Polyhydroxyalkanoates (PHA) production from synthetic waste using Pseudomonas pseudoflava : PHA synthase enzyme activity analysis from P. pseudoflava and P. palleronii

Polyhydroxyalkanoates production from synthetic waste using *Pseudomonas pseudoflava*: Polyhydroxyalkanoate synthase enzyme activity analysis from *P. pseudoflava* and *P. palleronii*

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Abstract

Synthetic wastewater (SW) at various carbon concentrations (5-60 g/l) were evaluated for polyhydroxyalkanoates (PHA) production using the bacteria *Pseudomonas pseudoflava.* Bacteria showed highest PHA production with 20 g/l (57±5%), and highest carbon removal at 5 g/l (74±6%) concentrations respectively. Structure, molecular weight, and thermal properties of the produced PHA were evaluated using various analytical techniques. Bacteria produced homo-polymer [poly-3-hydroxybutyrate (P3HB)] when only acetate was used as carbon source; and it produced co-polymer [poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) P(3HB-co-3HV)] by addition of co-substrate propionate. PHA synthase, the enzyme which produce PHA was extracted from two bacterial strains *i.e., P. pseudoflava* and *P. palleronii* and its molecular weight was analysed using SDS-PAGE. Protein concentration, and PHA synthase enzyme activity of *P. pseudoflava* and *P. palleronii* was carried out using spectrophotometer. Results denoted that *P. pseudoflava* can be used for degradation of organic carbon persistent in wastewaters and their subsequent conversion into PHA.

Keywords: *Pseudomonas pseudoflava*; SDS-PAGE; P(3HB-co-3HV); PHA synthase; Total organic carbon.

1. Introduction

Many of the plastics are generated by the use of non-renewable compounds and they are persisting in the environment due to their low degradable nature (Fradinho et al., 2014). To overcome this problem, research has been enduring for the replacement of conventional plastics by bioplastics. Polyhydroxyalkanoates (PHA) are bio-polyesters accumulated in cells by a wide range of bacteria. PHA polyesters have been studied intensively by academia and industry because they are biodegradable and their physical properties are similar to those of polypropylene (Sheu et al., 2009). Poly-3-hydroxybutyrate (P3HB) is one type of PHA produced by many bacteria (Kim et al., 2012). PHA can be used as a filler for non-biodegradable plastics, agriculture systems for prolonged release of fertilizers, agrochemicals and medicine (Sudesh et al., 2000). High cost of PHA economically limiting its application as alternative for traditional plastics (Fradinho et al., 2014; Ntaikou et al., 2009). Production costs of PHA should be reduced to compete with synthetic plastics. Low cost substrates such as molasses, plant oils and other fatty acids attracted the attention of various researches to reduce the PHA production cost (Ntaikou et al., 2009; Koller et al., 2005).

PHA synthase is the critical enzyme in PHA biosynthesis, and R-3-hydroxyacyl–coenzyme A (CoA) is the substrate. More than 59 PHA synthase genes have been cloned and analyzed from 45 bacteria (Sheu et al., 2009). PHA synthases have been assigned to three classes based on their substrate specificity and subunit composition (Rehm et al., 2001). The class I PHA synthases (*Ralstonia eutropha*) are composed of a single type of polypeptide chain and use mainly short chain length

hydroxyalkanoic acid CoA thioesters as substrates. The class III PHA synthases (*Allochromatium vinosum)* are composed by two different subunits, each of approximately 40 kDa (Rehm et al., 2001). The substrate specificity is similar with class I synthases, and some medium chain-length 3-hydroxyfatty acids are also incorporated. Class II PHA synthases are composed by one subunit Like class I enzymes, and found mainly in *Pseudomonas aeruginosa*. Substrate specificity is the main difference between class II and both class I and class III PHA synthases. Class II PHA synthases integrate specially 3-hydroxyfatty acids of medium chain length (C6-C14) into PHA, and the resulting product is a latex like polymer (Rehm et al., 2001). Class I PHA synthases synthesize higher molecular weight PHAs compared with class II PHA synthases (Rehm and Steinbüchel, 1999).

In the present study, capability of *Pseudomonas pseudoflava* to produce PHA at various synthetic wastewater (SW) concentrations was evaluated. PHA synthase the enzyme which produce PHA was extracted from two bacterial strains *i.e., P. pseudoflava* and *P. palleronii* and its molecular weight was analysed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration, and PHA synthase enzyme activity of *P. pseudoflava* and *P. palleronii* was carried out using spectrophotometer. Considering the importance of utilizing waste streams as feedstock for PHA production, the volatile fatty acids (VFA) tested in this work are common products of various wastewaters.

2. Materials and methods

2.1 Biocatalyst

Pseudomonas pseudoflava (NBRC-102513) was collected from the Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Japan. *P. pseudoflava* is a bacterium from the family of Comamonadaceae. Many authors reported about efficiency of this bacterium for production of PHA using sugars.

2.2 Media

For the growth of *P. pseudoflava* nutrient broth, and SW were used as the media. Composition of the SW was mentioned in Venkateswar Reddy et al. (2016). The carbon source composition of SW contains acetate and propionate. Different carbon concentrations (5-60 g/l) were used in SW. The pH of the medium was adjusted to 7 and autoclaved before adding to the flasks.

2.3 Growth curve studies

A loop of *P. pseudoflava* strain was initially inoculated into 50 ml of nutrient broth in 500 ml flasks, and kept in shaking incubator under dark condition at 30 ^{o}C for overnight at 180 rpm. For growth experiments 4 ml (4% v/v) of the overnight grown culture was inoculated into different shake flasks containing 100 ml of SW with different carbon concentrations *i.e.,* 5 to 60 g/l. The experiments were conducted for 192 h. Samples were collected at different time intervals, and growth was monitored spectrometrically by measuring the absorbance at 600 nm using UV-spectrometer (UV-1800, Shimadzu, Japan).

2.4 PHA production

Cultures grown with SW at different carbon concentrations were collected at 120 h and centrifuged. The resulting pellet was suspended in SW. The composition of SW was same as mentioned in 2.2 section, but low nitrogen and phosphorous concentrations (0.1 g/l) were used in order to create stress for accumulation of PHA granules. Also experiments were conducted by using only acetate (at 20 g/l) as carbon source in SW in order to know the difference in PHA composition. All the conditions were maintained as like in growth phase. Culture was collected and the PHA was extracted and analyzed at 72 h.

2.5 Analysis

2.5.1 TOC and VFA analysis

Total organic carbon (TOC) from clarified samples at different time intervals were analysed in a Shimadzu TOC automatic analyser to know the removal of carbon concentration in SW. Sodium acetate and propionate standards (10-500 mg/l) are used to produce the calibration curves. TOC was measured using NPOC (non-purgeable organic carbon) method. By sparging samples to which a small amount of acid has been added, the IC in the sample is converted to carbon dioxide. This carbon dioxide is removed, and the TOC is obtained by measuring the TC in the treated sample. VFA analysis was done as mentioned in Venkateswar Reddy et al. (2016). All results were presented as average and standard deviation of the data from three independent experiments.

2.5.2 Structure & molecular mass determination

Extraction and estimation of PHA was performed following the procedures reported (Law and lepecky, 1960; Venkata Mohan and Venkateswar Reddy, 2013; Venkateswar Reddy et al. 2016). ^{1}H

(500 MHz) NMR spectra was recorded on a JNM-ECA500 NMR spectrometer (JEOL, Japan) at 20 °C to know the structure of polymer. Samples of produced PHA and standard P3HB/ P(3HB-co-3HV) (Aldrich) were prepared by dissolving in deuterated chloroform ($CDCl_3$), and then were filtrated with cotton. The signals of tetramethylsilane (TMS) and $CDCl_3$ were used as the standards for chemical shift of ^1H spectra.

