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Production of Polyhydroxyalkanoates (pha) by *bacillus* and *pseudomonas* on Cheap Carbon Substrates

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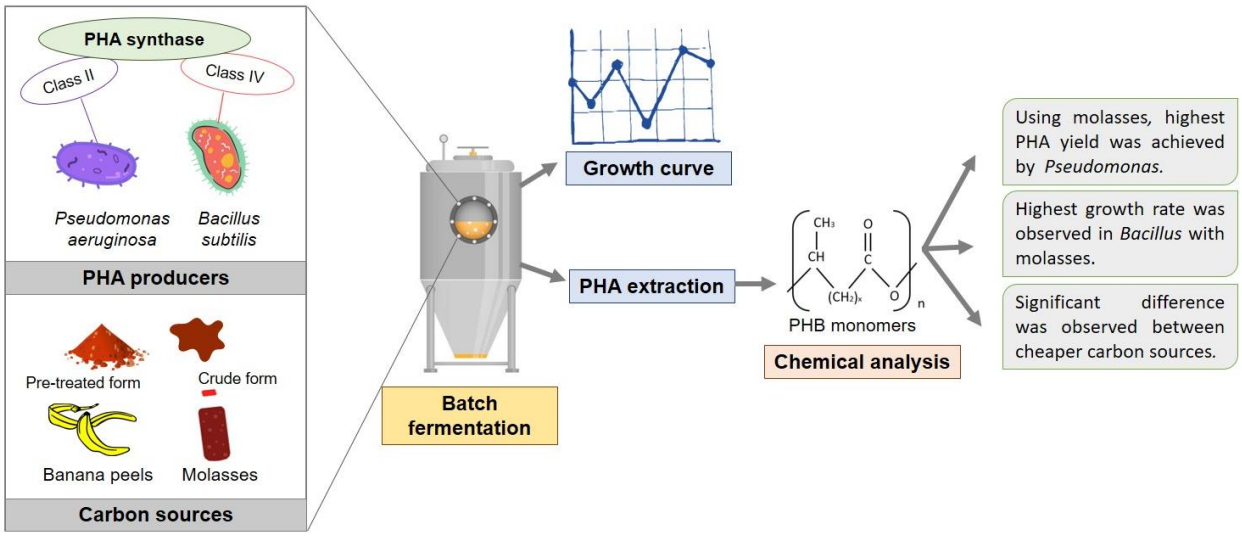
HIGHLIGHTS

- Compared agro-industrial wastes i.e. molasses and banana peels.
- Highest amount of PHA was produced by *Pseudomonas aeruginosa* utilizing molasses.
- Statistically, a significant difference was observed between cheaper substrates.
- FTIR analysis confirmed the extracted monomers as PHB.

Abstract: Polyhydroxyalkanoates are renewable, and biocompatible plastics having physiochemical properties similar to petrochemical plastics, and meet the Sustainable Development Goals (SDG). Two bacterial strains: *Pseudomonas aeruginosa* and *Bacillus subtilis* were selected to produce the PHA. The standard carbon source was glucose while molasses and banana peels were cheaper carbon substrates used in a medium to grow the bacterial strains. Sudan black B and Nile blue A staining were used to confirm the PHA production via spread plate assay. PHA granules were observed inside the bacterial cell microscopically, using Sudan stain. The carbohydrate concentration was estimated in molasses (29.4mg/mL) and banana peel extract (9.8mg/mL) by the Phenol-sulfuric acid test. In batch fermentation, time profiles of PHA production and growth of microbial strains for every 3hrs were performed up to incubation of 48hrs at 150rpm. PHA was extracted by sodium dodecyl sulfate digestion and chloroform extraction method. PHA production in *Pseudomonas aeruginosa* was 1.120g/L (66.82%) and 0.380g/L (44.60%) while in *Bacillus subtilis* was 0.476g/L (53.84%) and 0.288g/L (39.18%) utilizing molasses and banana peels, respectively after 48hrs. FTIR characterized the PHA monomer as polyhydroxybutyrate having a C=O bond at 1697.8cm^{-1} and 1750.0cm^{-1} and a CH_3 group at 1395.9cm^{-1} and 1458.3cm^{-1}. A statistical significance between the PHA productions was calculated by applying an unpaired t-test, for *Pseudomonas aeruginosa*, a significant difference was observed between glucose with both substrates, while for *Bacillus subtilis*, a significant difference was noted only between glucose and peel extract.

Keywords: *Bacillus*; Banana peels; Molasses; PHA yield; *Pseudomonas.*

GRAPHICAL ABSTRACT



INTRODUCTION

Human population growth increases the use of non-biodegradable plastics that have caused a drastic change on earth leading to plastic accumulations, resulting in environmental pollution [1]. The degradation rate of petrochemical-derived plastics is slow, resistant to microbial degradation, and releases harmful and toxic gasses during this process, and the plastic wastes take thousands of years to decompose in nature [2]. Oil resources are also diminishing due to increased plastic consumption, every year almost 150t plastic is consumed by the world's population [3]. Those materials that were readily wiped out from the environment in an eco-friendly manner could be used for producing plastics. Polyhydroxyalkanoates (PHA) have gained considerable attention as biodegradable alternatives to petroleum-based plastics. The chemical and structural properties of these bioplastics are similar to those of conventional plastics [4].

Several gram-negative, gram-positive, archaea, halotolerant, and halophilic bacteria can produce PHA. Prokaryotes produce PHA as intracellular granules as a source of energy under stressful conditions such as the limitation of potassium, magnesium, nitrogen, and the excess amount of carbon [5]. PHAs are the monomers of 3-hydroxy fatty acids having a diameter of 0.2-0.5µm, forming a polyester of 10^3 to 10^4 monomers. The composition, structure, size, and physiochemical properties of monomers vary among different PHA-producing bacterial strains and are also dependent on the carbon substrates either lipids or carbohydrates [6]. Chain lengths of polyesters are classified on the basis of carbon atom numbers, such as short chain length (C3-C5), medium chain length (C6-C14), and long chain length (>C14) [7]. Three genes are involved in encoding three enzymes for the production of PHA, *phaA* gene encodes the β-ketothiolase enzyme that forms acetoacetyl CoA, the condensation of two acetyl CoA molecules, *phaB* gene encodes NADPH-dependent acetoacetyl CoA reductase that reduced acetoacetyl CoA to (R)-3-hydroxybutyryl-CoA (for PHB production), and *phaC* gene encodes PHA synthase that polymerizes the monomers of (R)-3-hydroxybutyryl-CoA to form PHB [8]. The enzyme majorly involve during the biosynthesis of PHA is PHA synthase which is classified into four classes depending upon the subunits of the enzyme and synthesized chain length, class I is comprised of the *phaC*, class II contained *phaC*1 and *phaC*2, class III is composed of *phaC and phaE,* and class IV confined of *phaC* and *phaR* [9]. Among all classes, only class II synthesizes medium-chain length, and classes I, III, and IV form monomers of short-chain length, PHA can be degraded within 3-9 months by PHA depolymerase (*phaZ*), an enzyme secreted by microorganisms [10].

