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Production of poly-3-hydroxybutyrate (P3HB) and poly-3-(hydroxybutyrate-co-hydroxyvalerate) P(3HB-co-3HV) from synthetic wastewater using *Hydrogenophaga palleronii*

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Abstract

In the present study, synthetic wastewater (SW) was used for production of poly-3-hydroxybutyrate (P3HB) and poly-3-(hydroxybutyrate-co-hydroxyvalerate) P(3HB-co-3HV) using the bacteria *Hydrogenophaga palleronii*. SW at various carbon concentrations (5-60 g/l) was evaluated for the growth and biopolymer production using *H. palleronii*. Substrate degradation was analyzed using total organic carbon (TOC) analyzer and high pressure liquid chromatography (HPLC). *H. palleronii* showed highest and lowest removal of TOC at 5 g/l (88±4%) and 60 g/l (15±6%) respectively. Among all the concentrations evaluated, bacteria showed highest biopolymer production with 20 g/l (63±5%), followed by 30 g/l (58±3%) and 40 g/l (56±2%). Lowest biopolymer production was observed at 5 g/l concentration (21±3%). Structure, molecular weight, and thermal properties of the produced biopolymer were analyzed. These results denoted that the strain *H. palleronii* can be used for degradation of high concentration of volatile fatty acids persistent in wastewaters and their subsequent conversion into useable biopolymers.

Keywords: *Hydrogenophaga palleronii*; biopolymer; total organic carbon; gel permeation chromatography; sodium acetate.

1. Introduction

The exploitation of plastics by the public has been increasing in the last decades. Though their existence has indisputably improved our daily lives, most of the plastics are produced by the use of petroleum derived compounds (Srikanth et al., 2012; Venkata Mohan et al., 2010). Also, plastics incline to persist in the environment due to their low biodegradable nature (Fradinho et al., 2014; Khanna and Srivastava, 2005). To overcome this, approachability has ascended in relation to the replacement of synthetic plastics by biodegradable bioplastics. Polyhydroxyalkanoates (PHAs) are naturally synthesized by many bacteria and intracellularly accumulated as granules, presents characteristics similar to conventional plastics making it a promising material for biodegradable plastics production (Venkateswar Reddy et al., 2015; Fradinho et al., 2014; Laycock et al., 2013). Poly-3-hydroxybutyrate (P3HB) is one type of PHA, and polyester of 3-hydrobutyric acid that is accumulated by various bacteria (Kim et al., 2012).

The perspective areas of PHA applications include the use of PHA as a filler for non-biodegradable plastics, disposable packages, agriculture systems for prolonged release of fertilizers and agrochemicals and medicine (Sudesh et al., 2000). Currently PHA based biopolymers are commercially available, industrially produced using pure culture under aseptic conditions and supplied with defined mediums (Fradinho et al., 2014). However, costs associated to these operational conditions increase PHA prices, economically limiting PHA application as a substitute

for traditional plastics (Fradinho et al., 2014; Reis et al., 2011). To compete with synthetic plastics, the production costs of PHA have to be reduced. The cost of media contributes most significantly to the overall production cost of PHA. Inexpensive substrates such as starch, glycerol from biodiesel production, molasses, plant oils and other cheap fatty acids draw the consideration of various researches (Ntaikou et al., 2009; Koller et al., 2005).

Objective of the present study is conversion of volatile fatty acids (VFA) present in synthetic wastewater (SW) into useful biopolymers using *Hydrogenophaga palleronii*. In this study, examined the capacity of *H. palleronii* to produce PHA at various VFA concentrations. Effect of VFA concentration on bacterial growth and substrate degradation was also evaluated. Further, influence of VFA composition on structure and properties of PHA was evaluated. Considering the importance of utilizing waste streams as feedstock for PHA production, the VFA tested in this work are common products of waste and effluent fermentation (acetate, propionate, butyrate, lactate).

2. Materials and methods

2.1 Bacteria

Hydrogenophaga palleronii (also called as *Pseudomonas palleronii,* NBRC-102513) was collected from the Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Japan. *H. palleronii* is a bacterium from the family of Comamonadaceae, has the ability to degrade different types of organic pollutants. Many authors reported about efficiency of this bacterium for degradation of various toxic compounds, but there are no reports about degradation of VFA present

in wastewater using this bacterium.

2.2 Culture media

For the growth of *H. palleronii* nutrient broth, and SW were used as the media. Composition of the SW was slightly different from the originally proposed by Kourmentza et al. (2015). The SW contained 1.0 g $(NH_4)_2SO_4$, 1.0 g K_2HPO_4, 0.2 g NaH_2PO_4, 0.2 g $MgSO_4$. $7H_2O$, 0.05 g NaCl, 0.05 g $CaCl_2$, 8.3 mg $FeCl_3$. $6H_2O$, 1.4 mg $MnCl_2$.$4H_2O$, 1.17 mg Na_2MoO_4. $2H_2O$, and 1 mg $ZnCl_2$ per one liter of deionized water. One ml of a trace elements solution was added to one liter of SW. The trace elements solution contains 0.786 g $CuSO_4$. $5H_2O$, 5.0 g $FeSO_4$. $7H_2O$, 12.609 g $NaMoO_4$. $2H_2O$, 4.05 g $NiCl_2$. $6H_2O$, 4.398 g $ZnSO_4$. $7H_2O$, 2.453 g $CoCl_2$. $6H_2O$, 0.75 g KI, 3.0 g H_3BO_3, 5.0 g $MnCl_2$. $4H_2O$ and 5.0 g EDTA in one liter of distilled water (Kourmentza et al., 2015). The carbon source composition of SW contains acetate and propionate, among the two carbon sources acetate was used as the main carbon source (Table 1). Different carbon concentrations (5, 10, 15, 20, 30, 40, 50 and 60 g/l) were used in SW in order to identify the optimum concentration for bacterial growth (Table 1). Real wastewaters sometimes contains the higher concentration of VFA and will inhibit the bacterial growth. Hence in the present study we used higher concentrations (up to 60 g/l) of VFA to know the survival capacity of this bacteria. The pH of the medium was adjusted to 7 and autoclaved before adding to the flasks.

