

Biosynthesis and genetics of polyhydroxyalkanoates by newly isolated *Pseudomonas aeruginosa* IFS and 30N using inexpensive carbon sources

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Received: 5 August 2016/Revised: 26 December 2016/Accepted: 6 February 2017/Published online: 17 April 2017
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Abstract Poly(3-hydroxyalkanoates) (PHAs) are the bioplastics that are stored in many genera of bacteria as carbon and energy storage polyester granules. PHAs have established themselves as strong competitors to conventional plastics. This study reports the isolation of PHA-accumulating bacteria from local environment and their PHA characterization. Two potential strains identified as *Pseudomonas aeruginosa* strain IFS (Accession no. JQ041638) and *P. aeruginosa* strain 30N (Accession no. JQ041639) based on 16S rRNA gene sequence identity were cultivated under nitrogen limited conditions to study their PHA biosynthesis capabilities. The strain IFS and strain 30N produced 1.36 and 1.40 g l⁻¹ dry biomass with percentage PHA contents of 44.85 and 45.74%, respectively, when grown on glucose as carbon source. The PHA was identified as poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) by fourier transform infrared spectroscopy (FTIR) and gas chromatography coupled with mass spectrometry (GC-MS). The PHA synthase genes of these strains were isolated, sequenced and analyzed using bioinformatic tools that showed they belonging to type 2 PHA synthases and presented their evolutionary relationships with PHA synthases of other *Pseudomonas* species. The experimental results of this study highlight the importance of these strains for future use of bacterial biopolymer production growing on simple and inexpensive carbon sugars.

Keywords Carbon sources · Fourier transform infrared spectroscopy · Gas chromatography coupled with mass spectrometry · Polyhydroxyalkanoates · Polyhydroxyalkanoate synthase genes · *Pseudomonas*

Introduction

Poly(3-hydroxyalkanoates) (PHAs) are the biopolyesters that are produced by various groups of eubacteria and some members of Archaea. These PHAs are often produced under unfavorable conditions in the surrounding of these bacteria, such as low concentrations of N, P, O₂ or even the carbon source that makes them to be called as storage granules. PHA production is also related to the bacterial improvement for their environmental fitness under various stresses. PHAs resemble with the petro-chemically derived plastics in many physical and chemical properties and therefore called as bioplastics (Kahlon 2016). In the past few decades, the use of fossil fuels for the production of a variety of chemical and polymers has not only endangered the depletion of these natural resources but has also created major concern for the environment (Phukon et al. 2014). As compared to the conventional plastics, these bioplastics are biodegradable and environment friendly as well. The biggest problem in the biosynthesis of these biopolymers is their production cost, which is mainly due to the high prices of carbon sources fed to the bacteria. These high costs are responsible for the hindrance of the commercialization of these PHAs (Kim et al. 2016). Owing to their versatile properties, these PHAs find a number of applications in both packaging industry and biomedical field (Ali and Jamil 2016b).

PHAs can be divided into two major classes subject to the number of carbon atoms present in the side chains of

Editorial responsibility: X. Han.

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the subunits forming them, i.e., short-chain-length PHA (C4–C5) and medium-chain-length PHAs (C6–C14). The type and quantity of PHA produced depends upon various factors such as the nature of carbon source, type of bacterium used, conditions of microbial cultivation, strategy employed for microbial growth and feeding strategy of carbon source (Saratale and Oh 2015). PHA accumulation in bacteria is carried and controlled by the PHA synthase enzymes. There are four classes of PHA synthase, i.e., I to IV, depending upon the substrate specificity and gene subunits. Classes I, III and IV are mainly involved to incorporate short-chain subunits, whereas class II uses medium-chain subunits.

Fluorescent *Pseudomonas* has the ability to produce higher yields of PHAs with a variety of mcl monomers. *Pseudomonas* is a type genus to represent class II PHA synthases' structure and function. Various species such as *Pseudomonas* sp. 61–3, *Pseudomonas* sp. USM 4-55, *P. aeruginosa* PAO1, *P. putida* U, *P. oleovorans* and *P. mendocina* have been employed to study the PHA production from different carbon sources (Singh and Mallick 2009). A large number of unidentified bacteria are present in the environment that can be exploited for better PHA production and many other metabolic pathways.

This study was aimed to isolate and identify the bacteria from different environmental samples that could be used as potential strains for the production of polyhydroxyalkanoates (PHAs) from a variety of carbon sources and to characterize extracted PHA along with the evolutionary analysis of PHA synthase genes responsible for these PHAs production.

This study was carried out during 2014–2015 in Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore-54590, Pakistan.

