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Molecular Characterization of polyhydroxybutyrate producers from different mangrove forest soil rhizosphere of Kerala

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ABSTRACT

The ecological impact of non-bio-based plastics has accelerated the demand for natural bio-based resins like polyhydroxyalkanoates (PHAs), biodegradable compounds with industrial and medical applications. This study focuses on the isolation and molecular identification of PHB-synthesizing bacteria from mangrove forest ecosystems in Kerala, India. Thirty-two microbial species were isolated from four mangrove rhizospheric soil sites. Sudan Black B and Nile Blue A staining confirmed lipid inclusions and PHB accumulation in sixteen isolates. Bacillus velezensis, identified through Crotonic acid quantification and 16S rRNA sequence analysis, showed the highest PHB concentration. Phylogenetic analysis revealed Bacillus species dominated the mangrove soil biome. This is the first report of PHB-synthesizing bacteria isolated from Kerala's mangrove forests for potential biopolymer production.

Keywords: Mangroves, Polyhydroxybutyrate, Sudan Black B, Nile Blue A, Crotonic acid

1.INTRODUCTION

The mangrove ecosystem is deemed a rare environment exemplified by fluctuating tidal flows, scarcity of nutrients, hypoxia, high salinity, and ultraviolet radiation. Stressful conditions form reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals, hydrogen

peroxide, and singlet oxygen, which profoundly disrupt the ecosystem. However, mangroves encompass diverse bacterial communities that effectively harness the enzymatic and nonenzymatic scavengers to prevent the impact of detrimental free radicals. Ecological adaptation is the primary evolutionary driver enabling mangrove bacteria to develop specific metabolic pathways that mitigate natural stress. Bacteria thriving in mangrove environments have recently received considerable attraction for discovering novel biomolecules and untapped evolutionary mechanisms. Total genome sequencing and genome prospecting help to understand how bacterial genomes have adapted to new environmental niches. (1)

Plastics are ubiquitous in our everyday lives, transportation, electronics, medical devices, textiles, and packaging. They may take approximately 600 years to degrade fully. Petroleum-sourced plastics have detrimental environmental and social repercussions because of their non-perishable properties and the release of carcinogenic substances when subjected to scratching or heat (2). Overuse of plastics has led to depleting fossil fuels, endangering wildlife, global climate change, garbage buildup, and air and water quality degradation thereby creating harmful effects on all living organisms. (3)

Polymers obtained from renewable bioresources are usually biodegradable and are nontoxic (4). More than three hundred Gram-negative and Gram-positive bacterial species that are capable of producing PHAs have been isolated and identified. (3).

When exposed to nutrient-limiting conditions with abundant carbon, numerous Gram-positive and Gram-negative bacteria can produce PHAs as energy and carbon storage material.(5).

Polyhydroxybutyrate (PHB) is a compostable and biologically compatible thermoplastic synthesized by microorganisms, collectively called polyhydroxyalkanoates (PHAs). By replacing conventional plastics with biodegradable plastics many of the environmental pollution problems can be solved. Being a natural product, PHAs entirely hydrolyzes water and carbon dioxide by assorted microflora in the soil. These molecules can be used as biodegradable transporters of medicines, pest deterrents and plant eradication chemicals. They are additionally employed as osteosclerotic stimulants due to their piezoelectric characteristics, utilized in bone plates, surgical sutures, and substitutes for blood vessels. (6).

Despite the considerable potential of PHA-accumulating bacteria for the industrial production of bioplastics, the significant barrier lies in the elevated expenses associated with the raw materials utilized in the procedure. (7). Marine environment is a good source of polymer

production .Sterilized seawater can be used as the culture medium for marine bacteria in place of a mineral medium, which could lower the expense of PHA manufacturing. (8). Choosing bacterial strains with elevated PHA production capabilities, coupled with fine-tuned culture conditions, can alleviate barriers to cost-effective large-scale biosynthesis. Despite initiating industrial-scale production of this bioplastic many years ago, its widespread manufacturing

continues to face challenges due to factors such as the slow production rate of bacteria and the accessibility of raw materials used in its fabrication, ultimately leading to increased production costs of PHAs. (9). This research highlights the separation and molecular characterization of PHA-synthesizing bacteria from mangrove ecosystems. While possessing significant diversity, mangrove bacteria and their genomes are relatively understudied in comparison to their terrestrial and marine counterparts. The genome studies reflect the unusual characteristics of mangrove-associated bacteria that are exposed to natural selective pressures. The aim of the ongoing research was to isolate PHB-accumulating microbes from different mangrove forests in Kerala and identify the isolates exhibiting peak PHB production.

2.MATERIALS AND METHODS

2.1. Sample Collection

Rhizosphere soil samples were collected using soil probes aseptically in sterile zip lock polybags from four distinct forest mangrove sites in Kerala, India in July 2022. Soil samples were gathered from the mangrove forest of Mangalavanam (9°57'37.0488" N latitude and 76°17'5.4458" E longitude- Sample 1), Payannur (12°05'43.1" N latitude and 75°13'22.8" E longitude – Sample 2), Kumarakom (9°37'53.2" N latitude and 76°25'08.2" E longitude – Sample 3) and Kollam (8°53'45.5" N latitude and 76°35'07.7" E longitude – Sample 4). Quartering method of the sample was done for the appropriate identification of bacteria. The soil samples were labeled with information like location, season, date of collection and the name of the sampler. The soil samples were stored in aseptic plastic bags and were kept at 4°C for further use.