The purified carbohydrates kept the production cost higher than the synthetic plastic, the only drawback in producing PHA. Those substrates must be found that are less expensive and easily consumed by the microbial strains. Bacteria can synthesize the PHA from a comprehensive range of carbon sources, varying from plant oils to wastewater, alkanes, fatty acids, and carbohydrates [11]. Food-processing industries excrete a huge amount of waste and agricultural waste that can be used as a cheaper source for PHA

production. Sugar industries contain molasses, the waste of sugarcane, and sugarbeet which is rich in carbohydrates [12]. Typically, glucose costs about 33–50% more than molasses. Numerous agro-industrial wastes potato starch, pomegranate peels, wheat bran, bagasse, and banana peels were tested as carbon sources for PHA production [13]. Bananas are not seasonal fruits, and peels of bananas are available throughout the year to be the best economical option for producing PHA [14]. This research aimed to compare the PHA production by *Pseudomonas aeruginosa* belonging to class II of PHA synthase and *Bacillus subtilis* belonging to class IV of PHA synthase utilizing molasses and extract of banana peels, Time profiling of both strains for growth and production of PHA utilizing a sugar carbon source was observed, and chemical analysis of extracted PHA was carried out

MATERIALS AND METHODS

Materials

The banana peels and molasses were the raw materials. Banana peels were taken from the university canteen (University of Management and Technology, Lahore) and molasses was obtained from the local sugar mill (Nawan feeds, Kasur). Banana peels were pre-treated but molasses was used in a crude form as molasses contained a hydrolyzed form of sugar. Two bacterial strains were used: one is gram-positive, *Bacillus subtilis* strain AZR-1 (accession number: JQ398616) and the other is gram-negative *Pseudomonas aeruginosa* strain PA-1 (accession number: MG763232) obtained from the Institute of Microbiology and Molecular Genetics, University of the Punjab, Lahore. Both bacterial strains were characterized and reported [15, 16]. All the reagents and chemicals were purchased either from Sigma (USA) or Thermo fisher scientific (Maryland, USA).

Pre-treatment of Banana peels

The banana peels were pre-treated because these were large in size that contained cellulose a polysaccharide that had to be converted into the hydrolyzed form of carbohydrates (glucose) which microbes can simply utilize as the source of energy.

Preparation of Bran

There was an immersion of banana peels for 24hrs in a 1% v/v lime solution to prevent oxidation. Then, the peels were placed in a hot air oven for 24-48hrs at 60˚C, for drying. The dried peels were then crushed in a grinder to form the bran. Ethanol was used to wash the bran to eliminate the lipid content, then dried the bran for 24-48hrs at 60˚C in an oven. The 200-mesh sieve was used to sieve the bran to form the microparticles [17].

Chemical Treatment of Bran

The bran was treated to eliminate the components that are non-cellulosic in nature like pectin, hemicellulose, and lignin. An alkaline treatment was performed for solubilization of hemicellulose and pectin for that 5% w/v potassium hydroxide (KOH) solution having a ratio of 1:20 was used, under mechanical stirring for 14hrs at 25˚C. The bleaching treatment was done for delignification of insoluble residue with 1% v/v sodium hypochlorite (NaClO) solution at pH 5 (adjusted by 10% v/v CH_3COOH) for 1hrs at 70˚C, which displayed the chromophoric groups present in the lignin and broke down the phenolic compounds. The insoluble residue underwent acid hydrolysis for hydrolyzing the cellulose using 1% v/v sulfuric acid (H_2SO_4) solution for 1hrs at 80˚C. The resulting material contained the hydrolyzed form of cellulose. The insoluble residue was neutralized depending on the pH after each step using an acid solution (10% v/v CH_3COOH) or an alkaline solution (5% w/v KOH). The deionized water was used for washing the final residue and centrifuge (inno-MC20r, USA) the solution at 10,000 rpm for 20min, 5˚C until pH was neutralized, and the desired residue was stored at 4˚C in the form of suspension [17].

Carbohydrate Estimation test

The estimation of carbohydrate concentration present in both raw materials was performed by phenol-sulfuric acid test (quantitative analysis). The glucose stock solution (5mg/mL) was used to prepare the working standards along with the blank (distilled water). Two separate test tubes were also prepared for raw material. 1mL of 5%v/v phenol solution and 5mL of 96% sulfuric acid were added into the test tube, vortex the tubes to thoroughly mix the solution, incubated the test tubes at 25˚C for 20min, and measured the optical

density at 490nm using a spectrophotometer (AE-S60-2U, China). The glucose standard curve was plotted to calculate the concentration of glucose in raw materials [18].

Confirmation of Bacterial strains as PHA Producers

The ability of bacterial strains for producing PHA was confirmed by growing them on polyhydroxyalkanoate detection agar (PDA) [19] incubated for 24hrs at 37˚C to determine the turbidity of the colonies. The produced PHA was determined by adding 100µl of fat-soluble dye Sudan black B on each grown colony and after 30 min removing the extra stain using 95% ethanol to observe the blackish colonies [20]. Inside the bacterial cells, PHA granules were observed through microscope (XSZ-107BN, China), using Sudan black B as a primary stain, Xylene as a decolorizer, and Safranin as a counter stain [21]. The PHA granules were observed under 100X as bluish-black inclusions and the cytoplasmic region of cells was observed pink. Nile blue A is a more specific lipophilic dye for the determination of PHA, for that bacterial strains were grown on PDA by adding Nile blue A stain having a concentration of 0.5µg/mL, and the bluish fluorescence was observed under UV-Transilluminator (MD-20, 302nm, USA) [22].

Preparation of PHA Production Media

The preparation of standard inoculum was done by growing the *Bacillus subtilis* and *Pseudomonas aeruginosa* on 100 mL of nutrient broth and incubated in a rotatory shaker (THZ-98A, China) at 150rpm, 37˚C for 24hrs. The bacterial cells were re-suspended into the flask containing 300mL of PHA production media (g/L) that contained: $MgSO_4$. $7H_2O$ 0.5, $CaCl_2$. $2H_2O$ 0.01, Na_2HPO_4. $2H_2O$ 2.0, KH_2PO_4 2.0, $NaHCO_3$ 0.5, citric acid ($C_6H_8O_6$) 0.05, NH_4Cl 1.0, 3.6mL solution of trace elements (contained $ZnSO_4$. $7H_2O$ 0.08, $NiCl_2$. $6H_2O$ 0.02, $CoCl_2$. $6H_2O$ 0.2, $CuCl_2$. $2H_2O$ 0.01, H_3BO_3 0.3, $MnCl_2$. $4H_2O$ 0.03, Na_2MoO_4. $2H_2O$ 0.03). 2% of the carbon source (glucose, molasses, and banana peels) was added to the respective flask and kept the pH neutral by NaOH (10%) and HCl (10%) [19, 23]. Glucose used as a standard source of carbon while banana peels and molasses were the cheaper carbon substrates.

Time Profiling of Bacterial strains

The *Bacillus subtilis* and *Pseudomonas aeruginosa* were grown on a PHA production medium, and the growth pattern of each bacterial strain utilizing each carbon source was observed after 3-6hrs up to the incubation period of 48hrs, by measuring the turbidity using a spectrophotometer at 600nm. The medium was incubated in a rotatory shaker at 150rpm having a neutral pH and a temperature of 37˚C. Centrifuged the cultured medium in a pre-weighted tube for 10min at 4˚C, 10,000 rpm, and the cell pellet was washed with 10 mL of deionized water. The harvested cell pellet was dried at 105˚C for 48hrs and measured the dry cell weight.

