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Isolation And Characterization Of Polyhydroxyalkolates Producing Microbes

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ABSTRACT

Polyhydroxyalkanoates (PHAs) are biopolymers synthesized by numerous microorganisms as carbon and energy storage entities in the cell under conditions of limited nutrients and excess carbon source. These biopolymers are now of great interest for being biodegradable and biocompatible constituents for plastic materials that are considered eco-friendly alternatives for conventional petroleum-based plastics. Accordingly, any effort directed toward the isolating and characterizing of PHA-producing microbes is an important step toward improving the efficiency and commercialization potential of bioplastic production. In this review, we address different techniques used for the isolation of the potential PHA producers from various sources of environmental samples, such as soil and water to marine ecosystems. Standard screening techniques like Sudan Black B staining and Nile Blue A staining alongside modern molecular and genetic tools are discussed for the confirmation. Morphological, biochemical, and molecular characterizations further contribute to the identification of some chemically and physically robust strains that may possess certain industrial advantages. This review therefore emphasizes the importance of traditional culturable and advanced genomic methodologies on the selection of high-yielding putative microorganisms that are tolerant to environmental stressors. Research on PHA-producing microorganisms will thus bring forward greener technologies and eventually allow for the engineering of microorganisms for better biopolymer synthesis.

Keywords: PHAs, Biopolymers, Eco-friendly plastics, Petroleum-based plastics, Sudan Black B staining

1. INTRODUCTION

The continuous accumulation of man-made plastic waste on land and sea has become a major environmental concern [1]. Conventional plastics, derived from petrochemical sources, have been found to be resistant, which requires hundreds of years to completely degrade [2]. Not only does this biodegradation resistance aggravate land and sea pollution but also poses risks to biodiversity, human health, and planetary climate system stability [3].With the responsibility of such urgent issues, there is a strong need to move towards

sustainable, biodegradable options that can reduce environmental effects while ensuring material efficiency[4].

Many microorganisms, including bacteria and archaea, synthesise polyhydroxyalkanoates (PHAs), an important class of biodegradable polymers, especially when there is an abundance of carbon resources and nutritional shortages [5]. PHAs are intracellular storage molecules that store energy and carbon. They can be extracted and converted into bioplastics with a range of physicochemical characteristics [6]Of these, polyhydroxybutyrate (PHB) is the most studied and economically important PHA, but other members like polyhydroxyvalerate (PHV) and polyhydroxyhexanoate (PHH) are also investigated for their specific uses [7,8].

PHAs have many advantages over conventional polymers. They are made from renewable resources such industrial waste streams, municipal residues, and agricultural residues [11], and they are biodegradable in a variety of media [9] and biocompatible for use in medicine [10]. Depending on the microbial strain and substrate, PHAs can be tailored in terms of mechanical strength, thermal stability, and rate of breakdown [12,13].

Isolation and characterization of PHA-producing bacteria is a critical step in the creation of efficient economic bioplastic production systems [14] Natural environments like soil, sea, sewage, and compost possess heterogeneous microbial reservoirs of diversity, which can be used for effective and novel PHA producers [15,16]. Isolation of productive strains increases not just the efficiency of the production process but also diversity of raw material and operating conditions on which PHAs can be produced [17,18].

The current review aims to provide an overview of the strategies and methods used for the isolation and characterization of PHA-producing bacteria. It addresses traditional as well as modern techniques of bacterial screening, molecular identification, and analytical techniques for the detection and estimation of PHAs. Additionally, it discusses briefly recent developments and future prospects in microbial PHA research and emphasizes the integration of biotechnology with sustainable development goals to address plastic pollution [19,20].

2. PHA producing microbes and its application

Polyhydroxyalkanoates (PHAs) are a family of microbial polyesters that might serve as increasingly significant alternative biodegradable and renewable substitutes for ever-scarcer petrochemical plastics. These biopolymers synthesized by a variety of microorganisms are then stored intracellularly as reserve compounds during starvation of nutrients, particularly under conditions of excess carbon and shortage of nitrogen, phosphorus, or oxygen. Indeed, PHAs are stored in the cytoplasm as insoluble granules and metabolized by bacteria during starvation, and when the environment changes-accompanies this response from the metabolic natural of ever-evolved microbes.

Because of their considerable properties like biodegradability, biocompatibility, and non-toxicity, PHAs have gain much attention over the past decades. Indeed, such polymers have bright promises as the green and renewable alternative to petroleum-based plastics and thus could provide key solutions to the current pollution-wide challenge with plastics (Chen, 2009). Furthermore, PHAs can be synthesized from a wide range of renewable raw materials such as agricultural residues or by-products from food processing, proving at the same time economic and environmental feasibility, given that they can consider easy-cost carbon substrates.

