



## Biotechnology and Industrial Microbiology

# Evaluation of short-chain-length polyhydroxyalkanoate accumulation in *Bacillus aryabhatai*

Aneesh Balakrishna Pillai, Arjun Jaya Kumar, Kavitha Thulasi, Harikrishnan Kumarapillai\*

Rajiv Gandhi Centre for Biotechnology (RGCB), Environmental Biology Laboratory, Thiruvananthapuram, Kerala, India

### ARTICLE INFO

#### Article history:

Received 25 July 2016

Accepted 10 January 2017

Available online 11 March 2017

Associate Editor: Eleni Gomes

#### Keywords:

*Bacillus aryabhatai* PHB10

Polyhydroxybutyrate

Domestic sewerage

Polymer characterization

16S rRNA

### ABSTRACT

This study was focused on the polyhydroxybutyrate (PHB) accumulation property of *Bacillus aryabhatai* isolated from environment. Twenty-four polyhydroxyalkanoate (PHA) producers were screened out from sixty-two environmental bacterial isolates based on Sudan Black B colony staining. Based on their PHA accumulation property, six promising isolates were further screened out. The most productive isolate PHB10 was identified as *B. aryabhatai* PHB10. The polymer production maxima were 3.264 g/L, 2.181 g/L, 1.47 g/L, 1.742 g/L and 1.786 g/L in glucose, fructose, maltose, starch and glycerol respectively. The bacterial culture reached its stationary and declining phases at 18 h and 21 h respectively and indicated growth-associated PHB production. Nuclear Magnetic Resonance (NMR) spectra confirmed the material as PHB. The material has thermal stability between 30 and 140 °C, melting point at 170 °C and maximum thermal degradation at 287 °C. The molecular weight and poly dispersion index of the polymer were found as 199.7 kDa and 2.67 respectively. The bacterium *B. aryabhatai* accumulating PHB up to 75% of cell dry mass utilizing various carbon sources is a potential candidate for large scale production of bacterial polyhydroxybutyrate.

© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

## Introduction

Polyhydroxyalkanoates (PHAs) are reserves of carbon and energy found in bacteria in the form of intracellular inclusions. They are synthesized and deposited when bacterial cells

are cultured in a medium containing surplus amount of carbon source with inadequate supply of other nutrients.<sup>1</sup> These are biodegradable-biocompatible thermoplastics, non-toxic, hydrophobic, impermeable to gases, piezoelectric, enantiomerically pure and show a high degree of polymerization with molecular weights of 20,000 to 30 million Daltons.<sup>2–4</sup>

\* Corresponding author at: Environmental Biology Laboratory, Rajiv Gandhi Centre for Biotechnology (RGCB), Poojappura, Thycaud P. O., Thiruvananthapuram 14, Kerala, India.

E-mail: [harikrishnan@rgcb.res.in](mailto:harikrishnan@rgcb.res.in) (H. Kumarapillai).

<http://dx.doi.org/10.1016/j.bjm.2017.01.005>

1517-8382/© 2017 Sociedade Brasileira de Microbiologia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

PHAs have attracted a great deal of attention because of their bio-degradability and thermoplastic properties.<sup>5</sup> They show physical and material properties which make them suitable for applications in various fields such as manufacturing of packaging materials, as biomedical implant materials, as drug delivery carriers, as biofuels, as water resistant coatings on cardboard or paper, as additives in cosmetics and in food processing industries.<sup>2,6-8</sup> PHAs can be produced from renewable resources and they are considered as an alternative to non-biodegradable plastics produced from fossil oils.<sup>9</sup> Commercial production of PHA is limited by the high cost of production compared to conventional plastics. The main focus on the biopolymer research is to develop economically feasible methods for the large scale production of good quality biopolymer.

Most PHAs have been produced by prokaryotic microorganisms, including bacteria and archaea, although transgenic plants were reported to produce PHAs.<sup>10</sup> In prokaryotes PHA accumulation property is broadly distributed among the Gram-negative organisms such as *Cupriavidus*,<sup>11</sup> *Pseudomonas*,<sup>12</sup> etc., Gram-positive organisms such as *Bacillus*,<sup>12</sup> *Clostridium*,<sup>13</sup> *Corynebacterium*,<sup>14</sup> *Nocardia*, *Rhodococcus*,<sup>15</sup> *Streptomyces*,<sup>16</sup> *Staphylococcus*,<sup>17</sup> etc. and certain archaeal strains of *Halobacterium*,<sup>18</sup> *Haloarcula*,<sup>19</sup> *Haloquadratum*<sup>20</sup> and *Haloferax*.<sup>21</sup> *Bacillus* spp. are well known for their ability to accumulate poly-3-hydroxybutyrate (PHB) which is the most common and simplest form of PHA found in bacteria.<sup>22-25</sup> PHB is the first discovered and the most extensively studied biopolymer.<sup>26,27</sup>

*Bacillus aryabhatai* was first isolated from cryotubes used for collecting air from upper atmosphere.<sup>28</sup> The PHB accumulating property of this strain was reported by Van-Thuoc et al.<sup>29</sup> In this study we isolated a *B. aryabhatai* strain from soil and its molecular characterization was done. We also investigated the polyhydroxybutyrate biosynthetic property of the strain, variations in polymer accumulation in response to change in carbon source, time course analysis of polymer accumulation and its polymer characteristics.

## Materials and methods

### Sampling, isolation and maintenance of bacterial strains

Soil samples were collected from paddy fields, forests, riverbeds, sewerage system and estuaries of Kerala, India. Bacterial strains were isolated on nutrient agar medium (5 g of peptone, 5 g of sodium chloride, 1.5 g of beef extract, 1.5 g of yeast extract and 15 g of agar per liter at pH 7.4) (Himedia Laboratories, Mumbai, India) from serially diluted samples and incubated overnight. Since these are environmental bacterial isolates, incubation in initial experiments were done at room temperature (30 °C). The colonies were streaked several times for making them as pure cultures. The isolates were labeled as PHB series (PHB1-62) and were maintained on nutrient agar slants and stored at 4 °C.<sup>30,31</sup>

### Preparation of seed inoculum

One loop full of the culture from slant was inoculated in 5 mL of sterile nutrient broth (5 g of peptone, 5 g of sodium

chloride, 1.5 g of beef extract and 1.5 g of yeast extract per liter at pH 7.4) (Himedia Laboratories, Mumbai, India). After incubation for 24 h at room temperature, 1% (v/v) of culture having 10<sup>8</sup> cells/mL was aseptically transferred into 50 mL sterile nutrient broth and incubated for 18 h at room temperature. From this, inoculum was added at 1% level in all the polymer quantification experiments.

