Research Article

Open Access

Rafeya Sohail, Nazia Jamil*, Iftikhar Ali and Sajida Munir

Animal fat and glycerol bioconversion to polyhydroxyalkanoate by produced water bacteria

<https://doi.org/10.1515/epoly-2020-0011>

Received March 30, 2019; accepted November 15, 2019.

Abstract: Oil reservoirs contain large amounts of hydrocarbon rich produced water, trapped in underground channels. Focus of this study was isolation of PHA producers from produced water concomitant with optimization of production using animal fat and glycerol as carbon source. Bacterial strains were identified as *Bacillus subtilis* (PWA), *Pseudomonas aeruginosa* (PWC), *Bacillus tequilensis* (PWF), and *Bacillus safensis* (PWG) based on 16S rRNA gene sequencing. Similar amounts of PHA were obtained using animal fat and glycerol in comparison to glucose. After 24 h, high PHA production on glycerol and animal fat was shown by strain PWC (5.2 g/L, 6.9 g/L) and strain PWF (12.4 g/L, 14.2 g/L) among all test strains. FTIR analysis of PHA showed 3-hydroxybutyrate units. The capability to produce PHA in the strains was corroborated by PhaC synthase gene sequencing. Focus of future studies can be the use of lipids and glycerol on industrial scale.

Keywords: *Bacillus tequilensis*; Fourier transform infrared spectroscopy; *Pseudomonas aeruginosa*; polyhydroxyalkanoates (PHA); 16S rRNA sequencing

1 Introduction

Oil field operations produce large amount of waste during oil production. Most common waste is produced water accounting for almost 98% of fluid waste. Produced water is composed mainly of hydrocarbons especially phenols, polycyclic aromatic compounds, treating chemicals, radionuclides, dissolved oxygen and dispersed oil (1,2).

Overall, produced water has high carbon content and low nitrogen content, which makes it a selective habitat of many microorganisms that thrive in conditions of extreme environmental stress (3). These microorganisms include polyhydroxyalkanoate-producing bacteria, which require stressful and limiting conditions of nutrients, i.e. surplus carbon content and low nitrogen, magnesium and phosphorous content for their successful growth (4). Moreover, PHA producers thrive by conversion of hydrocarbons in produced water to carbon reserves – in form of inclusion bodies.

Polyhydroxyalkanoates (PHAs) are such inclusion bodies – used mainly as storage or energy reserves by the cell (5). These bio-polyesters are biodegradable as well as biocompatible (6). Most common PHAs, to date, are polyhydroxybutyrate (PHB) (7), produced by many bacterial genera including *Pseudomonas*, *Bacillus*, *Alcaligenes*, *Rhodococcus*, *Agrobacterium*, *Comamonas*, *Hydrogenophaga*, *Ralstonia* etc. (8). Approximately 150 types of PHA monomers have been reported (9). The properties of PHAs are strongly dependent on their monomeric composition and structures. Incorporation of specific monomers tend to enhance stability (10). Classification of PHA, based on monomeric structure, divides them into short chain length PHA (scl PHA), medium chain length PHA (mcl PHA) and long chain length PHA (lcl PHA) units (11). Scl PHA, ranging from C3 to C5, include 3-hydroxypropionate, 3-hydroxyvalerate etc. are produced mainly by *Ralstonia* and *Alcaligenes* species. Mcl PHA, ranging from C6 to C14, include 3-hydroxyhexanoate, 3-hydroxytetradecanoate etc. are produced mainly by *Pseudomonas* species. Whereas lcl PHA have >C14 PHA units (6). Some bacteria also produce copolymers of PHA (10,12). The chemical composition of PHA also depends on its biosynthetic pathway (13). There are four main classes of PHA synthases. However, the main enzyme needed for PHA production is PhaC synthase. Composition of PhaC synthase varies from species to species accounting for differences in PHA structure and composition (14). There are three main pathways of PHA production, i.e. the acetoacetyl-CoA pathway (for

* Corresponding author: Nazia Jamil, Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Punjab, Pakistan, email: nazia.mmg@pu.edu.pk

Rafeya Sohail, Iftikhar Ali and Sajida Munir, Department of Microbiology and Molecular Genetics, University of the Punjab, Quaid-e-Azam Campus, Lahore 54590, Punjab, Pakistan.

conversion of amino acids to mcl PHA), in situ fatty acid synthesis (for conversion of fatty acids to mcl PHA) and beta-oxidation cycles (for conversion of sugars to scl-PHA) (15,16). Polyhydroxyalkanoates produced by produced water bacteria are mostly mcl PHA, produced mainly by *Pseudomonas* and *Bacillus* spp. (10).

Polyhydroxyalkanoates (PHA) are biodegradable plastics that have the potential to effectively replace conventional synthetic and petrochemical-based plastics (6). The biodegradability of PHAs is the main property that is exploited in all commercial ventures (14), i.e. use as packaging material, agricultural implements and in surgical fields (6,9). Commercialization of PHA depends upon their successful production using low cost, effective practices (17). Production costs have to be reduced to the extent that the process is feasible. About 40-60%, production costs are concerned only with raw materials (17). Some approaches include use of low cost resources (6,15), use of biomass as feedstock, use of organic wastes as carbon source (18,19), use of genetic manipulation and recombinant methodologies. Use of process control strategies has also been employed to increase PHA production (18,20). Recent studies have also focused on manipulating biosynthetic pathways of production either to increase production (21) or to produce novel product (17,22). Gedikli et al. reported production of thermostable PHB by *Geobacillus kaustophilus* (23).