2.3 Growth curve analysis

A loop of *H. palleronii* strain was initially inoculated into 50 ml of nutrient broth in 500 ml flasks,

and kept in shaking incubator under dark condition at 30 ºC 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) was 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 bioplastics composition. All the conditions were maintained as like in growth phase. Culture was collected and the PHA was extracted and analyzed at 72 h as described in section 2.5.3.

2.5 Analysis

2.5.1 Total organic carbon (TOC) measurement

Total dissolved organic carbon from clarified samples at 0 h and 120 h was 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 TC and IC calibration curves.

2.5.2 Analysis of VFA utilization by bacteria

The concentration of acetate and propionate at different time intervals were analyzed on HPLC (Shimadzu) with an RI detector and Shim-pack SCR-102 (H) column (Shimadzu, Kyoto, Japan). Samples collected for HPLC analysis were acidified with phosphoric acid (10 %, wv^{-1}) to stop the biological reaction and centrifuged at 8,000×g for 10 min. The resulting supernatant was filtered and analyzed directly by HPLC. Filtered and degassed 5 mmol/l perchloric acid was used as mobile phase at a flow rate of 1.0 ml/min. The column was maintained at a temperature of 40 °C in a thermostat chamber. Acetate and propionate concentrations were calculated from the area of the curve obtained for 1 mM of the standards. All results were presented as average and standard deviation of the data from three independent experiments.

2.5.3 Extraction and estimation of PHA

Extraction and estimation of PHA was performed following the procedure reported with slight modification (Law and Slepecky, 1960; Venkata Mohan and Venkateswar Reddy, 2013). The biomass pellet was separated from the substrate by centrifugation (6000×g for 10 min at 10 ^{o}C) and the resulting pellet was washed with acetone and ethanol separately to remove unwanted materials. The pellet was suspended in 4% sodium hypochlorite and incubated at room temperature for 3 h. The resulting mixture was centrifuged (6000×g for 10 min at 10 ^{o}C) and the supernatant was discarded. The pellet with lysed cells after washing simultaneously with acetone and ethanol was dissolved in hot chloroform and was passed through filter paper (Whatman, cat no-1440-070, 7 cm Diameter, 8 Micron pore size) to separate the polymer from cell debris. PHA were separated from the chloroform

by filtration, and concentrated using Rotary evaporator (Eyela N-1000) followed by drying to a constant mass. The mass of PHA was calculated gravimetrically. Final PHA production was quantified as cell contents (wt %) and as a concentration (g/l). Chloroform filtrate obtained was precipitated with cold ethanol and used for analysis of structure, molecular weight and thermal properties. Spectrometric analysis was conducted according to the method described by Law and Slepecky, (1960) to identify P3HB concentration. The extracted biopolymer was dissolved in 10 ml of sulfuric acid (36 N), and the resultant solution was heated at 100 ^{o}C on a water bath for 10 min. Addition of sulfuric acid converts the P3HB to crotonic acid (Law and Slepecky, 1960). The solution was cooled and the absorbance was measured at 235 nm for determining the P3HB concentration against a sulfuric acid blank. Standard curve was prepared using pure P3HB (Sigma-Aldrich).

2.5.3.1 Nuclear Magnetic Resonance (NMR) spectroscopy

^{1}H (500 MHz) NMR spectra was recorded on a JNM-ECA500 NMR spectrometer (JEOL, Japan) at 20 °C. 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 ^{1}H spectra.

2.5.3.2 Molecular mass determination

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 ^{o}C and polystyrene standards (M_n = 1,680-3,065,000) were employed for calibration.

2.5.3.3 Thermal analysis

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.

3. Results and discussion

In the present study we used *H. palleronii* for degradation of VFA present in SW and subsequent conversion of their metabolites into PHA. This is an exceptional bacteria for studying the biosynthesis of PHA, because this bacterium has the ability to grow at higher concentration of VFA, and has the ability to degrade different types of organic pollutants present in waste streams, *i.e.,* 4-aminobenzenesulfonic acid (Noisommit-Rizzi et al., 1996), 2,4-dinitrotoluene (Nishino et al., 2000), and naphthalene (Xin et al., 2010).

3.1 Growth curve with SW

H. palleronii was cultivated in SW at 30 ^oC by supplementing with different concentrations (5 - 60 g/l) of carbon source. Growth curve analysis clearly showed that the VFA concentration showed significant influence on the bacterial growth (Figure 1A&B). Among all the concentrations, bacteria showed higher growth with 30 g/l (OD, 2.301) followed by 40 g/l (2.270), 50 g/l (2.207), 20 g/l (2.181), 60 g/l (2.143), 15 g/l (1.887), 10 g/l (1.688) and 5 g/l (1.461). Bacteria does not showed

maximum growth at same time interval for all the concentrations, it showed maximum growth at different time intervals based on the VFA concentration. Bacteria showed maximum growth at 80 h for lower concentrations (5 and 10 g/l), 120 h for 15 and 20 g/l substrate concentrations, 192 h for 30-60 g/l substrate concentrations. Bacteria at lower substrate concentrations (5-20 g/l) 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 80 h. When the growth was compared at 120 h, bacteria grown with 20 g/l showed highest growth (OD, 2.181), it was 4.45 times higher than 60 g/l, 2.12 times higher than 50 g/l, 1.96 times higher than 5 g/l, and 1.76 times higher than 10 g/l. Higher concentrations took more time to show maximum bacterial growth due to toxic nature of VFA. These results clearly indicates the effect of VFA concentration on the growth of bacteria.