Number and weight average molecular mass (M_n and M_w) of standard and sample P3HB/ P(3HB-co-3HV) were measured using GPC 900-1 (JASCO, Japan) equipped with two Shodex K-806L columns and an RI detector. Chloroform was used as an eluent at 40 ºC and polystyrene standards (M_n = 1,680-3,065,000) were employed for calibration.

2.5.3 Thermal properties determination

Thermogravimetric analysis (TG/DTA7300, Hitachi, Japan) was used to determine the decomposition temperature (T_d) of P3HB/P(3HB-co-3HV). PHA powder was added into an aluminum pan and subjected to a heating rate of 10 °C/min from 50 °C to 550 ºC. Differential scanning calorimetry (DSC, DSC7000X, Hitachi, Japan) was used to characterize the melting temperature (T_m). The temperature range for DSC varied from -50 ºC to 350 ºC at a heating rate of 10 ºC/min.

2.5.4 SDS-PAGE

P. palleronii and *P. pseudoflava* cells grown on MS medium containing acetate (19 g/l) and propionate (1 g/l) at 37 ^{O}C were harvested by centrifugation and whole cell extracts were prepared

by sonication. Cell extracts were prepared at 4 ^{O}C or on ice. Bacterial cells harvested from nitrogen limited cultures by centrifugation for 10 min at 10,000 × g, was washed two times with 50 mM Tris-HCl buffer (pH 8.3) and suspended in the same buffer (for 1 g of pellet, 5 ml of buffer was added). Cells were lysed in an ice using a Branson ultrasonic disrupter (Sonifer 250, Danbury, CT, USA) at one-minute flash for 10 min at output-3 and duty cycle-50%. Cell debris was removed by centrifugation at 20,000 × g for 20 min. The supernatant (crude extract) was stored at -20 ^{O}C until further enzyme assay and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The molecular mass of crude enzyme was determined by SDS-PAGE (Laemmli, 1970). SDS-PAGE was run on a 12.5% polyacrylamide gel. The molecular weights of proteins were estimated according to molecular weight of standards (Tefco co., Ltd, Japan).

2.5.5 Activity assay of PHA synthase

Assay of PHA synthase activity was carried out according to the methods described in literature (Miyake et al., 1997; Valentin and Steinbuchel, 1994). The assay mixtures (1 ml) contained 50 µl of 50 mM DL-β-hydroxybutyryl coenzyme A lithium salt (final concentration 2.5 mM) (Sigma-Aldrich, Cat No: H0261), 50 µl of 20 mM 5, 5`- dithio-bis (2-nitrobenzoic acid) (final concentration 1 mM) (DTNB, Wako pure chemicals, Japan, Cat No: 047-16401) in 870 µ1 of 1M Tris-HCl buffer. The reaction was started by addition of 30 µl of crude enzyme. The optical density of the thiobenzoate anion resulting from the reaction of CoA and DTNB was measured at 412 nm for 1 min at room temperature using spectrophotometer (UV-1800, Shimadzu, Japan). Protein

concentration was determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (Pierce, Rockford, IL, USA) as the standard at 595 nm using spectrophotometer (UV-1800, Shimadzu, Japan).

3. Results and discussion

3.1 Growth curve analysis

P. pseudoflava was cultivated in SW at 30 ^{o}C by supplementing with different concentrations (5 - 60 g/l) of carbon source. Growth curve analysis clearly showed that the carbon concentration had significant influence on the bacterial growth. Among all the concentrations, bacteria showed higher growth with 20 g/l (120 h OD, 2.239) followed by 15 g/l (120 h OD, 2.17), 40 g/l (80 h OD, 2.10), 30 g/l (80 h OD, 2.097), 60 g/l (168 h OD, 2.091), 50 g/l (120 h OD, 2.07), 10 g/l (120 h OD, 2.02) and 5 g/l (80 h OD, 1.53) (Fig. 1). Bacteria does not show maximum growth at same time interval for all the concentrations, it showed maximum growth at different time intervals based on the carbon concentration. Bacteria showed maximum growth at 80 h for 5 g/l, 30 g/l, and 40 g/l; 120 h for 10 g/l, 15 g/l, 20 g/l, and 50 g/l substrate concentrations, and 168 h for 60 g/l substrate concentration. The availability of acetate and propionate at different time intervals was considered to compare with growth curve results. For 5 g/l substrate concentration, 2.1 g/l of acetate and 0.13 g/l of propionate were available at 48 h; and 0.9 g/l of acetate and 0.06 g/l of propionate were available at 80 h. At 48 and 80 h, 5.2 g/l and 2.1 g/l of acetate and 0.29 g/l and 0.12 g/l of propionate were respectively available for 10 g/l concentration. For 15 g/l concentration, 9.4 g/l of acetate and 0.41 g/l of propionate were available at 48 h, after that availability was decreased to 4.1 g/l (acetate) and 0.23

g/l (propionate) at 80 h. Acetate and propionate were not available at 120 h for 5-15 g/l substrate concentrations. For 20 g/l concentration, 12.4 g/l of acetate and 0.71 g/l of propionate were available at 48 h, gradually the availability at 80 h was decreased to 6.3 g/l (acetate) and 0.42 g/l (propionate). At 120 h, 0.38 g/l of acetate was available, but propionate was not available. Acetate and propionate at the concentration of 15.1 g/l and 0.98 g/l were respectively available at 48 h for 30 g/l concentration. At 80 and 120 h, 11.2 g/l and 7.7 g/l of acetate and 0.26 g/l and 0.08 g/l of propionate were available respectively.

For 40 g/l concentration, availability of acetate was decreased from 24.2 g/l (48 h) to 9.24 g/l (120 h). Also propionate availability was decreased from 1.08 g/l (48 h) to 0.09 g/l (120 h). Similar trend was observed for 50 and 60 g/l substrate concentrations. For 50 g/l concentration, availability of acetate was decreased from 29.3 g/l (48 h) to 13 g/l (120 h). Also propionate availability was decreased from 1.29 g/l (48 h) to 0.2 g/l (120 h). Availability of acetate was decreased from 41.3 g/l (48 h) to 16 g/l (168 h) for 60 g/l concentration. Also propionate availability was decreased from 1.48 g/l (48 h) to 0.52 g/l (168 h).

Bacteria from 5 to 40 g/l substrate concentrations showed immediate growth *i.e.,* log phase started after 2 h, and for higher substrate concentrations it took more time *i.e.,* for 50 g/l log phase started after 10 h, for 60 g/l log phase started after 22 h. Higher concentrations took more time to show maximum bacterial growth due to toxic nature of VFA. These results clearly indicate the effect of

VFA concentration on the growth of bacteria. In our previous studies we used the bacteria *P. palleronii* and analysed growth curve results using similar VFA concentrations (Venkateswar Reddy et al., 2016). When the growth was compared for two bacteria strains, *P. palleronii* showed better growth than *P. pseudoflava* at higher concentrations. *P. palleronii* supplemented with 30-60 g/l VFA concentration showed 1.1-1.02 times higher growth than *P. pseudoflava.* Contrary to this, *P. palleronii* incubated at lower VFA concentrations showed lower growth than *P. pseudoflava. P. palleronii* supplemented with 5 g/l and 10 g/l concentrations showed 1.04 times and 1.2 times lower growth than *P. pseudoflava.* Even though two bacterial strains belongs to same genus, but they are two different species and they have different properties and substrate utilization capacity. Even though we provided similar concentration of substrates for both the bacteria, but they showed variation in their growth, it may depend on the nature and metabolic activities of bacteria. At present state, we don`t know the exact reason, to know that we need to do further in-depth analysis.