### Screening of isolates for PHA production

Bulk screening of isolates was done by colony staining on half strength nutrient agar (2.5 g of peptone, 2.5 g of sodium chloride, 0.75 g of beef extract, 0.75 g of yeast extract and 15 g agar per liter at pH 7.4) supplemented with 20 g/L glucose, after 48 h of incubation at room temperature.<sup>32</sup> *E. coli* colony was used as negative control. The bacterial colonies on Petri plates were flooded with Sudan Black B solution (0.05% in ethanol) and kept undisturbed for 30 min. The excess stain was washed out by sterile saline and the dark blue colored colonies were identified as PHA positive.

### Staining for PHA accumulation and microscopy

48 h old bacterial cells grown in basal medium (1.5 g of peptone, 1.5 g of yeast extract, 1 g of Na<sub>2</sub>HPO<sub>4</sub> and 0.2 g of MgSO<sub>4</sub>·7H<sub>2</sub>O per liter, pH 7.2) supplemented with 20 g/L glucose at room temperature were taken for staining and microscopic analysis. Sudan Black B stained smear was observed under 100× oil immersion objective lens of light microscope Nikon YS100 (Nikon Corporation, Tokyo, Japan).<sup>33</sup> Nile Red stained cell suspension was taken on a glass slide and was covered by a coverslip.<sup>34-36</sup> The cells were imaged on a Nikon A1R-Si laser scanning confocal spectral microscope with 50× magnification (Nikon Corporation, Tokyo, Japan) excited at 561 nm.

Scanning Electron Microscopy (SEM) analysis was performed according to Soo-Hwan et al. with some modifications.<sup>37</sup> Polymer accumulated bacterial cells were harvested, washed in phosphate buffered saline (PBS) and fixed overnight in 3% glutaraldehyde solution. The fixed cells were again washed in PBS to remove excess glutaraldehyde and successively dehydrated in 30%, 50%, 70%, 80% and 100% ethanol. 5 μL of this cell suspension was sputter coated with gold and analyzed in a Scanning Electron Microscope JEOL Model JSM – 6390LV (JEOL USA, Inc., MA, USA).

### Evaluation of bacterial strains for PHA production

From the isolates, six cultures producing considerable amount of PHA granules were inoculated in 1000 mL basal medium with 20 g/L of glucose and incubated at room temperature for 48 h at agitation rate of 150 rpm. The experiment was done in triplicate and the cells harvested were washed with sterile normal saline. The biomass obtained was lyophilized and the cell dry mass (CDM) was calculated. Polymer was extracted from the lyophilized cells, weighed and estimated the yield in percentage (w/w).<sup>34,38</sup>

### Morphological, biochemical and molecular characterization

The isolate was morphologically and biochemically characterized following the standard microbiological methods.<sup>39,40</sup> For molecular characterization, genomic DNA was isolated using Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, USA). 16S rRNA gene was amplified using the universal eubacterial primers 27F and 1492R.<sup>41</sup> Sequencing PCR was done with ABI PRISM Big Dye terminator v3.1 cycle sequencing kit and the sequencing was done in AB 3730 DNA analyzer (Life Technologies, CA, USA). The sequences obtained were viewed with ABI sequence scanner v1.0, compiled and aligned using BioEdit version 7.0.9.0.<sup>42</sup> Phylogenetic tree was constructed based on neighbor-joining method using MEGA 7.0.20 with bootstrap values for 1000 replicates.<sup>43,44</sup> *E. coli* and *Pseudomonas stutzeri* were used as outgroup. Reference sequences were retrieved from GenBank under the accession numbers indicated on the phylogenetic tree.

### Analysis of PHB accumulation at different temperature and carbon sources

20 g/L of glucose, fructose, maltose, starch and glycerol were supplemented independently as carbon sources in 1000 mL basal medium. The PHA accumulation property of the most productive isolate was evaluated using different carbon sources at temperatures 28, 31, 34, 37 and 40 °C for 48 h at agitation rate of 150 rpm. After incubation, the biomass was harvested, the polymer was extracted using the same method as described before and results were compared.<sup>34,38,45</sup>

### Growth rate analysis

In order to evaluate the growth pattern in a nutrient rich condition, 100 µL of the seed inoculum was added to 100 mL of nutrient broth at 31 °C and agitation rate of 150 rpm. At 1 h intervals, 100 µL of the culture sample was taken and measured the optical density at 600 nm in an Infinite 200 PRO multimode reader (Tecan Group Ltd., Maennedorf, Switzerland).<sup>39</sup>

### Time-course analysis of PHB production

Polymer accumulation rate of the isolate PHB10 at different time points were estimated by culturing in 1000 mL basal medium supplemented with 20 g/L glucose at 31 °C and agitation rate of 150 rpm.<sup>46</sup> The cells were harvested at each 2 h interval up to 72 h and the cell dry mass (CDM) and percentage PHA content were estimated.

### Polymer characterization

The proton Nuclear Magnetic Resonance Spectroscopy (<sup>1</sup>H NMR) and <sup>13</sup>C NMR spectra of the polymer were recorded after suspending the polymer in high purity deuteriochloroform (CDCl<sub>3</sub>).<sup>47</sup> <sup>1</sup>H NMR spectra were obtained in model Bruker Avance<sup>II</sup> 500 NMR spectrometer at 500 MHz and magnetic field strength of 11.7 T. For <sup>13</sup>C NMR spectra, model Bruker Avance<sup>III</sup> 400 NMR spectrometer was used at 400 MHz and 9.4 T (Bruker Corporation, Massachusetts, USA). Differential

Scanning Calorimetry (DSC) analysis of PHA sample was carried out in PerkinElmer DSC6000-Pyris Series instrument (PerkinElmer, Inc., Massachusetts, USA) under flowing nitrogen atmosphere at a heating rate of 10 °C per min using aluminum sample holder for taking thermogram.<sup>48</sup> 2–5 mg of sample was taken for analysis in order to ensure the uniformity of temperature and good reproducibility. Thermogravimetric analysis (TGA) was carried out using SDT Q600 V8.3 Build 101 thermal analyzer instrument (TA Instruments, Inc., Delaware, USA) over a temperature range from 28 to 600 °C at a heating rate of 10 °C/min.<sup>47</sup> The number-average molecular weight (M<sub>n</sub>), weight-average molecular weight (M<sub>w</sub>) and poly dispersion index (PDI) of the polymer were determined by Gel Permeation Chromatography (GPC) using Waters HPLC system with 600 Series Pump and Waters Styragel HR series HR5E/4E/2/0.5 column equipped with a 7725 Rheodyne injector and refractive index 2414 detector (Waters Corporation, Massachusetts, USA).<sup>49,50</sup> Chloroform was used as eluent at a flow rate of 1.0 mL/min. Polystyrene standards of molecular weight 1,865,000, 34,300, 685 were used for relative calibration.