Figure 1

3.2. TOC removal

TOC is an index of the total amount of organic substances present in water/wastewater. TOC measurement plays a key role in assessing the efficiency of a wastewater purification process. It is used in a wide array of applications from management of wastewater treatment plant influent and effluent, to drinking water supply management, and monitoring of impurities in process and surface waters. The determination of TOC content in water is useful as a measure of pollution. In the present study, TOC removal was noticed in SW irrespective of the carbon concentrations studied suggesting the system’s function towards treatment. Among the different carbon concentrations, 5 g/l illustrated

higher TOC removal (88±4%) followed by 10 g/l (86±4%), 15 g/l (80±6%), 20 g/l (68±5%), 30 g/l (47±5%), 40 g/l (28±6%), 50 g/l (25±4%) and 60 g/l (15±6%). Bacteria grown with lower carbon concentrations showed higher TOC removal than higher carbon concentrations. Lower TOC removal at higher carbon concentrations might be due to the inhibition of bacterial growth by VFA present in SW. This was supported by growth curve results, where lower substrate concentrations took short time, and higher substrate concentrations took more time to show maximum growth.

3.3 Degradation of VFA

Individual VFA composition of the SW at different concentrations (from 5 - 60 g/l) were analyzed at 0 h and 120 h using HPLC (Figure. 2). HPLC results showed that among two acids, acetic acid showed maximum removal than propionic acid. 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 95±3% removal of acetic acid, and 70±5% removal of propionic acid. Bacteria grown at 50 and 60 g/l concentration showed 64±6% and 52±6% removal of acetic acid; 52±5% and 39±4% removal of propionic acid respectively. Many authors reported about efficiency of bacteria *H. palleronii* for degradation of various toxic compounds, but little information available about degradation of VFA using this bacterium. Noisommit-Rizzi et al. (1996) reported that two bacterial species, *H. palleronii* and *Agrobacterium radiobacter* degraded 4-aminobenzenesulfonic acid in a mutualistic co-operation. Nishino et al. (2000) reported mineralization of 2,4-dinitrotoluene by *H. palleronii* strain JS863 when grew on 2,4-dinitrotoluene as

the sole source of carbon and nitrogen. Xin et al. (2010) reported that *H. palleronii* LHJ38 can grow on naphthalene as sole carbon and energy source, and it degraded the naphthalene (2 g/l) completely within 132 h.

Figure 2

3.4 Change in pH

Initial pH of the SW at different carbon concentrations was adjusted to 7 before being added to the flasks. The pH showed an increasing trend with time for all the carbon concentrations, which might be due to the utilization of VFA by bacteria (Figure 3). At 120 h, SW at 20 g/l concentration showed highest increment in pH (7 to 9.38), followed by 30 g/l (7 to 9.32), 15 g/l (7 to 8.98) and 10 g/l (7 to 8.32). SW at 50 g/l (7 to 7.89), and 60 g/l (7 to 7.21) concentrations showed little decrement in pH due to lower utilization of VFA by bacteria. Changes in the pH values were correlated well with the TOC and VFA removal pattern.

Figure 3

3.5 PHA production

In the present study we used *H. palleronii* for degradation of VFA present in SW and subsequent conversion of their metabolites into PHA. Even though these bacteria were able to tolerate and can grow with VFA, little information is available about the PHA production using this bacteria with feeding of high concentration of VFA. Renner et al. (1996) reported PHA production with *P. palleronii* using different carbon sources. They used 10 g/l of glucose, sodium acetate, and sodium butyrate individually as carbon sources. They reported that *P. palleronii* produced 26% of PHA with sodium acetate, 12% of PHA with glucose, and 8% of PHA with sodium butyrate. Timm and

Steinbuchel, (1990) used 15 g/l of sodium gluconate as carbon source for growth of *H. palleronii.* They reported *H. palleronii* produced 59.6% of CDM as polymer with composition of 98.3% of HB, and 1.7% of hydroxy octanoate. The bacteria *H. palleronii* we used in the present study is an exceptional candidate which can grow with higher concentrations of VFA present in wastewater. So, we selected these bacteria for production of PHA.

In this study, PHA was accumulated by *H. palleronii* when SW with different VFA concentrations were supplied. PHA production and CDM were evaluated at 120 h of time interval for all the carbon concentrations. Biomass levels (CDM) varied (0.89 to 1.62 g/l) according to the concentration of substrate (Table 1), and PHA was also accumulated to different levels ranging from 21% to 63% CDM (Table 1). The best accumulation of PHA (63±5%) was achieved with 20 g/l carbon concentration in SW. *H. palleronii* also accumulated the good level of PHA when supplied with other carbon concentrations like 30 g/l (58±3%), 40 g/l (56±2%), 15 g/l (54±4%), 50 g/l (51±6%), and 60 g/l (41±5%) (Table 1). On the other side *H. palleronii* accumulated lower PHA with lower substrate concentrations like 5 g/l (21±3%) and 10 g/l (38±2%). PHA accumulation ability of *H. palleronii* is carbon concentration dependent. *H. palleronii* at 20 and 30 g/l showed maximum CDM and PHA production; at 5 and 10 g/l showed lower CDM and PHA production. According to TOC results, at 5 g/l concentration 4.4 g was utilized by bacteria and 0.6 g was remained in the SW. At 10 g/l concentration 8.6 g was utilized by bacteria and 1.4 g was remained in the SW at 60 g/l concentration only 9 g was utilized by bacteria, and higher carbon concentration (51 g) was remained in the SW. In

this study PHA production and CDM were evaluated at 120 h of time interval for all the substrate concentrations, but based on these results we observed that higher substrate concentrations need more time for proper utilization of carbon source, and production of PHA.