2.2 **Isolation of bacteria**

For each soil sample taken from the Mangrove forests site, 1 gram of soil sample was dispersed in ten milliliters of sterile water and blended at 200 rpm. The samples were then progressively diluted with sterile water to 10-7. From each dilution, 100µl (10) was spread on a specialized agar medium containing the composition with (.3%) Beef Extract, (.5%) Peptone, (.8%) NaCl,

(1%) Glucose, (1.5%) Agar and the streaked plates were kept for growing at an incubator at 37°C for 24-48 hours (11). Morphologically unique colonies were chosen from the plates, and pure cultures of various isolates were stored at 4°C for subsequent screening to produce PHB, with maintenance achieved by sub-culturing the isolates every 4-6 weeks.

2.2. Primary assessment for PHB-producing microorganisms

2.2.1. Sudan Black B Dye method

A total of thirty-two bacterial isolates were evaluated for PHB-producing potential by dyeing the isolates with Sudan-black B dye on petri dishes, according to the procedure by El-Hamshary et al (2018) (12). The isolated bacterial colonies were cultured for 2-3 days at 37^{O}C in modified agar plates. Following incubation, the Sudan black B dye that is dissolved in .3% alcohol was applied evenly onto the bacterial cultures, and the plates were kept for 30 minutes. Later, the colonies were rinsed with ethanol (96%) to eliminate any surplus stain, and the outcomes were then examined. The lipid granules in bacterial isolates take up the stain and show a dark blue-black colour for PHB producers and were selected for further PHB production studies.

The cultures that exhibited positive results on Plate assay were grown and was stained on glass slides for the presence of lipid granules by the Sudan Black B staining method. The smears were prepared on a glass slide and fixed by heating, stained with Sudan black B (0.3% alcoholic solution) for 15 minutes. The slides were washed with xylene under running tap water to remove excess stains. The slides were differentially stained with 0.5% safranin solution for 1 minute and washed. The slides were then observed under oil immersion (11).

2.3. Secondary screening for PHB-producing microorganisms

2.3.1. Nile blue A dye viable colony method

Secondary screening of PHB-producing isolates was performed with Nile blue A using a modified method (13). The Nile blue A dye (25µg/mL) was directly incorporated in nutrient agar medium consisting of 1% glucose, 0.3% beef extract, 0.5% peptone,.8% sodium chloride , and 2% agar).Following the incubation, the cell smears were prepared and were heat fixed. The samples were stained with Nile blue A at 55°C for 10 minutes in a staining jar. The slide was then rinsed with water followed by 8% aqueous acetic acid for 1 minute. The positive isolates showed bright orange fluorescence under epifluorescence microscope at an excitation wavelength of 460nm.The presence of orange viable colonies were the positive indicators for the microorganisms that could be able to a potent PHB producer. Following Nile blue A

staining, colonies that accumulated PHB exhibited vivid orange fluorescence, with fluorescence intensity correlating positively with the bacterial cells' PHB content. The positive strains that showed bright orange fluorescence after Nile blue A viable colony method were selected

2.4. Estimation of PHB content

2.4.1. Crotonic acid assay

For quantitative screening of the accumulation of PHB, the crotonic acid assay was executed as per the modified protocol of Bhuwal et al., 2013 (14). 5 mL of 24 h old cultures (2×10^8 CFU/mL) were seeded to 250 mL Erlenmeyer flask containing 45 mL of the Zobell marine broth (2) and incubated for 72 hours at 37°C and 150 rpm, the extracted PHB was then quantified. Cell dry weight was calculated after centrifuging one OD culture at 8000 rpm at 28°C for 15 minutes and after subsequent washing is performed with distilled water in a pre-weighed centrifuge tube. For estimating the content of polyhydroxybutyrate, ten millilitre of sodium hypochlorite solution was mixed with the cell pellet. The mixture was incubated at 50°C for one hour for the lysis of cells. The pellet was centrifuged again at 12000rpm for 30 minutes and series of washing was done with distilled water and acetone. The pellet was dissolved in ten millilitre of chloroform and was kept for incubation at 50°C overnight and evaporated at room temperature. Following the evaporation, ten millilitre of sulphuric acid was added. The solution was placed for ten min at 100°C in a water bath. Then the centrifuge tube was allowed to cool. The polyhydroxybutyrate production was measured using spectrophotometer with absorbance at 235 nm and pure sulphuric acid as blank.

2.5. Molecular profiling of selected PHB-synthesizing isolates

2.5.1.DNA Extraction

The nucleic acid extraction of the bacterial cultures was done according to (Bacterial genomic DNA (spin), Chromous Biotech, Bangalore) following manufactures protocol. The genomic DNA was separated on an agarose gel (0.8%) made in 1X Tris Acetate buffer and stained by ethidium bromide. Agarose gel electrophoresis was performed at 70 V in an electrophoresis apparatus. The agarose gel was observed using a Gel documentation system (BIORAD Gel Doc™ XR+, USA) equipped with Image lab™ software.

2.5.2.PCR-based amplification of genomic DNA

PCR amplification of 16S ribosomal RNA gene of the isolates was done using universal bacterial primers which spans 27 forward and 1492 reverse strands of sixteen S ribosomal RNA genes (15) with an expected amplicon size of approximately 1500bp.