Materials and methods

Sample collection and isolation of bacteria

Samples of canal water (CW) from Lahore (31.523185 N°, 74.329759 E°) and wastewater from main drainage (MD) Kot Lakhpat, Lahore (31.445305 N°, 74.325403 E°) were collected and transported to laboratory in sterile containers. Physical parameters of the samples like pH, temperature and humidity were noted, respectively. All the chemicals of high purity were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA), whereas the media were obtained from Oxoid™ (Thermo Fisher Scientific, Waltham, USA). Standard procedures of serial dilutions were performed to isolate bacterial strains on LB agar medium as described previously (Ali and Jamil 2014). Bacterial load in terms of colony-forming unit (cfu) of the samples was calculated

followed by isolation and purification of various bacteria on the basis of colony morphology.

Screening of PHA-producing bacteria

Isolated bacterial colonies were cultured on nitrogen-deficient mineral salt medium (MSM) agar plates [(NH₄)₂SO₄, 2 g l⁻¹; KH₂PO₄, 13.3 g l⁻¹; MgSO₄·7H₂O, 1.2 g l⁻¹; citric acid, 1.7 g l⁻¹; agar 15 g l⁻¹ and trace elements solution 10 ml l⁻¹] supplemented with glucose 20 g l⁻¹ (111 mM) and Nile blue A dye (0.5 μg ml⁻¹) at 37 °C. The bacterial colonies were exposed with ultraviolet light to see any fluorescence expressed by PHA-accumulating bacteria (Chaudhry et al. 2011). Sudan black B staining was also performed to see the blue-black granules in PHA-accumulating bacteria (Ali and Jamil 2014). PHA-accumulating bacteria were selected for physical, biochemical characterization and PHA production studies.

Bacterial growth and PHA accumulation at different carbon sources

Isolated bacteria were grown in 500-ml Erlenmeyer flasks containing 100 ml of MSM supplemented with glucose at 20 g l⁻¹ and incubated at 37 °C on a reciprocal shaking incubator (200 rpm) for 48 h. Bacterial isolates IFS and 30N were also grown on MSM containing one of these carbon sources at a time (sucrose 20 g l⁻¹ (58.42 mM), fructose 20 g l⁻¹ (111 mM), molasses 20 g l⁻¹, lactose 20 g l⁻¹ (58.42 mM), octanoic acid 1% (60.16 mM), gluconic acid 1% (51 mM).

Extraction of PHA from bacteria

Bacterial broth samples were taken in sterilized tubes, centrifuged at 4000 g at 15 min at 8 °C (Sigma-Aldrich) to get pellets of bacterial wet biomass. These pellets were re-suspended in distilled water, re-centrifuged and finally freeze-dried. Bacterial dry cell weight (DCW) was calculated in terms of g l⁻¹ thereafter, followed by the treatment of sodium hypochlorite and chloroform extraction method (Ali and Jamil 2016a). The extracted PHA was dried at room temperature, and percentage PHA contents of bacterial cells were calculated as gram of PHA extracted per 100 g of DCW of bacterial cells.

Identification of extracted PHA

Extracted PHA (20 mg) was reacted with potassium bromide (sigma), and fourier transform infrared spectroscopy was carried out by PerkinElmer spectrum BX FTIR system (Beacon field Buckinghamshire HP9 IQA) with the range of 400–4000 cm⁻¹ and 32 sweeps realization by sample.

The graphs were studied to find out the functional groups present in PHA molecules (Naheed et al. 2012). The different functional groups were compared with transmittance peaks already reported (Khardenavis et al. 2009). The extracted PHA was also identified by gas chromatography–mass spectrometry (GCMS-QP2010 with D1) by the method as described previously (Ali and Jamil 2014).

16S rRNA gene amplification of isolated bacteria

The bacterial cultures grown in LB broth medium were centrifuged at 1000 *g* for 5 min, re-suspended in 0.85% NaCl aqueous solution, re-centrifuged, and pellets were obtained. These pellets were treated with digestion chemicals to extract genomic DNA (Ausubel 2002). Two common primers (27F and 1492R) were used to clone the 16S rRNA gene of the bacteria (by Macrogen, Inc. Korea).

Detection of PHA synthase genes in isolated bacteria

PHA synthase gene “*phaC*” was amplified using the general primers set for this gene [179–L (5′-ACA-GATCAAGTTCTACATCTTCGAC-3′) and 179–R (5′-GGTGTTCGTTCCAGTAGAGGATGTC-3′)]. The reaction mixture and PCR conditions were set as described previously using a Primus96 (PeQLab) PCR machine (Chaudhry et al. 2011). The amplified gene product was sequenced and nucleotide sequences were deposited to NCBI GenBank and accession numbers were obtained.

Nucleotide sequence analyses

The nucleotide sequences were compared by using BLASTn tool at NCBI database. The percentage identity matrix of the sequences was carried through multiple sequence alignment tool Clustal Omega at <http://www.ebi.ac.uk/Tools/msa/clustalo>. Phylogenetic trees were constructed for the 16S rRNA genes by the MEGA (version 5.0) employing the neighbor-joining method and with the respected sequences from NCBI GenBank. The partial protein sequence of *phaC* gene was analyzed by ORF finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The partial protein fragment was then compared with different protein sequences at NCBI database using BLASTp tool. It was followed by the multiple sequence alignment of the related sequences (<http://tcoffee.crg.cat/apps/tcoffee/do:regular>) by the T-Coffee software. Percentage identity matrix of the partial PHA synthase protein segment for strains 30N and IFS was calculated by Clustal2.1, followed by the construction of phylogenetic tree by MEGA as described above.