Table 1

The possibility of converting VFA into PHA has been previously reported by many authors using pure or mixed bacterial cultures (Table 2). Kourmentza et al. (2015) performed PHA production experiments under nitrogen limited conditions using enriched mixed culture and pure cultures belongs to the *Pseudomonas* group with synthetic wastewater contains acetate, propionate and butyrate at 0.8 g/l concentration. They reported that mixed culture PHA production was higher (64.4%) than the pure culture PHA production (varied from 5% to 23.8%). Amulya et al. (2014) reported 40% of PHA accumulation by *Bacillus tequilensis* using spent wash effluents contains acetate, propionate, butyrate and valerate as carbon source. Venkateswar Reddy and Venkata Mohan, (2012) reported 39.6% of PHA accumulation by mixed culture using fermented food waste contains VFA as carbon source.

Table 2

3.6 Carbon source influence on PHA composition

PHA composition was found to be directly related to the composition of the substrate used. 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) reported production of co-polymers of 3HB and 3HV in which the 3HV fraction increased agreeing to the propionate fraction in the substrate composition. Jiang et al. (2011) reported propionate encouraged mainly PHV production whereas acetate produced only PHB. They also reported experiments with acetate-propionate combinations produced PHB/PHV mixtures which directly related to the acetate and propionate utilization (Jiang et al., 2011). Dias et al. (2008) reported, the homo-polymer P3HB was produced when bacteria fed with acetate as sole substrate. When propionate was used as substrate, the composition of co-polymer was 20% HB and 80% HV. Dionisi et al. (2004) enriched a mixed culture on acetate, propionate and lactate mixture, and this culture was fed with propionate only, pure PHV was obtained.

3.7 Identification of PHA produced by *H. palleronii*

3.7.1 Structure determination with ^{1}H NMR

The ^{1}H NMR spectrum of $CDCl_3$ soluble part of P3HB and P(3HB-co-3HV) extracted from *H. palleronii* grown with SW was measured at 20 °C to deduce its chemical structure and primary sequence of polymer chain. 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 (5.25 ppm), methylene (2.59-2.48 ppm) and methyl (1.58 ppm) groups. In the case of P(3HB-co-3HV), protons in HV unit, *i.e.,* methyne (5.17 ppm), methylene on the main-chain (2.65-2.50 ppm), methylene on the side-chain (1.65-1.29 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 *H. palleronii* consist of HB and HB/HV units as their repeating units of the main-chains respectively.

3.7.2 Gel permeation chromatography (GPC)

To measure the molecular mass of homo-polymer (P3HB) and co-polymer P(3HB-co-3HV) produced from *H. palleronii*, we measured both number average molecular mass (M_n) and weight average molecular mass (M_w) of thin biopolymer solvent-cast films using by GPC. M_W and polydispersity index (PDI, M_w/M_n) of the P3HB produced by *H. palleronii* is 12 kDa and 2.3 respectively. The M_w of the standard P3HB (purchased from Aldrich) is 710 kDa, and PDI is 4.3. M_W and PDI of the co-polymer P(3HB-co-3HV) produced by *H. palleronii* is 46 kDa and 2.3 respectively. The M_w of the standard P(3HB-co-3HV) is 110 kDa, and PDI is 4.3. These results indicate that *H. palleronii* can produce biopolymers with relatively lower dispersity.

3.7.3 Thermogravimetric analysis (TGA)

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 (Figure 4). However the starting temperature for weight loss of the P3HB produced by *H. palleronii* was at around 170 °C and its T_{d5} was at 200 °C. In addition, the P3HB was completely not decomposed at 600 °C, 86% was decomposed at 540 °C indicating that the P3HB extracted from *H.*

palleronii composed of 14% 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. However the starting temperature for weight loss of the co-polymer P(3HB-co-3HV) produced by *H. palleronii* was at around 170 °C and its T_{d5} was at 205 °C, was completely not decomposed at 600 °C, 80% was decomposed at 540 °C. TGA analysis of co-polymer produced by *H. palleronii* 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 sample. This was supported by presence of large endothermic peak at 20-130 ^{o}C in DSC analysis.

Figure 4

3.7.4 Differential scanning calorimetry (DSC)

DSC was used to characterize the melting temperature (T_m) of the homo-polymer P3HB and co-polymer P(3HB-co-3HV) extracted from *H. palleronii*, and it was compared with respective standards (Figure 5). From the endothermic peaks in each DSC traces, it denoted that homo-polymer P3HB extracted from *H. palleronii* contains the T_m of 143-165 °C, it was matched with standard P3HB (T_m, 150-170 °C). However, co-polymer P(3HB-co-3HV) extracted from *H. palleronii* contains the T_m of 120-160 °C, it was matched with standard P(3HB-co-3HV) (T_m, 120-160 °C). In the standard co-polymer samples we observed two endothermic peaks, but in the co-polymer samples extracted from *H. palleronii* we observed three endothermic peaks, it may be due to impurities present in samples. Nevertheless, based on the results shown above, we concluded that the produced

polymer is P3HB and P(3HB-co-3HV).

Figure 5

4. Conclusion

In the present study, experiments were conducted to produce PHA from synthetic wastewater using the bacteria *Hydrogenophaga palleronii*. Results revealed that *H. palleronii* utilized volatile fatty acids present in SW as sole carbon and energy source, grew well and converted in to useful products. Among different concentration of SW, *H. palleronii* showed highest growth and PHA production at 20 and 30 g/l concentrations. Structure, molecular weight, and thermal properties of the produced PHA were compared with standard PHA. These findings indicate the possibility of feeding the *H. palleronii* with cheap VFA rich fermented wastes to produce PHA.

