology, 52(2), 715–726. https://doi.org/10.1007/s42770-021-00452-z

[2.] Vicente, D., Proença, D. N., & Morais, P. V. (2023). The role of bacterial polyhydroalkanoate (PHA) in a sustainable future: A review on the biological diversity. International Journal of Environmental Research and Public Health, 20(4), 2959. https://doi.org/10.3390/ijerph2004295

[3.] Naseem, A., Rasul, I., Ali Raza, Z., Muneer, F., ur Rehman, A., & Nadeem, H. (2024). Bacterial production of polyhydroxyalkanoates (PHAs) using various waste carbon sources. PeerJ, 12, e17936. https://doi.org/10.7717/peerj.17936

[4.] Thirumala, M., Krishna, E. S., Priya, P. S., & Reddy, S. V. (2022). Isolation and characterization of two PHA producing strains isolated from soil sample of Nalgonda District. Journal of Pure and Applied Microbiology, 16(4), 2738–2746. https://doi.org/10.22207/JPAM.16.4.44

[5.] Fukala, I., & Kučera, I. (2024). Natural polyhydroxyalkanoates—An overview of bacterial production methods. Molecules, 29(10), 2293. https://doi.org/10.3390/molecules29102293

[6.] Naseem, A., Rasul, I., Raza, Z. A., Muneer, F., ur Rehman, A., & Nadeem, H. (2024). Bacterial production of polyhydroxyalkanoates (PHAs) using various waste carbon sources. PeerJ, 12, e17936.
[7.] Cappuccino, J. G., & Sherman, N. (2013). Microbiology: A laboratory manual. Pearson Higher Ed.
[8.] Thakor, N., Patel, B., & Trivedi, U. (2005). Biosynthesis and characterization of polyhydroxyalkanoates produced by Alcaligenes eutrophus. Indian Journal of Biotechnology, 4(3), 276-283.
[9.] Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V., & Yadav, A. (2013). Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes. International Journal of Biomaterials, 2013, Article ID 752821. https://doi.org/10.1155/2013/752821
[10.] Kansagara, L., & Kothari, V. (2015). Sudan Black B and Nile Blue A staining methods for detection of intracellular polyhydroxyalkanoates (PHA) in bacteria. International Journal of Current Microbiology and Applied Sciences, 4(12), 720–729.
[11.] Swathi, K., & Ranjani, N. S. (2015). Production, isolation, screening and extraction of polyhydroxybutyrate (PHB) from Bacillus sps using treated sewage sample. International Journal of Pharmaceutical and Biological Sciences, 5, 58-64.
[12.] Bhuwal, A. K., Singh, G., Aggarwal, N. K., Goyal, V., & Yadav, A. (2013). Isolation and screening of polyhydroxyalkanoates producing bacteria from pulp, paper, and cardboard industry wastes. International Journal of Biomaterials, 2013(1), 752821.
[13.] Mesquita, D. P., Amaral, A. L., Leal, C., Oehmen, A., Reis, M. A., & Ferreira, E. C. (2015). Polyhydroxyalkanoate granules quantification in mixed microbial cultures using image analysis: Sudan Black B versus Nile Blue A staining. Analytica Chimica Acta, 865, 8-15.
[14.] Al-Hamdani, M. A., & Jaber, N. N. (2017). Production of polyhydroxy alkanoates by new strain Bacillus subtilis MANA18.
[15.] Muigano, M. N., Anami, S. E., Onguso, J. M., & Omare, G. M. (2023). The isolation, screening, and characterization of polyhydroxyalkanoate-producing bacteria from hypersaline lakes in Kenya. Bacteria, 2(2), 81-97.
[16.] Bektas, K. İ., Can, K., & Belduz, A. O. (2023). Isolation and screening of polyhydroxybutyrate (PHB) producing bacteria from soils. Biology Bulletin, 50(3), 319-328.
[17.] Saxena, N. K., Tyagi, P., & Sharma, A. (2023). Isolation and screening of gram-positive polyhydroxyalkanoate producing bacterial strains from pharmaceutical industry effluent. Research Journal of Pharmacy and Technology, 16(1), 373-376.
[18.] Devi, A. B., Nachiyar, C. V., Kaviyarasi, T., & Samrot, A. V. (2015). Characterization of polyhydroxybutyrate synthesized by Bacillus cereus. International Journal of Pharmacy and Pharmaceutical Sciences, 7(3), 140-144.
[19.] Hahn, S. K., Chang, Y. K., Kim, B. S., & Chang, H. N. (1994). Optimization of microbial poly-3-hydroxybutyrate recovery using dispersions of sodium hypochlorite solution and chloroform. Biotechnology and Bioengineering, 44, 256–261. https://doi.org/10.1002/bit.260440215