Results and discussion

Isolation, screening and biochemical characterization of PHA-producing bacteria

A total of 27 bacterial colonies were isolated and purified from the canal water (CW) and main drainage (MD) samples (Table 1 describes the physicochemical characteristics of samples). Bacterial load of 3.2×10^6 cfu per ml and 1.2×10^8 cfu per ml was found in CW and MD samples, respectively. Isolation of wild type of bacterial strains from different localities having more potential to produce PHAs can certainly help in bringing down their production prices. Numerous PHA-producing bacteria have been isolated from different locations such as sludge, soil, wastewater, Antarctic freshwater and other localities (Ciesielski et al. 2014). Sewage environments are commonly reported to have high BOD and COD due to more carbon compounds and low contents of N and P, making the environment more favorable for the growth of PHA-accumulating bacteria, as these PHAs are generally accumulated under high carbon to nitrogen/phosphorous ratios (Davis et al. 2013). Canal water and main drainage water samples are hence likely to be a rich source of PHA-producing bacteria.

From the CW sample, 3 out of 11 bacterial isolates were found to be positive in the Sudan black B staining and 4 out of 11 bacterial isolates were found to be positive for Nile blue A staining (Table 2). Only isolates IFS, C13 and C15 were found to be positive for both types of staining. While from MD sample, the bacterial isolates D9 and 30N passed the screening methods for PHA production out of total 16 isolated bacteria. Overall 4 out of 16 bacterial isolates showed positive staining for Sudan black B, and 2 out of 16 showed fluorescence for Nile blue A test (Table 2). Isolate D9 was found to be Gram-positive rod-shaped bacterium, while isolates C13, C15, IFS and 30N were found to be Gram-negative rods. Colony morphologies and biochemical characterizations of these five PHA-producing bacterial isolates are shown in Table 3. The bacterial isolates with more PHA-producing ability were selected for further studies. Both isolates showed almost similar pattern of growth having almost 8 h of lag phase. The bacteria entered into the stationary phase after 12 h of incubation (data not shown).

DNA-based identification of bacterial isolates

A nucleotide sequence of 1474 bp length was obtained for the 16S rRNA gene of strain 30N. This sequence showed 98.57% sequence identity (Fig. 3) to *Pseudomonas*

Table 1 Physicochemical characteristics of samples

Sample type	Location	pH	Temperature	Humidity
Canal water (CW)	Lahore (Latitude: 31.523185 N°, Altitude: 74.329759 E°)	7.2 ± 0.1	34.2 ± 0.2	67.5 ± 0.2
Wastewater from main drainage (MD)	Kot Lakhpat, Lahore (Latitude: 31.445305 N°, Altitude: 74.325403 E°)	6.0 ± 0.2	35.4 ± 0.3	49.5 ± 0.3

Table 2 Bacterial load and screening of PHA-producing bacteria from samples

Sample code	CFU (No. of cells per ml or g)	No. of isolates	Positive for Sudan black B	Positive for Nile blue A	PHA producers		Isolated bacterial strains
					Total no.	Percentage	
(CW)	3.2 × 10 ⁶	11	3	4	3	27.27	IFS, C13, C15
(MD)	1.2 × 10 ⁸	16	4	2	2	12.50	D9, 30N

Table 3 Colony morphology and biochemical characterization of isolated bacteria

Characteristics	Bacterial isolate				
	30N	IFS	D9	C13	C15
Colony shape	Circular	Circular	Irregular	Circular	Circular
Colony color	Green	Green	Yellow	Yellow	Blue
Colony size (mm)	2	2	4	3	3
Margins	Entire	Entire	Lobate	Entire	Entire
Elevation	Flat	Flat	Smooth	Raised	Flat
Gram reaction	–	–	+	–	–
Cell shape	Rods	Rods	Rods	Rods	Rods
Cell arrangement	Solitary	Solitary	Chain	Solitary	Solitary
Spore formation	–	–	+	–	–
Capsule staining	+	+	–	+	+
Catalase test	+	+	+	+	+
Motility	+	+	+	+	+
Oxidase test	+	+	+	+	+
Methyl red test	–	–	–	+	–
Voges–Proskauer test	–	–	+	–	–
Indole production	–	–	–	–	–
Urease	–	+	–	+	+
Citrate	+	+	+	–	+
Gelatin hydrolysis	+	+	+	–	+
Casein hydrolysis	–	+	+	–	+
Starch hydrolysis	–	–	–	–	–
Cellulase hydrolysis	–	–	+	–	–
Glucose utilization	+	+	+	+	+
Lactose	–	–	–	–	–
Fructose	+	+	+	+	–
Nitrate reduction	+	+	–	+	+