Polymerase chain reaction mixture of the 16s r ribosomal RNA gene with universal primers 27F(5′AGAGTTTGATCCTGGCTCAG3′) and1492R(5′TACGGYTACCTTGTTACGACTT-3′) consisted of 25 microliters of 2X premix Emerald Amp® GT PCR Master Mix (Takara), 4 microliters were taken from each set of Primers 27F and 1492R (10pmoles) and 5 microliters of sample DNA that contains target sequence, the reaction mixtures were made upto 50 microliters with Milli-Q water. The reactions were carried out using T100TM Thermal cycler made by Bio-Rad under the specified criteria: four minutes at 96°C, it was followed by 30 cycles at the duration of thirty seconds at a particular temperature of 94 °C, thirty seconds at a specific temperature of 58 °C, and one minute at 72 °C, and a finally the amplicon is extended at 72 °C for ten minutes. Portions of 10 microliters from each reaction were run on an Agarose gel (1.5%) suspended in 1X Tris - Acetate -EDTA buffer along with a molecular marker of 100bp and observed the gel on the Gel documentation system.

2.5.3.DNA sequencing and phylogenetic analysis

The amplified product was sequenced at GeneSpec (Kochi, Kerala). The evaluation of the sequences was performed using the nBLAST tool of NCBI. The evolutionary tree was developed using MEGA 11.0 (16) using the Neighbour-joining method (17) with bootstrapping (18) for one thousand replicates to assess the stability of tree topology. The evolutionary distances were calculated (19). Evolutionary tree was configured with Lactobacillus as an external reference for rooting. The organism was identified using the same process. The homologous DNA sequences were submitted in NCBI GenBank, and accession numbers were acquired. For this, a set of homologous sequences that showed the highest resemblance with the query sequence was obtained from the NCBI database and was aligned using the CLUSTAL W algorithm.

2.5.4. Accession numbers for nucleotide sequences

All the data from the present study with the universal 16S rRNA gene were uploaded to the NCBI.

3.RESULTS AND DISCUSSION

3.1. Isolation and Identification of PHB producers

The escalating global issue of nondegradable plastics has resulted in an urgent search for viable substitutes, with bioplastics emerging as a promising solution. In this study, the focus was on exploring the potential of microbial sources in the mangrove ecosystem in producing biodegradable PHAs.

PHAs are microbial products that exhibit similar properties to synthetic plastics like polypropylene. Numerous Gram + and Gram - bacteria can produce PHAs as energy and carbon resource material during conditions of nutrient scarcity with surplus carbon. (20). Mascarenhas & Aruna, (2017) (21) reported that mangrove ecosystems are typically abundant in organic material but poor in nitrogen and phosphorus. Halophilic and halotolerant bacteria strains collected from mangrove soil samples in Northern Vietnam are found to produce polyesters. The present study was carried out to screen and identify potent PHA-synthesizing bacteria, from the four distinct mangrove ecosystems.

3.2. Qualitative Analysis of PHB

For isolating PHA-synthesizing bacteria from nature, screening of specific isolates from a large collection of bacteria in a small duration of time by using dyes specific to PHA for the detection of lipid granules. In this study, isolates exhibited the presence of lipophilic granules as detected by both the Sudan Black B Plate assay and Sudan Black B glass slide staining. The potential PHB isolates stained bluish black with Sudan Black B, indicating the existence of lipid granules. Black granules were observed in the cultures by the Sudan Black B slide method which indicated that the isolated sixteen organisms are potential lipid producers. Mascarenhas & Aruna, (2017) (21) reported that Hartman (1940) (22) was one of the first Scientist to suggest the usage of Sudan Black B dye, as a bacterial lipid stain and the lipophilic dye has been widely acknowledged as a dye with a particularly high affinity for PHAs. Bhuwal et al., (2013) (14) used an alcoholic solution (0.02%) of both the dyes for the rapid detection and isolation of PHB-producing bacteria from paper industry sludge. A collective of 16 isolates exhibited a

black-blue hue upon staining with Sudan Black B, and these identical sixteen isolates also yielded affirmative outcomes with Nile Blue A staining.

Out of sixteen bacterial isolates collected and screened for their capacity to accumulate PHB, three bacterial isolates from Sample 1 (MSM2, MSM6, and MSM7), four isolates from Sample 2 (MSP2, MSP3, MSP5, MSP8), five isolates from Sample 3 (MSB1, MSB2, MSB3, MSB5, MSB8) and four isolates from Sample 4 (MSK2, MSK3, MSK4, MSK7) exhibited the potential to accumulate PHB using Sudan Black B staining on Petri dishes (Fig. 1). On an average, the cells of the strains of the Mangalavanam mangrove forest, Payyannur, Kumarakom and Kollam belonged to the Genus *Bacillus*. The potential PHB isolates stained bluish-black when dyed

with primary screening dye, showed the existence of lipid inclusions. Black granules were observed in all the above-mentioned isolates by the Sudan Black B slide method which indicated that the organisms are potential lipid producers. The Sudan Black B staining methodology was found to be an efficient method for the selection of PHB producers. Mostafa (et al., 2020b) (23) isolated unidentified strains of polyhydroxybutyrate producing bacteria from the rhizospheric soil of Mangroves of Red Sea, Kingdom of Saudi Arabia. The bacterial cultures were identified using Sudan Black-B stain, and out of the above 32 bacterial isolates, it was found that only 16 isolates were able to accumulate the PHB granules. Sudan Black-B lipophilic staining methodology demonstrates high sensitivity in screening for PHB. The stain unveiled the intracellular lipid substance as cytoplasmic inclusions or materials linked with the cellular structural components, which were previously unseen or even suspected.