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Table 1: Wet weight, cell dry mass (CDM), and PHA production from *H. palleronii*. Bacteria were incubated with different concentrations of volatile fatty acids in synthetic wastewater at 30 ºC.

Substrate concentration (g/l)	Substrate composition (g/l)		Wet weight (g/l)	Cell dry mass (g/l)	PHA production (%CDM)
	Acetate	Propionate			
5	4.75	0.25	1.81±0.2	0.89±0.15	21±3
10	9.5	0.5	2.54±0.1	1.13±0.20	38±2
15	14.25	0.75	3.26±0.3	1.44±0.13	54±4
20	19	1	3.53±0.3	1.61±0.18	63±5
30	28.75	1.25	3.62±0.4	1.62±0.15	58±3
40	38.5	1.5	3.14±0.6	1.43±0.25	56±2
50	48.25	1.75	3.12±0.5	1.39±0.31	51±6
60	58	2	2.25±0.6	0.94.±0.35	41±5

Table 2: Literature reports on PHA production from volatile fatty acids using various types of bacteria.

Name of bacteria	Type of Substrate	Substrate load	PHA production	Reference
Mixed culture	[1]SW contains acetate, propionate, butyrate	2.4 g/L	64.4 %CDM	Kourmentza et al., 2015
Pseudomonas sp.	SW contains acetate, propionate, butyrate	2.4 g/L	23.8 %CDM	Kourmentza et al., 2015
Bacillus tequilensis	SW contains acetate, propionate, butyrate	6 g/L	59 %CDM	Venkateswar Reddy et al., 2014
Bacillus tequilensis	[2]SWE contains acetate, propionate,butyrate, valerate	1.32 kg COD/m^3-day	40 %CDM	Amulya et al., 2014
Mixed culture	[3]FFW contains acetate, propionate, butyrate	1.11 kg COD/m^3-day	39.6 %CDM	Venkateswar Reddy and Venkata Mohan, 2012
Pseudomonas otitidis	SW contains acetate, propionate, butyrate	1.32 kg COD/m^3-day	58 %CDM	Venkateswar Reddy et al., 2012
Pseudomonas otitidis	[4]AE contains acetate, propionate, butyrate	1.98 kg COD/m^3-day	54 %CDM	Venkateswar Reddy et al., 2012
Mixed culture	[5]ARE contains acetate, propionate, butyrate	2.91 kg COD/m^3-day	25 %CDM	Venkata Mohan et al., 2010
Mixed culture	Acetate	22.14 g/L	89 %CDM	Johnson et al., 2009
Hydrogenophaga palleronii	Sodium acetate	10 g/L	26 %CDM	Renner et al., 1996
Hydrogenophaga palleronii	Sodium butyrate	10 g/L	8 %CDM	Renner et al., 1996
Hydrogenophaga palleronii	Sodium gluconate	15 g/L	59.6 %CDM	Timm and Steinbuchel, 1990
Hydrogenophaga palleronii	SW contains acetate, propionate	20 g/L	63 %CDM	This study

[1]SW: Synthetic wastewater; [2]SWE: Spent wash effluents; [3]FFW: Fermented food waste; [4]AE: Acidogenic effluents; [5]ARE: Acid rich effluents.

Figure captions

Figure 1: Growth curve of *H. palleronii* with different concentrations of synthetic wastewater, (A) 5 g/l - 20 g/l concentration; and (B) 30 g/l - 60 g/l concentration. Samples collected at different time intervals were used for measurement of OD at 600 nm.

Figure 2: Removal of volatile fatty acids at different concentrations in synthetic wastewater using *H. palleronii*.

Figure 3: pH variation of synthetic wastewater with respect to time at different volatile fatty acids concentrations using *H. palleronii*.

Figure 4: Thermogravimetric analysis of (A) homo-polymer P3HB; and (B) co-polymer P(3HB-co-3HV). – (a) is biopolymer extracted from *H. palleronii*; -- (b) is standard biopolymer purchased from Sigma.

Figure 5: Differential scanning calorimetry analysis of (A) homo-polymer P3HB; and (B) co-polymer P(3HB-co-3HV). (a) is biopolymer extracted from *H. palleronii*; (b) is standard biopolymer purchased from Sigma.