Extraction of Polyhydroxyalkanoates

The sodium dodecyl sulfate (SDS) digestion and chloroform ($CHCl_3$) extraction method was used for PHA extraction. 3mL of 5% w/v SDS solution was used to suspend the dried pellets and placed for 2hrs at 95˚C in a water bath (SH-WB-11GDN, South Korea) for complete digestion of cellular components. The SDS solution containing the pellet was centrifuged at 9000rpm, 25˚C for 10 min, discarding the supernatant to obtain the pellet contained granules of PHA and washing the pellet with 10mL of distilled water until SDS was completely removed. The suspension was transferred into a glass tube, 3mL of chloroform was added that dissolved PHA granules, and place the glass tube until an aqueous and organic layer formed. The organic layer was piped out to the pre-weighted glass plate, chloroform was air-dried, and dissolved granules of PHA were obtained [24, 25]. The glass plate was again weighed. The PHA yield and residual biomass were calculated by below formulas [26]:

PHA yield (%) = Dry weight of PHA extract (g/L) / Dry cell weight (g/L) ×100

Residual biomass (g/L) = Dry cell weight (g/L) - Dry weight of PHA extract (g/L)

Chemical Analysis of PHA

The chemical bonds present in an extracted PHA was analyzed by performing Fourier transform infrared (FTIR) spectroscopy. The solution was formed by dissolving the biopolymer into the chloroform, then added the solution into KBr pellets (optically transparent), and allowed the solvent to evaporate. The infrared spectra

of the extracted PHA were analyzed by FTIR spectrophotometer (Jasco FTIR-6100, Japan) under wave numbers ranging from 400cm^{-1} to 4000cm^{-1} [27].

Statistical Analysis (Unpaired Student T-test)

The statistical difference between the extracted PHA and also between the dry cell weight obtained from glucose, molasses, and banana peels from both bacterial strains was compared by applying an unpaired student t-test (independent groups). The applied test was set to two-tailed and the confidence level was 95% (p-value <0.05) using GraphPad Prism 8.0.2.

RESULTS

Bacterial strains

The identified bacterial strains: *Pseudomonas aeruginosa* and *Bacillus subtilis* were cultured on the nutrient agar medium and incubated at 37˚C for 24hrs. The colony morphology was observed after the incubation.

Carbohydrate Estimation test

The carbohydrate concentration of molasses and banana peels was estimated through the Phenol sulfuric acid test. A standard curve of glucose solution was plotted between the absorbance values taken at 490 nm along the vertical direction and the standard concentration of glucose on the horizontal axis. It was estimated through the standard curve that the ultimate concentration of glucose present was 9.8mg/mL and 29.4mg/mL in the banana peels and molasses, respectively.

Confirmation of Bacterial strains as PHA producers

The microscopic screening with Sudan Black B stain showed dark blue-black granules of polyhydroxyalkanoates inside the cells, confirming PHA-positive strains. While other parts of the cell appeared pink in the microscope due to the consumption of safranin as shown in Figure 1(a). In spread plate assay, Sudan stain imparted black color to the bacterial colonies that were grown on the PDA media. The blackish color confirms that both strains are PHA producers, as shown in Figure 1(b). The microbial strains that were cultivated on PDA media carrying Nile blue A solution represent bluish fluorescence under UV-Trans-illuminator. Bluish fluorescence indicates that both were PHA-producing strains. Figure 1(c).

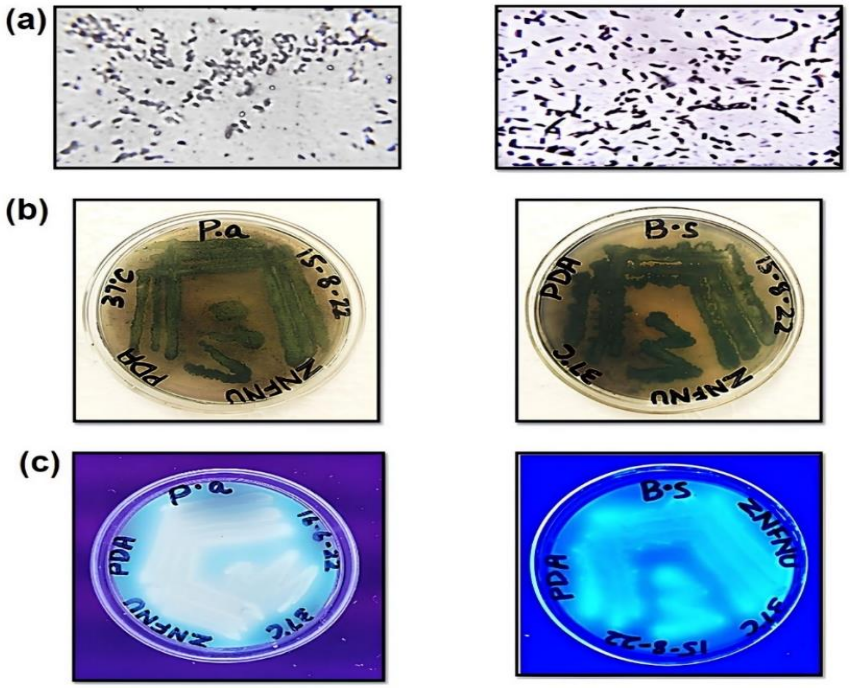


Figure 1. Bacterial strains (left) *Pseudomonas aeruginosa* (right) *Bacillus subtilis* confirmed as PHA producers **(a)** Sudan Black B staining, microscopic method **(b)** Sudan Black B staining, spread plate assay **(c)** Nile Blue A staining

Growth pattern of Bacterial strains

Compared with the glucose standard medium, *Pseudomonas aeruginosa* showed maximum growth in the molasses medium while the least growth was observed in banana peels containing medium. The lag phase persisted for up to 3-4hrs in glucose and molasses augmenting media. However, in the banana peels comprising medium, bacteria remain in their preparatory phase for 4-6hrs. The exponential phase started and continued for up to 5-33hrs in a glucose medium and 5-39hrs in a medium containing molasses while in a banana peels supplemented medium, the log phase was observed up to 7-33hrs. The growth pattern was shifted toward the stationary phase, this concluded that the growth rate stopped before 48hrs in all carbon substrates as shown in Figure 2 (a). *Bacillus subtilis* also showed the highest growth in molasses and the lowest growth in banana peels medium by comparing with glucose as a standard medium. Bacterial cells remained in the lag phase for up to 0-4hrs in the glucose and banana peels media while in the molasses-containing medium, the lag phase was extended for up to 0 to 6hrs. The exponential phase initiated after 4hrs and sustained for up to 48hrs in the glucose and banana peels media, however, the log phase for molasses augmented medium began after 6hrs and persisted for up to 48hrs as shown in Figure 2(b). It was investigated that the growth rate of *Bacillus subtilis* was higher as compared to *Pseudomonas aeruginosa* among all carbon substrates.

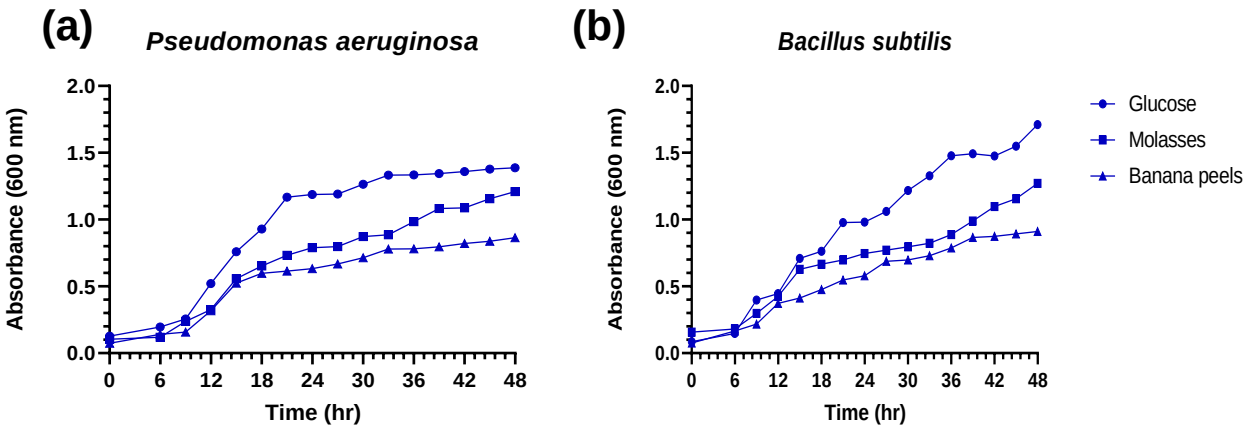


Figure 2. The growth curve of bacterial strains (a) *Pseudomonas aeruginosa* (b) *Bacillus subtilis* was determined every 3-6hrs for up to 48h of incubation at 600nm, grown in a PHA production media carrying different carbon sources in a batch fermentation at 150 rpm, having a temperature of 37˚C and pH 7.

