Poly (3-hydroxybutyrate) isolation and characterization of biopolymers of microbial origin towards a sustainable future

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ABSTRACT

Nowadays petroleum-based plastics have become a consequential part of our human lives. The non-degradable nature of petroleum-based polymers causes serious risks to the environment. Polyhydroxybutyrate (PHB), a storage granule of energy and carbon reserve in many bacteria attracts significant attention in recent years due to its biodegradable nature. Many bacterial strains (*Ralstonia eutropha*, *Alcaligenes latus*, *Pseudomonas putida* and *Aeromonas hydrophila*) are known for the natural synthesis of PHB under nutrient limited conditions. This study aims at identifying a novel microorganism that produces PHB naturally and to characterize the strains and synthesized polymer. Among 74 distinct isolates from various sources,5 strains (NAA1, NAA2, NAA3, NAA4 and NAA5) showed the presence of PHB in preliminary screening using Sudan Black B stain, Nile red, and Nile blue A staining methods. The microscopic examination of these strains using Sudan Black B stain and Safranin (counter stain) further confirmed the presence of PHB granules. The cell growth, pH change and PHB production of these 5 strains were evaluated under shake flask conditions. Among them, NAA2 and NAA4 produced 87.5% and 74% of PHB respectively from its total cell dry weight. The obtained polymers were extracted through sodium hypochlorite-chloroform extraction method and employed for the ^{1}H NMR and FTIR analysis. The16S rRNA sequencing revealed that the isolated strains were *Bacillus pacificus* and *Klebsiella quasipneumoniae* and the phylogenetic tree exhibiting their evolutionary relationship was constructed.

Keywords: *Petroleum-based polymer, Polyhydroxybutyrate, Sudan Black B, Nile Red, Nile Blue A, Sodium hypochlorite extraction*

INTRODUCTION

In everyday life, plastics have become an inevitable part of high commercial sense with wide applicability in almost every industrial sector starting from packaging to parts of industrial machinery [1].The demand for polymer materials has increased steadily since 2000, nearly doubling globally and outperforming all other bulk resources like steel, aluminium, or cement. Up to 12% of the world's oil consumption is fuelled by the manufacturing of more general plastics and associated commodities from petrochemical feedstocks [2].Conventional plastics are versatile in nature because of their highly stable and durable nature, ability to be cast into various shapes and sizes, transparency, good rheological properties, cost-

effectiveness, and excellent thermal properties. Generally, conventional plastics are petroleum-based products that take a more prolonged time to decompose. Some of the Petroleum-based plastics are nylon, Polyvinyl chloride (PVC), polyethylene (PE), Polypropylene (PP), Polystyrene (PS), and Poly ethyl terephthalate (PET) [1]. The increased production of plastic made from petroleum in recent decades has created a significant environmental problem for the management of solid wastes due to the plastic's biodegradation resistance [3]. Thus, dumping these petroleum-based plastics into the environment causes serious problems which include scarcity of fossil fuels, the meagreness of landfills, lack of effective post-use circularity technologies and also improper disposal of these non-biodegradable materials leads to devastation of air, land and marine ecology as well as damage to human life. Incineration of plastics leads to the emission of greenhouse gases which ultimately ended up in global warming. So, in the end, petroleum-based plastics are presented as a double-edged sword that has both positive and negative consequences for the environment [4].

The prevalence of environmental problems imposed by petroleum-based plastics accelerated the hunt for eco-friendly, novel biodegradable plastics [4,5]. Due to their non-toxicity, renewability, biocompatibility and rising demand as a replacement for synthetic plastics, bioplastics are in great demand [6]. Bioplastics are those which are derived from renewable sources [7,8] and can be transformed into carbon dioxide (CO_2), biomass and water by bacteria or fungi. The formed carbon dioxide will be taken up by the plants for photosynthesis for producing carbohydrates which are described as the carbon cycle of biodegradable polymers. Under anaerobic conditions, methane is also produced. Various sources such as plant-based proteins, polysaccharides, cellulose and starch-based plastics [9,10], polylactic acid (PLA) and polyhydroxyalkonate (PHA) [11-13] have been utilized to produce the biodegradable plastics. They are biocompatible and have qualities akin to those of ordinary polymers, are the most promising type of bioplastic. PHAs are biopolymers with a variety of structural variations [14-16]. Microbes having depolymerase enzymes can easily degrade the PHAs by catalyzing their ester bonds to water-soluble monomers and oligomers, and finally, these products will be broken down into CO_2 and H_2O. PHAs build up inside bacterial cells as stored energy as a defensive mechanism against stressful nutritional imbalances. Poly-3-hydroxybutyrate (PHB), the most prevalent type of PHAs, builds up in many bacteria by joining hydroxybutyrate monomers with ester linkages [17,18]. PHB has high yearly production rates and a lower universal manufacturing capacity than polypropylene, a plastic derived from petrochemicals, at around 30,000 tonnes per year. PHB is a green polymer [18,19] that has many features including thermostability, resistivity against UV, hydrolytic degradation, water insolubility and impermeable to oxygen. PHB is used as an interior material in a variety of technologies including automotive components, sanitary goods, electrical gadgets, coating materials, containers, packaging and disposable substances. Also, antimicrobial materials composed of PHB nanocomposites and silver nanoparticles [20,21] have been biosynthesized and employed as medication carriers in the biomedical industry for wound healing and tissue engineering applications.

Studies claiming that over 300 different bacterial strains are known for the natural synthesis of synthesize PHB compounds [2,13]. *Ralstonia eutropha* (also known as *Cupriavidus necator*), *Alcaligenes sp., Azotobacter sp., Bacillus sp., Nocardia sp., Pseudomonas sp., Rhizobium sp*. are some examples of extensively investigated strains towards the production of PHB, with *Ralstonia eutropha* being the most extensively studied microorganism. The majority of the PHB producers were isolated inanaerobic atmospheric condition which includes landfills, sewage, and anaerobic digesters as well as in aerobic atmospheres including soil, ponds, marine ecology and compost [22-24].

PHB is an intracellular granule, which serves as an energy reserve and during inimical conditions it safeguards the cell against heat shock and reactive oxygen species [25] PHBs are short-chain length polyhydroxy alkanoates where the production of PHB commences with glucose as the primary carbon source. The PHB production pathway comprises three steps. Two molecules of acetyl-CoA are abridged to create acetoacetyl-CoA in the first step, which is catalysed by the enzyme -ketothiolase, which is encoded by the *phaA* gene. The acetoacetyl-CoA reductase enzyme, which is encoded by the *phaB* gene, catalyses the reduction of acetoacetyl-CoA to (R)-3-hydroxybutyryl CoA monomer in the following step. The (R)-3-hydroxybutyryl CoA monomers are polymerized by the PHA synthase enzyme, which is encoded by the *phaC* gene, to produce PHB in the final step. Earlier research studies have shown that the high concentration of NADPH and acetyl-CoA, and the low concentration of Coenzyme A favours the production of PHB [2, 26].

Currently, however, only a few bacterial strains have been effectively employed for PHB synthesis on an industrial scale which is far minimal than the demand [23]. In order to produce PHB on a commercially viable scale in a sustainable and eco-friendly manner, it is essential to identify a natural producer, Current study aims at identifying a natural PHB producer which [19, 27-29] has the ability to attain higher growth in the minimal media with a capability to accumulate high amounts of PHB.

MATERIALS AND METHODS

1. Sample collection

The samples were immediately transported to the laboratory and kept chilled at 4°C for further testing after being aseptically collected from various natural sources in the Chennai area using a sterile zip-lock bag and ethanol-wiped sterilised spatula [30].