Produced water is a major waste of oil drilling processes (1). It has high hydrocarbon carbon content and serves as habitat for plethora of microbiota that flourishes in extreme environment. This microbiota mainly bacteria break down complex hydrocarbons and produce important biopolymers. Polyhydroxyalkanoate producing bacteria use hydrocarbons present in produced water for bioconversion of fatty acids to mcl PHA (24). Present study was planned in two main phases having separate objectives. First objective of this study was to isolate bacterial strains from produced water with the capability to utilize hydrocarbons for biopolymers production such as polyhydroxyalkanoates. Thereby, use the surplus amounts of produced water in a way that has potential for environmental conservation and producing environment friendly biodegradable polymers (25). In the first phase, produced water samples were collected from Potwar oil fields, Pakistan. Polyhydroxyalkanoate producers were screened by growth on PHA detection media and further confirmation was done by sequencing of *phaC* and *phaC1* gene. The bacterial strains with higher PHA production were identified as *Bacillus subtilis* (PWA), *Bacillus tequilensis* (PWF), *Bacillus safensis* (PWG) and *Pseudomonas aeruginosa* (PWC) by 16S rRNA sequencing.

Second objective of this study was to optimize PHA production using hydrocarbon sources that are similar in complexity to those found in produced water but cheaper, renewable, structurally diverse, and non-fossil fuel based, i.e. animal fat (26) and glycerol (27). This optimization by mapping PHA production, in a sustainable manner, has potential for industrial scale studies by lowering production costs (28). In the second phase, bulk production and extraction of polyhydroxyalkanoates was assessed using three different non-fossil fuel based carbon sources, as initiative to reserve fossil fuel based sources and minimize production costs (29).

2 Materials and methods

2.1 Sample collection, isolation and identification of bacterial strains

Produced water samples were collected in plastic sterilized bottles from Potwar oil fields and stored at 4°C. Sample was appropriately analyzed for many parameters including temperature, pH, odor, texture, and color. Qualitative characterization of sample was based on methodologies reported by Openshaw (30) and Dey (31). Isolation of bacterial strains was performed according to serial dilution method, as described by James and Natalie [32], using Luria-Bertani Agar as seed medium. Viable cell counts were measured after 24 h incubation. Bacterial colonies with distinguishing features were selected from the mixed culture plates to obtain pure colonies. Preliminary identification of isolates was done by microscopic measurements of bacterial cell, gram staining, spore staining, capsule staining, catalase activity test, oxidase test, DNase test, starch hydrolysis test, citrate utilization test, motility test and urease activity test etc. (32,33). Genomic DNA was isolated as described by Sambrooke et al. (34). 16S rRNA gene sequencing of selected bacterial strains was done as commercial service by Macrogen Inc., Seoul, Korea, (https://dna.macrogen.com/eng/support/ces/guide/universal_primer.jsp). Forward and reverse sequences were provided separately. Reverse sequence was converted to complementary sequence with Chromas Pro 2.6.5 software (35). Forward and reverse sequences were aligned and assembled to obtain consensus sequence using Cap3 software (36). Sequences were inspected for maximum homology against GenBank using BlastN (37). Phylogenetic trees were constructed for sequences using MEGA4 by neighbor joining method (38).

2.2 Screening of polyhydroxyalkanoate (PHA) producers

Isolated bacterial strains were screened for PHA production ability by using PHA detection media (39) agar plates (40) supplemented with Nile blue A (41-44) or Nile red (45-47) for direct screening. After 24 h incubation, all plates were observed under UV light. Ability of strains to produce PHA was confirmed by staining of screened colonies with Sudan black B dye to visualize PHA granules (43). After direct screening and staining, PHA production was further verified by culturing selected strains on PHA detection media supplemented with Nile blue (42).

2.3 Optimization of polyhydroxyalkanoate (PHA) production

Three unrelated carbon sources namely glucose, glycerol and animal fat oil were used for optimization of PHA production. Glucose was selected as a monomeric, easily available carbon source, to compare production kinetics (48). Glucose solution was prepared and autoclaved for sterilization. Glycerol and animal fat oil were selected as biochemically, structurally complex carbon sources (49,50). Waste glycerol, an industrial byproduct, was collected and sterilized by autoclaving. Animal fat oil was extracted by heating animal fat (51). The residue obtained was decanted and filtered to obtain oil. Each carbon source was used in 2% v/v concentration in one liter of PHA detection media. Growth kinetic studies of PHA producers conducted in 500 mL flasks containing 300 mL PHA detection media supplemented with 2% carbon source (glucose, glycerol or animal fat oil), were repeated three times, to obtain mean values. Culture densities were recorded at 600 nm using spectrophotometer (52).

2.4 Polyhydroxyalkanoate (PHA) extraction

Culture broth was collected and centrifuged at 4000 rpm for 15 min. Supernatant was discarded and tubes containing biomass pellet were placed at -4°C overnight. Dry pellet was obtained by lyophilizing at 0.011 mbar and -60°C and dry cell weight (biomass) was weighed. Pellet was treated with 0.25% SDS at 25°C and pH 10 for 15 min, followed by treatment with 5.25% sodium hypochlorite at room temperature and pH 10 for 5 min. Mixture was centrifuged;

pellet was washed with acetone and centrifuged again. Crude PHA pellet was suspended in chloroform (10 times the volume of pellet) to dissolve PHA and incubated for 48 h at room temperature. After incubation, PHA layer was separated by filtration. Chloroform was dried by evaporation and weight of dried PHA films was measured in grams (53). Percentage of PHA (% PHA) was calculated as follows:

$$\% \text{ PHA} = \frac{\text{Weight of PHA}}{\text{Weight of biomass}} \times 100 \quad (1)$$

Timely variations in culture densities, biomass, and PHA production were recorded, over a period of 96 h, in triplicate studies. Standard error for mean values was calculated.

2.5 Fourier transform infrared (FT-IR) spectroscopy

Fourier transform infrared (FT-IR) spectroscopic analysis of extracted PHA samples was conducted (54,55), to identify the functional groups and to record PHA spectrum around scan range 400 to 4000 cm^{-1} , at Research Centre, Lahore Women's University.