Fig. 1

3.2. Carbon removal

TOC removal was noticed in SW irrespective of the carbon concentrations studied suggesting the system's function towards treatment. Among the different time intervals, 5 g/l substrate concentration illustrated higher TOC removal rate at 120 h (3.7 g/l), followed by 80 h (2.6 g/l) and 48 h (1.9 g/l). Higher TOC removal rate of 7 g/l (at 120 h) followed by 4.9 g/l (at 80 h) and 2.5 g/l (at 48 h) was observed for 10 g/l substrate concentration. *P. pseudoflava* at 15 g/l substrate concentration illustrated higher TOC removal rate at 120 h (10.2 g/l) followed by 80 h (7.8 g/l), and 48 h (3.1 g/l).

For 20 g/l substrate concentration, higher TOC removal rate of 11.6 g/l was observed at 120 h, followed by 7.8 g/l at 80 h, and 4.4 g/l at 48 h. *P. pseudoflava* at 30 g/l concentration illustrated higher TOC removal rate of 13.2 g/l at 120 h followed by 80 h (9.2 g/l) and 48 h (5.9 g/l). Bacteria grown at 40 and 50 g/l substrate concentrations illustrated higher TOC removal rates of 17.2 g/l and 25.5 g/l respectively at 120 h. Bacteria at 60 g/l substrate concentration illustrated higher TOC removal rate at 120 h (24.6 g/l) followed by 80 h (18.8 g/l), and 48 h (6.3 g/l).

When the TOC removal was compared for two bacteria strains, *P. palleronii* showed higher TOC removal than *P. pseudoflava* at lower substrate concentrations (5-30 g/l). *P. palleronii* at 5 g/l and 30 g/l showed 1.18 and 1.06 times higher TOC removal than *P. pseudoflava*. Mass balance analysis was performed for all the substrate concentrations at 120 h. Initially 5 g/l substrate was given as carbon source for PHA production experiments. From that 3.7 g/l of substrate was utilized and 1.3 g/l was present as residual carbon source in the solution. Out of the 3.7 g/l of substrate, 1.6 g/l was retained in the form of biomass and PHA, and 2.1 g/l of carbon remained in the form of unknown organic matter. For 10 g/l substrate concentration, 7 g/l was utilized and out of it 2.5 g/l was remained in the form of biomass and PHA. 10.2 g/l of substrate was utilized from 15 g/l of total substrate concentration. From total concentration of 20 g/l, 11.6 g/l of substrate was utilized and 8.4 g/l was remained as residual carbon source in the solution. Out of the 11.6 g/l of substrate, 0.38 g/l was retained in the form of acetate, 3.9 g/l was retained in the form of biomass and PHA. From total concentration of 30 g/l, 13.2 g/l of substrate was utilized and 16.8 g/l was remained as residual carbon source in the solution. Out of the 13.2 g/l of substrate, 7.7 g/l was retained in the form of acetate and 0.08 g/l were retained in the form of propionate, 3.3 g/l was retained in the form of biomass and PHA, and 2.12 g/l of carbon remained in the form of unknown organic matter. From total concentration of 40 g/l substrate, 17.2 g/l of substrate was utilized and 22.8 g/l was remained as

residual carbon in the solution. Out of the 17.2 g/l of substrate, 9.24 g/l was retained in the form of acetate, 0.09 g/l was retained in the form of propionate, 3.7 g/l was retained in the form of biomass and PHA. From 50 g/l substrate concentration, 25.5 g/l of substrate was utilized. Out of the 25.5 g/l of substrate, 13 g/l was retained in the form of acetate, 0.2 g/l was retained in the form of propionate, and 4.8 g/l was retained in the form of biomass and PHA. From total concentration of 60 g/l, 24.6 g/l of substrate was utilized and 35.4 g/l of substrate was existed as residual carbon source in the solution. Out of the 24.6 g/l of substrate, 18 g/l was retained in the form of acetate, 0.7 g/l was retained in the form of propionate, 4.2 g/l was retained in the form of biomass and PHA, and 1.7 g/l of carbon remained in the form of unknown organic matter. Jin and co-workers reported that along with PHA production other products like glycerol and different amino acids *i.e.*, serine, glycine and glutamine might also be generated (Jin et al., 2013). Hence the unknown organic matter may contribute to byproducts such as glycerol and amino acids.

3.3 VFA removal

Individual VFA composition of the SW at different concentrations (from 5 - 60 g/l) were analyzed at different time intervals using HPLC (Table 1). Among all the carbon concentrations, bacteria grown at 5, 10 and 15 g/l showed complete removal (100%) of both acetic and propionic acids within 120 h. Bacteria grown at 20 g/l concentration showed 98±3% removal of acetic acid, and 100±5% removal of propionic acid. Bacteria grown at 50 and 60 g/l concentration showed 73±4% and 58±5% removal of acetic acid; 89±4% and 65±5% removal of propionic acid respectively. Many authors reported about efficiency of bacteria *P. pseudoflava* for degradation of various sugars, but little information was available about degradation of VFA using this bacterium.

Table 1

3.4 pH increment

The pH showed an increasing trend with time which might be due to the utilization of VFA by *P. pseudoflava* (Fig. 2). Lower carbon concentrations showed high increment of pH due to complete utilization of acids. At 120 h, SW at 5 g/l concentration showed highest increment in pH (7 to 9.34) with TOC removal rate of 3.7 g/l. SW at 10 g/l concentration showed pH increment from 7 to 9.22 with TOC removal rate of 7 g/l. The pH increment of 7 to 9.07 was observed with 10.2 g/l of TOC removal rate at 15 g/l substrate concentration. At 120 h, SW at 20 g/l concentration showed pH increment of 7 to 8.71 with TOC removal rate of 11.6 g/l. For 40 g/l, 50 g/l and 60 g/l substrate concentrations, pH increment of 7 to 8.01; 7 to 7.65; and 7 to 7.48 with TOC removal rates of 17.2 g/l; 25.5 g/l; and 24.6 g/l were observed respectively.

Fig. 2

3.5 Accumulation and composition of PHA

Degradation of VFA and subsequent conversion of their metabolites into PHA was studied by *P. pseudoflava*. In this study, PHA accumulation was studied at 15 g/l, 20 g/l and 30 g/l carbon concentrations. PHA production and CDM were evaluated at 120 h of time interval. The best accumulation of PHA (57±5%) was achieved with 20 g/l carbon concentration in SW. *P. pseudoflava* also accumulated the good level of PHA when supplied with 30 g/l (53±4%) and 15 g/l (51±4%) carbon concentrations. *P. palleronii* showed 1.09 times and 1.05 times higher PHA at 30 g/l and 15 g/l substrate concentrations respectively than *P. pseudoflava*. In this study PHA production and CDM were evaluated at 120 h of time interval for all the substrate concentrations.