2. Isolation of pure bacterial cultures

Following a standard serial dilution approach, the collected soil samples were serially diluted to identify the most prevalent cultivable bacterial isolates after being thawed at room temperature. One gram of the collected soil sample was added to the first test tube and thoroughly mixed with 10 mL of sterile distilled water. Again, 1mL of the sample from the first test tube was taken and properly mixed to provide a dilution factor of 10^{-1} in the second test tube, which contained 9 mL of sterile distilled water. The dilution factor was increased by this process until it reached 10^{-5}. By inoculating roughly 50 µL of 10^{-5} dilutions of collected soil samples onto sterile nutrient agar plates, the spread plate was performed. It was

then incubated for 48 hours at 37°C alongside a sterile control. Following quadrant streaking, the dominant and individual colonies were separated from the master plate, sub-cultured for purification, and stored at 4°C for additional analysis.

Screening of PHB producers

Three screening procedures (Sudan black B test [31], Nile red test [32], and Nile blue A test [31]) were performed to detect the presence of PHB-producing bacterial isolates.

a. Sudan Black B staining

The ability of the 74 dominant bacterial isolates to actively grow on nutrient agar was considered when choosing them. Sudan black B lipophilic stain was used to further analyse the isolates for PHB production. Briefly, 2 mL of Sudan black B stain (0.05%) was applied to the colonies on the nutrient agar plate. The colonies were then incubated at room temperature for 30 minutes before the excess stain was removed with 60 % ethanol. The stained culture plates were then incubated once more for 30 minutes, at which point colonies with this colour change were determined to be PHB positive [33,34].

b. Nile red and Nile blue A staining

For screening the bacterial colonies that produce PHB, Nile red and Nile blue stains are more reliable. Therefore, Sudan black B positive colonies were rescreened using these stains. Nile red stain and Nile blue A stain were each dissolved in 0.25 mg in 1 mL of dimethyl sulfoxide (DMSO) to create the stock solutions for the stains (per mL medium). After the proper incubation times, the agar plates were exposed to 312 nm ultraviolet light to look for the accumulation of PHAs [35].

c. Microscopic examination of PHB Granules

Sudan black B was used to stain a thin smear that was prepared, air dried, and placed on microscopic slides. Additionally, the stained slides were left alone for 10 to 15 minutes. The slides were counter stained with safranin for 10 seconds after being cleaned with distilled water. With distilled water once more, the slides were cleaned, and tissue paper was used to blot dry them. Additionally, PHB granules were initially checked for on the slides using a microscope (blue coloured) [36].

4.Shake flask production

The chosen isolates were used to produce PHB in the Mineral Salts Medium (MSM), which contains the following ingredients (in g/L): Urea (1.0), Yeast extract (0.16), $CaCl_2$ (0.02), KH_2PO_4 (1.52), $MgSO_4{\cdot}7H_2O$ (0.52), Na_2HPO_4 (4.0) Glucose (40) and trace element solution 0.1 mL $ZnSO_4{\cdot}7H_2O$ (0.13), $FeSO_4{\cdot}7H_2O$ (0.02), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.06), and H_3BO_3 are all present in the trace elements solution in (g/L) (0.06). Prior to the inoculation, the trace element solution and glucose were both autoclaved separately and reconstituted [23]. The isolates were subcultured twice in nutrient broth to create the pure culture. Then, to estimate the biomass and PHB production, 1 mL of a 24 h incubated culture was inoculated

into 100 mL of production media in three sets, and it was incubated for 48, 60, and 72 h, respectively, at 37 °C and 150 rpm. Both the optical density and pH were measured over a period of 6 hours. Additionally, samples gathered during the appropriate time period were centrifuged at 10,000 rpm for 15 minutes and their respective amounts of dry biomass were measured. The pellet of collected biomass was dried at a temperature of 55 °C [37].

5.Extraction of PHB

PHB was extracted using the sodium hypochlorite-chloroform method. The solution was centrifuged after the process was finished, producing three phases. Chloroform with cell debris is present in the middle phase, while hypochlorite solution is present in the upper phase. The hot chloroform was used to extract the PHB-containing bottom phase, which was then collected and precipitated with ethanol and acetone (1:1). To obtain PHB crystals, the precipitate was allowed to evaporate at 30 °C [38].

The amount of PHB present in the dry cell weight is used to estimate the percentage of intracellular PHB accumulation [39].

$$\text{PHB accumulation (\%)} = \frac{\text{Dry weight of extracted PHB (g/mL)}}{\text{Dry Cell weight (g/mL)}} \times 100$$

6. Characterization of selected bacterial strain

a. Gram staining

Based on the morphological observations of colonies, shape of bacteria, and using gram staining technique the microorganisms were segregated. A thin smear of the bacterial isolate was placed on the glass slide. After heat fixing the sample, crystal violet (primary stain) was flooded over the smear and let stand for 1 min. After washing with distilled water, iodine (mordant) was added to the smear and stood for 2 min. Then washed with ethanol (decolourizer). The safranin (counter stain) was added to the smear and let stand for a minute and then rinsed with distilled water. The sample was air dried and observed under the microscope. Gram-positive bacteria stains blue with crystal violet dye and gram-negative bacteria stains red with safranin dye.

b. **16s rRNA sequencing and phylogenetic tree construction**

Using 16S rRNA gene sequencing, the isolates that produced a significant amount of PHB were identified. The screened pure culture samples were sent to IMMUGENIX BIOSCIENCES, Chennai for 16S rRNA sequencing. The genomic DNA was isolated from the samples using the IGB DNA extraction kit method (column based). Then the samples were PCR amplified using broad range pan eubacterial primers in veriti 96-well thermal cycler (Applied Biosystem, USA). The amplification of genes was confirmed by running in agarose gel electrophoresis using a 100bp DNA marker. The amplified products were purified using FavorPrep PCR purification mini kit and sequencing was performed by ABI 3730XL sequencer (Applied Biosystem, USA) using ABI PRISM® BigDye™ Terminator. The specimen's sequence similarity was determined using the GenBank database

(http://www.ncbi.nlm.nih.gov/genbank/). The MEGA 11 software tool's Maximum Composite Likelihood method was used to analyse the sequence's divergence.

7.Characterization of PHB

a) Fourier transform-infrared spectroscopic analysis

Following the general process outlined by Sholkamy *et al.*, (2015) the extracted PHB was analysed using FT-IR analysis. The FTIR analysis was performed on the extracted PHB, wherein the 1mg of the extracted sample was dissolved in 7 mL of chloroform. The dissolved PHB in chloroform was subjected for FTIR analysis using KBr pellet and the spectra were recorded in the range of 4000-400 cm^{-1} [40].

b) ^{1}H NMR analysis

In accordance with Sholkamy *et al.*, (2015) technique, ^{1}H-NMR was used to study the PHB extract dynamic components, individual monomers, and molecular organization. A Bruker ACF300 spectrophotometer was used to record the sample's spectra at 300 MHz with phase and frequency resolutions of > 0.1 degree and >0.1 Hz, respectively, using tetramethyl saline as an internal reference [40].