PHAs are produced by bacteria belonging to different species. These include Cupriavidus (previously known as Ralstonia), Pseudomonas, Bacillus, Alcaligenes, etc. (Keshavarz & Roy, 2010). They show metabolic diversity and can utilize a wide variety of carbon substrates, for example, simple sugars, fatty acids, alcohols, and sometimes even complex organic wastes. These factors particularly warrant isolation of new and high-yield PHA producers in nature and a characterization of their physiological and metabolic capabilities, which would be necessary for optimizing microbial PHA production for further industrial application.

2.1. Isolation of PHA Producing microbes

Isolation of microorganisms producing polyhydroxyalkanoates (PHAs) is usually done by the sampling of environments rich in microbial and organic matter such as soils, compost heaps, municipal solid waste dumps, activated sludge from wastewater treatment facilities, and marine sediments. These environments have been shown to contain bacteria that have adapted to grow under nutrient-limited but carbon-rich conditions and are thus potential PHA producers (Lee, 1996). These environments are dynamic microbial communities that immerse microbes in a variety of organic substrates and stressors.

To selectively stimulate and enrich potential PHA producers, environmental samples were incorporated into enrichment media supplemented with supplements of high carbon sources, such as glucose, fructose, molasses, or vegetable oils, while depriving nitrogen or phosphorus. This imbalance nutrient mimics the natural stressful situations that trigger microbes to accumulate PHA as a survival strategy (Keshavarz & Roy, 2010). This stage assists in the enrichment of that microbial population which possesses metabolic bias towards accumulating

PHAs.After the enrichment, the cultures were serially subcultured onto nutrient-poor agar plates for the isolation of individual colonies. To assess for PHA production, colonies were stained with dyes such as Sudan Black B and Nile Blue A, which are concentrated selectively in intracellular PHA granules. Thus, examining the cultures by light microscopy, Sudan Black B will stain the PHA granules as black or dark blue inclusions, while Nile Blue A will fluoresce strongly in UV light and facilitate their identification (White et al., 1994). These qualitative staining techniques are quick, simple, and efficient for high-throughput screening of many bacterial cultures for PHA accumulation.

Following staining, presumptive PHA-producing colonies are further tested for PHA production and subculture for purity. Next, further physiological, biochemical, and molecular characterizations can be performed on the isolates to determine their efficiencies, substrate specificities, and suitability for PHA manufacturing at a commercial scale. The isolation and identification of new strains from natural and extreme habitats help enhance accessibility to PHA-producing organisms, thus enhancing the overall potential of microbial bioplastic production systems.

2.2. Characterization of PHA Producing Microbes

The next important step after identifying the potential PHA-producing bacterial isolates via primary screening is extensive characterization of these isolates that would ensure authentication of PHA accumulation and their taxonomic and physiological characteristics, which would subsequently prove the significance of characterization for the purposes of establishing not just the ability of the strain to produce polymer but also its commercial potential.

Microscopic analysis forms the basis to verify intracellular PHA accumulation. Sudan Black B, which stains PHA granules very darkly within the cytoplasm when viewed with a light microscope, is an example of lipid-specific dyes usually employed for such analysis to enable rapid visual identification of PHA granules. Nile Blue A is more specific because, when immobilized on PHAs and irradiated by UV, it gives bright fluorescence and can then be detected more precisely by fluorescence microscopy (Sudesh et al., 2000). These methods really help in distinguishing between high and low producers of PHA at an early stage.

In addition to qualitative microscopy, quantitative solvent-extraction measures are also applied for the measurement of PHA content. Due to the lack of earlier methods discussed, usually, the biomass of the bacterium is harvested through centrifugation or filtration for drying purposes before organic solvents such as chloroform are used to extract the intracellular PHAs from the biomass. The polymer thus extracted is then collected, dried, and weighed to estimate total PHA content by gravimetric method. It is analyzed further on its recovery with methanolysis to produce methyl esters and later analyzed chromatographically through gas chromatography (GC). This technique provides information on the monomer composition, concentration, and purity of the PHAs. Such type of information is vital in establishing the value of a particular strain for specific bioplastic applications. Anderson & Dawes, 1990.

Culture and characterization of bacterial isolates are based on most of classical methods, however, molecular techniques such as the sequencing of the 16S ribosomal RNA (rRNA) gene are now popular methods of identification and classification of bacterial isolates. The method allows for a correct taxonomic placement by comparison of the nucleotide sequence of the isolate's 16SrRNA gene with those in databases of previously known species of bacteria (Lee, 1996). The phylogenetic trees generated using this information will help in the understanding of evolutionary relationships besides indicating potential novel strains.