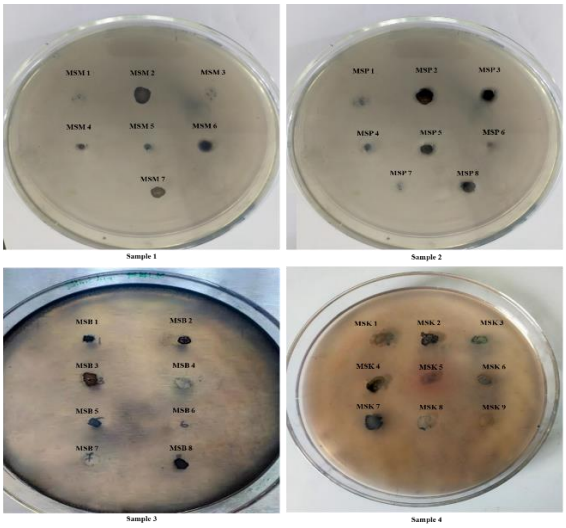


Figure 1 Bacterial isolates stained with Sudan Black B. Colonies-coloured dark blue indicated PHB production.

The Nile Blue stain represents a superior and specific method for confirming the accumulation of PHAs. The procedure followed the protocols outlined by (13, 24, 25) Smears were prepared on glass slides, fixed through heating, and then stained with a 1% aqueous solution of Nile Blue A (Sigma-Aldrich, Germany) at 55°C for approximately 10 minutes. Subsequently, the slides were rinsed with sterile water and dried in the presence of air. Examination was conducted using a fluorescence microscope (ZEISS, GERMANY) at 460 nm. The selected isolates were inoculated on carbon-rich nutrient agar medium. Isolates demonstrating vibrant orange fluorescence under an epifluorescent microscope following Nile blue A staining were chosen as PHB accumulators. Three bacterial isolates from Sample 1 (MSM2, MSM6, and MSM7), four isolates from Sample 2 (MSP2, MSP3, MSP5, MSP8), five isolates from Sample 3 (MSB1, MSB2, MSB3, MSB5, MSB8) and four isolates from Sample 4 (MSK2, MSK3, MSK4, MSK7) exhibited the potential to accumulate PHB using Nile Blue A staining on glass slides. These samples exhibited bright orange fluorescence, when it was viewed under an epifluorescent microscope at an excitation wavelength of 460nm.Sample 1(MSM2), Sample 2 (MSP2, MSP3), Sample 3 (MSB1, MSB2), and Sample 4 (MSK2, MSK3) exhibited bright orange fluorescence and most of the producers identified belonged to the genus *Bacillus* (Fig 2). (26) isolated fifty bacteria from a mangrove habitat, in Kingdom of Saudi Arabia, and were separated for potential polyhydroxybutyrate producers using the Nile Blue dye method. All fifty bacterial strains were identified as positive for polyhydroxybutyrate production, of which one strain showed a maximum of 137 mgL^{-1} and it was identified as *Bacillus thuringiensis.*