The possibility of converting VFA into PHA has been previously reported by many authors using pure or mixed bacterial cultures (Kourmentza et al. 2015; Amulya et al. 2014; Venkateswar Reddy and Venkata Mohan, 2012). Many authors used various substrates for PHA production using *P. pseudoflava*. According to Timm and Steinbuchel, *P. pseudoflava* produced 14.3% of CDM as polymer with composition of 99.2% of 3-hydroxy butyrate (3HB), and 0.8% of 3-hydroxyl octanoate (3HO) by using 15 g/l of sodium gluconate as carbon source (Timm and Steinbuchel, 1990). Studies conducted by Povolo et al. (2013) revealed that *P. pseudoflava* achieved 5.7 g/l of dry biomass with 20 g/l of sucrose as carbon source, and at 72 h it produced 53% of PHA with composition of P(3HB-co-2.5%HV). They also reported 2.4 g/l of dry biomass achieved by *P. pseudoflava* with 20 g/l of lactose as carbon source, and at 48 h it produced 27% of PHA with composition of P(3HB-co-3%HV). *P. pseudoflava* synthesized copolymer P(3HV-co-4HB) by using valerolactone plus butyrolactone as a carbon source in a mineral salts medium (Choi et al., 2013). Dietrich et al. reported that *P. pseudoflava* was determined to produce PHB with glucose (DCW-4.2 g/l; PHB-1.7 g/l at 24 h), mannose (DCW-3.5 g/l; PHB-1.2 g/l at 24 h), xylose (DCW-2.3 g/l; PHB-0.1 g/l at 72 h) and arabinose (DCW-0.2 g/l; PHB-0.2 g/l at 72 h) at 10 g/l individual initial substrate concentrations (Dietrich et al., 2013). Other than bioplastics production, *P. pseudoflava* has the property of colonization and mineralization of palmitic acid (Thomas and Alexander, 1987).

Experiments were conducted to know the difference in PHA composition by using only acetate (20 g/l) as carbon source, and mixture of acetate-propionate (19:1) as carbon source in SW. Experiments

with only acetate produced homo-polymer P3HB, experiments with acetate-propionate mixtures produced co-polymer P(3HB-co-3HV). It was supported with previous reports by various authors. (Takabatake et al. 2000; Jiang et al. 2011). *P. pseudoflava* produced PHA from pentoses (Bertrand et al., 1990). This organism was able to use a hydrolysate from the hemiceliulosic fraction of poplar wood as a carbon and energy source for its growth. When *P. pseudoflava* was grown on the major sugars present in hemicelluloses in batch cultures, P4HB accumulated when glucose, xylose, or arabinose was the sole carbon source, with the final PHB content varying from 17% of the biomass dry weight on arabinose to 22% of the biomass dry weight on glucose and xylose. PHB weight-average molecular weights were 640,000 on arabinose and 1,100,000 on glucose and xylose. Copolymers of HB and, HV were produced when propionic acid was added to shake flasks containing 10 g of glucose per liter. The HV monomer content attained a maximum of 45 mol% when the initial propionic acid concentration was 2 g per liter. Povolo et al. (2013) reported production of PHA containing 3HB, 3HV and 4HB as co-monomers through the use of inexpensive carbon sources such as whey from dairy industry. Ramsay et al. (1990) reported that *P. pseudoflava* under nitrogen-limited conditions, was found to accumulate 43% DCW of PHA at 48 h when supplied with glucose (10 g/l) and propionic acid (1 g/l). The produced PHA contains P(HB-co-HV) copolymer with 45% HV content.

3.6 NMR and GPC analysis

The ^{1}H NMR spectrum of $CDCl_3$ soluble part of P3HB and P(3HB-co-3HV) extracted from *P.*

pseudoflava grown with SW was measured at 20 ^{O}C to deduce its chemical structure and primary sequence of polymer chain (Fig. S1). Based on their peak positions, splitting patterns and integral ratio of these signals, each peak of P3HB can be assigned to the protons on methyne (5.30-5.22 ppm), methylene (2.65-2.45 ppm) and methyl (1.35-1.15 ppm) groups. In the case of P(3HB-co-3HV), protons in HV unit, *i.e.*, methyne (5.30-5.03 ppm), methylene on the main-chain (2.65-2.43 ppm), methylene on the side-chain (1.60-1.30 ppm), and methyl group (0.92-0.83 ppm) were observed with the peaks of protons in P3HB unit. These spectra were almost same as both P3HB and P(3HB-co-3HV) standards measured at the same conditions. The results suggested that P3HB and P(3HB-co-3HV) produced by *P. pseudoflava* consist of HB and HB/HV units as their repeating units of the main-chains respectively.

To measure the molecular mass of homo-polymer (P3HB) and co-polymer P(3HB-co-3HV) produced from *P. pseudoflava*, we measured both number average molecular mass (M_n) and weight average molecular mass (M_w) of biopolymer using by GPC. M_W and polydispersity index (PDI, M_w/M_n) of the P3HB produced by *P. pseudoflava* is 17.63 kDa and 3.3 respectively. M_W and PDI of the co-polymer P(3HB-co-3HV) produced by *P. pseudoflava* is 52.33 kDa and 5.7 respectively. The M_w of the standard P(3HB-co-3HV) is 110 kDa, and PDI is 4.3. These results indicate that *P. pseudoflava* can produce biopolymers with relatively lower dispersity. The M_w of the P3HB produced from *Cupriavidus* sp. CY-1 (Venkateswar Reddy et al., 2015a), *Bacillus* sp. CYR1 (Venkateswar Reddy et al., 2015b), and *P. palleronii* (Venkateswar Reddy et al., 2016) were

compared with P3HB produced by *P. pseudoflava* (Table 2).

Regarding the molecular weights of PHA synthesized in biological systems it seems obvious that type I PHA synthases synthesize PHA with molecular weights ranging from approximately 500000 to several millions compared with type II PHA synthases which synthesize PHAs with molecular weights ranging from approximately 50000 to 500000, respectively (Rehm and Steinbüchel, 1999). Type III PHA synthases seem to synthesize PHAs with molecular weights that are in between. The molecular weight of PHAs depends on several factors, *i.e.*, the physiological background is important with respect to the provision of HA-CoA thioesters and concentration of the substrates of the PHA synthases and also with respect to the availability of enzymes that hydrolyze PHAs such as PHA depolymerases or unspecific esterases and lipases (Rehm and Steinbüchel, 1999). If the physiological background does not provide such enzymes, then PHAs of higher molecular weight might be produced. Level of expression of active PHA synthase protein in the cells is also very important. The higher the concentration of active PHA synthase protein in the cells, the lower the molecular weight of the accumulated polyester (Rehm and Steinbüchel, 1999).

Table 2

3.7 TGA and DSC analysis

TGA was used to evaluate the thermal stability of polymers, *i.e.*, decomposition temperature, especially focus on the temperature of 5% weight loss (T_{d5}). The weight loss of standard homo-polymer P3HB started at around 240 °C and its T_{d5} was at 269 °C, completely decomposed at 295 °C (Fig. 3A). However the starting temperature for weight loss of the P3HB produced by *P.*

pseudoflava was at around 160 °C and its T_{d5} was at 180 °C. In addition, the P3HB was completely not decomposed at 550 °C, 75% was decomposed at 540 °C indicating that the P3HB extracted from *P. pseudoflava* composed of 25% inorganic material which may be came from bacterial dry mass. The weight loss of standard co-polymer P(3HB-co-3HV) started at around 140 °C and its T_{d5} was at 260 °C, completely decomposed at 285 °C (Fig. 3B). However, the starting temperature for weight loss of the co-polymer P(3HB-co-3HV) produced by *P. pseudoflava* was at around 170 °C and its T_{d5} was at 210 °C, and it was completely not decomposed at 550 °C, around 70% was decomposed at 540 °C. TGA analysis of co-polymer produced by *P. pseudoflava* showed that initial decomposition started at 20-130 ^{o}C this was not due to the polymer, we think this may be due to the solvents or low molecular weight material present in the sample.