## Results

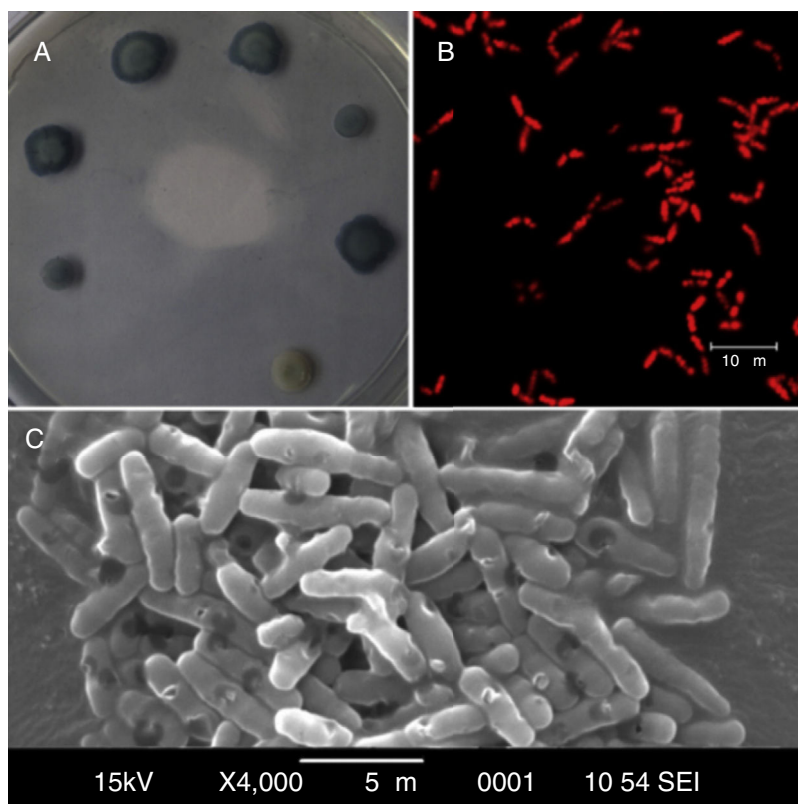
A large number of white, yellow, pink and peach colored colonies were grown on nutrient agar plates after plating the environmental samples. 62 bacterial colonies were isolated and out of these, 24 PHA accumulating isolates which showed darkly stained colony morphology were screened out by Sudan Black B colony staining (Fig. 1A). From these positive isolates, six isolates were selected based on the size of their granules as seen under microscope for yield analysis. Isolate named PHB10 was the best among the six with a polymer level 63% of CDM (Fig. S1) and was selected for further studies. Nile red staining showed bright red fluorescence (Fig. 1B) and Scanning Electron Micrograph (SEM) showed bacillus morphology (Fig. 1C).

### Strain identification

Morphological, biochemical characterizations and antibiogram of the isolate PHB10 are given in Table 1. Based on these characteristics, along with 16S rRNA gene sequence homology (99%) with *B. aryabhatai* strain CM44 and was referred to as *B. aryabhatai* PHB10. The 16S rRNA sequence of this isolate has been submitted to NCBI gene bank (Accession no. KF056892) and the bacterial strain was deposited in MTCC (Accession no. 12561). The phylogenetic position of the isolate is depicted in Fig. 2.

### Analysis of PHA accumulation at different temperature and carbon sources

When glucose was used as carbon source at 31 °C, the cell dry mass and polymer yield were obtained at its maximum of 4.36 g/L and 74.89% (3.26 g/L) respectively (Table 2). At incubation temperatures above and below this point PHB10 exhibited a decreasing trend in biomass production as well as PHA accumulation. In presence of other carbon sources such as



**Fig. 1 – Staining for PHA accumulation and microscopy (A) Sudan Black B colony staining for PHA accumulation, negative control (*E. coli*) and six PHA positive isolates in anticlockwise order, (B) confocal fluorescence micrograph of polymer accumulated in PHB10 cells, (C) Scanning Electron Micrograph of PHB10 cells.**

fructose, maltose, starch and glycerol, the strain showed maximum polymer yield of 65.37% (2.18 g/L), 51.15% (1.47 g/L), 49.15% (1.742 g/L) and 65.92% (1.786 g/L) respectively at 34 °C.

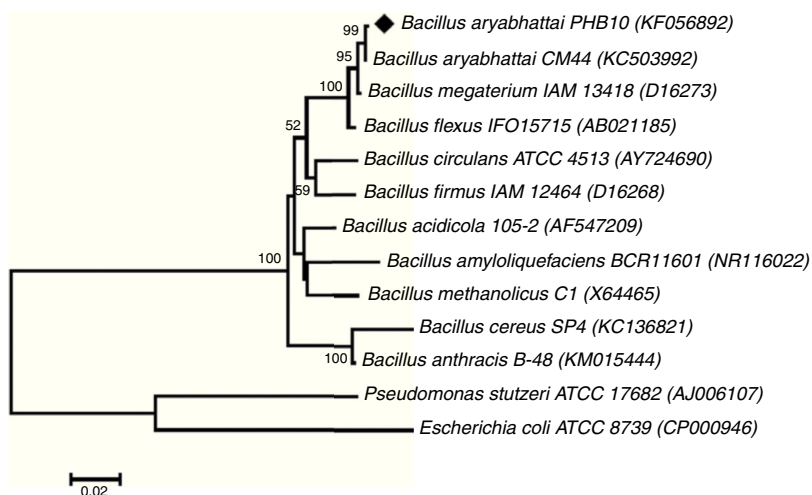
#### Growth rate analysis

The isolate was grown in nutrient broth and reached its log phase in 2 h, mid-log phase at 8 h and attained its maximum