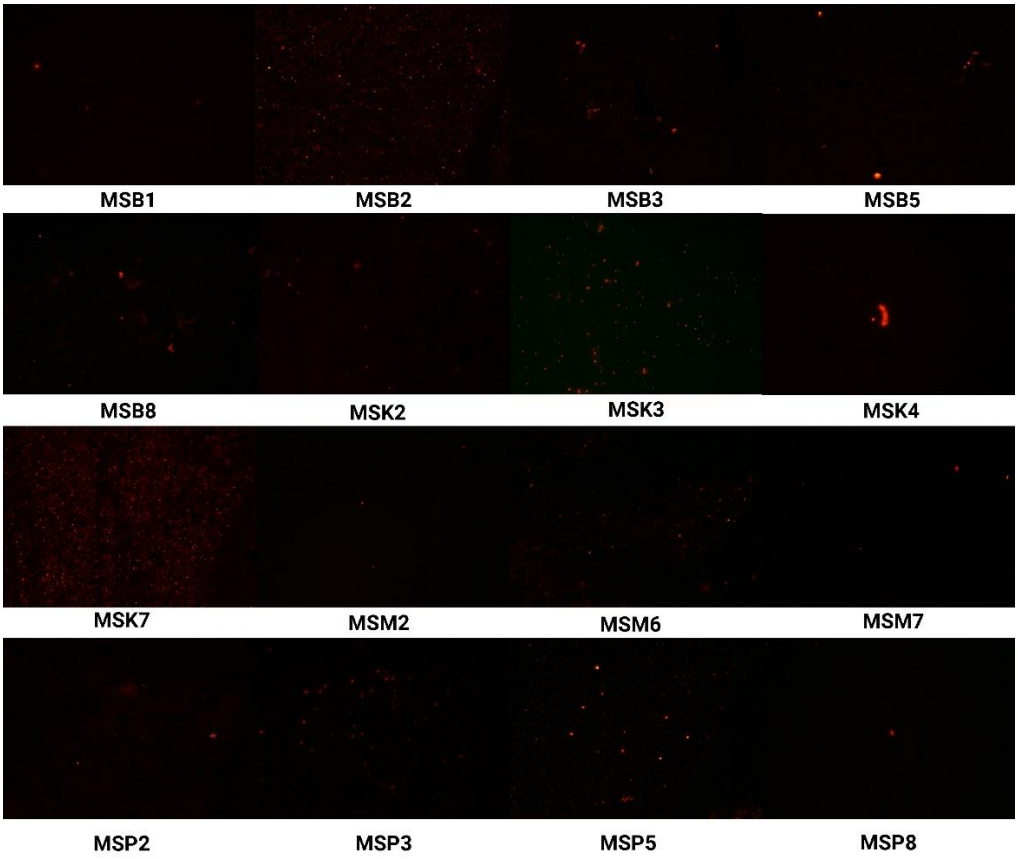


Figure 2 Nile blue dyeing of the potent bacterial isolates as seen under fluorescence microscope.

3.3. Quantification of PHB content by Crotonic acid assay

The amount of PHB in the sample was determined by crotonic acid assay. A standard curve was drawn between crotonic acid concentrations, and its respective absorbance measured at 235 nm. The observed relationship between the above parameters was linear. The slope was found to be y=0.0165x - 0.0018 where y equals the absorbance of the measured sample and x represents the crotonic acid concentrations (µg/ml) in the sample solution which is equivalent to the PHB concentration of the sample. The amount of crotonic acid per milliliter of the sample was calculated. In the present study, it was observed that among the isolates MSB3 (33.56%), MSB8 (32.54%), and MSM2 (36.04%) exhibited the highest production of PHB than other bacterial isolates (Table 1, Figure 3). All three bacterial isolates were identified as *Bacillus species*. The highest potent producer among the isolates was MSM2 (36.04%) was identified as ***Bacillus velezensis***

(Penkhrue et al., 2020) (27) reported that the Gram +bacteria, *Bacillus* was the first identified polyhydroxybutyrate producing bacteria. Amidst the identified polyhydroxybutyrate

producing bacteria, *Bacillus megaterium* (S29) and *Bacillus* sp. (IPCB-403) had the outstanding ability to collect more than 70% PHB content with respective to cell dry weight in its respective standardized conditions. *Bacillus aryabhattai* (T34-N4) had the ability to harness waste starch for the direct production of polyhydroxybutyrate. Bacillus *cereus* is reported to yield up to 48% of polyhydroxybutyrate content by using starch as the only reserve of carbon. Among microbial genera, *Bacillus group* is found to be more suitable for polyhydroxybutyrate production since it delivers maximum PHB output and doesn't demand any stringent controls for fermentation. The genus *Bacillus* seems to be a potential candidate to produce PHB due to its better polymer yields and does not require strict fermentation conditions. (Thirumala et al., 2009) (28) reported that Gram + bacteria, precisely Bacillus and *Streptomyces*, have been extensively used for industrial purposes. Gram - bacteria has lipopolysaccharides which co-purify with the polyhydroxyalkanoates and cause immunologic responses and are currently the only commercial source of PHA. But Gram + bacteria does not have lipopolysaccharide, a differentiating factor that promotes valuable and extensive research into their PHA production. The Polyhydroxyalkanoate synthase discovered from the *Bacillus* genus is able to incorporate both short-chain length and medium-chain length PHA, revealing that the genus Bacillus can be a potential producer of new and known PHA with divergent ranges of monomeric compositions. The new polyhydroxyalkanoate synthase gene discovered from the gram + *Bacillus* group is able to incorporate both short-chain length and medium-chain length PHA, showing that the specific group can be a prospective producer of new and known PHA with a wide range of monomeric structures.

Sl. No	**Isolates**	**PHB Concentration (µg/mL)**
1	MSB1	10.89
2	MSB2	8.41
3	MSB3	33.56
4	MSB5	23.13
5	MSB8	32.54
6	MSM2	36.04

7	MSM6	16.53
8	MSM7	12.47
9	MSK2	8.77
10	MSK3	7.26
11	MSK4	10.23
12	MSK7	22.89
13	MSP2	14.71
14	MSP3	9.01
15	MSP5	22.35
16	MSP8	12.16

Table. 1 PHB concentration of the bacterial isolates

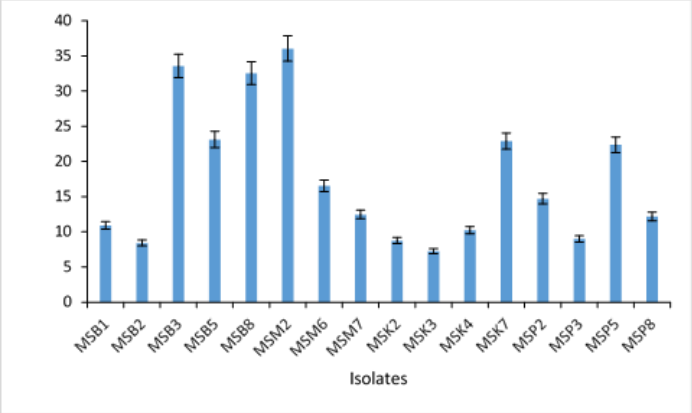


Figure 3 PHB concentration among the isolates.