RESULTS AND DISCUSSION

1. Sample collection, isolation and screening:

As the PHB producers are reported to be present in wide range of sites, the soil and water samples collected from various locations were analysed for the presence of natural PHB producers using standard microbiological methods [30]. The serial dilution and plating yielded number of colonies in nutrient agar plates among which a total of 74 bacterial colonies with various morphological features were chosen to identify the natural ability to accumulate intracellular PHB. The intracellular presence of PHB was initially identified using various staining methods such as Sudan black B test, Nile red and Nile blue A tests. Among the 74 isolated colonies, 30 colonies showed the positive results while subjected to Sudan black staining test. Sequentially, these 30 colonies were further subjected Nile red test for the confirmation of the presence of PHB. Among 30 clones, only 18 showed the luminescence while exposed to the UV light and confirmed PHB presence. These positive 18 clones were further tested with Nile blue A from which 5 colonies were found to exhibit fluorescence under UV light (Fig.1 & Fig.2). Based on these results, these colonies were labelled as NAA1, NAA2, NAA3, NAA4 and NAA5 and chosen for microscopical examinations. The microscopic observation revels the presence of PHB granules (Fig. 3).

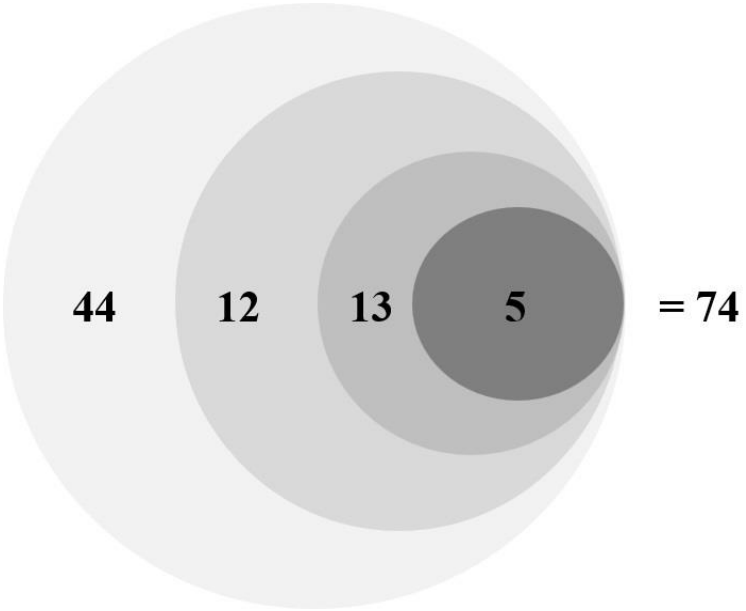

Fig. 1 Screening of PHB producing microorganisms using Sudan black B, Nile red and Nile blue A staining methods. The figure reperesents the stepwise selection of PHB positive isolates. [=Total isolates (74), =Sudan black B positive isolates (30), =Nile red positive isolates (18), =Nile blue A positive isolates (05)]

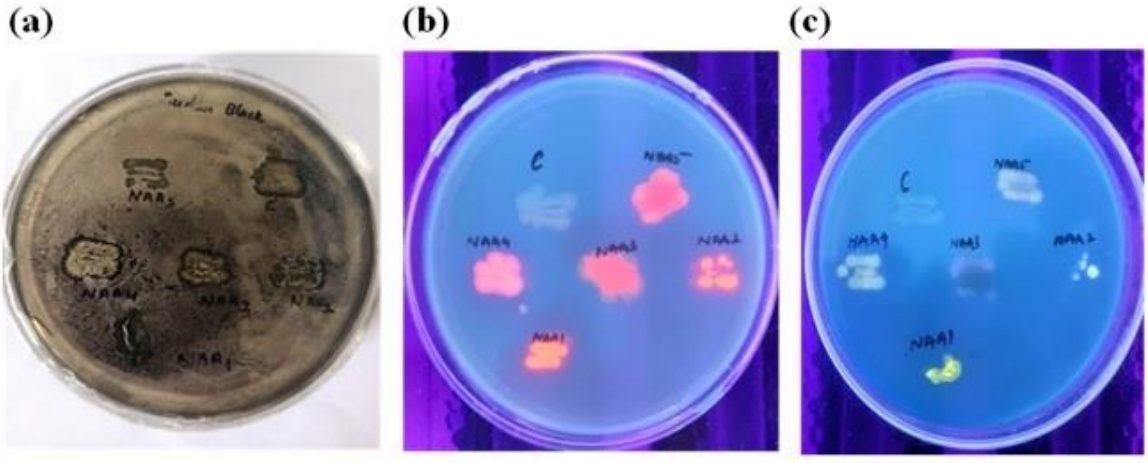

Fig. 2 Validation of PHB production for the clones exhibiting the positive results for all the staining methods. (a) Sudan Black staining – the isolates absorbed the dye and exhibited blue-black colonies, (b) Nile Red staining – the isolates exhibited fluorescence when exposed to UV light (312 nm) (c) Nile Blue staining – the isolates exhibited fluorescence under UV light (312 nm).

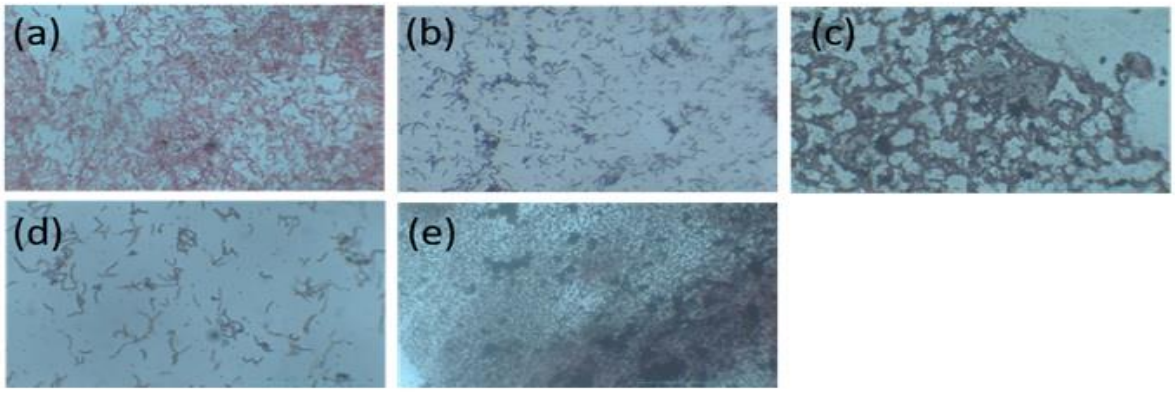

Fig. 3 Microscopic images different strain stained with Sudan Black-B: (a) NAA1, (b) NAA2, (c) NAA3, (d) NAA4 and (e) NAA5 exhibiting PHB granules.

2. Production of PHB under shake flask conditions

In the initial screening, 5 potential isolates were screened using different staining methods. These strains were employed for the shake flask production in MSM (Mineral salt media) along with *R. eutropha* (positive control), natural PHB producer and *E. coli* (negative control). In addition to the production, the growth capability of the newly isolated strains was also evaluated in the media containing glucose as sole carbon source. From the 5 isolates, NAA4 showed superior growth rates from the beginning and ended up with the highest OD_{600}, whereas NAA2 outperformed other strains (including positive and negative controls) except NAA4. Though NAA1 showed similarity with NAA2 in the final cell density, the growth rate was found to be relatively slow. Meanwhile, NAA3 and NAA5 exhibited lower growth rate as well as final cell density. Interestingly, all the newly isolated strains displayed better growth characteristics than the controls. *E. coli*, the negative control registered the lowest growth in MSM among all other strains (Fig. 4). This could be due to the rapid drop in the pH which could be due to the production of other acidic by-products such as acetic acid etc.