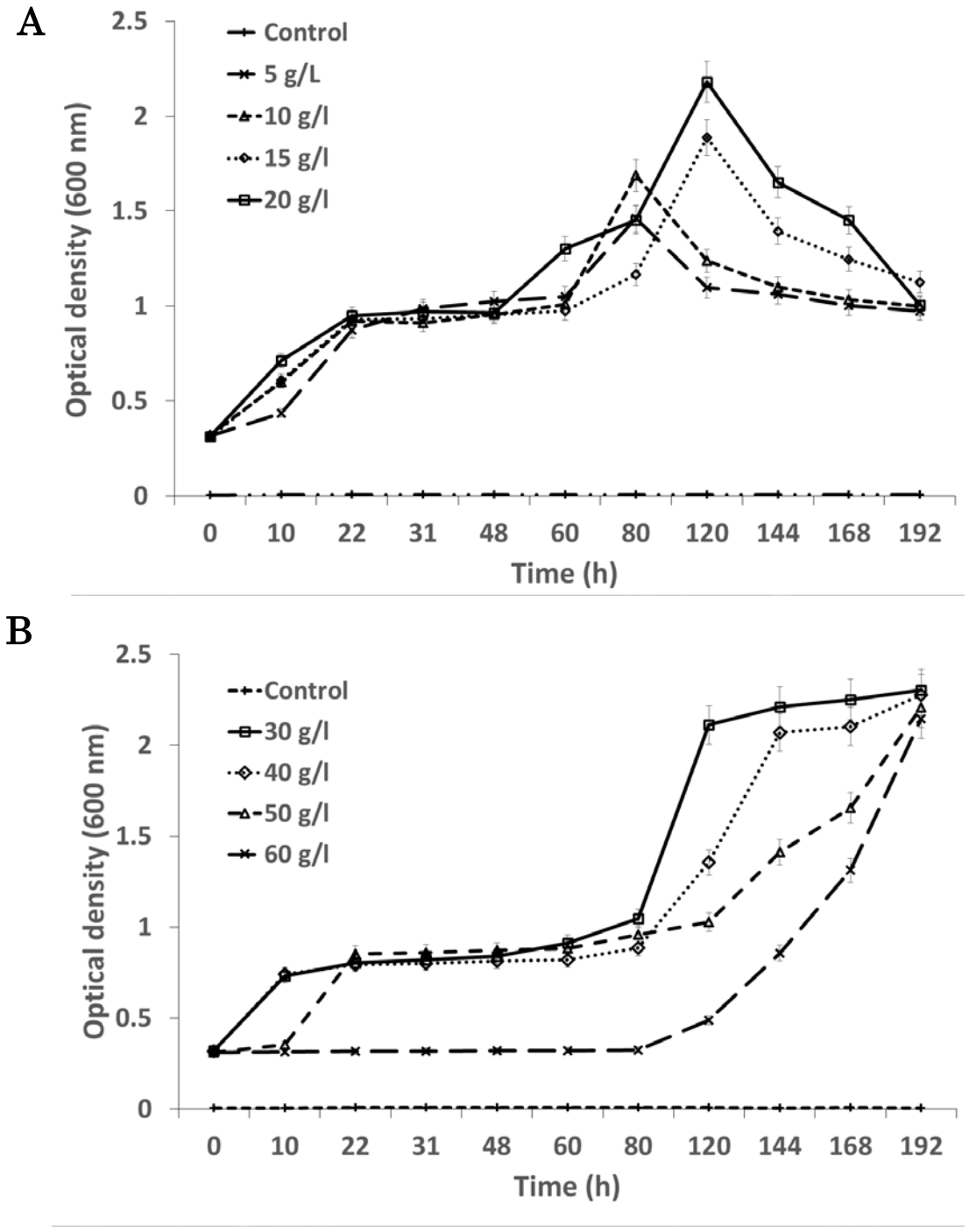
A
Control
5 g/L
10 g/l
15 g/l
20 g/l
Optical density (600 nm)
Time (h)
B
Control
30 g/l
40 g/l
50 g/l
60 g/l
Optical density (600 nm)
Time (h)

Figure 1

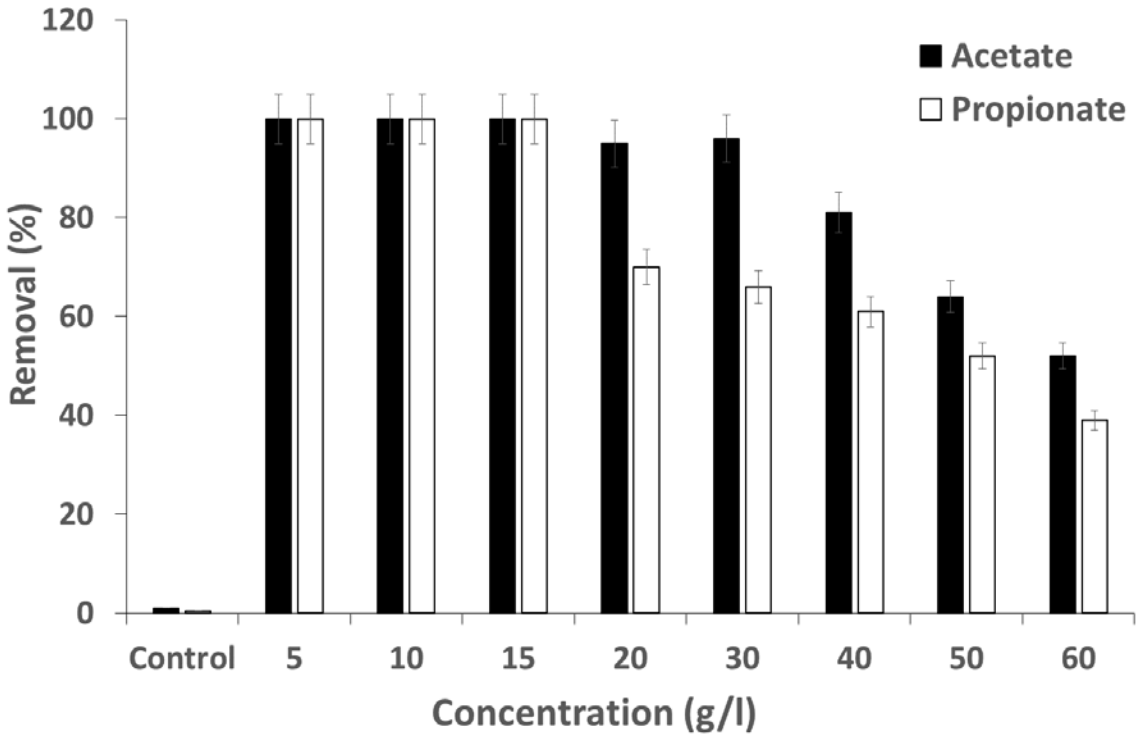
120
100
80
60
40
20
0
Removal (%)
Acetate
Propionate
Control
5
10
15
20
30
40
50
60
Concentration (g/l)