Polyhydroxyalkanoate (PHA) polymers possess some physicochemical characteristics: Fourier-transform infrared spectroscopy (FTIR) and nuclear magnetic resonance (NMR) are among the most commonly employed methods for elucidating properties of the substances themselves. FTIR is used to find functional groups in the polymer to define its chemical nature, and NMR gives direct access to the monomer structure in the polymer. All analyses make it possible to make differentiation among the various classes of PHAs such as homopolymer-like poly(3-hydroxybutyrate) [P(3HB)] and copolymer, including poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-co-3HV)] (Chen, 2009).

2.3. Optimization and Industrial Relevance

Potential metabolic activity of PHA biosynthetic microorganisms varies considerably among species, strains, and environmental circumstances. Maximizing growth parameters becomes, therefore, imperative when producing polymers and for the whole productivity. It is thus important to precisely fine-tune factors such as carbon source type and concentration, nitrogen or phosphorus levels, pH, temperature, aeration rate, and incubation time for all Keshav properties (Keshavarz & Roy, 2010). Small-scale laboratories shake flasks are first developed to test these modifications before being scaled up into bioreactor models imitating industrial environments.

Carbon source has a straightforward impact on PHA yields and contents. Microorganisms can usually utilize inexpensive carbon sources such as molasses, glycerol (a waste by-product in biodiesel production), starch, vegetable oils, and even lignocellulosic hydrolysates. Such raw materials not only diminish production costs but also promises sustainable waste treatment. Some bacterial strains have the ability to grow using agricultural waste and food residues; this is a way that can be environmentally friendly and economically competitive for large-scale production of PHA (Chen et al., 2018).

Some research areas have focused on fed-batch fermentation and continuous fermentation processes to prolong microbial action and increase PHA synthesis. Such approaches improve biomass concentrations and overall yields of polymers compared to ordinary batch culture. Genetic modifications and metabolic pathway optimization have also been employed for the alteration of the present strains or to establish recombinant strains that exhibit enhanced carbon assimilation and incorporation of PHA (Madison & Huisman, 1999).

On an industrial scale, PHAs are fast becoming popular, considering their mechanical flexibility and tailorability. The physical characteristics of PHAs such as tensile strength, crystallinity, and elasticity change with the types of monomers used. These properties can be controlled either by fermentation conditions or choice of microbial strains which natively produce different types of PHAs (Sudesh et al., 2000).

2.5. Industrial significance of PHAs includes a wide range of applications:

-Biodegradable disposables and packaging.

-Medical applications such as sutures, implants, drug carriers, and scaffolds for tissues.

-Agricultural uses like slow-release fertilizers and mulching film.

Because of their inherent biocompatibility and degradation products that are non-toxic, PHAs are very promising in some biomedical applications. With increasing demand for green construction materials worldwide, there has been more investment in technology by industries that use PHAs from microbial fermentation as substitutes for petrochemical or petroleum plastics.