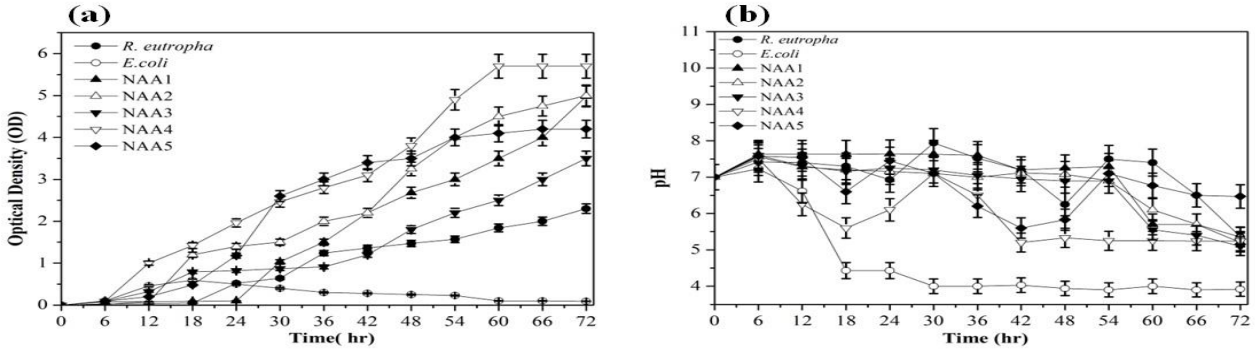

Fig. 4 Growth of the various strains at different incubation times (a) cell growth with respect to incubation time (b) pH change with respect to incubation time. The standard deviation for the growth of different strains and pH measurements were < 5%. (closed circle -*R. eutropha*, open

circle - *E. coli*, closed triangle -NAA1, open triangle -NAA2, inverted closed triangle-NAA3, inverted open triangle -NAA4 and closed square-NAA5)

The isolates along with control strains were further analysed for the PHB production profile, with varying incubation period of 48 h, 60 h &72 h. Though the cell growth and pH were evaluated at every 6 h intervals, the intracellular accumulation of PHB was estimated at 48 h, 60 h and 72 h by harvesting and lysing the cells respectively (Fig. 4). By employing the harvested cells to sodium hypochlorite-chloroform method of extraction, the percentage accumulation of PHB was determined. From the results, it was observed that the accumulation of PHB was at high with NAA2 followed by NAA4 which are found to be higher than the positive control *R. eutropha*. The amount of PHB accumulation was relatively much lower with NAA1 and NAA3. The similar trend was noticed with NAA2 and NAA4 at 60 h, but NAA1 showed better PHB synthesis than NAA3 in contrast to 48 h. interestingly, *R. eutropha* and NAA5 showed comparably similar levels of PHB in both 48 h and 60 h extractions. In 72 h extraction results also showed the comparable fashion with 60 h. On the other hand, the amount of PHB synthesis was gradually decreasing with the increase in time, i.e. the highest producer NAA2 exhibited ~88% at 48 h which drastically reduced to ~70% at 60 h and 72 h. The exact trend was evidenced with all other strains except NAA1. The decreasing PHB trend with respect time could be due to the reutilization of PHB by the cells for the cell maintenance [23,41]. From these experimental results, it was evident that NAA2 and NAA4 were found to be the best producers among other isolates including positive control, *R. eutropha* (Fig. 5).

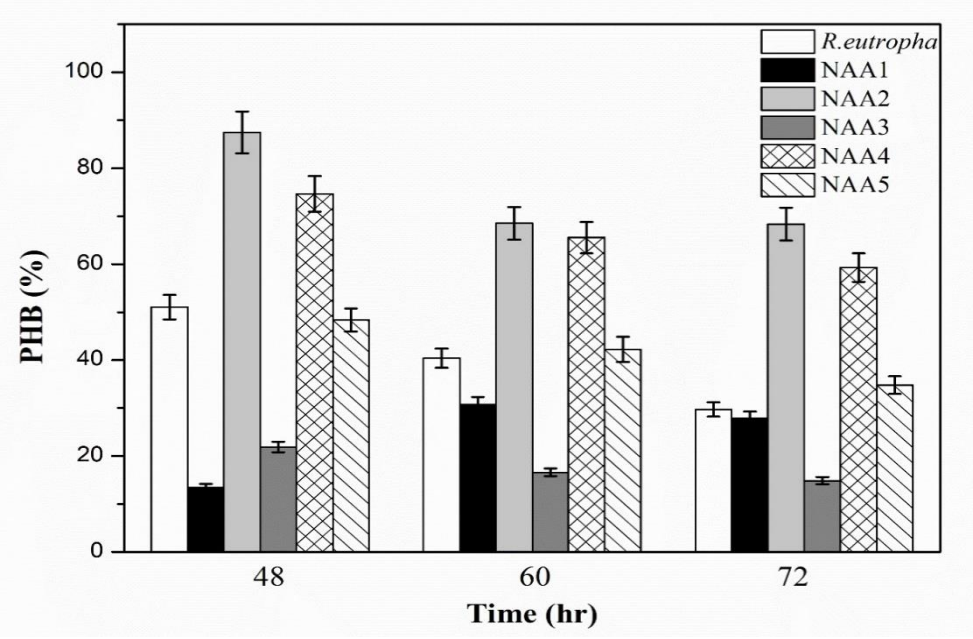

Fig. 5 PHB production of different strains with varying incubation period of 48 h, 60 h &72 h. The standard deviation for the PHB production of different strains were < 5%. (White bar -*R. eutropha*, Black bar-NAA1, Light grey bar-NAA2, Dark grey bar-NAA3, Checked bar-NAA4 and Striped bar-NAA5)

Furthermore, the extracted sample of PHB from NAA2 and NAA4 using sodium hypochlorite method were air dried and stored (Fig. 6(a)). The incubational time impact on the production of PHB from the strains NAA2 and NAA4 exhibited higher PHB production at 48 h (Fig. 6(b)). The amount of PHB production decreases as the time increases in a uniform fashion and this can be attributed due to utilization of PHB by cells in response to nutrient depletion with increased time. Complimentarily the microscopic observation of PHB precipitate in chloroform exhibited the polymeric property and the same sample was observed as crystalized PHB (Fig.7).

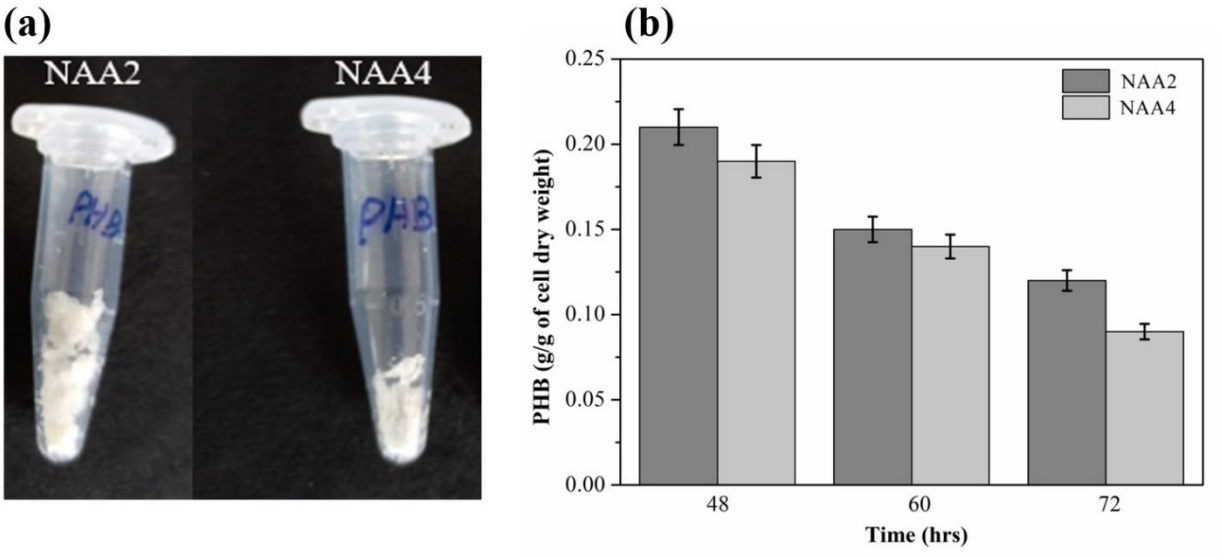

Fig. 6 (a) Extracted and dried PHB using sodium hypochlorite method from strain NAA2 and NAA4, **(b)** PHB production from strains NAA2 & NAA4 with varying incubation period of 48 h, 60 h &72 h. The standard deviations of the PHB production were < 5%. (Dark grey bar-NAA2, Light grey bar-NAA4)