aeruginosa strain APKGN (Accession no. KC967220). 16S rRNA gene sequence (1471 bp) of strain IFS showed 99.93% identity (Fig. 4) with *Pseudomonas aeruginosa* strain CS 182 (Accession no. JQ433551). Both strains showed 98.54% sequence identity to each other. Hence, these strains were identified and named as *Pseudomonas aeruginosa* strain IFS (Accession no. JQ041638) and *Pseudomonas aeruginosa* strain 30N (Accession no. JQ041639). Strains 30N and IFS belong to *P. aeruginosa* of genus “*Pseudomonas*” that contains members capable of using a wide ranging of various compounds and localizing under varied ecological conditions. *Pseudomonas aeruginosa* have been classified under the group 1 based on rRNA homology that also include *P. putida*, *P. mendocina*, *P. fluorescens*. *Pseudomonas* belongs to the subclass gamma of proteobacteria. Many species of *Pseudomonas* genus have the ability to accumulate PHAs as their storage granules, of which *P. putida*, *P. mendocina*, *P. fluorescens*, *P. syringae* and *P. nitroreducens*, *P. stutzeri*, *P. oleovorance*, and *P. aeruginosa* have been well described in the recent studies for the PHA accumulation (Kahlon 2016). *Pseudomonas* has been also grouped under Genus 1 of family *Pseudomonadaceae* that also include four other genera, i.e., *Azotobacter*, *Azomonas*, *Azorhizophilus* and *Cellvibrio* (Moore et al. 2006). Different species of these genera produce PHA granules utilizing diverse carbon sources as well.

Extraction and quantitative analysis of PHAs using different carbon sources

Higher PHA contents in terms of percentage of total dry cell weight were obtained from isolate 30N and isolate IFS grown in minimal salt medium supplemented with glucose as sole carbon source. The isolate 30N produced 1.40 g l^{-1} of DCW as compared to isolate IFS (1.36 g l^{-1}) and isolate

C13 (1.34 g l^{-1}). Isolate C15 produced 0.81 g l^{-1} DCW, while isolate D9 produced the lowest DCW of 0.32 g l^{-1} (Fig. 1). The amount of PHA contents were found to be highest among isolates 30N and IFS with values of 45.74 and 44.85%, respectively. Isolates C13 and C15 accumulated 38.72 and 24.86% PHA in their cells. Isolate D9 produced only 5.74% PHA. The isolates 30N and IFS were further grown on different carbon sources in MSM (Table 4). Different carbohydrates and carboxylic acids were given as sole carbon sources, and it was found that gluconic acid was more favored by both IFS and 30N strains. Strain 30N produced 1.34 g l^{-1} of DCW with 262.37 mg l^{-1} of accumulated PHA (19.58%). The strain IFS also produced 24.08% PHA (257.17 mg l^{-1}) and 1.06 g l^{-1} of DCW. Strain IFS accumulated more PHA when grown on octanoic acid (21.38%) as compared to strain 30N (18.16%). Fructose was found to be more suitable for bacterial growth of 30N and IFS (0.86 and 0.92 g l^{-1} , respectively). Sucrose and molasses could not be utilized for bacterial growth as efficiently as other carbon sources. Although strains 30N and IFS utilized lactose for their growth (0.64 and 0.48 g l^{-1} , respectively) but could not accumulate sufficient amounts of PHA (2.64 and 8.42%, respectively). Overall, it can be established that the strains 30N and IFS generally favor the short-chain carbon monomers such as glucose, fructose and gluconic acid for their growth and PHA accumulation and especially glucose for the maximum PHA production. It has been reported that poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) is accumulated by *Pseudomonas putida* Gpp104 strain using 6 carbon monomers like gluconate or glucose (Qiu et al. 2005). Genetically engineered *P. aeruginosa* PAO1 and *P. oleovorans* harboring different vectors can produce up to 51 and 46% PHA, respectively, when grown on gluconate (Rehm et al. 1998), whereas these indigenous strains could produce comparative amounts of PHA when grown on

Fig. 1 Growth and PHA production of bacterial strains grown in minimal salt medium with glucose as carbon source (37 °C and 200 rpm). Values are the averages of triplicate findings, while error bars represent the standard deviation