solate	Organism	DWC (g/L)	PHA Concentration (g/L)	PHA Content	PHA Yield (g/g)
ME31	Bacillus safensis strain Ter61	2.881	0.151	5.24%	0.008
ME32	Bacillus sp. strain CHA410	2.117	0.090	4.25%	0.005
ME33	Bacillus pumilus strain 37	1.890	0.215	11.38%	0.011
ME42	Rhodobaca sp. strain HJB301	1.984	0.150	7.56%	0.008
ME51	Bacillus sp. E-127	1.678	0.110	6.56%	0.006
ME54	Bacillus sp. strain JSM 1684086	0.935	0.085	9.09%	0.004
ME81	Bacillus sp. E-127	1.718	0.150	8.73%	0.008
ME82	Bacillus sp. ISO_06_Kulunda	1.610	0.196	12.17%	0.010
ME88	Bacillus sp. ISO_09_Wadi-Natrun	0.877	0.080	9.12%	0.004
MO12	Bacillus sp. KVD-DM52	1.025	0.090	8.78%	0.005
MO15	Bacillus sp. KVD-DM52	1.043	0.078	7.48%	0.004
MO22	Bacillus sp. strain JSM-1684023	1.515	0.290	19.14%	0.015
MO25	Rhodobaca sp. strain ZN9W	2.619	0.108	4.12%	0.005
MS42	Bacillus sp. ISO_06_Kulunda	1.292	0.170	13.16%	0.009
MS44	Exiguobacterium aurantiacum GBRS02	1.888	0.144	7.63%	0.007
MS52	Bacillus sp. ISO_06_Kulunda	1.305	0.095	7.28%	0.005
MW27	Bacillus sp. A-09	3.506	0.275	7.84%	0.015
MW52	Bacillus sp. BAB-1831	0.877	0.055	6.27%	0.003
MW54	Bacillus sp. (in: firmicutes)	1.458	0.064	4.38%	0.003
SE42	Paracoccus sp. TMN-21-1	1.130	0.079	6.99%	0.004
SE83	Exiguobacterium sp. QZS4_8	1.411	0.062	4.39%	0.003
SE84	Arthrobacter sp. strain C15	0.689	0.052	7.55%	0.003
SE89	Paracoccus sp. TMN-21-1	1.082	0.085	7.86%	0.004
SE93	Bacillus sp. ISO_06_Kulunda	1.360	0.151	11.10%	0.008
SO13	Bacillus sp. 01105	1.194	0.078	6.53%	0.004
SO31	Bacillus subtilis strain OTPB28	1.401	0.052	3.71%	0.003
SO75	Bacillus sp. strain FA2-253	1.232	0.082	6.66%	0.004
SW32	Bacillus pumilus strain 37	1.772	0.235	13.26%	0.012
SW36	Halomonas alkalicola EXT	2.412	0.397	16.46%	0.020
SW38	Bacillus sp. strain MEB205	1.942	0.189	9.73%	0.009
SW62	Bacillus subtilis strain QD9	3.520	0.188	5.34%	0.009

Table 1:- Preliminary production of polyhydroxyalkanoates by bacteria isolated from Lakes Simbi and Magadi. DCW and PHA concentrations represent means of triplicates. DCW = dry cell weight; PHA content = % of DCW. Isolates designated S and M were isolated from Lake Simbi and Lake Magadi, respectively.(41)

3. Methods used in isolation and characterization of PHA producing microbes

3.1 Sample Collection

Soil samples from different waste-related environments were collected for the possible isolation of PHA-producing microorganisms. The sites for the study included agricultural waste sites, food waste dump sites, and industrially polluted soils-all of which had observable organic waste deposits and a documented history of waste deposition.

Sterile spatulas were used to take aseptic soil samples at a depth of 5-10 cm. About 100 grams of soil from each point were then scooped into sterile polythene bags. Multiple points were taken from each site so that their combinations gave composite samples that represented the greatest possible diversity of microbial distribution.

Samples immediately transported to laboratory in insulated containers, in order to minimize changes in microbial activity. The samples were processed within 24 hours after storing them at 4°C upon arrival.

Very strict aseptic techniques were observed during sampling. Instruments and gloves used were sterile to prevent external contamination. Also, every single sample was properly labeled with the identity of the site, date, time of collection, as well as important environmental conditions.

3.2 Isolation of Pure Cultures

In the laboratory, 1 gram of all soil samples were suspended in 9 mL sterile 0.85% saline solution. Serial dilutions to 10^{-6} were made by transferring 1 mL aliquots into new tubes of saline.

100 µL aliquots from the dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were spread uniformly over nutrient agar plates with sterile L-shaped spreaders. Plates were incubated at [30°C/37°C] for 24–48 hours to provide time for the development of colonies.

After incubation, colonies showing clear differences in color, size, margin, elevation, and texture were picked carefully. The colonies were streaked again on fresh nutrient agar plates to isolate pure cultures. Streaking was repeated successively until even colony morphology established the purity of the isolates.

Each purified isolate was stored on nutrient agar slants at 4°C for short-term preservation. Exhaustive records were maintained with a codification of each isolate systematically according to its location and colony number. Preliminary observations on the colony morphology were recorded in great detail to aid identification in the future.

3.3 Screening for PHA-Producing Microbes

PHA-producing micro-organisms are screened

All pure isolates were inoculated into nutrient broth and shaken (150 rpm) for 24 hours; this was done to provide active inoculum cultures.

In the PHA production screening, isolates were inoculated in a specialized PHA induction media, containing limited nitrogen and excess sources of carbon such as glucose, starch, or molasses, for PHA accumulation.

After incubation, 1ml cultures were centrifuged and the pellets spread onto clean glass slides and thereafter, heat fixed and stained for ten minutes with Sudan Black B solution. The excess stain was then wiped off with xylene and the slides counter-stained using safranin.

At 100x oil immersion microscopy, intracellular black or dark blue granules were identified as PHA producers. Isolates that showed clear granules of PHA were shortlisted for further biochemical and morphological characterization.