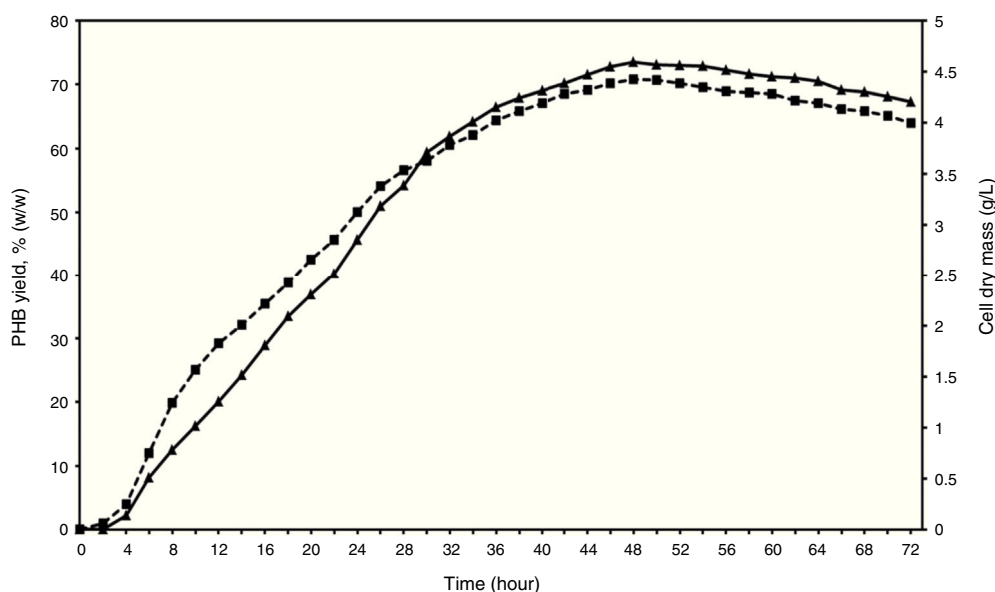
rate at 18 h of incubation. The culture entered its declining phase after 21 h of incubation (data not shown).

#### Time-course analysis of polymer production

The time-course analysis of polymer production revealed that PHA production in *B. aryabhattai* is growth associated. The polymer accumulation begins along with the cell growth, i.e.,



**Fig. 2 – Phylogenetic tree depicting the position of PHB10 based on 16S rDNA sequence. Neighbor-Joining tree was constructed with MEGA 7.0.20 with bootstrap values for 1000 replicates. *E. coli* and *P. stutzeri* were used as outgroup.**



**Fig. 3** – Time-course analysis of cell growth and polymer yield of PHB10 in basal medium supplemented with 20 g/L glucose at 31 °C, closed squares – cell dry mass (g/L), closed triangles – PHB yield (% w/w).

**Table 1** – Morphological and biochemical characteristics of the isolate PHB10.

Characteristic	Result
Isolation source	Domestic sewerage soil
Colony diameter (mm)	5–9
Colony shape	Entire, round and flat
Colony color	Ivory
Oxidase	+
Catalase	+
Urease	+
$\beta$ -Galactosidase	+
Gelatin hydrolysis	–
Starch hydrolysis	+
Reduction of nitrate to nitrite	+
Indole production	–
Methyl red test	+
Voges-Proskauer test	+
Malonate utilization	+
Citrate utilization	–
Acid production from	
Glucose	+
Lactose	+
Maltose	+
Sucrose	+
Antibiotic sensitivity	
Penicillin	R
Ampicillin	R
Tetracycline	IR
Co Trimoxazole	S
Cephadrine	R
Gentamycin	S
Colistin	R
Cephataxime	R
Streptomycin	IR
Doxycycline	R
Chloramphenicol	S

R, resistant; IR, intermediate resistant; S, sensitive.

from the log phase itself. The polymer accumulation rate increased exponentially up to stationary phase at 36 h of incubation and reached its maximum level after 60 h, contributing 65% of CDM (Fig. 3).

#### Polymer characterization

Fig. 4A shows the  $^1\text{H}$  NMR spectrum from polymer synthesized in the presence of 20 g/L glucose. The spectrum showed the expected resonances for PHB between 5.22–5.28 ppm, 2.45–2.62 ppm and 1.26–1.28 ppm. Scans of  $^{13}\text{C}$  NMR (Fig. 4B) showed prominent peaks at 169.13, 67.62, 40.81 and 19.76 ppm. The NMR spectra were compared with standard PHB (Sigma–Aldrich, MO, USA) (Figs. S2 and S3). Through DSC analysis the melting point of the polymer was found as 170 °C and it was thermally stable in a temperature range of 30 °C to 140 °C (Fig. 5A). Fig. 5B shows the TGA thermogram of the PHA film. Thermal degradation of the polymer occurs at 247 °C and maximum degradation at 287 °C whereas the standard PHB (Sigma–Aldrich) showed the respective degradations at 212 °C and 266 °C. GPC analysis suggests that the polymer has a number average molecular weight ( $M_n$ ) of 74.874 kDa and a weight average molecular weight ( $M_w$ ) of 199.740 kDa with poly dispersion index (PDI) 2.67.

#### Discussion

Since petroleum-based plastics are becoming a major cause of environmental pollution, the biodegradable plastics have been drawing much attention the world over for their unique properties as thermoplastics. But the production of these bioplastics is limited due to their higher cost of production. Hence studies in search of bacterial strains with better productivity and optimization of their culture conditions are of prime importance to minimize the cost of PHB production.