3.4. Molecular identification of the PHB-producing isolates

The DNA extraction of sixteen isolates was done and was run on gel that was prepared with agarose (0.8%) dissolved in 1x Tris acetate EDTA buffer solution. DNA was observed as distinct bright bands (Figure: 3). Molecular identification of PHB-producing isolates revealed sixteen bacterial isolates viz. *Priestia endophytica* (MSB1), *Priestia megaterium* (MSB2), *Bacillus cereus* (MSB3), *Bacillus pumilus* (MSB5), *Bacillus cereus* (MSB8), *Bacillus velezensis* (MSM2), *Rahnella aquatilis* (MSM6), *Stenotrphomonas maltophilia* (MSK2), *Priestia endophytica* (MSK3), *Bacillus cereus* (MSK4), *Bacillus thuringiensis* (MSK7), *Bacillus* (MSP2), *Bacillus subtilis* (MSP3), *Niallia circulans* (MSP5). (Table: 1). (Getachew & Woldesenbet, 2016) (29) isolated PHB-producing bacteria in different locations in the vicinity of Arba Minch city and studied PHB production by using agricultural waste materials. It was found that production of PHB using agricultural - wastes, especially sugar-cane bagasse by *Bacillus* sp is feasible. This can decrease production expenses and mitigate the disposal

challenges of these agricultural wastes. The production of PHB can be enhanced through the optimization of production parameters and substrates. Currently, approximately forty PHA synthase genes are studied from various microorganisms. The identification of an innovative PHA synthase gene can synthesize a new type of PHA (28). The DNA banding pattern of PCR amplification in agarose gel electrophoresis is shown in Figure 4.

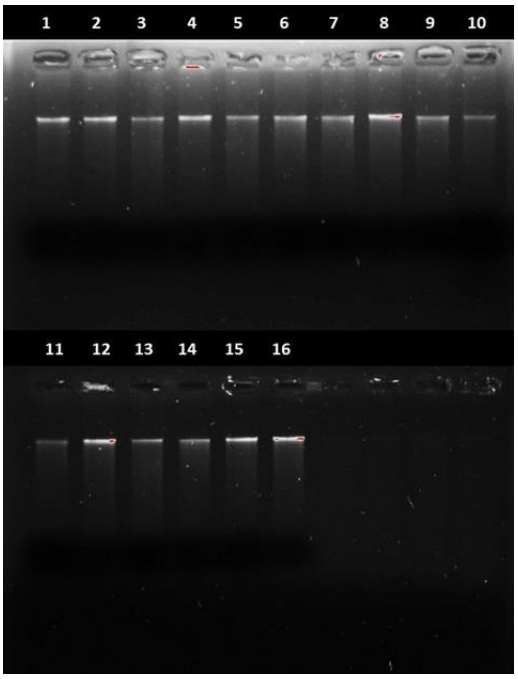


Figure 4 Gel electrophoresis showing extracted DNA from sixteen samples

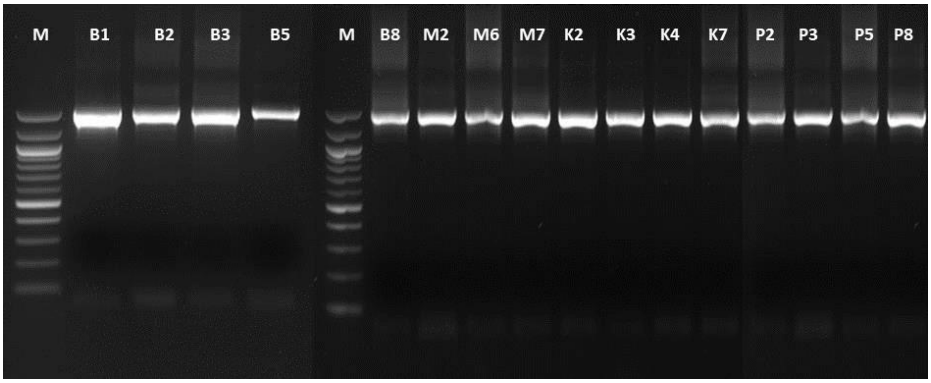


Figure 5 Amplification of PCR Products in 1.5% agarose gel

DNA banding pattern after the PCR amplification cycle of 16S rDNA gene where lane 1 = 1.0 kb ladder, lanes B1- B5 samples isolated from Kumarakom Bird sanctuary mangrove,

Lanes B8 from Bird sanctuary, then M2, M6 &M7 from Mangalavanam Mangrove, Lanes K2to K7 from Kollam Mangrove forests, Lanes P2 to P8 from Payannur mangrove forests.