Fig. 3

DSC was used to characterize the melting temperature (T_m) of the homo-polymer P3HB and co-polymer P(3HB-co-3HV) extracted from *P. pseudoflava*, and it was compared with respective standards (Fig. 4AB). From the endothermic peaks in each DSC traces, it denoted that homo-polymer P3HB extracted from *P. pseudoflava* contains the T_m of 140-165 °C, it was matched with standard P3HB (T_m, 150-170 °C). In the standard co-polymer samples, we observed two endothermic peaks, one peak denotes the T_m (155 °C) and another peak denotes the T_g (140 °C). We observed one peak at 135 ^{O}C from co-polymer samples extracted from *P. pseudoflava*, it may be due to glass transition temperature (T_g) of amorphous nature of the polymer. We did not find T_m for our co-polymer, because of its non-crystalline form. Nevertheless, based on the results shown above, we

concluded that the produced polymer is P3HB and P(3HB-co-3HV).

Fig. 4

3.8 Molecular weight determination and enzyme assay

Fig. 5 shows the electrophoretic pattern of the SDS-PAGE analysis of PHA synthase in *P. palleronii* and *P. pseudoflava*. Based on the SDS-PAGE analysis, the PHA synthases isolated from the both bacteria corresponded to a molecular mass of 63 kDa. It was supported by existing literature. Tsuge et al. (2004) reported that SDS-PAGE analysis revealed that the PHA synthase ($PhaC_{Da}$) isolated from the recombinant *E. coli* had molecular mass of 69 kDa. According to Rehm and Steinbüchel, PHA synthase proteins belonging to classes I and II exhibit molecular masses of approximately 63-73 kDa, whereas class III consist of two different subunits PhaC and PhaE with molecular masses of about 40 kDa (Rehm and Steinbüchel, 1999).

Fig. 5

P. palleronii showed higher protein concentration (0.68±0.05 mg/ml) than *P. pseudoflava* (0.57±0.03 mg/ml). Also higher enzyme activity was observed in *P. palleronii* (0.142±0.02 Unit/ml) than *P. pseudoflava* (0.054±0.004 Unit/ml). Unit activity was defined as the amount of enzyme required for conversion of µM of substrate in to product per minute. Higher enzyme activity of *P. palleronii* than *P. pseudoflava* was supported by presence of strong band in SDS-PAGE analysis and also higher PHA production. Miyake et al. (1997) reported that the cyanobacterium *Synechococcus* sp. MA19, under nitrogen-deprived condition showed 0.24 µM/min/mg protein of PHB synthase enzyme activity. Tsuge et al. (2004) reported about 350 U/g of PHA synthase activity in recombinant *E. coli* JM109.

4. Conclusion

Bacteria *P. pseudoflava* utilized VFA present in SW and converted in to useful products. Structure, molecular weight, and thermal properties of the produced PHA were analysed. SDS-PAGE analysis showed that the enzyme PHA synthases isolated from the *P. pseudoflava* and *P. palleronii* corresponded to a molecular mass of 63 kDa. *P. palleronii* showed higher PHA synthase enzyme activity than *P. pseudoflava*. These findings indicate the possibility of feeding the *P. pseudoflava* with cheap VFA rich fermented wastes to produce PHA.

Acknowledgements

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The authors have declared no conflict of interest.

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Table 1: Acetate and propionate removal at different concentrations of synthetic wastewater using *P. pseudoflava* at 120 h time interval.

Initial substrate concentration (g/l)	**Acetate removal (%)**	**Propionate removal (%)**
5	100±6	100±6
10	100±4	100±4
15	100±5	100±5
20	98±5	100±5
30	73±6	94±5
40	76±6	88±5
50	73±4	89±5
60	58±5	65±5

Table 2: Comparison of physical and thermal properties of PHB produced from different bacterial strains in our previous studies.

Bacteria name	**Molecular Weight**	**Polydispersity index**	**Weight loss started at**	**T_{d5}**	**Tm**	**Reference**
Cupriavidus sp. CY-1	269 KDa	2.9	100 ^{O}C	160 ^{O}C	160 ^{O}C	31
Bacillus sp. CYR1	709 KDa	2.2	100 ^{O}C	180 ^{O}C	171 ^{O}C	32
P. palleronii	12 KDa	2.3	170 ^{O}C	180 ^{O}C	165 ^{O}C	30
P. Pseudoflava	52 KDa	5.7	160 ^{O}C	180 ^{O}C	160 ^{O}C	This study
Standard PHB	725 KDa	4.2	230 ^{O}C	274 ^{O}C	178 ^{O}C	31

T_{d5} is decomposition temperature where polymer showed 5% weight loss.

Figure captions

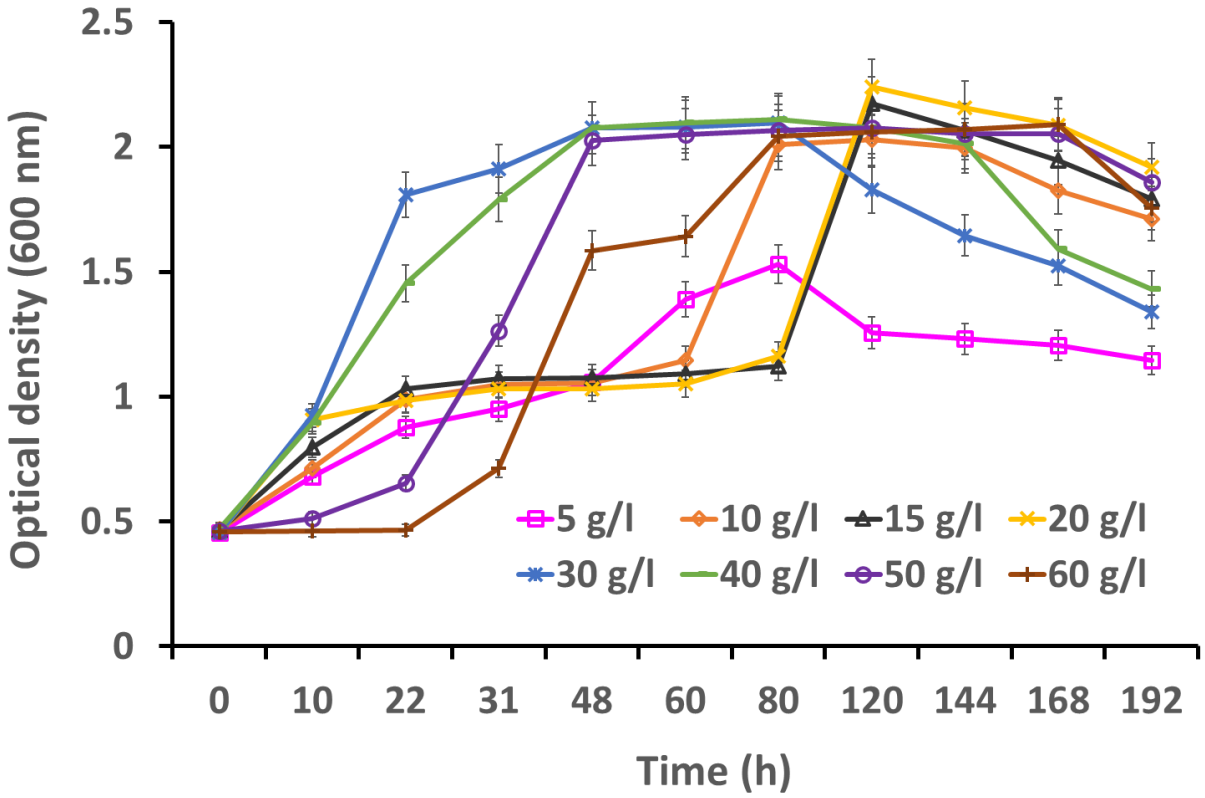
Fig. 1: Growth curve of *P. pseudoflava* using various concentrations (5 - 60 g/l) of synthetic wastewater.