2.6 Molecular analysis of synthase gene

Polyhydroxyalkanoate synthase gene was amplified using F-gen (CCGCAATTGAACAAGTTCTACCT) and R-gen (CGGGAGACGCGTGGTGTCTTG) primers by PCR (56). Initial denaturation was at 95°C for 10 min. Final extension was at 72°C for 10 min. PCR was run for 35 cycles. Each cycle consisted of denaturation at 95°C for 45 s, annealing at 60.7°C for 45 s and extension at 72°C for 1 min. Amplicons were sequenced by Sanger dideoxy sequencing. Partial sequences of PHA synthase genes, *phaC* and *phaC1* were inspected for maximum homology using BlastN and submitted to GenBank under accession numbers MH384823 to MH384825 and MH400895. Sequences were translated using ExPASy to determine reading frames. Translated sequences were aligned with similar sequences by using BioEdit 7.2.6 software to corroborate biological PHA production capability. Sequences were also analyzed for determination of conserved domains against NCBI conserved domain database (57,58).

3 Results

3.1 Sample collection, isolation and identification of bacterial strains

Produced water sample had light brown color, diesel like smell and oily texture. Temperature and pH of sample were noted as 27°C and 6.0, respectively. Positive results for qualitative characterization of sample were obtained indicating the presence of nitrogen, halogens, sulfur, lipids, aldehydes, alcohols, phenols, carboxylic acids, and dissolved carbon dioxide. Bacterial colony forming unit of each dilution of sample was calculated and was observed as highest in dilution 10^{-1} , which had 263 discrete colonies. Out of thirteen bacterial isolates, eleven were gram-positive rods (Figure 1a), while remaining two were gram-negative rod (Figure 1b) and gram-positive cocci (Figure 1c). According to morphological and biochemical characterization results, these isolates belong to genus *Pseudomonas*, *Bacillus* and *Rhodococcus* (Supplementary data Table 1). Strains PWA, PWC, PWF, and PWG were identified as *Bacillus subtilis* (MH142143), *Pseudomonas aeruginosa* (MH142144), *Bacillus tequilensis* (MH142145) and *Bacillus safensis* (MH142146) respectively by 16S rRNA gene sequencing (Table 1). Strain PWA was identified as *Bacillus subtilis* (MH142143) as it showed 100% homology to *Bacillus subtilis* (MG434569.1). Strain PWC, identified as *Pseudomonas aeruginosa* (MH142144), showed 99% homology to *Pseudomonas aeruginosa* (MG818964.1).

Strain PWF showed 99% homology to *Bacillus tequilensis* (MF521563.1) and was identified as *Bacillus tequilensis* (MH142145), whereas strain PWG identified as *Bacillus safensis* (MH142146) showed 99% homology to *Bacillus safensis* (MG432700.1).

3.2 Screening of polyhydroxyalkanoate (PHA) producers

Bacterial strains were screened for PHA production and six out of thirteen were found positive. These six strains gave fluorescence on Nile blue and Nile red supplemented PHA detection media. On Nile blue A supplemented plates, blue fluorescence was observed (Figures 2a and 2b) while on Nile red supplemented plates; green fluorescence was observed, due to binding of dye molecules to PHA granules (Figure 2c). On Sudan Black B staining of these strains, black granules of PHA were observed against pink background (Figure 2d). Results for verification of PHA production indicated strains PWF and PWC as the most potent PHA producing bacteria.

3.3 Kinetics of polyhydroxyalkanoate (PHA) production

All strains showed highest growth on glucose, followed by growth on animal fat oil. While lowest growth

Figure 1: Gram staining micrograph of (a) *Bacillus subtilis* shows gram-positive bacilli in chains. (b) *Pseudomonas aeruginosa* shows gram-negative bacilli. (c) *Rhodococcus* shows gram-positive cocci.

Table 1: PHA producing bacteria isolated from Potwar oil field produced water.

Strain name	GenBank accession number	Closest related classified organism	Sequence similarity (%)
PWA	MH142143	<i>Bacillus subtilis</i> (MG434569.1)	100%
PWC	MH142144	<i>Pseudomonas aeruginosa</i> (MG818964.1)	100%
PWF	MH142145	<i>Bacillus tequilensis</i> (MF521563.1)	99%
PWG	MH142146	<i>Bacillus safensis</i> (MG432700.1)	99%

was observed on glycerol (Supplementary material – Figure S1). Strain PWA (MH142143) showed almost similar growth rates on PHA detection media supplemented with glucose, glycerol, or animal fat oil. Strain PWC (MH142144) and PWF (MH142145) showed higher growth rates on animal fat oil, followed closely by growth rates on glycerol supplemented PHA detection media. However, growth rates on all carbon sources were almost same up to 48 h. Strain PWG (MH142146) showed higher growth rate on glycerol, but comparatively lower growth rates on animal fat oil. PHA production rates on animal fat oil followed closely. PHA production by PWA and PWG increased exponentially. PHA productions by PWA on glycerol and animal fat oil after 24 h were 4.6 g/L (11%) and 4.0 g/L (9%), respectively. PHA productions by PWG on glycerol and animal fat oil after 24 h were

6.4 g/L (27%) and 6.1 g/L (27%), respectively. Highest PHA production was shown by PWF (as shown in Figure 3) followed by PWC (as shown in Figure 4). After 24 h of incubation, PWC and PWF showed 5.2 g/L (15%) and 12.4 g/L (32%) production on glycerol, respectively. Polyhydroxyalkanoate production by PWF on glycerol increased exponentially and was highest (49.4 g/L; 42%) after 96 h. Polyhydroxyalkanoate production by PWC (MH142144; *Pseudomonas aeruginosa*) increased exponentially from 24 h mark. Mapping production statistics showed 8.7 g/L (17%), 14.8 g/L (20%) and 27.9 g/L (26%) production by PWC and 22.3 g/L (36%), 32.4 g/L (37%) and 49.4 g/L (42%) production by PWF, after 48, 72, and 96 h, respectively. PHA production, after 24 h, on animal fat oil by PWC was 6.9 g/L (20%). Highest PHA production (14.2 g/L; 40%) by strain PWF

Figure 2: PHA production in live cells and under light microscope. (a,b) Screening on Nile blue containing PHA detection media demonstrates blue fluorescence under UV light by *Bacillus subtilis* and *Bacillus tequilensis* respectively. (c) Screening on Nile red containing PHA detection media demonstrates green fluorescence under UV light by *Pseudomonas aeruginosa*. (d) Sudan Black B staining micrograph of *Bacillus subtilis* shows presence of PHA granules (as indicated by arrows).