PHA Production by Bacterial strains

PHA production increased with time but the total yield of PHA decreased after a particular time of incubation. For *Pseudomonas aeruginosa*, in a glucose-supplemented medium, the maximum yield of PHA among all carbon sources was observed at 27hrs of 84.50% and PHA production was 0.589g/L. In a molasses-enriched medium, the top PHA accumulation was 76.70% determined at 21hrs having PHA production of 0.688g/L. In a medium containing banana peels, the major rise in PHA yield was 53.28% observed at 30hrs of incubation with PHA production of 0.308g/L. Banana peels produced the lowest PHA yield of 53.28% among all carbon sources. After the 48hrs of incubation, the PHA yield observed was 69.03%, 66.82%, and 44.60% carrying the PHA production of 0.856g/L, 1.120g/L, and 0.380g/L for glucose, molasses, and banana peels, respectively as represented in Figure 3(a). For *Bacillus subtilis*, the highest PHA yield among all carbon sources was observed with a glucose-supplemented medium at the incubation of 21hrs of 65.77% carrying 0.392g/L PHA production. In a molasses-augmented medium, the maximum value of PHA yield was observed at 27hrs of 61.82% and 0.413g/L PHA production. In a medium containing banana peels, the peak value of PHA yield was shown at 33h of 46.03% containing PHA production of 0.232g/L. The PHA yield observed at 48hrs of incubation was 57.28%, 53.84%, and 39.18% while the PHA production was 0.472g/L, 0.476g/L, and 0.288g/L for glucose molasses and banana peels, respectively as shown in Figure 3(b).

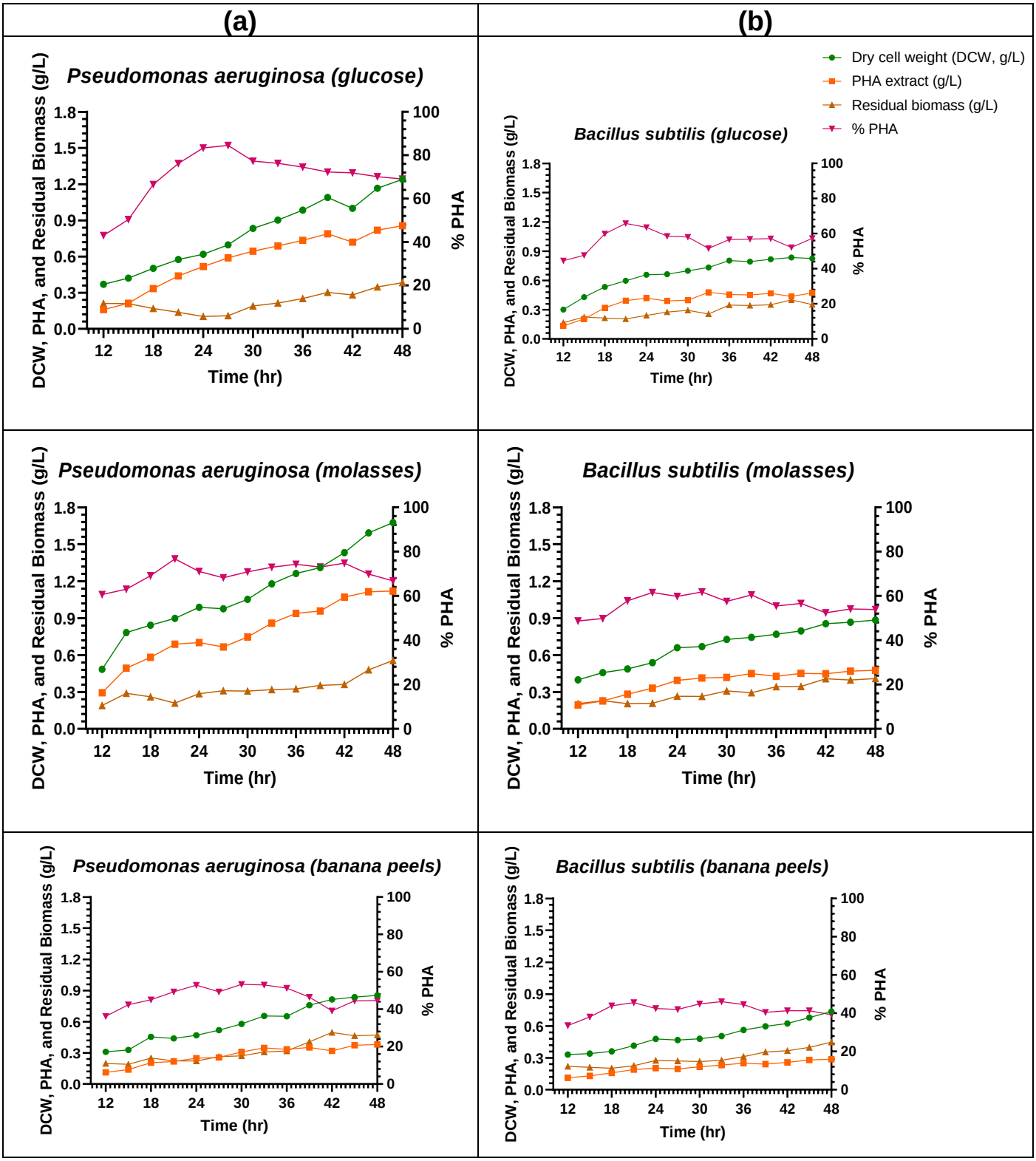
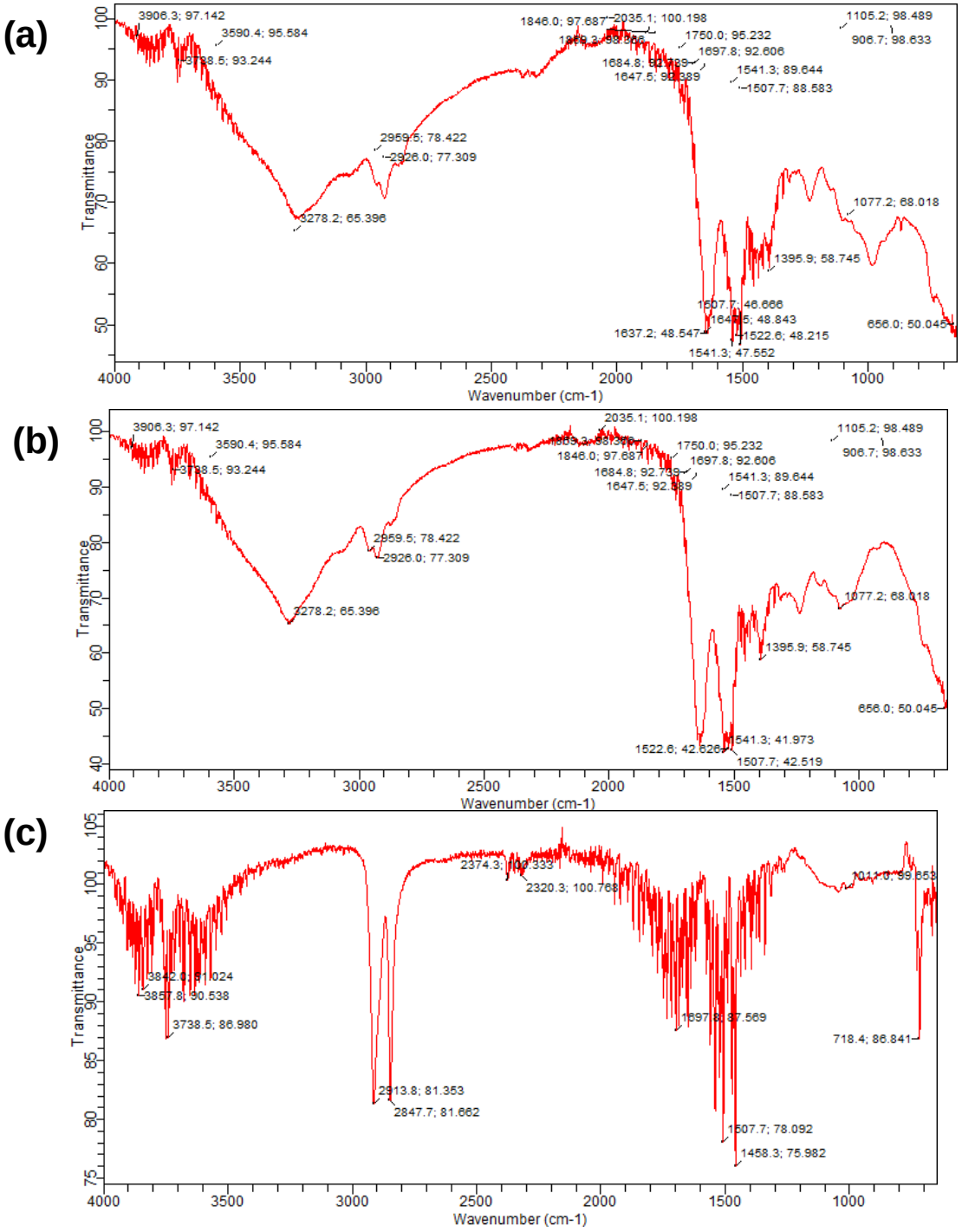