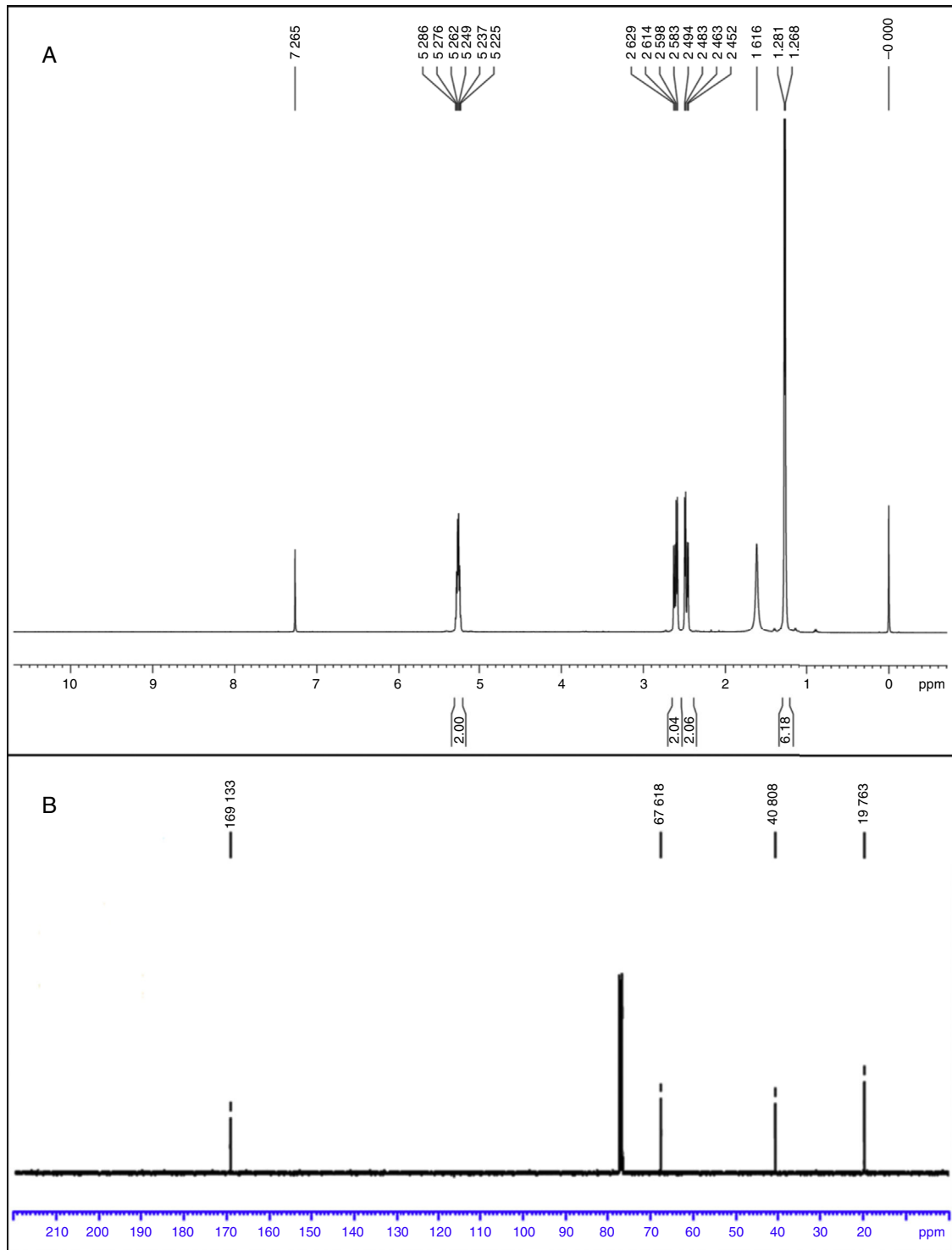
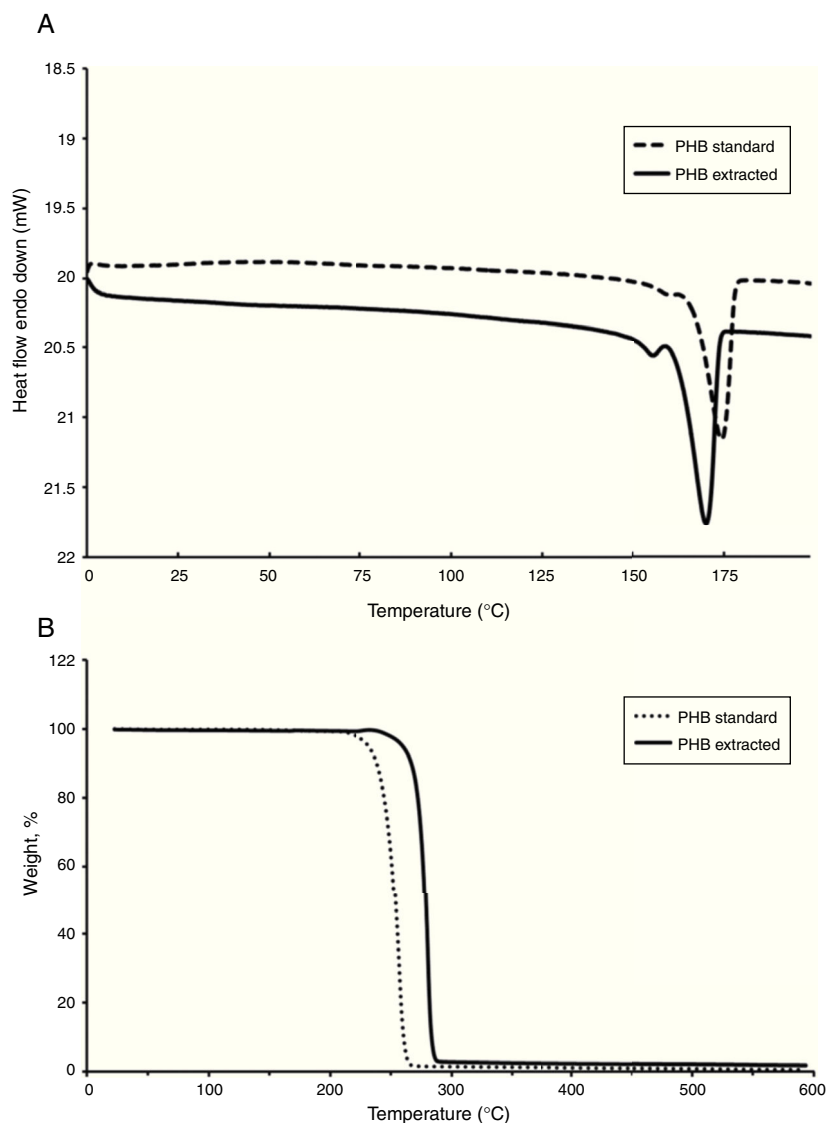


Fig. 4 – NMR spectrum of polymer obtained from PHB10. (A)  $^1\text{H}$  NMR spectrum, (B)  $^{13}\text{C}$  NMR spectrum.

There are currently more than 300 different bacterial species, isolated from diverse environmental conditions known for PHA accumulation.<sup>51</sup> In search of better PHA producing bacteria, we selected five different environmental sources. The strains were grown, established as pure cultures and stored on nutrient rich medium. Usually, bacteria accumulate PHAs under nutrient imbalanced growth conditions.<sup>1</sup>

Hence the isolates were subsequently screened on growth medium with limited nitrogen compounds and abundant supply of glucose, for PHA accumulation. The colony staining is a simple method for rapid detection and isolation of PHA accumulating bacteria from environment.<sup>32</sup> During the study, we screened out 24 PHA accumulating bacteria through the colony staining. Secondary screening of these isolates resulted



**Fig. 5 – (A) DSC curves of polymer. (B) TGA curves. Solid curve – polymer from PHB10, dotted curve – standard PHB.**

in obtaining a bacterial isolate PHB10 with much better PHB accumulation potential, which was originally isolated from soil collected from a local domestic sewerage. Nile red staining showed bright red fluorescence which further confirmed multiple PHA granules within the cytoplasm of the strain PHB10.<sup>36</sup> The 16S rRNA gene sequence of this isolate resembled that of *B. aryabhatai* and the biochemical and morphological characteristics matched with early reports of Shivaji et al.<sup>28</sup> The phylogenetic tree revealed the similarity of the isolate with another strain of the same species and its position among other *Bacillus* spp.