Fig. 2: Variation of pH with respect to time at different volatile fatty acids concentrations using *P. pseudoflava*.

Fig. 3: Thermogravimetric analysis of (A) homo-polymer P3HB; and (B) co-polymer P(3HB-co-3HV). – (a) is biopolymer extracted from *P. pseudoflava*; -- (b) is standard biopolymer (Sigma-Aldrich).

Fig. 4: Differential scanning calorimetry analysis of (A) homo-polymer P3HB; and (B) co-polymer P(3HB-co-3HV). (a) is biopolymer extracted from *P. pseudoflava*; (b) is standard biopolymer (Sigma-Aldrich).

Fig. 5: SDS-PAGE analysis of crude protein isolated from bacteria, Lane 1: Crude proteins extracted from *P. pseudoflava*; 2: Crude proteins extracted from *P. palleronii*; M: molecular size markers.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2
1.5
1
0.5
0
0
10
22
31
48
60
80
120
144
168
192
Time (h)
5 g/l
10 g/l
15 g/l
20 g/l
30 g/l
40 g/l
50 g/l
60 g/l

Figure 1

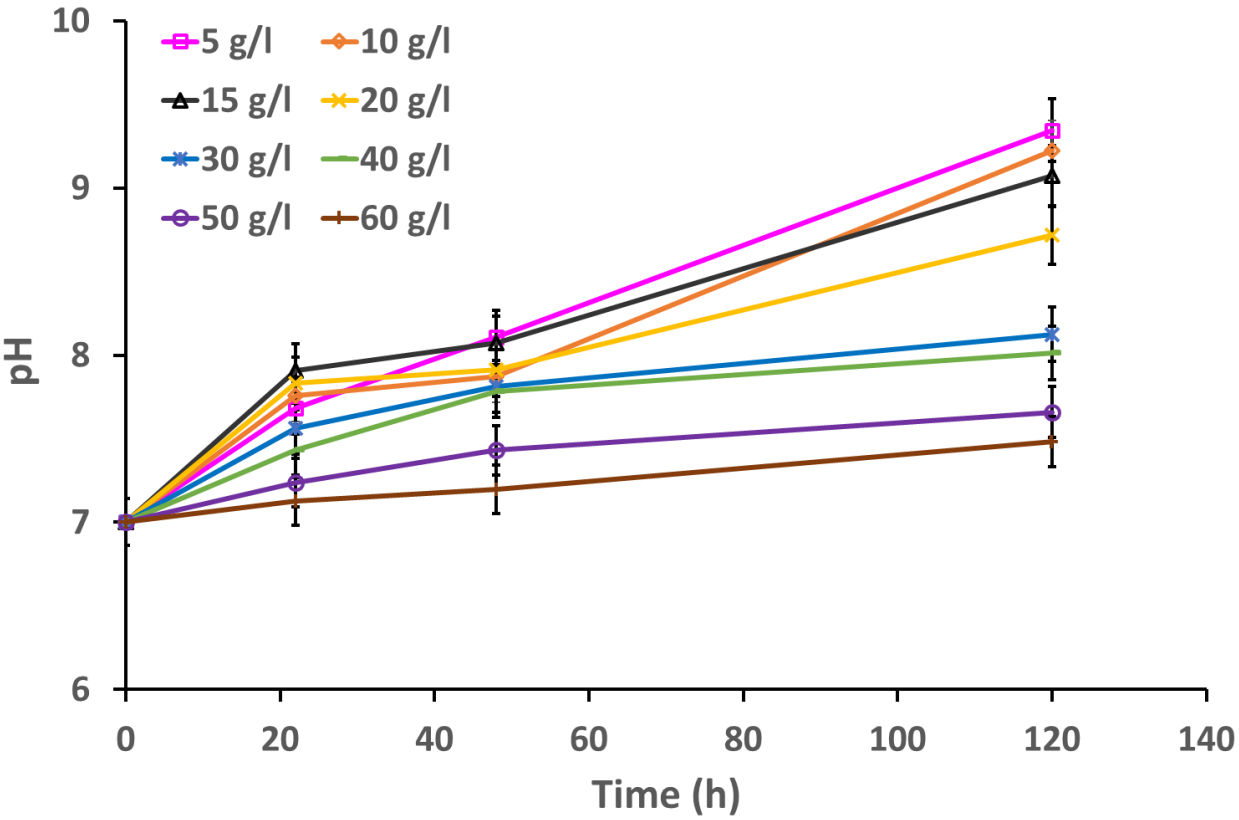
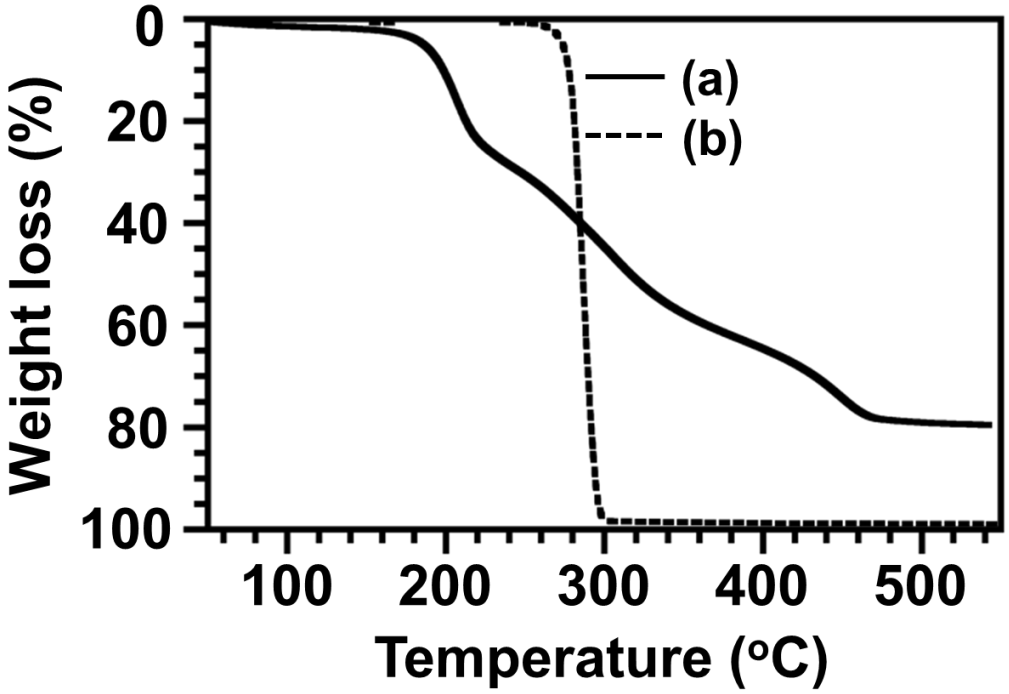