Figure 3: PHA production by strain PWF (*Bacillus tequilensis*; MH142145). PHA production by strain PWF (*Bacillus tequilensis*; MH142145) was highest, after 96 h on glycerol and after 24 h on animal fat oil. Biomasses and percentage PHA plotted against time are the mean of values recorded, during triplicate experiment. Standard error was calculated.

Figure 4: PHA production by strain PWC (*Pseudomonas aeruginosa*; MH142144). PHA production by strain PWC (*Pseudomonas aeruginosa*; MH142144) on all carbon sources was highest after 96 h. Biomasses and percentage PHA plotted against time are the mean of values recorded, during triplicate experiment. Standard error was calculated.

was on animal fat oil after 24 h. After 24 h, percentage PHA decreased gradually. PHA production on animal fat oil resulted in 10.4 g/L (18%), 16.2 g/L (22%) and 29.4 g/L (26%) production by PWC and 24.5 g/L (39%), 35.4 g/L (37%) and 42.1 g/L (36%) production by PWF, after 48, 72 and 96 h, respectively.

3.4 FT-IR analysis of polyhydroxyalkanoate (PHA)

FT-IR spectroscopy results indicated PHA samples from *Pseudomonas aeruginosa* (see Figure 5) and *Bacillus tequilensis* (see Figure 6) having 3-hydroxybutyrate units, identifying PHA as polyhydroxybutyrate (PHB) (55). Absorption bands of 1720.53 cm^{-1} and 1721.50 cm^{-1} (reported to be PHA marker bands) were assigned to stretching vibrations of carbonyl (C=O) ester bond. FTIR spectrum absorption bands at 3582.62 cm^{-1} and 3744.86 cm^{-1} were assigned to hydroxyl group (OH). Absorption at 2929.92 cm^{-1} was assigned to lateral monomeric chains asymmetric $\text{CH}_2\text{-CH}_3$. Absorption at 1456.28 cm^{-1} was assigned to the intracellular amide (–CO–N–) II found in bacteria. Absorption peak at 1379.13 cm^{-1} was assigned to terminal CH_3 groups. While absorption peaks at 1277.86 cm^{-1} and 1274.97 cm^{-1} were assigned to stretching vibrations of asymmetric C–O–C. Series of absorption bands from 605.66 cm^{-1} to 1101.3 cm^{-1} and 603.66 cm^{-1} to 1101.37 cm^{-1} were assigned to C–O and C–C stretching vibrations.

3.5 Molecular analysis of synthase gene

PHA Synthase gene *phaC* of strains PWA (MH384823), PWF (MH384824), PWG (MH384825) showed 91% homology to Bacterium TERI PHA synthase (*phaC*) gene (GU196137.1). While *phaC1* gene of strain PWC showed 100% homology to *Pseudomonas aeruginosa* PHA synthase (*phaC1*) gene (LT883143.1) (Table 2). Aligning of sequences using BioEdit 7.2.6 software determined presence of variable and constant regions in *phaC* and *phaC1* genes. Presence of conserved domains in sequences determined against NCBI conserved sequence domain (57,58) indicated that strains PWA, PWC, PWF, and PWG contain conserved domains of N terminal of Poly-beta-hydroxybutyrate from nucleotide 2 to 502, 59 to 502, 1 to 504, and 36 to 505, respectively.

4 Discussion

It is well known that produced water has high quantities of dissolved crude oil, petroleum and related hydrocarbons (1). This carbon rich composition also makes it an ideal environment for many polyhydroxyalkanoates producing bacterial species since PHA inclusion bodies are produced as energy reserves in the presence of high carbon content (2,59). In the current study, isolation of PHA producing bacteria from produced water presents significant two-fold results in environmental studies (60). Firstly, produced water was utilized for isolation of bacteria resulting in biological clean-up of environment

Figure 5: FTIR spectrum of PHA produced by strain PWC (*Pseudomonas aeruginosa*; MH142144) showing absorption band at 1720.53 cm^{-1} which is a reported PHA marker band (C=O bond).

Figure 6: FTIR spectrum of PHA produced by strain PWF (*Bacillus tequilensis*; MH142145) showing absorption band at 1721.50 cm^{-1} which is a reported PHA marker band (C=O bond).

(61). Secondly, these bacteria demonstrate the ability to produce large quantities of useful biopolymer PHA. Out of thirteen isolates, 46% were PHA producers. Among selected strains, PWC; *Pseudomonas aeruginosa* and PWF; *Bacillus tequilensis* were able to grow efficiently on PHA detection media supplemented with all carbon sources. Glucose, because of its monomeric composition provides a readily available, easily replenishable supply of carbon. Overall, highest growth rates were observed on glucose as it is easily accessible and bacteria can readily utilize it (48). In comparison, glycerol (49) and animal fat oil (50,51) were utilized as feedstock to reduce production costs. Glycerol is a main byproduct of biodiesel industry (62). While animal fat is the main agro-industrial waste. It was selected especially due to its complex hydrocarbon rich content, which provides an environment similar in composition to that of produced water. Use of animal fat oil verified the fatty acid biodegradative activity of isolated strains (50,51). Microbiota of produced water especially PHA producers can successfully degrade complex fatty acids and utilize catabolic byproducts as a carbon source (59). Thus, growth rates on animal fat oil were higher than growth rates on glucose. Lowest growth rates were observed on glycerol except by strain PWG. This is because glycerol is only catabolized into simple components by some microorganisms. Therefore, use of glycerol imparts an evolutionary and biodegradability advantage to these PHA producers due to bacterial selectivity for glycerol (49).