Figure 2

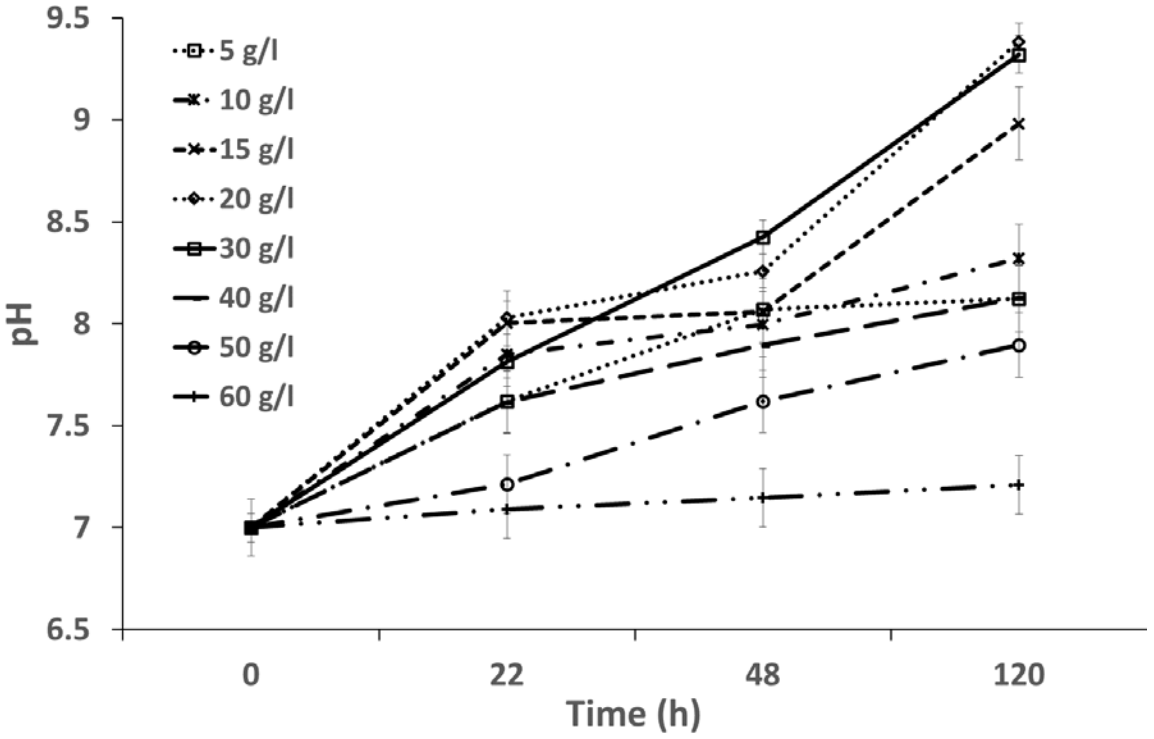
9.5
9
8.5
8
7.5
7
6.5
pH
5 g/l
10 g/l
15 g/l
20 g/l
30 g/l
40 g/l
50 g/l
60 g/l
0
22
48
120
Time (h)

Figure 3

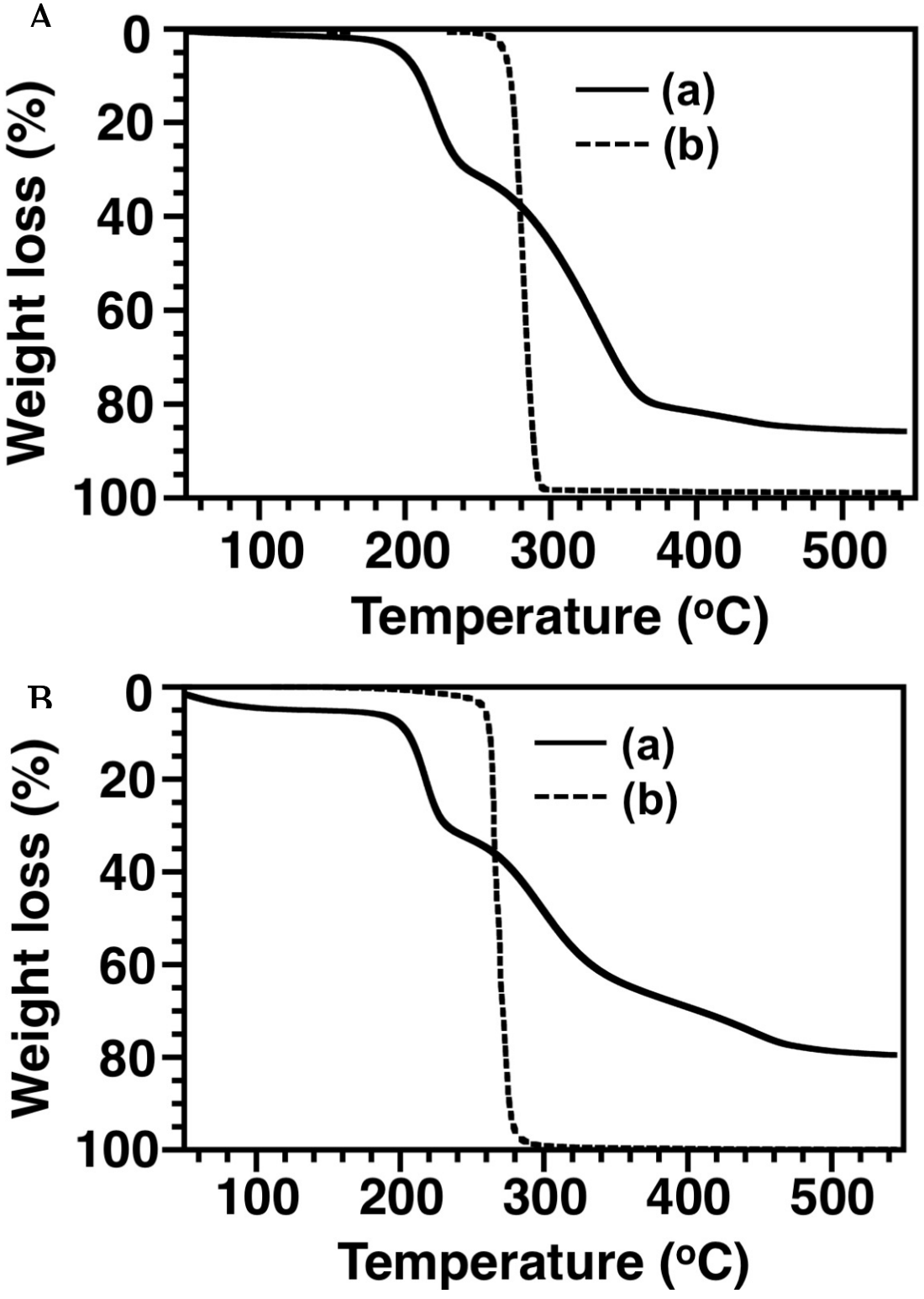
A
Weight loss (%)
0
20
40
60
80
100
100
200
300
400
500
Temperature (°C)
(a)
(b)
B
Weight loss (%)
0
20
40
60
80
100
100
200
300
400
500
Temperature (°C)
(a)
(b)