Figure 3. Time profiling of Dry cell weight (DCW), Residual biomass, PHA production, and % PHA of bacterial strains **(a)** *Pseudomonas aeruginosa* **(b)** *Bacillus subtilis* grown in a rotatory shaker at 150rpm, at a temperature of 37 ˚C and pH 7 up to 48hrs of incubation with glucose, molasses, and banana peels.

Chemical analysis of PHA

The FTIR analysis represents different peaks for various functional groups that are present in the sample. The analysis of the polymer produced by *Pseudomonas aeruginosa* and *Bacillus subtilis* revealed the sharp peaks under the wavelength range studied for PHAs in literature. The IR spectrum of the polymer indicated the marked peaks at the wavenumber 1697.8 cm^{-1} and 1750.0 cm^{-1} for the ester (C=O) carbonyl groups. Polymeric bonds (C-O-C) were also observed between the absorbance ranges of 1011.0 cm^{-1} and 1077.2 cm^{-1}. For O-H bending groups, intense absorption was observed at 3278.2 cm^{-1} and 3738.5 cm^{-1}. Certain other bands were also recorded at the wavelength of 1395.9cm^{-1} and 1458.3cm^{-1} which shows the presence of the CH_3 group. C-H stretching bonds derived from the alkane group were also revealed at the absorption bands of 2847.7 cm^{-1} and 2959.5 cm^{-1}. **Figure 4** shows the Fourier transform infrared analysis of *Pseudomonas aeruginosa* and *Bacillus subtilis*.



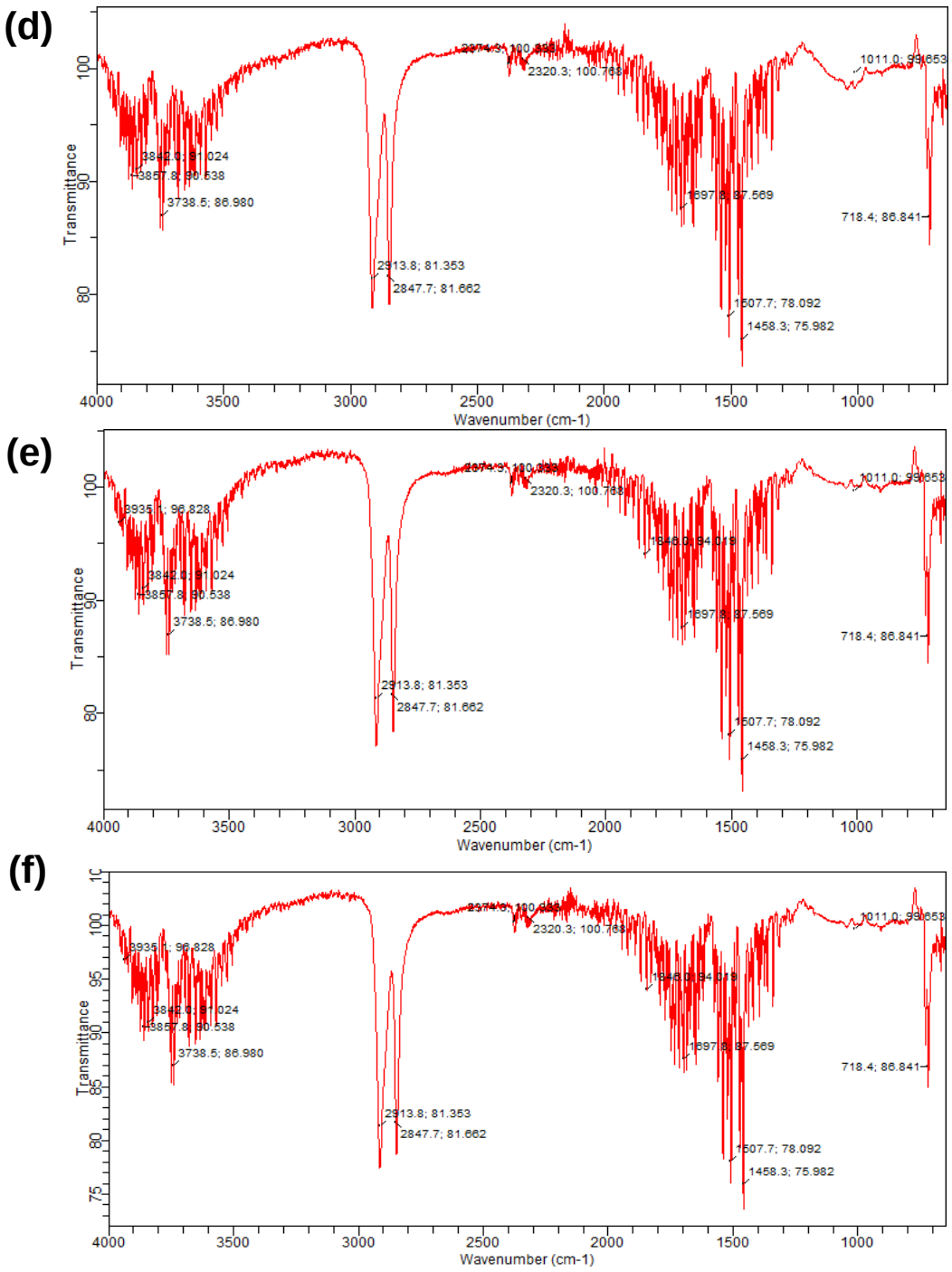


Figure 4. Functional group analysis of extracted PHA by bacterial strains using different carbon sources **(a)** *Pseudomonas aeruginosa*, glucose **(b)** *Bacillus subtilis*, glucose **(c)** *Pseudomonas aeruginosa*, molasses **(d)** *Bacillus subtilis*, molasses **(e)** *Pseudomonas aeruginosa*, banana peels **(f)** *Bacillus subtilis*, banana peels.