PHA production by bacterium PWA increased exponentially after 24 h on both glycerol (4.6 g/L; 11%) and animal fat oil (4.0 g/L; 9%). Mohapatra et al. reported 3.09 g/L PHA production using different carbon and nitrogen compositions (63). Ray et al. reported 0.455 g/L PHA production by mixed *Bacillus subtilis* culture using crude glycerol (64). Mohandas et al. reported 2.54 ± 0.07 g/L PHA yield by *Bacillus cereus* using glycerol (65). Differences in production kinetics could be due to differences in metabolic activity of PHA synthase enzymes. PHA production by PWG remained almost same up to 96 h, although biomass increased exponentially. On glycerol and animal fat oil, production after 24 h was 6.4 g/L (27%) and 6.1 g/L (27%), respectively. Madhumathi et al. reported 6.41 g/L PHA production after 48 h. Comparatively, PWG shows high production, which could be because of its selective ability to utilize glycerol efficiently (66).

Strain PWC produced 26% PHA after 96 h on both glycerol (27.9 g/L) and animal fat oil (29.4 g/L) (Figure 4). In a similar study, Abid et al. reported 50.27% PHA production by *Pseudomonas aeruginosa*

using soybean oil (67). Gatea et al. reported 100 mg/L PHA production by *Pseudomonas aeruginosa* using waste cooking oil (68). Although both previous studies and this study use fatty acids for PHA production, bioconversion pathways for vegetable oil and animal fat oil could be different (24,69).

Polyhydroxyalkanoate production by strain PWF (MH142145; *Bacillus tequilensis*) was highest amongst all isolated strains (Figure 3). Moralejo-Garate et al. also reported high PHA production (80%) on glycerol by *Bacillus tequilensis* (70). High PHA production rates on glycerol could be due to increased enzymatic activity. Glycerol has been reported to enhance PHA production in *Pseudomonas putida* by Fontaine et al. (71) and in *Cupriavidus eutrophus* by Volova et al. (72). Chandani et al. reported 87% to 52% PHA production by *Bacillus tequilensis* (73). Reddy et al. reported 59% PHA production on fatty acid waste by *Bacillus tequilensis* (74). This comparative decrease in production could be due to exhaustion of fatty acids in media after initial burst of PHA bioconversion. It could be due to adaption of bacteria from carbon rich environment of produced water to limited carbon media. Oliveira et al. have reported effect on PHA production rates due to carbon exhaustion in media (75).

PHA samples of strains PWC (Figure 5) and PWF (Figure 6) were identified as polyhydroxybutyrate (PHB) due to presence of 3-hydroxybutyrate units. Absorption bands at 1720.53 cm^{-1} and 1721.50 cm^{-1} (reported PHA marker bands) were observed. Hassan et al. reported peaks for ester carbonyl group at 1721 cm^{-1} (76).

Strains PWA (MH142143), PWC (MH142144), PWF (MH142145), and PWG (MH142146) were identified as *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Bacillus tequilensis*, and *Bacillus safensis* by 16S rRNA sequences (Table 1). PCR amplicons of PHA genes showed resemblance to *phaC* of PHA synthases Group IV and *phaC1* of Group II (Table 2). Homology against conserved domains showed resemblance to N terminal of poly-beta-hydroxybutyrate polymerase (PhaC) (58).

PHA is a very significant product of microorganisms, having a plethora of advantages in environmental sectors as well as petroleum and biodiesel industries. Productions of high quantities of PHA are needed to replace their synthetic counterparts. High production of PHA over a wide range of renewable carbon sources such as animal fat oil, therefore, goes a long way to further their advantage over the fuel consuming production of their counterparts. *Bacillus tequilensis* and *Pseudomonas aeruginosa* isolated from produced water, in this study, can be used for high yield of PHA utilizing low cost resources and practices.

Table 2: Sequencing of gene *phaC* in produced water isolates.

Strain	GenBank accession number	Closest related classified organism	Gene	Group of PHA genes	Sequence similarity (%)
PWA	MH384823	Bacterium TER1 (GU196137.1)	<i>phaC</i>	Group IV	91%
PWC	MH400895	<i>Pseudomonas aeruginosa</i> (LT883143.1)	<i>phaC1</i>	Group II	100%
PWF	MH384824	Bacterium TER1 (GU196137.1)	<i>phaC</i>	Group IV	91%
PWG	MH384825	Bacterium TER1 (GU196137.1)	<i>phaC</i>	Group IV	91%

5 Conclusion

In this study, produced water was found to be a rich source for successful isolation of polyhydroxyalkanoate producing bacteria. Additionally, the use of cheap, readily renewable, non-fossil fuel based carbon sources, i.e. glycerol and animal fat for production optimization was explored, with significant results shown by bacterial strains PWC and PWF for PHA production. FTIR results and *phaC* gene sequences corroborated the capability to produce PHA by the analyzed bacterial strains. Future studies can focus on identifying other such carbon sources and designing strategies based on reducing cost of production. The potential of strains isolated in the current study can be explored for industrial scale studies defining a low cost, resource conserving innovative.