Figure 4

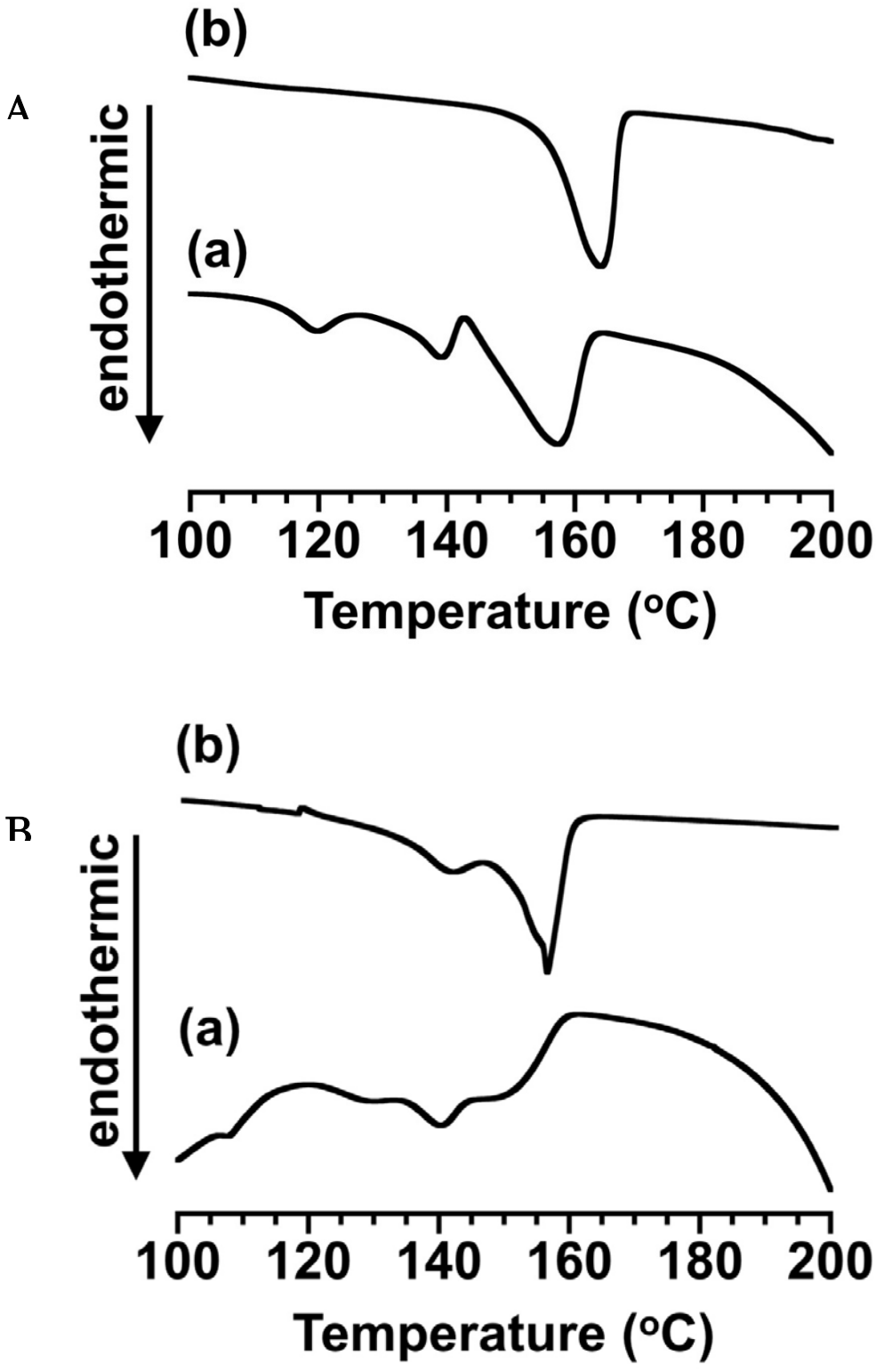
A
(b)
(a)
endothermic
100
120
140
160
180
200
Temperature (°C)
B
(b)
(a)
endothermic
100
120
140
160
180
200
Temperature (°C)

Figure 5