Statistical Analysis (Unpaired Student t-test)

The unpaired student t-test was applied for both bacterial strains to determine the statistical difference between dry cell weight and extract of PHA derived from all carbon sources. Two independent groups of carbon sources were compared. The significance level (p-value) for all the data sets was 0.05, the degree of freedom was 24, and the critical value was 2.064. To determine the statistical t-value for the dry cell weight of *Pseudomonas aeruginosa*, the mean of DCW of glucose was compared with the means of DCW of molasses and also with the DCW of banana peels which were 2.530 and 2.200, respectively. Similarly, the statistical t-value was determined for the PHA extracts of *Pseudomonas aeruginosa*, by comparing the mean of the PHA extract of glucose with the means of the PHA extract of molasses and banana peels which were 2.222 and 4.414, respectively. The t- values of DCW and PHA extract after comparing molasses and banana peels were 4.873 and 6.907, respectively. From the above values, it was determined that the critical value

was less than the statistical t-value of all data sets. Therefore the null hypothesis was rejected and there was a significant difference between the dry cell weights of glucose with molasses as well as with banana peels and also between molasses and banana peels. Likewise, a significant difference was also observed between the PHA extract of glucose with both carbon substrates and also between both sources. For *Bacillus subtilis*, the statistical t-value was 0.1893 between the mean of DCW of glucose and molasses, this showed the critical value was greater than the t-value therefore, the null hypothesis was accepted and no significant difference was observed between them. However, the t-value for the DCW of glucose and banana peels was 2.801 therefore, there exists a significant difference. The statistical t-value for the comparison between the mean of the PHA extract of glucose and molasses was 0.06634 and with banana peels was 5.237 which showed no significant difference between glucose and molasses while between the glucose and banana peels, a significant difference was observed. The t-values 3.033 and 5.671 were obtained after comparing the DCW and PHA extract of molasses and banana peels, respectively showed a significant difference between both cheaper substrates. The p-value that was obtained by the comparison between carbon sources had shown in Table 1.

Table 1. Unpaired student t-test depicting the significant difference between carbon sources utilized by *Pseudomonas aeruginosa* and *Bacillus subtilis.* (*) showed the statistical significance, the higher the number of asterisks greater the significant difference.

Bacterial strains		Carbon sources	Unpaired t-test p-value (<0.05)
Pseudomonas aeruginosa	Dry cell weight (g/L)	glucose/molasses	0.0184*
		glucose/banana peels	0.0377*
		molasses/banana peels	<0.0001****
	PHA extract (g/L)	glucose/molasses	0.0360*
		glucose/banana peels	0.0002***
		molasses/banana peels	<0.0001****
Bacillus subtilis	Dry cell weight (g/L)	glucose/molasses	0.8514
		glucose/banana peels	0.0099*
		molasses/banana peels	0.0057**
	PHA extract (g/L)	glucose/molasses	0.9477
		glucose/banana peels	<0.0001****
		molasses/banana peels	<0.0001****

DISCUSSION

The purified carbon sources are expensive to use as a carbon substrate for PHA production at an industrial scale, this quickened the search for cheaper carbon substrates to low the production cost of PHA [28]. Therefore, this study targeted the sugar raw materials banana peels and molasses, both have sufficient sugar content to use in bacterial media as a carbon source and also managed the agro-industrial wastes. Banana peels are rich in cellulose [29] to increase the content of hydrolyzed sugars peels were pretreated. The crude form of molasses was used because it contained a hydrolyzed form of carbohydrates and other nutritional supplements [23].

Several gram-positive and gram-negative bacteria have the capability to produce PHA, another selection criterion was the class of PHA synthase enzyme present in bacterial strains that polymerizes granules of PHA, *Pseudomonas aeruginosa,* and *Bacillus subtilis* were selected to produce PHA belonging to class II [30] and class IV [31] of PHA synthase, respectively. *Pseudomonas aeruginosa* exhibited the finest growth in the medium consisting of molasses as a carbon substrate, the lag phase was appeared early in the media containing glucose and molasses but the bacterial medium comprising banana peels as a carbon source extended the lag phase as it was influenced by the metabolic activity of *Pseudomonas aeruginosa* to utilize different carbon substrates at different time periods*. Pseudomonas putida* had the potential to metabolize an extensive range of carbon sources, and it showed growth phases at different time points while utilizing different substrates [32]. The exponential phase was observed at different time periods then the strain shifted the growth towards the stationary phase due to the depletion of nutrients as sugar substrates were the sole energy source while other nutrients were also limited under batch fermentation. *Pseudomonas fluorescens* in batch fermentation shifted the cell growth towards the stationary phase after 24hrs of cultivation [33]. *Bacillus subtilis* showed an exponential phase up to incubation of 48hrs this indicated that it utilized the PHA during the division of cells. *Bacillus cereus* consumed the PHA reserves due to the lessening of

carbohydrates after 48hrs of cultivation [34]. A media supplemented with glucose, and molasses exhibited the best growth than banana peels-supplemented medium as utilization of pre-treated peels was more difficult than readily available carbon sources. *Bacillus cereus* strain ARY73 showed a high growth rate utilizing glucose instantly [35]. The growth rate of *Bacillus subtilis* increased even after 48hrs but *Pseudomonas aeruginosa* stopped the growth before 48hrs, for the comparative analysis between the bacterial strains the growth pattern was stopped observing after 48hrs. *Bacillus subtilis* showed the highest growth rate than *Pseudomonas aeruginosa* in all carbon sources under the same conditions but *Pseudomonas aeruginosa* exhibited the highest PHA yield as compared to *Bacillus subtilis* in all sugar substrates, this clearly indicated the utilization of PHA granules by *Bacillus subtilis* after 48hrs that increase its growth rate.