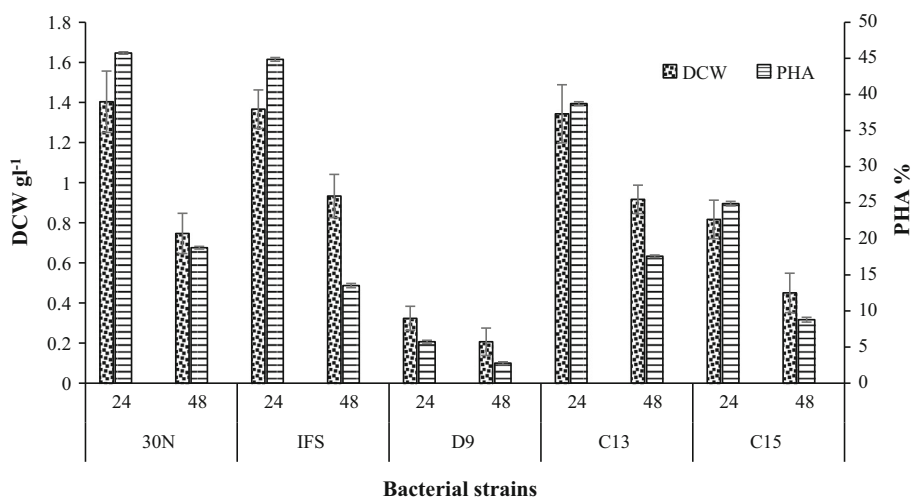


Table 4 PHA production by *Pseudomonas aeruginosa* strains IFS and 30N on various carbon sources

Strain	Growth medium	Carbon source	Cultivation time (h)	DCW g l^{-1}	PHA (%)	PHA mg l^{-1}	PHA monomers
30N	MSM	Sucrose 20 g l^{-1}	24	0.36	10.62	38.65	3HB
	MSM	Fructose 20 g l^{-1}	24	0.86	22.64	195.60	3HB
	MSM	Lactose 20 g l^{-1}	24	0.64	2.64	16.98	3HB
	MSM	Molasses 20 g l^{-1}	24	0.22	13.56	30.91	3HB
	MSM	Octanoic acid 60.16 mM	24	0.73	18.16	133.29	92.6% 3HO, 7.4% 3HD
	MSM	Gluconic acid 51 mM	24	1.34	19.58	262.37	3HX
IFS	MSM	Sucrose 20 g l^{-1}	24	0.15	8.18	12.76	3HB
	MSM	Fructose 20 g l^{-1}	24	0.92	17.6	162.27	3HB
	MSM	Lactose 20 g l^{-1}	24	0.48	8.42	40.92	3HB
	MSM	Molasses 20 g l^{-1}	24	0.36	12.8	47.10	3HB
	MSM	Octanoic acid 60.16 mM	24	0.85	21.38	182.15	91.8% 3HO, 8.2% 3HD
	MSM	Gluconic acid 51 mM	24	1.06	24.08	257.17	3HX

glucose. *P. putida* KT2440 and *P. oleovorans*, however, can produce more PHA when supplied with volatile fatty acids (Timm and Steinbuchel 1992). Strains IFS and 30N produced more dry mass and PHA contents as compared to PHA production by *P. aeruginosa* ATCC9027, *P. putida* W619 and *P. fluorescens* 555 (10.8, 25 and 34% PHA, respectively) grown on short-chain monosaccharides (Davis et al. 2013). *P. oleovorans* DSM1045 has shown the ability to produce 7.7% PHB of dry cell mass in shake flasks having glucose as sole carbon source (Timm and Steinbuchel 1992). Strains IFS and 30N also produces higher PHA contents when grown on gluconate and glucose as compared to *P. aeruginosa* PAK and *P. aeruginosa* JQ866912 strains (Phukon et al. 2014). Cellular biomass of strains IFS and 30N is although lower than that of *P. corrugata* (1.52 g l^{-1} DCW) grown on glucose but the PHA contents are almost 1.5 times, i.e., 44.85 and 45.74% as compared to 31% by *P. corrugata* (Solaiman et al. 2002). However, *P. oleovorans* strain NRRL B-778 and *P. aeruginosa* MTCC7925 can grow very well on glucose (~ 3.4 and 2.88 g l^{-1} DCW, respectively) but they produce only 31 and 24% PHA, respectively (Singh and Mallick 2009). A high biomass production (5.8 g l^{-1}) by *P. aeruginosa* 47T2 has been reported but it can only produce 9.5% PHA (Haba et al. 2007). It can be established that strains IFS and 30N prefer glucose for their growth and PHA accumulation.

Characterization of extracted PHAs

Since the first report on FTIR use for the PHA detection (Jüttner et al. 1975), it has been reported by various researchers for PHA quantification and identification as it requires minimal sample pretreatment. FTIR was used to