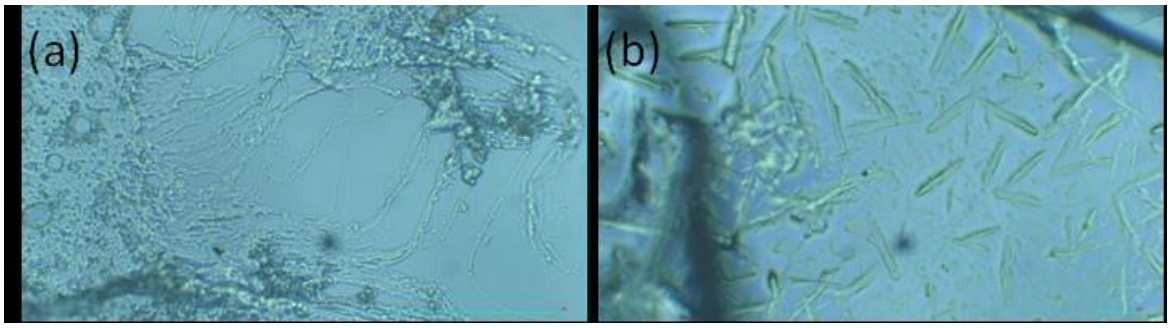

Fig. 7 Microscopic images of extracted PHB from *Bacillus pacificus* via sodium hypochlorite-Chloroform method: **(a)** precipitated PHB crystals in the presence of chloroform exhibiting elastic nature, **(b)** extracted PHB crystals formed when dried at 40°C for overnight.

3. Characterization of PHB positive isolate

From the production studies, NAA2 and NAA4 were found to be the best producers and they were selected for the forthcoming characterization studies. The morphological analysis of these isolates comprises the shape and colour of the colonies, their response to the gram staining and the spore forming ability and briefed in Table 1.

Table 1: Morphological observation of strains NAA2 & NAA4

Morphological Observation	NAA2	NAA4
Colony shape	Circular	Mucoid and large
Colony colour	White	White
Gram's stain	Positive	Negative
Spore Formation	Spore forming	Non- spore forming

16S rRNA analysis

The PHB producing isolates (NAA2 and NAA4) were subjected to 16S rRNA sequencing using the commercial service from IMMUGENIX BIOSCIENCES, Chennai. Around 930 bp of the gene products were amplified using the PCR and then sequenced[38]. Using the Basic Local Alignment Search Tool (BLAST) programme, the obtained sequences were compared and identified as *Bacillus pacificus and Klebsiella quasipneumoniae* through 100% and 99% homology, respectively. The phylogenetic evolution trees of the isolates were constructed using MEGA11 software. The evolutionary history was predicted using the Neighbour-Joining method (Fig. 8). The maximum composite likelihood method was used to compute the evolutionary distances. 18 nucleotide sequences were involved for this analysis. The positions greater than 95% coverage were used in the process whereas others with 5% alignment gap, missing data and ambiguous base were eliminated by partial data elimination method [42-45].

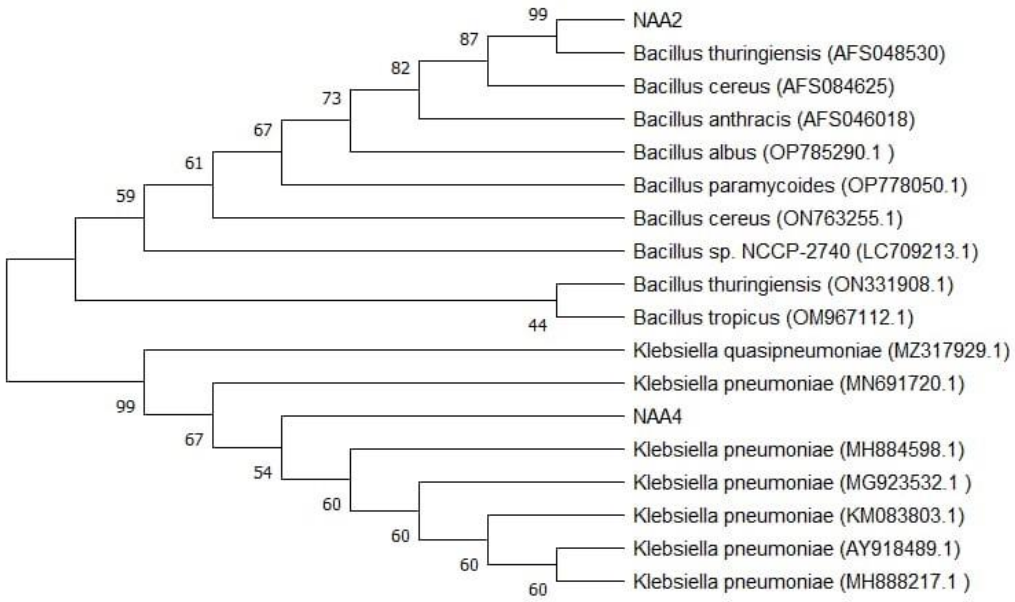

Fig. 8 Phylogenetic tree of the best PHB producing organisms based on 16S rRNA sequencing using MEGA11 tool

4. Polymer characterization

Several analytical techniques were used to evaluate the extracted biopolymers chemical structure. The spectra for the extracted PHB showed excellent agreement with

spectra from both pure PHB and previously published samples. The PHB from the *Bacillus pacificus* and *Klebsiella quasipneumoniae* were successfully extracted and analysed their structural characteristics through FT-IR, ^{1}H NMR analysis.

Fourier-transform infrared spectroscopy analysis

The FTIR spectral analysis of the PHB polymer shows peaks at 1722.18 cm^{-1} and 1279.39 cm^{-1}which are attributed to the rotations around carbon atoms contributed from the functional groups (Fig. 9). The vibrational peak at 1722.18 $cm^{-1}$is attributed to the C=O stretch contributed from the ester group of the PHB. Moreover, the carbonyl group (C=O) is identified as the most prevalent group among all the types of PHB. Furthermore, the peak at t 1279.39 cm^{-1} attributed to the methylene group (CH).

The resultant spectrum contains a strong and broad vibrational peak at 3401.51 cm^{-1} which is contributed by the O-H stretching and the vibrational peak at 2924.03 cm^{-1} is attributed to the C-H stretching from alkane group. The presence of the vibrational peaks at 172.18 cm^{-1} and 1101.01 cm^{-1} confirms the presence of C=O and C-O stretching due to the ester group [46, 47].