The highest yield of PHA was observed in *Pseudomonas aeruginosa* at particular times in different substrates and then PHA yields started to decrease after incubating the culture broth under batch fermentation due to the reduction of the nutrients. It has been reported that *Pseudomonas* sp. increased the biomass up to 72hrs; however, the accumulation of PHA was reduced after 48hrs, this occurred due to the utilization of PHA by the bacterial strain when the carbon source was depleted in a medium [36]. The PHA yield obtained utilizing banana peels was 53.28% by *Pseudomonas aeruginosa* after 30hrs of cultivation. The *Pichia kudriavzevii*, a halophilic yeast strain produced 79.68% PHA yield utilizing chicken feathers and banana peels as the source of nitrogen and carbon after 96hrs of incubation [37]. The PHA yield acquired after 48hrs was reduced in all substrates, the decrease in the PHA was due to the increase in the viscosity of the media that limited the oxygen. It has been documented that PHB yield was depleted after cultivation of 48hrs due to unfavorable conditions as viscosity in a media was observed at 72, 96, and 120hrs, viscous media increased the production of exopolysaccharides and restricting the oxygen transfer thus decreasing the synthesis of PHB [38]. The PHA production was higher in molasses and lesser in banana peels compared with the standard glucose after 48hrs, a significant variance was observed by comparing the PHA extract obtained from the glucose with the PHA extract obtained from cheaper carbon substrates having a p-value <0.05 by applying unpaired t-test, this revealed that *Pseudomonas aeruginosa* consumed even the crude form of molasses more quickly than pre-treated peels. The PHA yield of 39.06% was produced using molasses as a carbon source after incubating for 7 days by *Pseudomonas fluorescens* [39].

Bacillus subtilis produced the highest PHA yield utilizing molasses at 27hrs and utilizing peels extract at 33hrs of incubation. It is reported that *Bacillus megaterium* after 48hrs of incubation produced a yield of 46.2% [40]. *Bacillus cereus* produced a 67.4% yield utilizing sugarcane molasses [41]. PHB was produced 47.3% by the novel strain *Zobellela* sp. utilizing banana peel extract [42]. The PHA yield after 48hrs was decreased but the growth rate was increased. The yield was lessened as a high concentration of PHA could show negative feedback that declines the production and shifts the enzymatic pathway towards cell growth or probably due to the destruction of the bacterial enzymes that are involved in the biosynthesis of PHA. *Bacillus subtilis* declined PHA production after 96hrs utilizing molasses due to the destruction of PHA-producing enzymes [23]. The PHA production at 48hrs by *Bacillus subtilis* utilizing molasses was 0.476g/L approximately equal to glucose 0.472g/L, but banana peels produced 0.288g/L showed a substantial variance from standard. A significant difference was confirmed between the PHA production of banana peels with glucose and molasses by an unpaired t-test having a p-value <0.0001. *Bacillus siamensis*, *Staphylococcus aureus*, and *Cupriavidus necator* utilized banana peels as an inexpensive carbon source [43].

FTIR analysis had shown the peaks of the diverse functional groups as the samples were not purified and analyzed in crude form. The obtained chemical bonds were compared with the wavenumbers reported in the study [44]. The wavenumber of samples was also compared with standard ranges [45], and the nearest found wavenumbers were identified as functional groups of polyhydroxybutyrate (PHB). The wavenumber represented the carbonyl group depicting the ester bond, and the methyl groups (CH_3) indicated at 1395.9cm^{-1} and 1458.3cm^{-1} signified the presence of PHB, short-chain length monomers produced by both bacterial strains. *Bacillus subtilis* usually synthesized short-chain length monomers as it belongs to class IV of PHA synthase utilizing sugar substrates [46]. *Pseudomonas aeruginosa* belongs to class II of PHA synthase that usually produced medium-chain length utilizing lipids [47] but can also be synthesized short-chain length in unusual conditions, in the presence of sugar carbon sources [30].

CONCLUSION

The bacterial strains were significant PHA producers, both strains had produced PHB. Comparing both strains among all carbon sources showed that, *Pseudomonas aeruginosa* produced the highest PHA yield but the growth rate was higher in *Bacillus subtilis.* A significant difference was found between molasses and banana peels, which concluded molasses even in the crude form contains a higher amount of sugar than peel extract. Bacterial PHAs are advantageous due to their excellent biodegradability and biocompatibility

therefore they are utilized in making biomedical devices, pharmaceutical, and tissue engineering products. PHA can reduce the environmental pollution caused by petrochemical plastics, and using agro-industrial wastes as a source of energy can also play an important role in waste management. PHA accumulation can be further improved by using genetic engineering tools.

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REFERENCES

1. Tripathi AD, Paul V, Agarwal A, Sharma R, Hashempour-Baltork F, Rashidi L, et al. Production of polyhydroxyalkanoates using dairy processing waste–a review. Bioresour Technol. 2021;326:124735.
2. Brodhagen M, Peyron M, Miles C, Inglis DA. Biodegradable plastic agricultural mulches and key features of microbial degradation. Appl Microbiol Biotechnol. 2015;99(3):1039-56.
3. Gao Q, Yang H, Wang C, Xie X-Y, Liu K-X, Lin Y, et al. Advances and trends in microbial production of polyhydroxyalkanoates and their building blocks. Front Bioeng Biotechnol. 2022;10:966598.
4. Chavan S, Yadav B, Tyagi RD, Drogui PJBT. A review on production of polyhydroxyalkanoate (PHA) biopolyesters by thermophilic microbes using waste feedstocks. Bioresour Technol. 2021;341:125900.
5. Vu DH, Wainaina S, Taherzadeh MJ, Åkesson D, Ferreira JAJB. Production of polyhydroxyalkanoates (PHAs) by Bacillus megaterium using food waste acidogenic fermentation-derived volatile fatty acids. Bioengineered. 2021;12(1):2480-98.
6. Khamkong T, Penkhrue W, Lumyong SJP. Optimization of production of polyhydroxyalkanoates (PHAs) from newly isolated Ensifer sp. strain HD34 by response surface methodology. Processes. 2022;10(8):1632.
7. Koller MJF. A review on established and emerging fermentation schemes for microbial production of polyhydroxyalkanoate (PHA) biopolyesters. Fermentation. 2018;4(2):30.
8. Li H-F, Wang M-R, Tian L-Y, Li Z-JJM. Production of polyhydroxyalkanoates (PHAs) by Vibrio alginolyticus strains isolated from salt fields. Molecules. 2021;26(20):6283.
9. Sathya A, Sivasubramanian V, Santhiagu A, Sebastian C, Sivashankar RJJoP, Environment t. Production of polyhydroxyalkanoates from renewable sources using bacteria. J Polym Environ. 2018;26:3995-4012.
10. Zou H, Shi M, Zhang T, Li L, Li L, Xian M. Natural and engineered polyhydroxyalkanoate (PHA) synthase: key enzyme in biopolyester production. Appl Microbiol Biotechnol. 2017;101(20):7417-26.
11. Tarrahi R, Fathi Z, Seydibeyoğlu MÖ, Doustkhah E, Khataee AJIjobm. Polyhydroxyalkanoates (PHA): From production to nanoarchitecture. Int J Biol Macromol. 2020;146:596-619.
12. Morgan-Sagastume F, Bengtsson S, De Grazia G, Alexandersson T, Quadri L, Johansson P, et al. Mixed-culture polyhydroxyalkanoate (PHA) production integrated into a food-industry effluent biological treatment: A pilot-scale evaluation. J Environ Chem Eng. 2020;8(6):104469.
13. Chaudhry WN, Jamil N, Ali I, Ayaz MH, Hasnain S. Screening for polyhydroxyalkanoate (PHA)-producing bacterial strains and comparison of PHA production from various inexpensive carbon sources. Ann Microbiol. 2011;61(3):623-9.
14. Katagi VN, Bhat SG, Paduvari R, Kodavooru D, Somashekara DM. Waste to value-added products: an innovative approach forsustainable production of microbial biopolymer (PHA)-emphasis on inexpensive carbon feedstock. Environ Technol Rev. 2023;12(1):570-87.
15. Alvi IA, Asif M, Tabassum R, Aslam R, Abbas Z. RLP, a bacteriophage of the family Podoviridae, rescues mice from bacteremia caused by multi-drug-resistant Pseudomonas aeruginosa. Arch Virol. 2020;165(6):1289-97.
16. Ali I, Jamil N. Biosynthesis and characterization of poly3-hydroxyalkanote (PHA) from newly isolated bacterium Bacillus sp. AZR-1. Iran J Sci Technol Trans A Sci. 2018;42(2):371-8.
17. Tibolla H, Pelissari FM, Menegalli FC. Cellulose nanofibers produced from banana peel by chemical and enzymatic treatment. LWT - Food Sci Technol. 2014;59(2):1311-8.
18. Agrawal N, Minj DK, Rani K. Estimation of total carbohydrate present in dry fruits. IOSR J Environ Sci Toxicol Food Technol. 2015;1:24-7.
19. Sharma L, Singh AK, Panda B, Mallick N. Process optimization for poly-β-hydroxybutyrate production in a nitrogen fixing cyanobacterium, Nostoc muscorum using response surface methodology. Bioresour Technol. 2007;98(5):987-93.
20. Susianingsih E, Kadriah I, editors. Screening and isolation of PHB (Poly-β-hydroxybutyrate) producing bacteria as an alternative material for disease prevention on the shrimp culture. IOP Conference Series: Earth and Environmental Science; 2020: IOP Publishing.
21. Wei Y-H, Chen W-C, Huang C-K, Wu H-S, Sun Y-M, Lo C-W, et al. Screening and evaluation of polyhydroxybutyrate-producing strains from indigenous isolate Cupriavidus taiwanensis strains. Int J Mol Sci. 2011;12(1):252-65.
22. Arshad A, Ashraf B, Ali I, Jamil N. Biosynthesis of polyhydroxyalkanoates from styrene by Enterobacter spp. isolated from polluted environment. Front Biol. 2017;12(3):210-8.