To verify the staining procedure, known PHA-producing and non-PHA-producing strains were employed as positive and negative controls, respectively.

3.4 Biochemical characterization of isolates

Shortlisted isolates were investigated using a series of primary biochemical tests to enable preliminary identification:

Gram Staining was used to determine the Gram nature and cell morphology.

Catalase Test entailed the addition of 3% hydrogen peroxide to a smear of bacteria; formation of bubbles showed a positive result.

Oxidase Test utilized oxidase reagent, while a dark purple color change indicated a positive result.

Indole Production Test was carried out in tryptone broth, and a red layer upon addition of Kovac's reagent showed indole production.

Methyl Red (MR) and Voges-Proskauer (VP) Tests differentiated the glucose fermentation pathways; a red color in MR indicated mixed acid fermentation, while VP positivity showed production of acetoin.

The Citrate Utilization Test was performed to check for utilization of citrate as the only C-source, growth, and color change to blue on Simmon's citrate agar.

Starch Hydrolysis Test

The starch agar plate is inoculated with an isolated test organism, streaking with a sterile inoculating loop in a single straight line.

Incubate the plate for 24 to 48 hours between 35 and 37 °C.

After incubation, flood the plate with an iodine solution and let it stand for some minutes.

Observation of the plate for clear zones surrounding bacterial growth is then made.

The biochemical identification keys (e.g., those presented in the Bergy Manual of Systematic Bacteriology) were then used to interpret the experimental results. These tests aided in limiting the identification of candidate PHA-producing genera for further in-depth studies.

3.5 Morphological Characterization

The morphological characterization matched the biochemical findings.

At the colony level, observations made included size (small, medium, large), shape (circular, irregular), color (white, yellow, cream, etc.), margin (entire, undulate), elevation (flat, raised, convex), and surface texture (smooth, rough, wrinkled).

Microscopically, Gram-stained preparations were examined to identify the cellular shape (cocci, bacilli, spirilla) and arrangement (single, paired, chains, or clusters).

Motility was determined by both the hanging drop technique and motility agar. Cells that were non-motile did not move, whereas motile organisms had direction or tumbling motion.

All morphological findings were documented in tabular format and kept photographic records of the stained slides and colony types for future reference and comparative study.

3.6 Ethical Considerations

All experimental manipulations followed routine biosafety practices to maintain laboratory safety and environmental stewardship. Soil sampling was done with minimal disruption to the local ecosystems, and biohazardous waste produced during research was disposed of in accordance with established guidelines.

3.7 PHA Extraction

Cultivation for Biomass Production:

The isolates are recovered in a medium optimized for the production of PHA and under continuous shaky condition at 30°C for 48 hours-72 hours for biomass accumulation and PHA biosynthesis.

Harvesting of Cells:

After the end of the incubation period, the cultures were harvested by centrifugation at 8000 rpm for 10 minutes and the cell biomass was collected. These cell pellets were washed with sterile distilled water to remove any media components which are left over.

Cell Disruption and PHA Recovery:

Such washed pellets were treated with sodium hypochlorite solution within 10-15%(v/v) and kept at room temperature for 1-2 hours to lyse the cells and release PHA granules stored intracellularly.

PHA Extraction:

The suspension obtained was centrifuged again at 8000 rpm for 10 minutes to get the pellet, rich in PHA. This pellet is washed alternately with distilled water, acetone, and ethanol to remove non-PHA impurities. Granules were washed and solubilized in chloroform for further treatment.

PHA Precipitation:

Purified PHA is given by PHA, e.g., through precipitation with cold methanol addition, or by evaporating chloroform under vacuum conditions.

Storage:
The PHA was air-dried under aseptic conditions and kept in sterile vials at room temperature for further characterization.

Precautions:
The whole extraction procedure was performed aseptically, and precautions, such as the use of fume hoods, were taken for the entire duration of the exercise.

CONCLUSION

The isolation and characterization of some PHA-producing microorganisms represent an important foundation for the establishment of eco-friendly bioplastic production systems. Consequently, through strategic screening and identification techniques, novel strains with a high potential for PHA accumulation and favorable growth characteristics might be uncovered. Using traditional staining techniques and molecular tools integratively forms a comprehensive framework for the identification and validation of such efficient producers. Future work should relate to the assessment of extremely unique and poorly explored environments to isolate unconventional microbial candidates. In parallel, synergistically linking metabolic engineering and synthetic biology to classical isolation strategies shows promise in making PHA production economically viable. Science on the whole in this area deserves to prosper to forward the cause of a sustainable and plastic-free future.

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