10
9
8
7
6
pH
5 g/l
10 g/l
15 g/l
20 g/l
30 g/l
40 g/l
50 g/l
60 g/l
0
20
40
60
80
100
120
140
Time (h)

Figure 2

A


Weight loss (%)
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20
40
60
80
100
100
200
300
400
500
Temperature (°C)
(a)
(b)

B

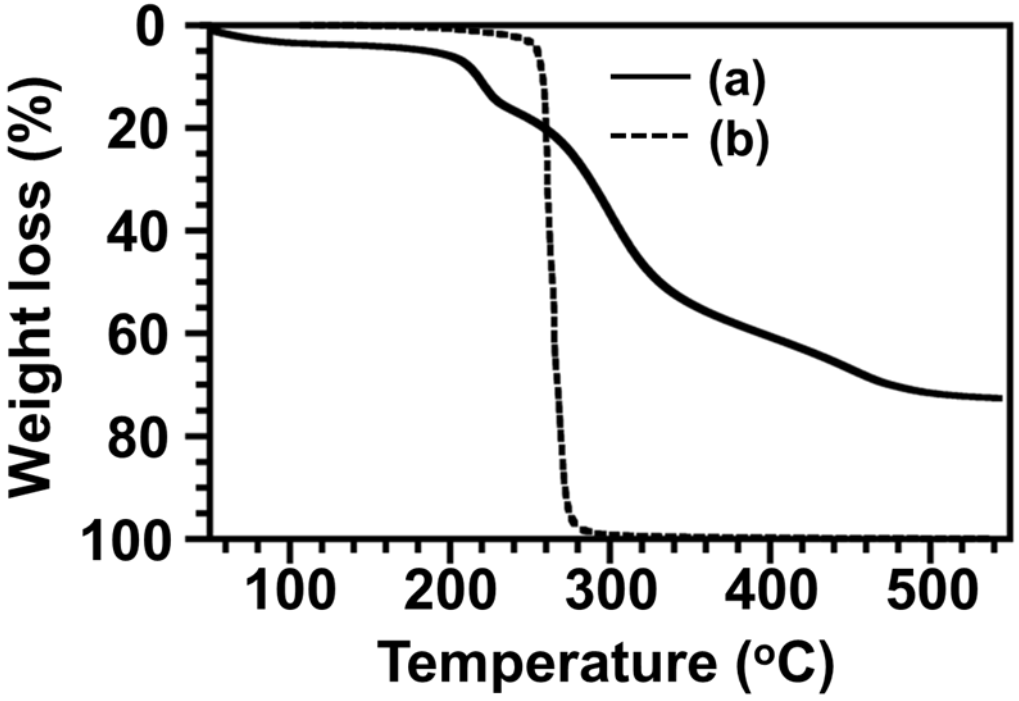
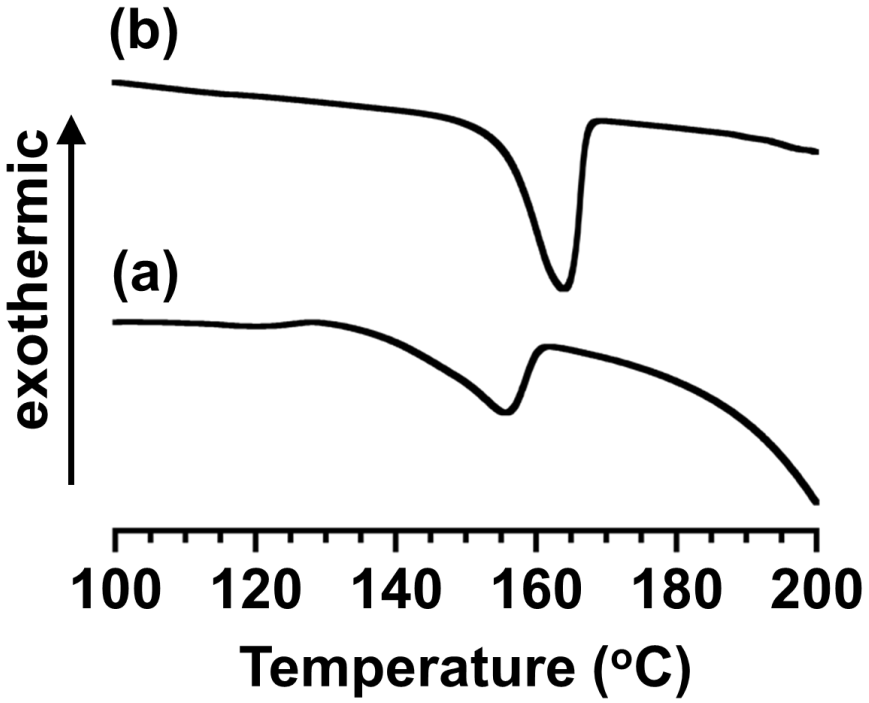

Weight loss (%)
0
20
40
60
80
100
100
200
300
400
500
Temperature (°C)
(a)
(b)

Figure 3

A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(a)
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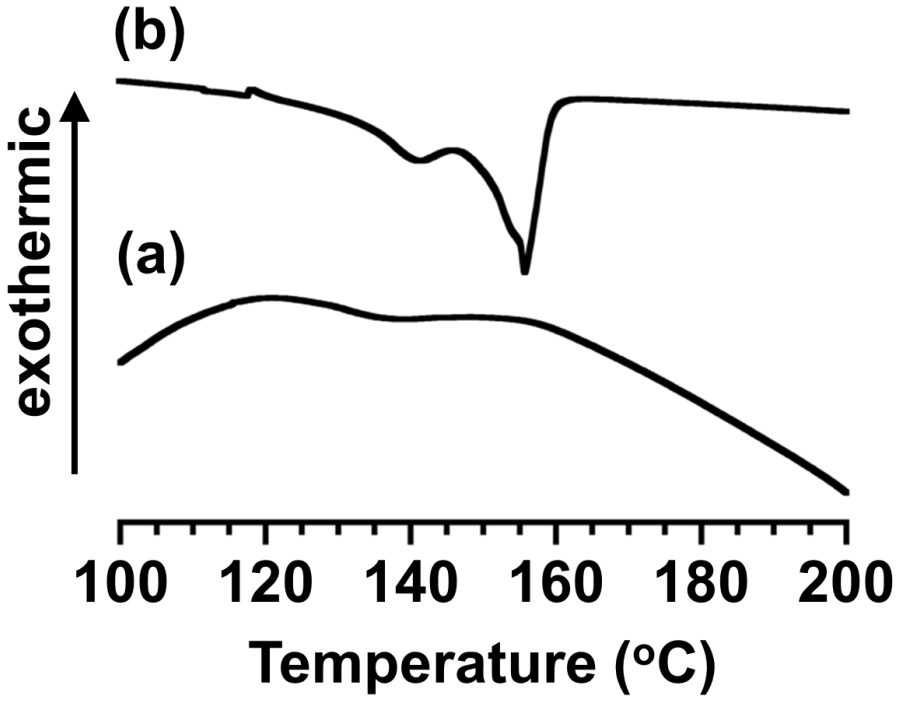
Temperature (°C)