identify the structure of PHA. IR spectrum of pure PHA extracted from strain 30N grown on glucose is presented in Fig. 2. The absorbance peaks of the extracted PHA show similarities with the PHBV signals as reported in the literature (Poupart et al. 2015). The presence of functional groups at specific positions is in accordance with the subjected peaks of PHBV. The ester linkage C–O can be seen at 1277.6 cm^{-1} , while ester carbonyl group (–C=O) can be seen at 1720.2 cm^{-1} . The ester carbonyl peak in this range also indicates the crystalline nature of extracted PHA. The presence of peaks at 2954.4, 2923.5 and 2861.8 cm^{-1} corresponds to the –C–H aliphatic group at various symmetrical, anti-symmetrical and asymmetrical positions. Peak at 3490.5 cm^{-1} represents the –O–H stretching band in the polymer. CH vibrations of –CH₂ and –CH₃ functional groups are indicated by the peaks at 1450.2 and 1376.9 cm^{-1} , respectively. These signals confirm the production of PHBV type of polymer by bacteria cultured on glucose. The use of shorter carbon precursor results in the formation of copolymers of PHB-co-HV, which is characteristic of organisms like *Ralstonia eutropha* and *Alcaligenes latus* (Kim et al. 2016). The fact that *P. oleovorans* NRRL B-778 has also shown to produce copolymer when grown on glucose (Singh and Mallick 2009) suggests that *P. aeruginosa* strains IFS and 30N also possess a slightly different PHA synthase enzyme. GC–MS confirmed the PHBV nature of polymer from both strains when fed with glucose. However, the 3HB and 3HV monomers were in relatively similar ratios for both strains, i.e., 3HB % was above ~ 98 . These strains mainly produced scl-PHA when grown on sucrose, lactose, fructose, molasses and gluconic acids, whereas 3HO and 3HD monomers were produced for octanoic acids (Table 4).

Fig. 2 FTIR spectrum of PHA extracted from strain 30N when grown on glucose as carbon source (a=3490.5, b 2954.4, c=2923.5, d=2861.8, e=1720.2, f=1450.2, g=1376.9, h= 1277.6)

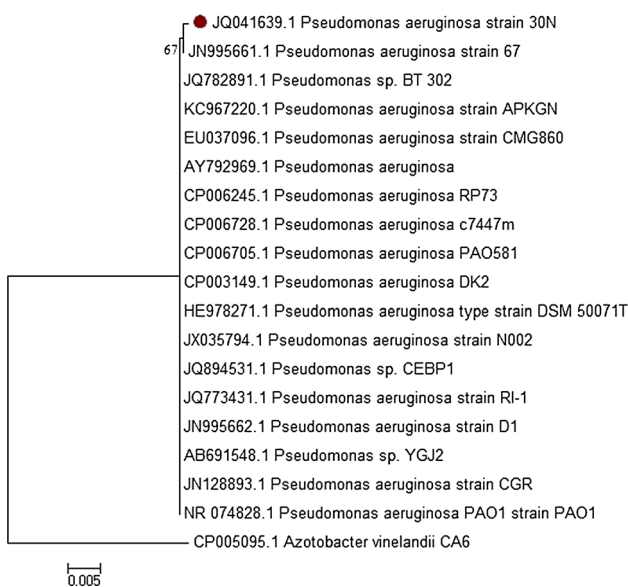
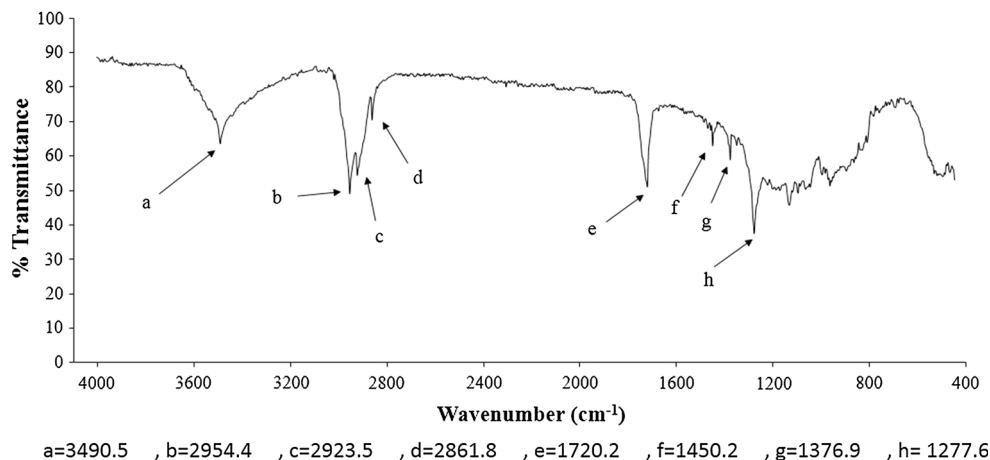


Fig. 3 Phylogenetic tree of bacterium *Pseudomonas aeruginosa* strain 30N. Mega 5.2 software was used to construct evolutionary tree by neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The evolutionary distances were computed using the Tamura–Nei method. *Azotobacter vinelandii* CA6 was taken as an out group

Evolutionary analyses of PHA synthase genes of strains IFS and 30N

Amplification of the PHA synthase genes resulted in amplicons of size 471 bp (Accession no. KJ146056) and 452 bp (Accession no. KJ146057) nucleotide sequences for the strains IFS and 30N, respectively. Open reading frames (136 aa and 154 aa for strains 30N and IFS, respectively) for partial subunit of PHA synthase proteins (Accession no. AHL43543 and AHL43542 for strains 30N and IFS) were analyzed with BLASTp tool at NCBI database. It was found that PHA synthase of strain 30N showed 98.54%