Sixteen bacterial strains were isolated that meet the selection criteria are MSB1, MSB2, MSB3, MSB5, MSB8, MSM2, MSM6, MSM7, MSK2, MSK3, MSK4, MSK7, MSP2, MSP3, MSP5, and MSP8. According to the obtained results MSB1, MSB2, MSB3, MSB5, MSB8, MSM2, MSM6, MSM7, MSK2, MSK3, MSK4, MSK7, MSP2, MSP3, MSP5, and MSP8 & fourteen isolates were identified as *Priestia endophytica (MSB1), Priestia megaterium (MSB2), Bacillus cereus (MSB3), Bacillus pumilus (MSB5), Bacillus cereus (MSB8), Bacillus velezensis (MSM2), Rahnella aquatilis (MSM6), Stenotrphomonas maltophilia (MSK2), Priestia endophytica (MSK3), Bacillus cereus (MSK4), Bacillus thuringiensis (MSK7), Bacillus (MSP2), Bacillus subtilis (MSP3), Niallia circulans (MSP5)* respectively. MSB3, MSB5, MSB8, MSM2, MSK4, MSK7, MSP2, &MSP3 were classified as *Bacillus sp.* Among these isolates *MSB3, MSB5, &MSB8* were found to be *Bacillus cereus.* The isolates MSB1, MSB2, &MSK3 were found to be *Priestia* spp. ((Kola et al., 2023)(30) isolated a PHA-producing strain from soil sample of agricultural land. The isolation was characterized by biochemical tests and identified by using 16S rRNA sequencing. The isolate exhibited sequence similarity with *Priestia aryabhattai* B8W22 and was designated as *Priestia aryabhattai* VITJK01.

3.5. Phylogenetic relationship

The phylogenetic study of PHB-producing bacterial isolates based on 16S rRNA sequences revealed evolutionary relationships utilizing the Neighbor-joining algorithm. The optimal tree based on 24 nucleotide sequences and 1220 positions in the final dataset demonstrated evolutionary distances measured in base substitutions per site using the Tajima-Nei method.

The analysis conducted in Mega 11, depicted the relationships among taxa. Bootstrap support percentages on nodes generated from 1000 resampled datasets provided confidence in the clustering. The scaled bar represented 0.10 nucleotide substitutions per location in the sequence illustrating the genetic divergence among isolates.

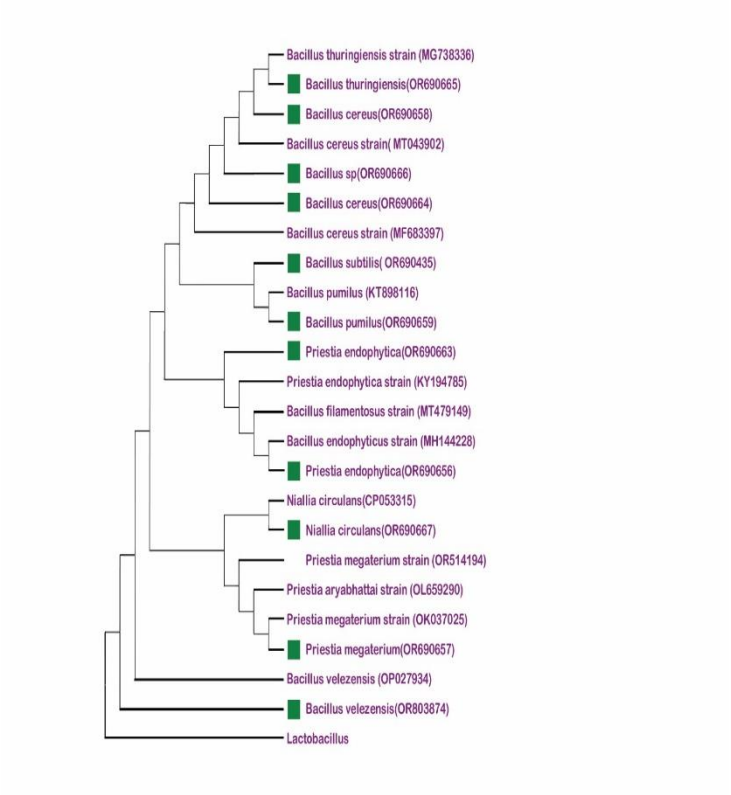
The top hit, determined by factors such as E value, bit score, percentage identity, and query coverage, was chosen, indicating both statistical and biological significance in the BLAST result. Our query sequences were identified as closely associated with the 16S sequence of Bacillus species. Subsequent multiple sequence alignment and phylogenetic analysis situated our query sequence within the Bacillus cluster. So, among the sixteen bacterial isolates, eight bacteria were confirmed as *Bacillus* sp., three bacteria were confirmed as *Priestia*, and one bacterium was identified as *Niallia* and the variance in percentage identity compared to the closest organism hints at the possibility of it being a novel strain.

The two bacteria isolated were found to be Gram negative and was found to be close to Rhanella species and Stenotrophomonas respectively.