Generally bacteria belonging to the genera *Bacillus* accumulate short chain length polyhydroxyalkanoates such as PHB.<sup>16</sup> In order to identify the most suitable carbon source for PHB production, PHB10 was cultured using different carbon sources and the effect on PHB accumulation was studied. PHB10 accumulated PHB up to 75% and 4.36 g/L of dry weight after 48 h, in shake flask culture. PHB10 recorded much higher rate of polymer accumulation when compared to earlier

reports of 26% in 10 g/L glucose and 58% in 20 g/L sweet sorghum juice.<sup>29,52</sup> The study reveals the better ability of *B. aryabhatai* PHB10 in terms of PHB accumulation and this is the best yielding strain of this species reported till date. These findings suggested that glucose was the best carbon source for PHB accumulation among the carbon sources used in this study and the optimum temperature for maximum productivity was 31 °C.

Interestingly, the isolate showed good polymer accumulation in basal medium with glycerol, next to the production level in glucose. After 48 h of incubation, *B. aryabhatai* PHB10 accumulated 66% (1.79 g/L) of polymer using glycerol and it was found to be comparable with that of *Bacillus sonorensis* SM-P-1S (2.0 g/L, 71.8% after 96 h) as reported by Shrivastav et al.<sup>31</sup> Hence, for large scale production of the polymer using the strain PHB10, glycerol may be a better option as it is cheaper than glucose and can be obtained as a by-product from industries such as biodiesel production.<sup>31</sup> The polymer accumulation in starch containing medium

**Table 2 – Analysis of polymer accumulation in PHB10. Values are means from three independent fermentations in 1 L basal medium with 20 g/L of carbon sources (standard deviation in parenthesis).**

Temperature (°C)	Glucose		Fructose		Maltose		Starch		Glycerol	
	PHB weight (g/L)	PHB %	PHB weight (g/L)	PHB %	PHB weight (g/L)	PHB %	PHB weight (g/L)	PHB %	PHB weight (g/L)	PHB %
28	1.754 (±0.118)	57.35 (±3.12)	0.239 (±0.05)	17 (±4.19)	0.979 (±0.084)	50.04 (±5.58)	0.608 (±0.089)	34.46 (±5.11)	1.073 (±0.105)	42.07 (±5.33)
31	3.264 (±0.164)	74.89 (±3.89)	0.089 (±0.025)	14.41 (±3.77)	1.1 (±0.085)	45.63 (±4.24)	0.364 (±0.078)	25.47 (±4.34)	1.617 (±0.112)	53.29 (±2.81)
34	1.156 (±0.131)	63.18 (±3.18)	2.181 (±0.169)	65.37 (±4.87)	1.47 (±0.171)	51.15 (±4.94)	1.742 (±0.276)	49.15 (±5.7)	1.786 (±0.043)	65.92 (±4.08)
37	0.754 (±0.094)	51.43 (±3.73)	1.103 (±0.065)	44 (±4.13)	0.774 (±0.037)	35.04 (±4.26)	0.937 (±0.105)	34.81 (±4.02)	1.557 (±0.172)	59.48 (±5)
40	0.049 (±0.031)	7.73 (±4.22)	0.011 (±0.013)	2.88 (±3.58)	0.021 (±0.02)	4.47 (±4.44)	0.215 (±0.106)	6.27 (±3.14)	0.963 (±0.169)	49.14 (±5.42)

(1.742 g/L) indicates the isolate can utilize starch and can convert them to bioplastics. This property of simultaneous saccharification and fermentation of starch can aid in better resource utilization and may reduce the polymer production cost considerably. For a large scale polymer production process, PHB accumulation property of this strain within a minimum period of incubation, utilizing inexpensive and easily available raw materials may reduce the production cost.

Gouda et al. studied the effect of different carbon sources on the production of PHB by *Bacillus megaterium* and observed that maximum PHB accumulation obtained when glucose was used as sole carbon source and maximum cell dry mass was obtained in maltose.<sup>53</sup> Similarly, in this study among the carbon compounds used, excepting glucose, fructose was the best for polymer accumulation while starch for cell growth. Maltose was found as a poor carbon source for cell growth as well as polymer production.

In this study the optimum temperature for polymer production was found to be 31 °C and 34 °C in various carbon sources. These results are in agreement with Grothe et al. who found that incubation temperature affects polymer accumulation at a range of 25–37 °C and over this range, the effect of temperature is negligible.<sup>54</sup>

The growth of PHB10 cells in nutrient rich medium was found faster than in basal medium and attained exponential, stationary and death phases immediately. This is because of the extra nitrogen compounds supplied by the nutrient medium than by the limiting basal medium.<sup>55</sup> The time-course analysis of PHB production revealed that PHB production in *B. aryabhatai* is growth associated. PHB accumulation begins along with the cell growth, i.e., from the log phase itself. PHB accumulation rate increased exponentially up to stationary phase at 36 h of incubation and reached to the maximum at 48 h. These results are consistent with those reported by Tanamool et al.<sup>52</sup> A small drop in the level of CDM after 66 h coincided with a small decline in PHA content, which may indicate the presence of an intracellular PHA depolymerase.<sup>50</sup>

The <sup>1</sup>H NMR spectrum showed the expected resonances for PHB as demonstrated by the methine group (–CH–) between 5.22 and 5.28 ppm, a methylene group (–CH<sub>2</sub>–) between 2.45 and 2.62 ppm, and the methyl group (–CH<sub>3</sub>) between 1.26 and 1.28 ppm as in standard PHB. Scans of <sup>13</sup>C NMR showed peaks at 169.13, 67.62, 40.81 and 19.76 ppm which represents the carbonyl carbon (–C–), ester (–O–CH–) group, methylene (–CH<sub>2</sub>–) and the methyl (–CH<sub>3</sub>) groups as shown in the standard. These results confirm the material as a homopolymer of 3-hydroxybutyrate, i.e., poly-3-hydroxybutyrate (PHB).<sup>56</sup> The melting point at 170 °C and thermal stability at a temperature range of 30–140 °C suggest that the PHB obtained is of good quality. From the TGA data, it can be assumed that the thermal degradation of the extracted polymer occurs mainly in one step, at around 242–287 °C where 96.08% of the original mass was lost. The polymer sample showed better thermal stability than that of standard PHB. The higher thermal stability of the extracted polymer seemed to be due to the more crystalline morphology than the standard.<sup>57</sup>

The bacterial isolate *B. aryabhatai* PHB10 can assimilate various carbon sources and it is a good producer of short chain



length PHA. Currently this bacterial isolate is being studied in detail for the optimized polymer production using cheap carbon sources for the further reduction in production cost and for large scale production of PHB.