B


(b)
exothermic
(a)
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Temperature (°C)

Figure 4

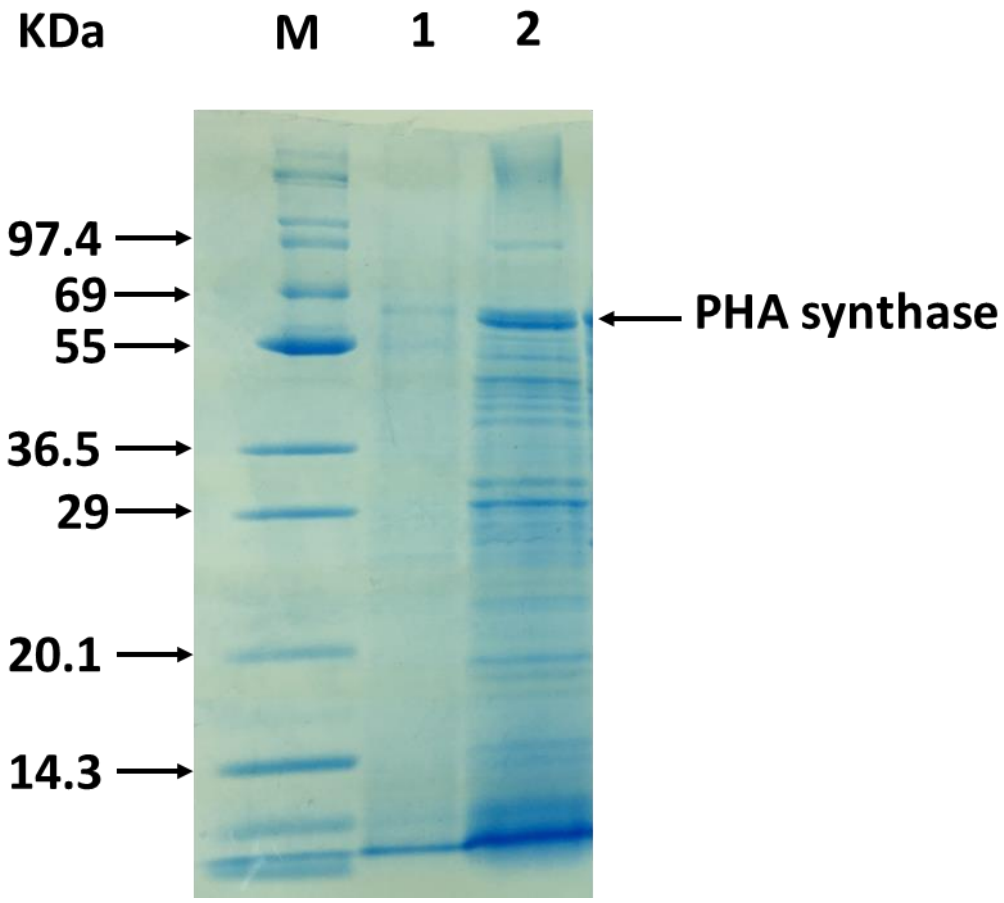

Figure 5

Polyhydroxyalkanoates production from synthetic waste using *Pseudomonas pseudoflava*:

Polyhydroxyalkanoate synthase enzyme activity analysis from *P. pseudoflava* and *P. palleronii*

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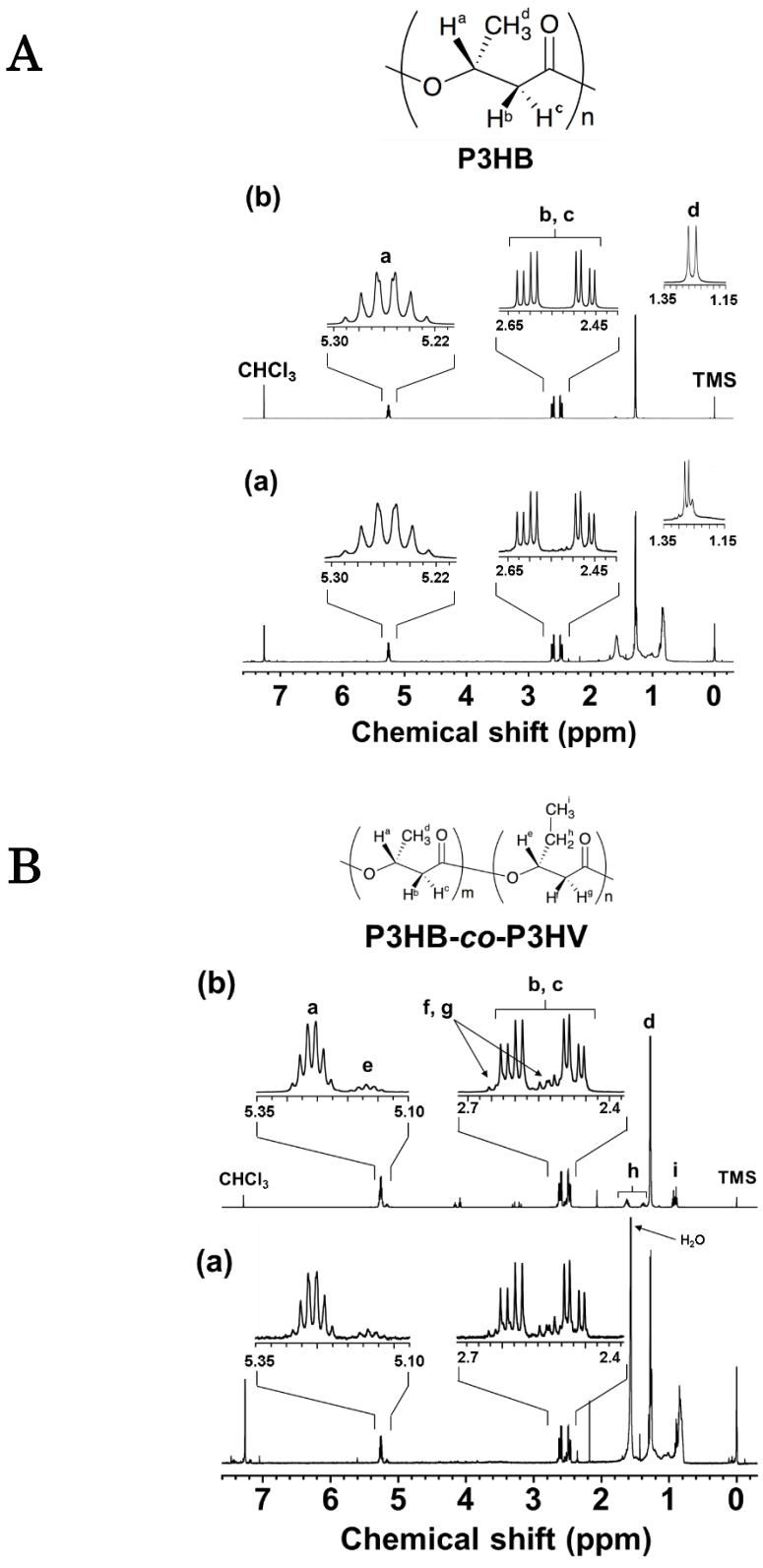

Figure S1: ^{1}H NMR spectra of (A) homo-polymer; and (B) co-polymer extracted from *P. pseudoflava*. (a) is ^{1}H NMR spectra of biopolymer extracted from *P. pseudoflava*; (b) is ^{1}H NMR spectra of standard biopolymer purchased from Sigma.