The isolated bacterial strains revealed a high degree of similarity. The phylogenetic relationships of the isolated strains are represented (Figure:6) and their accession numbers are also represented (Table: 2). The three isolates MSB3, MSB5 and MSB8 obtained from Kumarakom Bird Sanctuary mangrove soil, MSM2, obtained from Mangalavanam mangrove soil, MSK4, and MSK7 obtained from Kollam mangrove soil and MSP2, &MSP3 obtained from Payyannur Mangrove soil belonged to the Genus-Bacillus. The five isolates obtained from Kumarakom Bird Sanctuary Mangrove were respectively *Priestia endophytica, Priestia megaterium*, *B. cereus*, *B. pumilus*, and *B. cereus*. The two isolates obtained from Mangalavanam forests were respectively of *B. velezensis*, and *Rahnella aquatilis*. The four isolates obtained from Kollam were respectively of *Stenotrphomonas maltophilia*, *Priestia endophytica*, *B. cereus*, and *B. thuringiensis*. The three isolates from Payyannur forests were respectively of Bacillus sp, *B. subtilis*, and *B. circulans*. Kumarakom Bird Sanctuary, Mangalavanam, Kollam and Payyannur mangrove forests had identified four different genera, and the isolates were identified as, MSB2, MSM6, MSK7, MSP2. PHA is natural polyester that acts as a carbon and energy storage in microorganisms. PHA is composed of 600 to 35000 monomers of (R)-hydroxy fatty acids. PHA is important for the survival of bacteria under adverse environmental circumstances such as UV exposure, desiccation and osmotic pressure. When there is an abundant carbon supply and less availability of nitrogen, sulphur, oxygen and other nutrients, microorganisms synthesize PHA. PHAs are accumulated in the cytoplasm of several bacteria such as *Bacillus, methylotrophs, Alcaligenes* and *Pseudomonas* species. Scientific reports suggest that the genus *Bacillus* is generally preferred for biopolymer synthesis, over Gram-negative bacteria because of their genetic stability, rapid growth, utilization of cheap carbon sources and manufacture of PHB free from endotoxin (30).

(Mohandas et al., 2018) (8) reported that PHAs generated by Bacillus sp. are preferable for a range of biomedical uses, as Gram-positive bacteria do not possess pyrogenic lipopolysaccharide (LPS) endotoxin, which has the potential to induce immunogenic reactions.

Enterococcus sp. NAP11 exhibits promising potential for the industrial synthesis of PHB using wastewater derived from the cardboard industry. (14).



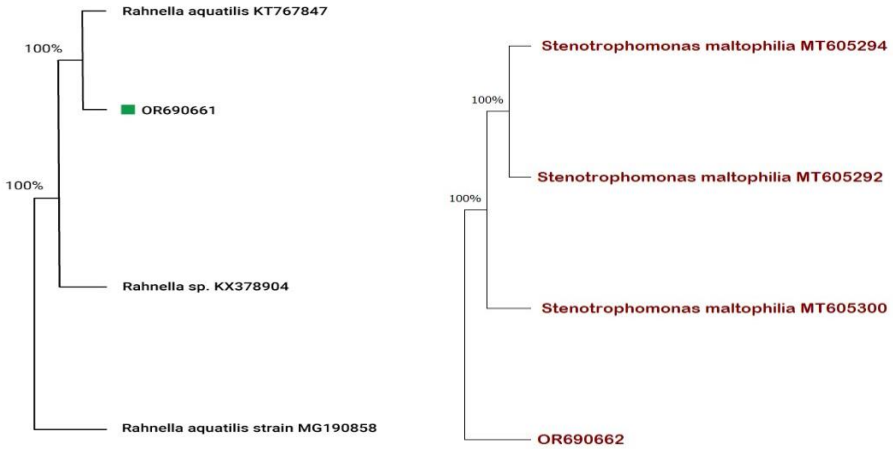


Figure 6 Phylogenetic relationship representation of the bacterial isolates from the mangrove sediments using Neighbor Joining algorithm.

SL.NO	Strain ID	Nearest relative sequences of the amplicon in NCBI	Gen bank Accession number	Similarity (%)	GenBank number of the isolate
1	MSB1	**Priestia endophytica**	MT588728	100	**OR690656**
2	MSB2	**Priestia megaterium**	PP262555	100	**OR690657**
3	*MSB3*	***Bacillus cereus***	*MT043902*	*100*	***OR690658***
4	MSB5	**Bacillus pumilus**	KT898116	99.87	**OR690659**
5	MSB8	**Bacillus cereus**	MF360050	100	**OR690660**
6	MSM2	**Bacillus velezensis**	OP027934	100	**OR803874**
7	MSM6	**Rhanella aqualitis**	KT767847	100	**OR690661**
8	MSK2	**Stenotrophomonas maltophilia**	MT605300	100	**OR690662**
9	MSK3	**Priestia endophytica**	KY194785	99	**OR690663**
10	MSK4	**Bacillus cereus**	MF683397	99.73	**OR690664**
11	MSK7	**Bacillus thuringiensis**	MG738336	99.89	**OR690665**
12	MSP2	**Bacillus sp.**	MF319430	100	**OR690666**
13	MSP3	**Bacillus subtilis**	OK189715	100	**OR690435**
14	MSP5	**Niallia circulans**	CP053315	96	**OR690667**

Table 2 NCBI BLAST examination of 16S rRNA gene sequences from the isolates.

4.Conclusion

Our investigation aimed to identify proficient PHA producers within various Mangrove sources in Kerala. The findings from this study highlight the presence of highly efficient PHB-producing bacteria within Mangrove ecosystems. Specifically, strains belonging to Bacillus, Priestia, Rahnella, and Stenotrophomonas genera exhibited substantial PHB production capabilities. These results underscore the promising potential of these bacterial isolates to produce biodegradable plastics as a sustainable alternative to nondegradable counterparts.

There exists a viable pathway for developing eco-friendly PHB-based materials by tapping the potential of microbes from Mangrove ecosystems. Harnessing the capabilities of these identified bacterial strains could significantly contribute to mitigating the environmental challenges posed by nondegradable plastics.

5. **Conflict of Interest**

The authors whose names are listed certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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