### Conflicts of interest

The authors declare no conflicts of interest.

### Acknowledgements

The authors are grateful to Prof. M. Radhakrishna Pillai, Director, RGC B for the facilities provided. This work was supported by Department of Biotechnology, Govt. of India. Aneesh B would like to acknowledge Council of Scientific and Industrial Research (CSIR), India for awarding research fellowship (SRF; 09/716(0149)/2012-EMR-I).

### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bjm.2017.01.005.

### REFERENCES

- Andreeben B, Lange AB, Robenek H, Steinbüchel A. Conversion of glycerol to poly(3-hydroxypropionate) in recombinant *Escherichia coli*. *Appl Environ Microbiol*. 2010;76(2):622–626.
- Chen GQ. Plastics completely synthesized by bacteria: polyhydroxyalkanoates. In: Chen GQ, ed. *Plastics from Bacteria: Natural Functions and Applications*, Microbiology Monographs. Berlin Heidelberg: Springer-Verlag; 2010:17–37.
- Hocking J, Marchessault RH. Biopolyesters. In: Griffin G, ed. *Chemistry and Technology of Biodegradable Polymers*. London: Chapman and Hall; 1994:48–96.
- Muller HM, Seebach D. Polyhydroxyalkanoates: a fifth class of physiologically important biopolymer? *Angew Chem Int*. 1993;32(4):477–502.
- Yoshihiro A, Koichi Y, Yoshiharu D. Thermal degradation of poly [(R)-3-hydroxybutyrate], poly[e-caprolactone], and poly[(S)-lactide]. *Polym Degrad Stab*. 2002;76(1):53–59.
- Anderson AJ, Dawes EA. Occurrence, metabolism, metabolic role, and industrial use of bacterial polyhydroxyalkanoates. *Microbiol Rev*. 1990;54(4):450–472.
- Poli A, Di Donato P, Abbamondi GR, Nicolaus B. Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by archaea. *Archaea*. 2011;693253:1–13.
- Steinbüchel A, Fuchtenbush B. Bacterial and other biological systems for polyester production. *Trends Biotechnol*. 1998;16(10):419–427.
- Steinbüchel A, Lütke-Eversloh T. Metabolic engineering and pathway construction for biotechnological production of relevant polyhydroxyalkanoates in microorganisms. *Biochem Eng J*. 2003;16(2):81–96.
- Poirier Y. Polyhydroxyalkanoate synthesis in plants as a tool for biotechnology and basic studies of lipid metabolism. *Prog Lipid Res*. 2002;41(2):131–155.
- Reinecke F, Steinbüchel A. *Ralstonia eutropha* strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. *J Mol Microbiol Biotechnol*. 2008;16(1–2):91–108.
- Philip S, Keshavarz T, Roy I. Polyhydroxyalkanoates: biodegradable polymers with a range of applications. *J Chem Technol Biotechnol*. 2007;82(3):233–247.
- Valentin HE, Dennis D. Production of poly (3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. *J Biotechnol*. 1997;58(1):33–38.
- Jo SJ, Matsumoto KI, Leong CR, Ooi T, Taguchi S. Improvement of poly (3-hydroxybutyrate) [P(3HB)] production in *Corynebacterium glutamicum* by codon optimization, point mutation and gene dosage of P(3HB) biosynthetic genes. *J Biosci Bioeng*. 2007;104(6):457–463.
- Alvarez HM, Kalscheuer R, Steinbüchel A. Accumulation of storage lipids in species of *Rhodococcus* and *Nocardia* and effect of inhibitors and polyethylene glycol. *Eur J Lipid Sci Tech*. 1997;99(7):239–246.
- Valappil SP, Boccaccini AR, Bucke C, Roy I. Polyhydroxyalkanoates in Gram-positive bacteria: insight from the genera *Bacillus* and *Streptomyces*. *Antonie van Leeuwenhoek*. 2007;91(1):1–17.
- Wong PAL, Cheung MK, Lo WH, Chua H, Yu PHF. Investigation of the effects of the types of food waste utilized as carbon source on the molecular weight distributions and thermal properties of polyhydroxybutyrate produced by two strains of microorganisms. *e-Polymers*. 2004;4(1):324–334.
- Fernandez-Castillo R, Rodriguez-Valera F, Gonzalez-Ramos J, Ruiz-Berraquero F. Accumulation of poly ( $\beta$ -hydroxybutyrate) by halobacteria. *Appl Environ Microbiol*. 1986;51(1):214–216.
- Han J, Lu Q, Zhou L, Zhou J, Xiang H. Molecular characterization of the phaEChm genes, required for biosynthesis of poly (3-hydroxybutyrate) in the extremely halophilic archaeon *Haloarcula marismortui*. *Appl Environ Microbiol*. 2007;73(19):6058–6065.
- Legault BA, Lopez-Lopez A, Alba-Casado JC, et al. Environmental genomics of “*Haloquadratum walsbyi*” in a saltern crystallizer indicates a large pool of accessory genes in an otherwise coherent species. *BMC Genomics*. 2006;7(1):171.
- Lillo JG, Rodriguez-Valera F. Effects of culture conditions on poly ( $\beta$ -hydroxybutyric acid) production by *Haloferax mediterranei*. *Appl Environ Microbiol*. 1990;56(8):2517–2521.
- Labuzek S, Radecka I. Biosynthesis of PHB tercopolymer by *Bacillus cereus* UW85. *J Appl Microbiol*. 2001;90(3):353–357.
- Mizuno K, Ohta A, Hyakutake M, Ichinomiya Y, Tsuge T. Isolation of polyhydroxyalkanoate producing bacteria from a polluted soil and characterization of the isolated strain *Bacillus cereus* YB-4. *Polym Degrad Stab*. 2010;95(8):1335–1339.
- Shamala TR, Chandrashekar A, Vijayendra SVN, Kshama L. Identification of polyhydroxyalkanoate (PHA)-producing *Bacillus* spp. using the polymerase chain reaction (PCR). *J Appl Microbiol*. 2003;94(3):369–374.
- Singh M, Patel SKS, Kalia VC. *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. *Microb Cell Fact*. 2009;8:38.
- Lemoigne M. Products of dehydration and of polymerization of  $\beta$ -hydroxybutyric acid. *Bull Soc Chem Biol*. 1926;8:770–782.
- Volova TG. *Polyhydroxyalkanoates: Plastic Materials of the 21st Century: Production, Properties, Applications*. New York, USA: Nova publishers Inc.; 2004. ISBN: 1-59033-992-4.
- Shivaji S, Chaturvedi P, Begum Z, et al. *Janibacter hoylei* sp. nov., *Bacillus isronensis* sp. nov. and *Bacillus aryabhatai* sp. nov., isolated from cryotubes used for collecting air from the upper atmosphere. *Int J Syst Evol Microbiol*. 2009;59(12):2977–2986.
- Van-Thuoc D, Huu-Phong T, Thi-Binh N, Thi-Tho N, Minh-Lam D, Quillaguamán J. Polyester production by halophilic and halotolerant bacterial strains obtained from