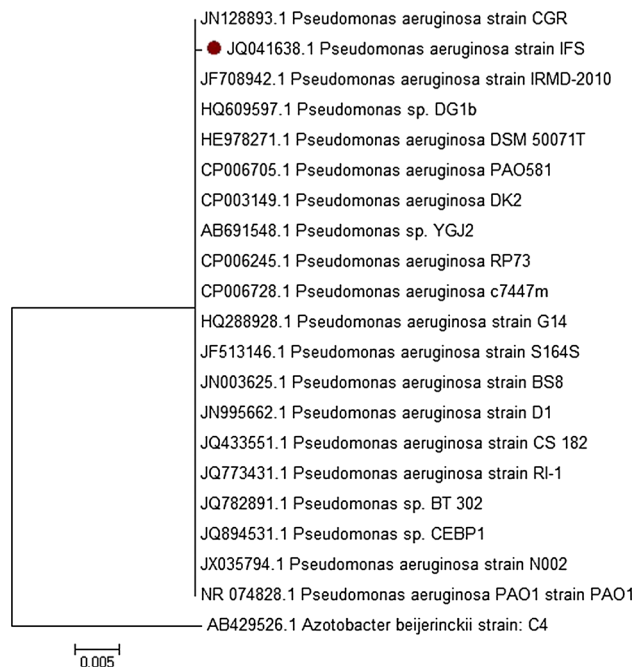


Fig. 4 Phylogenetic tree of bacterium *Pseudomonas aeruginosa* strain IFS. Mega 5.2 software was used to construct evolutionary tree by neighbor-joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown above the branches. The evolutionary distances were computed using the Tamura–Nei method. *Azotobacter beijerinckii* strain: C4 was taken as an out group

homology with PHA synthase of *P. aeruginosa* PA38182 (CDI93634), whereas PHA synthase of IFS strain showed 97.81% homology with the same strain PA38182 (Fig. 5). The PHA synthase proteins of both strains were 96.35% structurally identical. It can be concluded that PHA polymerizing genes of strain 30N and strain IFS belong to PHA synthases of class II (Rehm and Steinbüchel 2005). The class II PHA synthase has been reported to occur as phaC1 and phaC2 synthase genes with phaZ (PHA depolymerase) gene present in between them. These genes have the ability

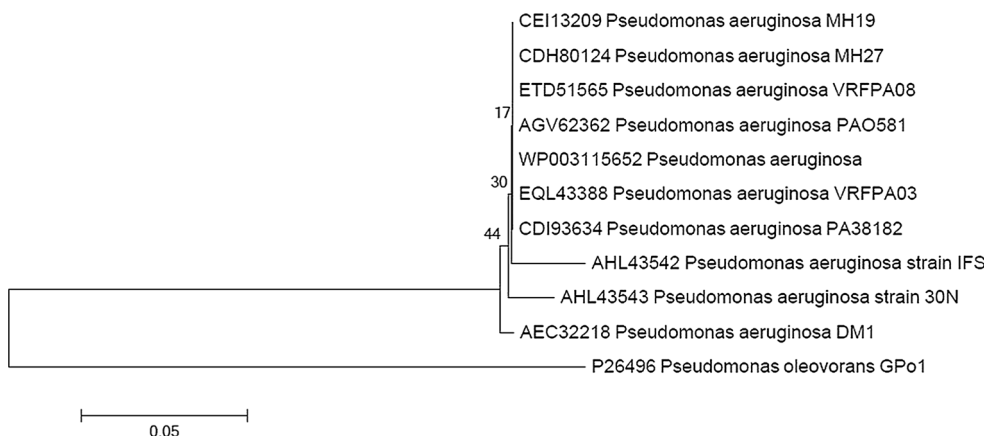


Fig. 5 Phylogenetic tree of PHA synthases' partial amino acid sequences of *Pseudomonas aeruginosa* strains IFS and 30N. Mega 5.2 software was used to construct evolutionary tree by neighbor-joining method. The percentage of replicate trees in which the associated taxa

clustered together in the bootstrap test (1000 replicates) are shown above the branches. The evolutionary distances were computed using the Tamura-Nei method. *P. oleovorans* GPo1 strain was taken as an out group