23. Gomaa EZ. Production of polyhydroxyalkanoates (PHAs) by Bacillus subtilis and Escherichia coli grown on cane molasses fortified with ethanol. Braz Arch Biol Technol. 2014;57:145-54.
24. Choi Ji, Lee SY. Efficient and economical recovery of poly (3-hydroxybutyrate) from recombinant Escherichia coli by simple digestion with chemicals. Biotechnol Bioeng. 1999;62(5):546-53.
25. Kim M, Cho K-S, Ryu HW, Lee EG, Chang YKJBl. Recovery of poly (3-hydroxybutyrate) from high cell density culture of Ralstonia eutropha by direct addition of sodium dodecyl sulfate. Biotechnol Lett. 2003;25(1):55-9.
26. Fauzi AHM, Chua ASM, Yoon LW, Nittami T, Yeoh HKJPS, Protection E. Enrichment of PHA-accumulators for sustainable PHA production from crude glycerol. Process Saf Environ Prot. 2019;122:200-8.
27. Shamala T, Chandrashekar A, Vijayendra S, Kshama L. Identification of polyhydroxyalkanoate (PHA)-producing Bacillus spp. using the polymerase chain reaction (PCR). J Appl Microbiol. 2003;94(3):369-74.
28. Jo SY, Sohn YJ, Park SY, Son J, Yoo JI, Baritugo K-A, et al. Biosynthesis of polyhydroxyalkanoates from sugarcane molasses by recombinant Ralstonia eutropha strains. Korean J Chem Eng. 2021;38(7):1452-9.
29. Khawas P, Deka SC. Comparative nutritional, functional, morphological, and diffractogram study on culinary banana (Musa ABB) peel at various stages of development. Int J Food Prop. 2016;19(12):2832-53.
30. Aremu M, Layokun S, Solomon B. Production of Poly (3-hydroxybutyrate) from cassava starch hydrolysate by Pseudomonas aeruginosa NCIB 950. Am J Sci Ind Res. 2010;1(3):421-6.
31. Reddy MV, Watanabe A, Onodera R, Mawatari Y, Tsukiori Y, Watanabe A, et al. Polyhydroxyalkanoates (PHA) production using single or mixture of fatty acids with Bacillus sp. CYR1: Identification of PHA synthesis genes. Bioresour Technol Rep. 2020;11:100483.
32. Wang Q, Nomura CT. Monitoring differences in gene expression levels and polyhydroxyalkanoate (PHA) production in Pseudomonas putida KT2440 grown on different carbon sources. J Biosci Bioeng. 2010; 110(6):653-9.
33. Gamal RF, Abdelhady HM, Hassan EA, El-Tayeb T, Aboutaleb KA. Production of Polyhydroxyalkanoate (PHAs) and Copolymer [P (HB-co-HV)] by soil bacterial isolates in batch and two-stage batch cultures. Egypt J Microbiol. 2011;46(1):109-23.
34. Bharathi B, Gowdhaman D, Ponnusami V. Isolation and Identification of Polyhydroxybutyrate (PHB) Producing Bacillus cereus BB613-A Novel Isolate. Int J Chemtech Res. 2016;9:224-8.
35. Yasin AR, Al-Mayaly IK. Study of the Fermentation Conditions of the Bacillus Cereus Strain ARY73 to Produce Polyhydroxyalkanoate (PHA) from Glucose. J Ecol Eng. 2021;22(8).
36. Munir S, Iqbal S, Jamil N. Polyhydroxyalkanoates (PHA) production using paper mill wastewater as carbon source in comparison with glucose. J Pure Appl Microbiol 2015;9:453-60.
37. Ojha N, Das N. Process optimization and characterization of polyhydroxyalkanoate copolymers produced by marine Pichia kudriavzevii VIT-NN02 using banana peels and chicken feather hydrolysate. Biocatal Agric Biotechnol. 2020;27:101616.
38. Belal EB. Production of poly-β-hydroxybutyric acid (PHB) by Rhizobium elti and Pseudomonas stutzeri. Curr Res J Biol. 2013;5(6):273-28.
39. Hendrawan Y, Alvianto D, Sumarlan SH, Wibisono Y. Characterization of Pseudomonas fluorescens polyhydroxyalkanoate produced from molasses as a carbon source. Adv Food Sci. 2020;3(1):1-10.
40. 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):201-7.
41. Suryawanshi SS, Sarje SS, Loni PC, bhujbal S, Kamble PP. Bioconversion of sugarcane molasses into bioplastic (polyhydroxybutyrate) using Bacillus cereus 2156 under statistically optimized culture conditions. Anal Chem Lett. 2020;10(1):80-92.
42. Maity S, Das S, Mohapatra S, Tripathi A, Akthar J, Pati S, et al. Growth associated polyhydroxybutyrate production by the novel Zobellellae tiwanensis strain DD5 from banana peels under submerged fermentation. Int J Biol Macromol. 2020;153:461-9.
43. Vijay R, Tarika K. Banana peel as an inexpensive carbon source for microbial polyhydroxyalkanoates (PHA) production. Int Res J Env Sci. 2018;7(1):1-8.
44. Joyline M, Aruna KJIJRSR. Production and characterization of polyhydroxyalkanoates (PHA) by Bacillus megaterium strain JHA using inexpensive agro-industrial wastes. Int J Rec Sci Res. 2019;10(7):33359-74.
45. Nandiyanto ABD, Oktiani R, Ragadhita R. How to read and interpret FTIR spectroscope of organic material. Indones J Sci Technol 2019;4(1):97-118.
46. 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 Technol. 2019;16(7):3497-512.
47. Tanikkul P, Sullivan GL, Sarp S, Pisutpaisal N. Biosynthesis of medium chain length polyhydroxyalkanoates (mcl-PHAs) from palm oil. Case Stud Chem Environ Eng. 2020;2:100045.