- mangrove soil samples located in Northern Vietnam. *Microbiologyopen*. 2012;1(4):395–406.
30. Singh P, Kim YJ, Nguyen NL, et al. *Cupriavidus yeoncheonense* sp. nov., isolated from soil of ginseng. *Antonie van Leeuwenhoek*. 2015;107(3):749–758.
  31. Shrivastav A, Mishra SK, Shethia B, Pancha I, Jain D, Mishra S. Isolation of promising bacterial strains from soil and marine environment for polyhydroxyalkanoates (PHAs) production utilizing *Jatropha* biodiesel byproduct. *Int J Biol Macromol*. 2010;47(2):283–287.
  32. Liu M, González JE, Willis LB, Walker GC. A novel screening method for isolating exopolysaccharide-deficient mutants. *Appl Environ Microbiol*. 1998;64(11):4600–4602.
  33. Burdon KL. Fatty materials in bacteria and fungi revealed by staining dried, fixed slide preparations. *J Bacteriol*. 1946;52(6):665–678.
  34. Aneesh BP, Arjun JK, Kavitha T, Hari Krishnan K. Production of short chain length polyhydroxyalkanoates by *Bacillus megaterium* PHB29 from starch feed stock. *Int J Curr Microbiol App Sci*. 2016;5(7):816–823.
  35. Greenspan P, Mayer PE, Fowler DS. Nile Red: a selective fluorescent stain for intracellular lipid droplets. *J Cell Biol*. 1985;100(3):965–973.
  36. Jendrossek D, Selchow O, Hoppert M. Poly(3-hydroxybutyrate) granules at the early stages of formation are localized close to the cytoplasmic membrane in *Caryophanon latum*. *Appl Environ Microbiol*. 2007;73(2):586–593.
  37. Soo-Hwan K, Hyeong-Seon L, Deok-Seon R, Soo-Jae C, Dong-Seok L. Antibacterial activity of silver-nanoparticles against *Staphylococcus aureus* and *Escherichia coli*. *Korean J Microbiol Biotechnol*. 2011;39(1):77–85.
  38. Shi H, Shiraiishi M, Shimizu K. Metabolic flux analysis for biosynthesis of poly( $\beta$ -hydroxybutyric acid) in *Alcaligenes eutrophus* from various carbon sources. *J Ferment Bioeng*. 1997;84(6):579–587.
  39. Cappuccino JG, Sherman N. *Microbiology: A Laboratory Manual*. 9th ed. San Francisco, Halaman: Pearson Benjamin Cummings; 2011.
  40. Holt JG, Krig NR, Sneath PHA, Staley JT, Williams ST. *Bergey's Manual of Determinative Bacteriology*. 9th ed. Baltimore, MD: Williams and Wilkins; 1994.
  41. Lane DJ. 16S/23S rRNA sequencing. In: Stackebrandt E, ed. *Nucleic Acid Techniques in Bacterial Systematics*. Chichester, UK: John Wiley and Sons; 1991:115–175.
  42. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl Acids Symp Ser*. 1999;41:95–98.
  43. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4(4):406–425.
  44. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol*. 2016;33:1870–1874.
  45. Kourmentza C, Ntaikou I, Lyberatos G, Kornaros M. Polyhydroxyalkanoates from *Pseudomonas* sp. using synthetic and olive mill wastewater under limiting conditions. *Int J Biol Macromol*. 2015;74:202–210.
  46. Sangkharak K, Prasertsan P. Nutrient optimization for production of polyhydroxybutyrate from halotolerant photosynthetic bacteria cultivated under aerobic-dark condition. *Electron J Biotechnol*. 2008;11(3):83–94.
  47. Salgaonkar BB, Mani K, Braganca JM. Characterization of polyhydroxyalkanoates accumulated by a moderately halophilic salt pan isolate *Bacillus megaterium* strain H16. *J Appl Microbiol*. 2013;114(5):1347–1356.
  48. Gunaratne LMWK, Shanks RA, Amarasinghe G. Thermal history effects on crystallisation and melting of poly(3-hydroxybutyrate). *Thermochim Acta*. 2004;423(1):127–135.
  49. Su WF. *Polymer Size and Polymer Solution*. In *Principles of Polymer Design and Synthesis*. New York: Springer; 2013:9–26.
  50. Qi Q, Rehm BH. Polyhydroxybutyrate biosynthesis in *Caulobacter crescentus*: molecular characterization of the polyhydroxybutyrate synthase. *Microbiology*. 2001;147(12):3353–3358.
  51. Liu C, Wang H, Xing W, Wei L. Composition diversity and nutrition conditions for accumulation of polyhydroxyalkanoate (PHA) in a bacterial community from activated sludge. *Appl Microbiol Biotechnol*. 2013;97(21):9377–9387.
  52. Tanamool V, Imai T, Danvirutai P, Kaewkannetra P. Biopolymer generation from sweet sorghum juice: screening, isolation, identification and fermentative polyhydroxyalkanoate production by *Bacillus aryabhattai*. *Turk J Biol*. 2013;37(3):259–264.
  53. Gouda MK, Swellam AE, Omar SH. Production of PHB by a *Bacillus megaterium* strain using sugarcane molasses and corn steep liquor as sole carbon and nitrogen sources. *Microbiol Res*. 2001;156(3):201–207.
  54. Grothe E, Moo-Young M, Chisti Y. Fermentation optimization for the production of poly-( $\beta$ -hydroxybutyric acid) microbial thermoplastic. *Enzym Microb Tech*. 1999;25(1):132–141.
  55. Yao Z, Davis RM, Kishony R, Kahne D, Ruiz N. Regulation of cell size in response to nutrient availability by fatty acid biosynthesis in *Escherichia coli*. *Proc Natl Acad Sci U S A*. 2012;109(38):E2561–E2568.
  56. Bonthron KM, Clauss J, Horowitz DM, Hunter BK, Sanders JKM. The biological and physical chemistry of polyhydroxyalkanoates as seen by NMR spectroscopy. *FEMS Microbiol Rev*. 1992;103:269–278.
  57. Hahn SK, Chang YK, Lee SY. Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. *Appl Environ Microbiol*. 1995;61(1):34–39.