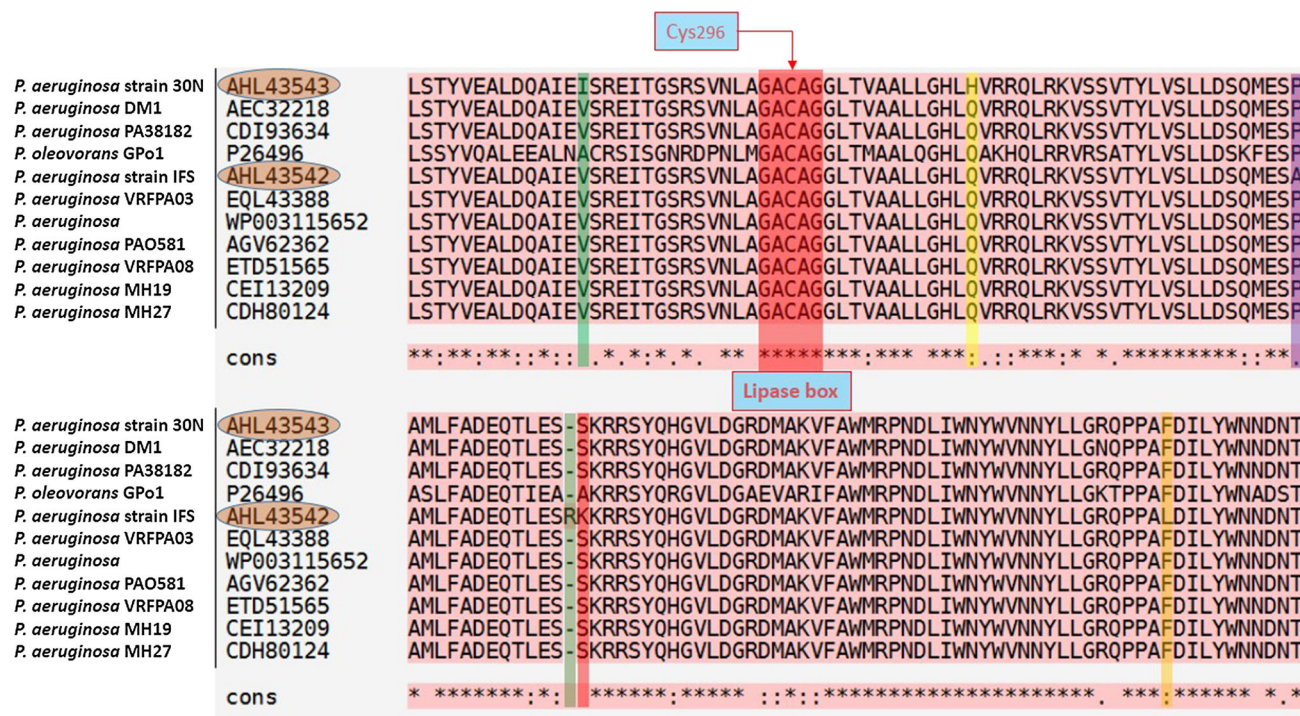


Fig. 6 Multiple sequence alignment of amino acids of PHA synthases from strain IFS and 30N with the related PHA synthases of other *P. aeruginosa* spp. A clear difference can be observed between them and *P. oleovorans* GPo1 (P26496)

to utilize the short-chain carbon compounds, e.g., glucose as carbon source for the scl-PHA production and to metabolize long-chain carbon monomers such as octanoic acid, nonanoic acid and various vegetative oils that contain the long-chain fatty acids to produce mcl-PHAs (Vo et al. 2015).

Multiple sequence alignment (MSA) not only gives an insight of the secondary structure prediction of a protein

but also suggests the probable structure that relates its function to some already know proteins, making MSA a significant tool for the finding of taxonomy (Chen et al. 2014). It has been reported that the PHA synthase II enzyme belongs to the α/β hydrolase superfamily in which a lipase box (G-X-C-X-G) is present at location 294–298. It was found that both strains IFS and 30N contain the G-A-C-A-G residues in their lipase boxes (Fig. 6). The

mutagenesis in conserved amino acids results in the change of substrate specificity. Cysteine residue at location 296 of PHA synthase enzymes actually plays a crucial role for the formation of a catalytic triad along with Asp451 and His479, as it plays the main nucleophilic attack and polymerizes the substrate for PHA polymers (Chen et al. 2014).

Conclusion

This study reports the isolation and identification of wild-type bacterial strains 30N and IFS belonging to *Pseudomonas* genus having the ability to utilize different carbon sources for the production of PHAs. The *Pseudomonas aeruginosa* strains IFS and 30N can grow well on glucose and other similar carbon sources under the same growth conditions to produce PHA. The production of PHBV by these strains on glucose as carbon source suggests that their PHA synthase has the ability to incorporate 4 carbon and 5 carbon monomers to polymerize. It can be concluded that these strains may be used as good candidates for higher PHA production from different inexpensive carbon sources. Future work is aimed to study the fermentation of glucose by these strains in lab-scale fermenters to maximize the PHA yield under best feeding strategy.

Acknowledgement Authors are thankful to University of the Punjab, Pakistan, and Higher Education Commission, Pakistan, under Indigenous scheme to support and conduct this study.

Compliance with ethical standards

Conflict of interest Authors declare no conflict of interest.

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