References

1. Igundu E.T., Chen G.Z., Produced water treatment technologies. LCT, 2012, 9(3), 157-177.
2. Akob D.M., Cozzarelli I.M., Dunlap D.S., Rowan E.L., Lorah M.M., Organic and inorganic composition and microbiology of produced waters from Pennsylvania shale gas wells. Appl. Geochem., 2015, 60, 116-125.
3. Hashemi S.Z., Fooladi J., Ebrahimipour G., Khodayari S., Isolation and Identification of Crude Oil Degrading and Biosurfactant Producing Bacteria from the Oil-Contaminated Soils of Gachsaran. Appl. Food. Biotechnol., 2016, 3(2), 83-89.
4. Elain A., Le Grand A., Corre Y.-M., Le Fellic M., Hachet N., Le Tilly V., et al., Valorisation of local agro-industrial processing waters as growth media for polyhydroxyalkanoates (PHA) production. Ind. Crop. Prod., 2016, 80, 1-5.
5. Muhammadi S., Afzal M., Hameed S., Bacterial polyhydroxyalkanoates-eco-friendly next generation plastic: production, biocompatibility, biodegradation, physical properties and applications. Green Chem. Lett. Rev., 2015, 8(3-4), 56-77.
6. Povolo S., Romanelli M.G., Basaglia M., Ilieva V.I., Corti A., Morelli A., et al., Polyhydroxyalkanoate biosynthesis by *Hydrogenophaga pseudoflava* DSM1034 from structurally unrelated carbon sources. New Biotechnol., 2013, 30(6), 629-634.
7. Santhanam A., Sasidharan S., Microbial production of polyhydroxy alkanotes (PHA) from *Alcaligenes* spp. and *Pseudomonas oleovorans* using different carbon sources. Afr. J. Biotechnol., 2010, 9(21), 3144-3150.
8. Lee E.Y., Kang S.H., Choi C.Y., Biosynthesis of poly (3-hydroxybutyrate-co-3-hydroxyvalerate) by newly isolated *Agrobacterium* sp. SH-1 and GW-014 from structurally unrelated single carbon substrates. J. Ferment. Bioeng., 1995, 79(4), 328-334.
9. Shah Tejas V., Vasava Dilip V., A glimpse of biodegradable polymers and their biomedical applications. e-Polymers, 2019, 19(1), 385.
10. Khosravi-Darani K., Mokhtari Z.-B., Amai T., Tanaka K., Microbial production of poly (hydroxybutyrate) from C 1 carbon sources. Appl. Microbiol. Biot., 2013, 97(4), 1407-1424.
11. Singh A., Mallick N., Enhanced production of SCL-LCL-PHA copolymer by sludge-isolated *Pseudomonas aeruginosa* MTCC 7925. Lett. Appl. Microbiol., 2008, 46(3), 350-357.
12. Anjum A., Zuber M., Zia K.M., Noreen A., Anjum M.N., Tabasum S., Microbial production of polyhydroxyalkanoates (PHAs) and its copolymers: a review of recent advancements. Int. J. Biol. Macromol., 2016, 89, 161-174.
13. Koller M., Vadjla D., Braunegg G., Atlić A., Horvat P., Formal-and high-structured kinetic process modelling and footprint area analysis of binary imaged cells: Tools to understand and optimize multistage-continuous PHA biosynthesis. EuroBiotech. J., 2017, 1(3), 203-211.
14. Pantazaki A.A., Papaneophytou C.P., Lambropoulou D.A., Simultaneous polyhydroxyalkanoates and rhamnolipids production by *Thermus thermophilus* HB8. AMB Express, 2011, 1(1), 17.
15. Tsuge T., Metabolic improvements and use of inexpensive carbon sources in microbial production of polyhydroxyalkanoates. J. Biosci. Bioeng., 2002, 94(6), 579-584.
16. Chen G.-Q., Jiang X.-R., Guo Y., Synthetic biology of microbes synthesizing polyhydroxyalkanoates (PHA). Synth. Syst. Biotechnol., 2016, 1(4), 236-242.
17. Mahishi L., Tripathi G., Rawal S., Poly (3-hydroxybutyrate) (PHB) synthesis by recombinant *Escherichia coli* harbouring *Streptomyces aureofaciens* PHB biosynthesis genes: effect of various carbon and nitrogen sources. Microbiol. Res., 2003, 158(1), 19-27.
18. Vargas A., Montañó L., Amaya R., Enhanced polyhydroxyalkanoate production from organic wastes via process control. Bioresource Technol., 2014, 156, 248-255.