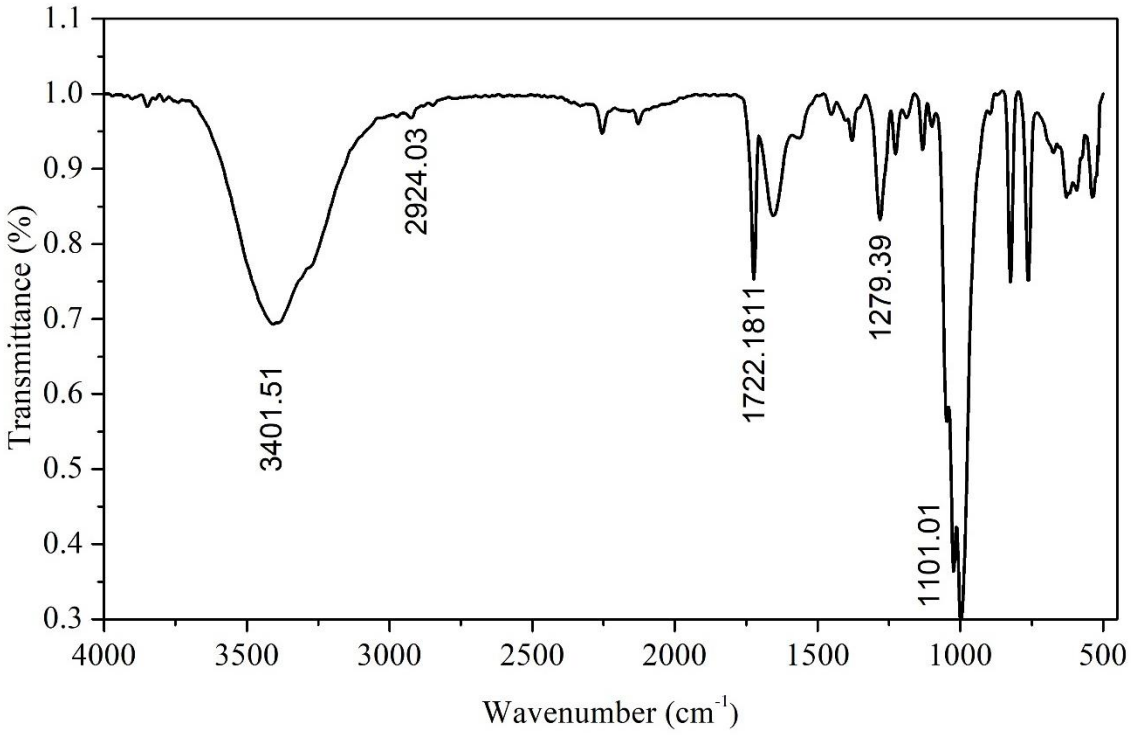

Fig. 9 FT-IR spectra of PHB produced by *Bacillus pacificus.* The FTIR spectral analysis of the PHB polymer shows peaks at 1722.18 cm^{-1} and 1279.39 cm^{-1} which are attributed to the rotations around carbon atoms contributed from the functional groups. The resultant spectrum contains a strong and broad vibrational peak at 3401.51 cm^{-1} which is contributed by the O-H stretching and the vibrational peak at 2924.03 cm^{-1} is attributed to the C-H stretching from alkane group.

^{1}H NMR

A key technique for accurately identifying biopolymer structures is NMR spectroscopy. The structural details of *Bacillus pacificus* and *Klebsiella quasipneumoniae* produced PHB were possibly analysed by ^{1}H NMR [33]. The PHB structure was confirmed by the ^{1}H NMR spectrum, which displayed bands for the CH_3, CH_2, and CH_4 groups.

The methyl group coupled to one proton is responsible for the doublet in the spectrum at 1.77 ppm. The methylene group next to an asymmetric carbon atom with one atom is responsible for the doublet of quadruplet at 2.33 ppm. The methylene group is represented by the multiplet at 5.36 ppm. Two additional signals are seen, including a broad water signal at 1.56 ppm (Fig. 10). Similar findings were previously reported by Bonthrone *et al.*,1992; and Jan *et al.*, 1996 [48,49].

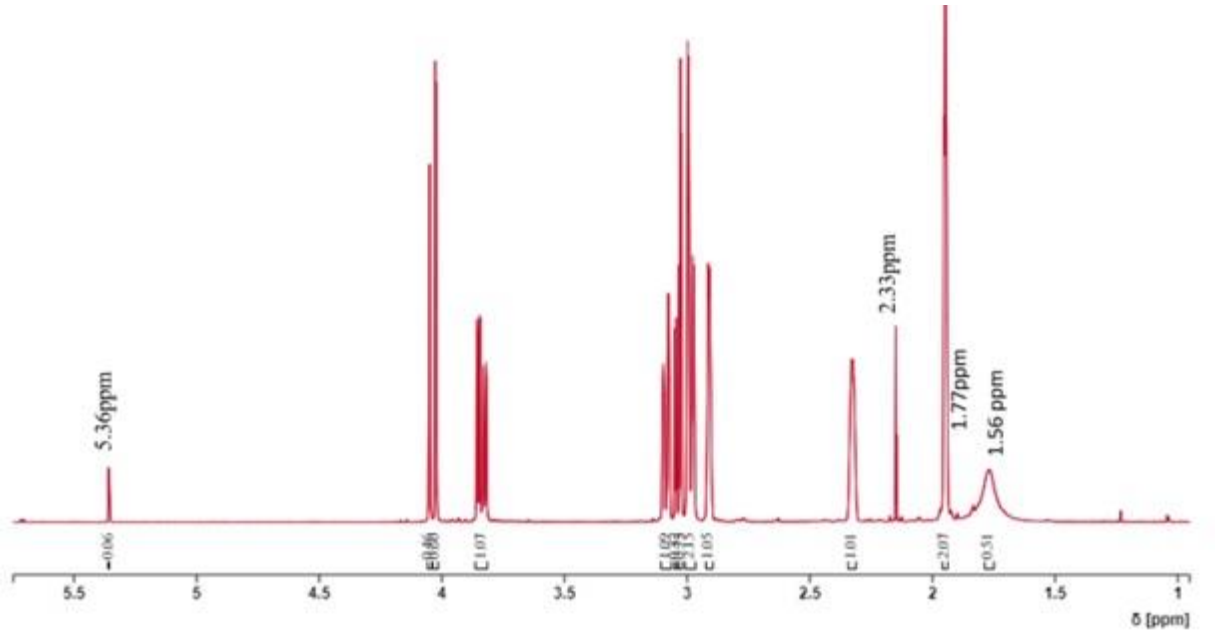

Fig. 10 ^{1}H NMR spectrum of PHB produced by *Bacillus pacificus*. (^{1}H NMR (600 MHz): δ 1.77 (1H, m), 1.94-1.96 (2H, 1.95 (t, *J* = 3.29 Hz), 1.94 (s)), 2.33 (1H, m), 2.91 (1H, m), 2.99 (2H, m), 3.03 (1H, d, *J* = 2.29 Hz), 3.05 (0H, d, *J* = 2.31 Hz), 3.08 (1H, m), 3.84 (1H, ddd, *J* = 15.48, 6.68, 1.24 Hz), 4.02 (1H, s), 4.05 (0H, s), 5.36 (0H, dt, *J* = 1.08, 0.51 Hz), 6.01 (1H, ddd, *J* = 6.94, 1.47, 0.54 Hz), 6.34 (1H, dd, *J* = 9.02, 1.35 Hz), 7.30 (1H, m))

5. Conclusion

In this study, different bacterial strains were isolated from lake soil sample and screened for polyhydroxybutyrate production using MSM media. From 74 colonies, 5 colonies showed challenging results in various staining techniques. The shake flask production studies revealed that NAA2 and NAA4 as potential PHB producing candidates. When evaluated under shake flask for growth and PHB biosynthesis, both of the chosen isolates performed well, accumulating PHB up to 87.5% and 74% of the total biomass, respectively. The morphological and biochemical characterization led to the identification of NAA2 and NAA4 as *Bacillus pacificus and Klebsiella quasipneumoniae*, respectively. The FTIR and NMR characterization studies confirmed the presence of PHB extracted from the fermentation of

NAA2 and NAA4. The development of process for the enhanced synthesis of PHB is under progress.