19. Hassan M., Bakhiet E., Hussein H., Ali S., Statistical optimization studies for polyhydroxybutyrate (PHB) production by novel *Bacillus subtilis* using agricultural and industrial wastes. *Int. J. Environ. Sci. Te.*, 2019, 16(7), 3497-3512.
20. Porras M.A., Ramos F.D., Diaz M.S., Cubitto M.A., Villar M.A., Modeling the bioconversion of starch to P (HB-co-HV) optimized by experimental design using *Bacillus megaterium* BBST4 strain. *Environ. Technol.*, 2019, 40(9), 1185-1202.
21. Dhangdhariya J.H., Dubey S., Trivedi H.B., Pancha I., Bhatt J.K., Dave B.P., et al., Polyhydroxyalkanoate from marine *Bacillus megaterium* using CSMCRI's Dry Sea Mix as a novel growth medium. *Int. J. Biol. Macromol.*, 2015, 76, 254-261.
22. Shi H., Shiraishi M., Shimizu K., Metabolic flux analysis for biosynthesis of poly (β -hydroxybutyric acid) in *Alcaligenes eutrophus* from various carbon sources. *J. Ferment. Bioeng.*, 1997, 84(6), 579-587.
23. Gedikli S., Çelik P.A., Demirbilek M., Mutlu M.B., Denkbaş E.B., Çabuk A., Experimental Exploration of Thermostable Poly (β -Hydroxybutyrate)s by *Geobacillus kaustophilus* Using Box-Behnken Design. *J. Polym. Environ.*, 2019, 27(2), 245-255.
24. Magdouli S., Brar S.K., Blais J.-F., Tyagi R.D., How to direct the fatty acid biosynthesis towards polyhydroxyalkanoates production? *Biomass Bioenerg.*, 2015, 74, 268-279.
25. Kumar P., Ray S., Kalia V.C., Production of co-polymers of polyhydroxyalkanoates by regulating the hydrolysis of biowastes. *Bioresource Technol.*, 2016, 200, 413-419.
26. Garcia N.H., Strazzera G., Frison N., Bolzonella D., Volatile Fatty Acids Production from Household Food Waste. *Chem. Engineer. Trans.*, 2018, 64, 103-108.
27. Burniol-Figols A., Varrone C., Daugaard A.E., Le S.B., Skiadas I.V., Gavala H. N., Polyhydroxyalkanoates (PHA) production from fermented crude glycerol: Study on the conversion of 1, 3-propanediol to PHA in mixed microbial consortia. *Water Res.*, 2018, 128, 255-266.
28. Koller M., Maršálek L., de Sousa Dias M.M., Brauneegg G., Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnol.*, 2017, 37, 24-38.
29. Cruz M.V., Freitas F., Paiva A., Mano F., Dionísio M., Ramos A.M., et al., Valorization of fatty acids-containing wastes and byproducts into short-and medium-chain length polyhydroxyalkanoates. *New Biotechnol.*, 2016, 33(1), 206-215.
30. Openshaw H.T., A laboratory manual of qualitative organic analysis. At The University. Cambridge, 1948.
31. Dey B., Raman M., Laboratory manual of organic chemistry. G. Srinivasachari and Sons, Madras, 1941.
32. James C., Natalie S., Microbiology. A laboratory manual. Pearson Education, 2014.
33. Harley J.P., Prescott L.M., Laboratory exercises in microbiology. McGraw-Hill, 2005.
34. Sambrook J., Fritsch E.F., Maniatis T., Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, 1989.
35. McCarthy C., Chromas: version 2.0. Technelysium PTY, Australia, 1996, 1(1), 39.
36. Huang X., Madan A., CAP3: A DNA sequence assembly program. *Genome Res.*, 1999, 9(9), 868-877.
37. Dumontier M., Hogue C.W., NBLAST: a cluster variant of BLAST for NxN comparisons. *BMC Bioinformatics*, 2002, 3(1), 13.
38. Kumar S., Nei M., Dudley J., Tamura K., MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. *Brief. Bioinform.*, 2008, 9(4), 299-306.
39. Rehman S., Jamil N., Husnain S., Screening of different contaminated environments for polyhydroxyalkanoates-producing bacterial strains. *Biologia*, 2007, 62(6), 650-656.
40. Chaudhry W.N., Jamil N., Ali I., Ayaz M.H., Hasnain S., Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. *Ann. Microbiol.*, 2011, 61(3), 623-629.
41. Oshiki M., Satoh H., Mino T., Rapid quantification of polyhydroxyalkanoates (PHA) concentration in activated sludge with the fluorescent dye Nile blue A. *Water Sci. Technol.*, 2011, 64(3), 747-753.
42. Ostle A.-G., Holt J., Nile blue A as a fluorescent stain for poly-beta-hydroxybutyrate. *Appl. Environ. Microb.*, 1982, 44(1), 238-241.
43. Phanse N., Chincholikar A., Patel B., Rathore P., Vyas P., Patel M., Screening of PHA (poly hydroxyalkanoate) producing bacteria from diverse sources. *IJB*, 2011, 1, 27-32.
44. Kitamura S., Doi Y., Staining method of poly (3-hydroxyalkanoic acids) producing bacteria by Nile blue. *Biotechnol. Tech.*, 1994, 8(5), 345-350.
45. Spiekermann P., Rehm B.H., Kalscheuer R., Baumeister D., Steinbüchel A., 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.*, 1999, 171(2), 73-80.
46. Greenspan P., Mayer E.P., Fowler S.D., Nile red: a selective fluorescent stain for intracellular lipid droplets. *J. Cell. Biol.*, 1985, 100(3), 965-973.
47. Serafim L.S.S., Lemos P.C., Levantesi C., Tandoi V., Santos H., Reis M.A., Methods for detection and visualization of intracellular polymers stored by polyphosphate-accumulating microorganisms. *J. Microbiol. Meth.*, 2002, 51(1), 1-18.
48. Kim G.J., Lee I.Y., Yoon S.C., Shin Y.C., Park Y.H., Enhanced yield and a high production of medium-chain-length poly (3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. *Enzyme Microb. Tech.*, 1997, 20(7), 500-505.
49. Mothes G., Schnorpfeil C., Ackermann J.U., Production of PHB from crude glycerol. *Eng. Life Sci.*, 2007, 7(5), 475-479.
50. Ashby R., Foglia T., Poly (hydroxyalkanoate) biosynthesis from triglyceride substrates. *Appl. Microbiol. Biot.*, 1998, 49(4), 431-437.
51. Akiyama M., Taima Y., Doi Y., Production of poly (3-hydroxyalkanoates) by a bacterium of the genus *Alcaligenes* utilizing long-chain fatty acids. *Appl. Microbiol. Biot.*, 1992, 37(6), 698-701.
52. Teeka J., Imai T., Cheng X., Reungsang A., Higuchi T., Yamamoto K., et al., Screening of PHA-producing bacteria using biodiesel-derived waste glycerol as a sole carbon source. *J. Water Environ. Tech.*, 2010, 8(4), 373-381.
53. Van Doan T., Hoi L.T., Phong T.H., Recovery of Poly (3-hydroxybutyrate) from *Yangia* sp. ND199 by Simple Digestion with sodium hypochlorite. *Vietnam J. Sci. Technol.*, 2015, 53(6), 706.
54. Shah K., FTIR analysis of polyhydroxyalkanoates by a locally isolated novel *Bacillus* sp. AS 3-2 from soil of Kadi region, North Gujarat, India. *J. Biochem. Technol.*, 2012, 3(4), 380-383.
55. Gumel A.M., Annur M.S.M., Heidelberg T., Biosynthesis and characterization of polyhydroxyalkanoates copolymers produced by *Pseudomonas putida* Bet001 isolated from palm oil mill effluent. *PLoS One*, 2012, 7(9), e45214.