6. References

[1] I. S. Sidek, S. F. S. Draman, S. R. S. Abdullah, and N. Anuar, 'Current development on bioplastics and its future prospects: an introductory review', *in wascon Technology Magazine*, 2019, doi: 10.26480/itechmag.01.2019.03.08.

[2] B. McAdam, M. B. Fournet, P. McDonald, and M. Mojicevic, 'Production of polyhydroxybutyrate (PHB) and factors impacting its chemical and mechanical characteristics', *Polymers*, vol. 12, no. 12. 2020. doi: 10.3390/polym12122908.

[3] M. Okan, H. M. Aydin, and M. Barsbay, 'Current approaches to waste polymer utilization and minimization: a review', *Journal of Chemical Technology and Biotechnology*, vol. 94, no. 1. 2019. doi: 10.1002/jctb.5778.

[4] K. P. Rajan, S. P. Thomas, A. Gopanna, and M. Chavali, 'Polyhydroxybutyrate (PHB): A standout biopolymer for environmental sustainability', in *Handbook of Ecomaterials*, vol. 4, 2019. doi: 10.1007/978-3-319-68255-6_92.

[5] B. García*et al.*, 'Novel Biodegradable Aromatic Plastics from a Bacterial Source', *Journal of Biological Chemistry*, vol. 274, no. 41, 1999, doi: 10.1074/jbc.274.41.29228.

[6] J. Carruthers, 'Technology Overview Biocomposites', *Technology Strategy Board*, 2012.

[7] C. Forman, 'Plastics for Barrier Packaging', 2017.

[8] A. Iordanskii, 'Bio-Based and biodegradable plastics: From passive barrier to active packaging behavior', *Polymers*, vol. 12, no. 7. 2020. doi: 10.3390/polym12071537.

[9] E. Zanchetta*et al.*, 'Algal cellulose, production and potential use in plastics: Challenges and opportunities', *Algal Research*, vol. 56. 2021. doi: 10.1016/j.algal.2021.102288.

[10] Z. Wang, Y. Xu, R. Liu, and X. Zhu, 'Planstic: Biodegradable Plastic with High-Entropy Fibers Made from Waste Plastic and Plant Leaves', *ACS Appl Polym Mater*, vol. 3, no. 11, 2021, doi: 10.1021/acsapm.1c01242.

[11] Muhammadi, Shabina, M. Afzal, and S. Hameed, 'Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: Production, biocompatibility, biodegradation, physical properties and applications', *Green Chem Lett Rev*, vol. 8, no. 3–4, 2015, doi: 10.1080/17518253.2015.1109715.

[12] C. S. K. Reddy, R. Ghai, Rashmi, and V. C. Kalia, 'Polyhydroxyalkanoates: An overview', *Bioresource Technology*, vol. 87, no. 2. 2003. doi: 10.1016/S0960-8524(02)00212-2.

[13] R. A. J. Verlinden, D. J. Hill, M. A. Kenward, C. D. Williams, and I. Radecka, 'Bacterial synthesis of biodegradable polyhydroxyalkanoates', *Journal of Applied Microbiology*, vol. 102, no. 6. 2007. doi: 10.1111/j.1365-2672.2007.03335.x.

[14] V. C. Kalia, *Biotechnological applications of polyhydroxyalkanoates*. 2019. doi: 10.1007/978-981-13-3759-8.

[15] V. C. Kalia, S. Ray, S. K. S. Patel, M. Singh, and G. P. Singh, 'The dawn of novel biotechnological applications of polyhydroxyalkanoates', in *Biotechnological Applications of Polyhydroxyalkanoates*, 2019. doi: 10.1007/978-981-13-3759-8_1.

[16] M. E. Grigore, R. M. Grigorescu, L. Iancu, R. M. Ion, C. Zaharia, and E. R. Andrei, 'Methods of synthesis, properties and biomedical applications of polyhydroxyalkanoates: a review', *Journal of Biomaterials Science, Polymer Edition*, vol. 30, no. 9. 2019. doi: 10.1080/09205063.2019.1605866.

[17] L. Yañez, R. Conejeros, A. Vergara-Fernández, and F. Scott, 'Beyond Intracellular Accumulation of Polyhydroxyalkanoates: Chiral Hydroxyalkanoic Acids and Polymer Secretion', *Frontiers in Bioengineering and Biotechnology*, vol. 8. 2020. doi: 10.3389/fbioe.2020.00248.

[18] Y. S. Mostafa, S. A. Alrumman, S. A. Alamri, K. A. Otaif, M. S. Mostafa, and A. M. Alfaify, 'Bioplastic (poly-3-hydroxybutyrate) production by the marine bacterium Pseudodonghicolaxiamenensis through date syrup valorization and structural assessment of the biopolymer', *Sci Rep*, vol. 10, no. 1, 2020, doi: 10.1038/s41598-020-65858-5.

[19] R. Turco, G. Santagata, I. Corrado, C. Pezzella, and M. di Serio, 'In vivo and Post-synthesis Strategies to Enhance the Properties of PHB-Based Materials: A Review', *Frontiers in Bioengineering and Biotechnology*, vol. 8. 2021. doi: 10.3389/fbioe.2020.619266.

[20] A. Jayakumar, K. Prabhu, L. Shah, and P. Radha, 'Biologically and environmentally benign approach for PHB-silver nanocomposite synthesis and its characterization', *Polym Test*, vol. 81, 2020, doi: 10.1016/j.polymertesting.2019.106197.

[21] J. L. Castro-Mayorga, F. Freitas, M. A. M. Reis, M. A. Prieto, and J. M. Lagaron, 'Biosynthesis of silver nanoparticles and polyhydroxybutyrate nanocomposites of interest in antimicrobial applications', *Int J BiolMacromol*, vol. 108, 2018, doi: 10.1016/j.ijbiomac.2017.12.007.

[22] C. C. Chien, C. C. Chen, M. H. Choi, S. S. Kung, and Y. H. Wei, 'Production of poly-β-hydroxybutyrate (PHB) by Vibrio spp. isolated from marine environment', *J Biotechnol*, vol. 132, no. 3, 2007, doi: 10.1016/j.jbiotec.2007.03.002.

[23] A. Getachew and F. Woldesenbet, 'Production of biodegradable plastic by polyhydroxybutyrate (PHB) accumulating bacteria using low cost agricultural waste material', *BMC Res Notes*, vol. 9, no. 1, 2016, doi: 10.1186/s13104-016-2321-y.

[24] A. R. Reddy, R. B. Kumar, and K. V. Prabhakar, 'Isolation and Identification of PolyHydroxyButyrate (PHB) producing bacteria from Sewage sample', *Res J Pharm Technol*, vol. 10, no. 4, 2017, doi: 10.5958/0974-360x.2017.00193.7.

[25] S. Obruca*et al.*, 'The presence of PHB granules in cytoplasm protects non-halophilic bacterial cells against the harmful impact of hypertonic environments', *N Biotechnol*, vol. 39, 2017, doi: 10.1016/j.nbt.2017.07.008.

[26] Y. Jiang, L. Marang, R. Kleerebezem, G. Muyzer, and M. C. M. van Loosdrecht, 'Polyhydroxybutyrate production from lactate using a mixed microbial culture', *BiotechnolBioeng*, vol. 108, no. 9, 2011, doi: 10.1002/bit.23148.

[27] S. Kliem, M. Kreutzbruck, and C. Bonten, 'Review on the biological degradation of polymers in various environments', *Materials*, vol. 13, no. 20. 2020. doi: 10.3390/ma13204586.

[28] J. Lee, H. J. Park, M. Moon, J. S. Lee, and K. Min, 'Recent progress and challenges in microbial polyhydroxybutyrate (PHB) production from CO2 as a sustainable feedstock: A state-of-the-art review', *Bioresource Technology*, vol. 339. 2021. doi: 10.1016/j.biortech.2021.125616.

[29] J. C. C. Yeo, J. K. Muiruri, W. Thitsartarn, Z. Li, and C. He, 'Recent advances in the development of biodegradable PHB-based toughening materials: Approaches, advantages and applications', *Materials Science and Engineering C*, vol. 92. 2018. doi: 10.1016/j.msec.2017.11.006.

[30] M. Narayanan, S. Kandasamy, S. Kumarasamy, K. Gnanavel, M. Ranganathan, and G. Kandasamy, 'Screening of polyhydroxybutyrate producing indigenous bacteria from polluted lake soil', *Heliyon*, vol. 6, no. 10, 2020, doi: 10.1016/j.heliyon.2020.e05381.

[31] O. I. M. El-Hamshary, H. A. Kadi, and N. H. Al-Twaty, 'Molecular Characterization and Uv Improvement of Some Bioplastic-Producing Bacteria Isolated From Plants in Taif City, Saudi Arabia.', *Pharmacophore*, vol. 9, no. 2, 2018.

[32] J. Juengert, S. Bresan, and D. Jendrossek, 'Determination of Polyhydroxybutyrate (PHB) Content in Ralstoniaeutropha Using Gas Chromatography and Nile Red Staining', *Bio Protoc*, vol. 8, no. 5, 2018, doi: 10.21769/bioprotoc.2748.

[33] M. Nishida, T. Tanaka, Y. Hayakawa, and M. Nishida, 'Solid-State nuclear magnetic resonance (NMR) and nuclear magnetic relaxation time analyses of molecular mobility and compatibility of plasticized polyhydroxyalkanoates (PHA) copolymers', *Polymers (Basel)*, vol. 10, no. 5, 2018, doi: 10.3390/polym10050506.

[34] R. Rendón-Villalobos, A. Ortíz-Sánchez, E. Tovar-Sánchez, and E. Flores-Huicochea, 'The Role of Biopolymers in Obtaining Environmentally Friendly Materials', in *Composites from Renewable and Sustainable Materials*, 2016. doi: 10.5772/65265.

[35] P. Spiekermann, B. H. A. Rehm, R. Kalscheuer, D. Baumeister, and A. Steinbüchel, 'A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds', *Arch Microbiol*, vol. 171, no. 2, 1999, doi: 10.1007/s002030050681.

[36] A. A. Alarfaj, M. Arshad, E. N. Sholkamy, and M. A. Munusamy, 'Extraction and characterization of polyhydroxybutyrates (PHB) from Bacillus thuringiensis KSADL127 isolated from mangrove environments of Saudi Arabia', *Brazilian Archives of Biology and Technology*, vol. 58, no. 5, pp. 781–788, 2015, doi: 10.1590/S1516-891320150500003.

[37] B. Hungund, V. S. Shyama, P. Patwardhan, and A. M. Saleh, 'Production of polyhydroxyalkanoate from paenibacillusdurus BV-1 isolated from oil mill soil', *J MicrobBiochemTechnol*, vol. 5, no. 1, 2013, doi: 10.4172/1948-5948.1000092.

[38] P. Singh and N. Parma, 'Isolation and characterization of two novel polyhydroxybutyrate (PHB)-producing bacteria', *Afr J Biotechnol*, vol. 10, no. 24, 2011.

[39] M. R. Zakaria *et al.*, 'Biosynthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer from wild-type Comamonas sp. EB172', in *Polymer Degradation and Stability*, 2010, vol. 95, no. 8. doi: 10.1016/j.polymdegradstab.2010.01.020.

[40] A. A. Alarfaj, M. Arshad, E. N. Sholkamy, and M. A. Munusamy, 'Extraction and characterization of polyhydroxybutyrates (PHB) from Bacillus thuringiensis KSADL127 isolated from mangrove environments of Saudi Arabia', *Brazilian Archives of Biology and Technology*, vol. 58, no. 5, 2015, doi: 10.1590/S1516-891320150500003.

[41] W. N. Chaudhry, N. Jamil, I. Ali, M. H. Ayaz, and S. Hasnain, 'Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources', *Ann Microbiol*, vol. 61, no. 3, 2011, doi: 10.1007/s13213-010-0181-6.

[42] N. Saitou and M. Nei, 'The neighbor-joining method: a new method for reconstructing phylogenetic trees.', *Mol BiolEvol*, vol. 4, no. 4, 1987, doi: 10.1093/oxfordjournals.molbev.a040454.

[43] J. Felsenstein, 'Confidence Limits on Phylogenies: An Approach Using the Bootstrap', *Evolution (N Y)*, vol. 39, no. 4, 1985, doi: 10.2307/2408678.

[44] K. Tamura, M. Nei, and S. Kumar, 'Prospects for inferring very large phylogenies by using the neighbor-joining method', *Proc Natl Acad Sci U S A*, vol. 101, no. 30, 2004, doi: 10.1073/pnas.0404206101.

[45] K. Tamura, G. Stecher, and S. Kumar, 'MEGA11: Molecular Evolutionary Genetics Analysis Version 11', *Mol BiolEvol*, vol. 38, no. 7, 2021, doi: 10.1093/molbev/msab120.

[46] R. Sindhu *et al.*, 'Production and characterization of poly-3-hydroxybutyrate from crude glycerol by Bacillus sphaericus NII 0838 and improving its thermal properties by blending with other polymers', *Brazilian Archives of Biology and Technology*, vol. 54, no. 4, 2011, doi: 10.1590/s1516-89132011000400019.

[47] S. Dubey, P. Bharmoria, P. S. Gehlot, V. Agrawal, A. Kumar, and S. Mishra, '1-Ethyl-3-methylimidazolium Diethylphosphate Based Extraction of Bioplastic "polyhydroxyalkanoates" from Bacteria: Green and Sustainable Approach', *ACS Sustain Chem Eng*, vol. 6, no. 1, 2018, doi: 10.1021/acssuschemeng.7b03096.

[48] K. M. Bonthrone, J. Clauss, D. M. Horowitz, B. K. Hunter, and J. K. M. Sanders, 'The biological and physical chemistry of polyhydroxyalkanoates as seen by NMR spectroscopy', *FEMS Microbiol Lett*, vol. 103, no. 2–4, 1992, doi: 10.1016/0378-1097(92)90320-N.

[49] S. Jan, C. Roblot, J. Courtois, B. Courtois, J. N. Barbotin, and J. P. Séguin, '1H NMR spectroscopic determination of poly 3-hydroxybutyrate extracted from microbial biomass', *Enzyme MicrobTechnol*, vol. 18, no. 3, 1996, doi: 10.1016/0141-0229(95)00096-8.