56. Cameron S., Phenotypic and genotypic investigations into fluoroquinolone resistance in the genus *Acinetobacter*. PhD thesis, University of Dundee, 2002.
57. Metzker M.L., Caskey C.T., Polymerase chain reaction (PCR). eLS, 2009.
58. Marchler-Bauer A., Derbyshire M.K., Gonzales N.R., Lu S., Chitsaz F., Geer L.Y., et al., CDD: NCBI's conserved domain database. *Nucleic Acids Res.*, 2014, 43(D1), D222-D226.
59. Goudarztalejerdi A., Tabatabaei M., Eskandari M., Mowla D., Iraj A., Evaluation of bioremediation potential and biopolymer production of pseudomonads isolated from petroleum hydrocarbon-contaminated areas. *Int. J. Environ. Sci. Te.*, 2015, 12(9), 2801-2808.
60. Rajagopalan R., Environmental studies: from crisis to cure. Oxford University Press, 2015.
61. Kuppusamy S., Palanisami T., Megharaj M., Venkateswarlu K., Naidu R., Ex-situ remediation technologies for environmental pollutants: a critical perspective. *Rev. Environ. Contam. T.*, 2016, 236.
62. Cavaleiro J.M., de Almeida M.C.M., Grandfils C., Da Fonseca M., Poly (3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochem.*, 2009, 44(5), 509-515.
63. Mohapatra S., Mohanta P., Sarkar B., Daware A., Kumar C., Samantaray D., Production of polyhydroxyalkanoates (PHAs) by *Bacillus* strain isolated from waste water and its biochemical characterization. *Proc. Natl. Acad. Sci. India. Sect. B. Biol. Sci.*, 2017, 87(2), 459-466.
64. Ray S., Sharma R., Kalia V.C., Co-utilization of crude glycerol and biowastes for producing polyhydroxyalkanoates. *Indian J. Microbiol.*, 2018, 58(1), 33-38.
65. Mohandas S.P., Balan L., Jayanath G., Anoop B., Philip R., Cubelio S.S., et al., Biosynthesis and characterization of polyhydroxyalkanoate from marine *Bacillus cereus* MCCB 281 utilizing glycerol as carbon source. *Int. J. Biol. Macromol.*, 2018, 119, 380-392.
66. Madhumathi R., Muthukumar K., Velan M., Optimization of polyhydroxybutyrate production by *Bacillus safensis* EBT1. *CLEAN-Soil Air Water*, 2016, 44(8), 1066-1074.
67. Abid S., Raza Z.A., Hussain T., Production kinetics of polyhydroxyalkanoates by using *Pseudomonas aeruginosa* gamma ray mutant strain EBN-8 cultured on soybean oil. *3 Biotech.*, 2016, 6(2), 142.
68. Gatea I.H., Haider N.H., Khudair S.H., Bioplastic (Poly-3-Hydroxybutyrate) production by local *Pseudomonas aeruginosa* isolates utilizing waste cooking oil. *World J. Pharm. Res.*, 2017, 6(8), DOI:10.20959/wjpr20178-8631.
69. Jimenez-Diaz L., Caballero A., Segura A., Pathways for the Degradation of Fatty Acids in Bacteria. *Aerobic Utilization of Hydrocarbons, Oils and Lipids*, 2017.
70. Moralejo-Gárate H., Mar'atusalihat E., Kleerebezem R., van Loosdrecht M.C., Microbial community engineering for biopolymer production from glycerol. *Appl. Microbiol. Biot.*, 2011, 92(3), 631-639.
71. Fontaine P., Mosrati R., Corrolier D., Medium chain length polyhydroxyalkanoates biosynthesis in *Pseudomonas putida* mt-2 is enhanced by co-metabolism of glycerol/octanoate or fatty acids mixtures. *Int. J. Biol. Macromol.*, 2017, 98, 430-435.
72. Volova T., Demidenko A., Kiselev E., Baranovskiy S., Shishatskaya E., Zhila N., Polyhydroxyalkanoate synthesis based on glycerol and implementation of the process under conditions of pilot production. *Appl. Microbiol. Biot.*, 2019, 103(1), 225-237.
73. Chandani N., Mazumder P., Bhattacharjee A., Production of polyhydroxybutyrate (biopolymer) by *Bacillus tequilensis* NCS-3 isolated from municipal waste areas of Silchar, Assam. *Int. J. Sci. Res.*, 2014, 3(12), 198-203.
74. Reddy M.V., Amulya K., Rohit M., Sarma P., Mohan S.V., Valorization of fatty acid waste for bioplastics production using *Bacillus tequilensis*: integration with dark-fermentative hydrogen production process. *Int. J. Hydrogen Energ.*, 2014, 39(14), 7616-7626.
75. Oliveira C.S., Silva C.E., Carvalho G., Reis M.A., Strategies for efficiently selecting PHA producing mixed microbial cultures using complex feedstocks: Feast and famine regime and uncoupled carbon and nitrogen availabilities. *New Biotechnol.*, 2017, 37, 69-79.
76. Hassan M.A., Bakhiet E.K., Ali S.G., Hussien H.R., Production and characterization of polyhydroxybutyrate (PHB) produced by *Bacillus* sp. isolated from Egypt. *J. Appl. Pharm. Sci.*, 2016, 6(4), 46-51.