Study on Sustainable Recovery and Extraction of Polyhydroxyalkanoates (PHAs) Produced by *Cupriavidus necator* Using Waste Glycerol for Medical Applications

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The current study shows that *Cupriavidus necator* has the ability to grow on waste glycerol as carbon source, and can synthesize a highly thermostable copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). Batch cultivation on waste glycerol showed accumulation of 6.76 g L^{-1} biomass containing 4.84 g L^{-1} poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer with 3-hydroxyvalerate content of 24.6 mol%. A novel recovery strategy was developed for the extraction of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer from *Cupriavidus necator* using recyclable solvents, i.e., propylene carbonate, butyl acetate, isoamyl alcohol, and ethyl acetate. Propylene carbonate demonstrated the recovery efficiency of 90 % and polymer purity of 95 %, at 120 °C after 30 min. Ethyl acetate exhibited a higher efficiency than butyl acetate in terms of recovering the copolymer from cells. Ethyl acetate extraction demonstrated a recovery yield of 96 % and purity of 93 % at 100 °C. Efficacy of an anionic surfactant, linear alkylbenzene sulfonic acid (LAS), was also tested for extraction, and it showed maximum yield of 84 % and purity of 90 % at 80 °C and pH 5.0. Extraction of copolymer using these solvents could help in replacing generally used chlorinated toxic solvents, such as 1,2 dichloroethane and chloroform. Further, GPC, TGA and DSC analysis revealed that the thermo-physical properties were not significantly affected by the extraction method. However, the molecular weight distribution of the polymer showed a variation depending on the type of solvent used for extraction. Subsequently, endotoxins were removed efficiently to less than 5 EU g^{-1} of copolymer using alkali at optimized conditions of 6 h digestion time and 2.5 N NaOH concentration for medical applications.

Keywords:
polyhydroxyalkanoates, *Cupriavidus necator*, extraction, recovery yield, endotoxin

Introduction

An imprudent and continuous use of environmentally toxic synthetic plastics and depletion of fossil fuels have shifted research focus towards the synthesis of bioplastics – polyhydroxyalkanoates (PHAs)[1]. PHAs are considered as best substitutes for conventional plastics, since they are produced by the fermentation of renewable feedstock, and are completely biodegradable upon disposal as opposed to petroleum-derived polymers[2]. PHAs are polyesters of hydroxyalkanoates, which are synthesized by prokaryotic microbes intracellularly as energy reserve compounds[3]. Depending upon their monomeric units' composition, they can be used in various products, including packaging items, household objects, sutures, vascular stents, and medical scaffolds[4].

Poly(3-hydroxybutyrate) (PHB) is the most widely studied candidate of PHAs family. However, PHB has poor mechanical properties, such as brittleness, stiffness, and high crystallinity, which seems to restrict its industrial application. Introduction of different monomer units like 3-hydroxyvalerate (3HV) into PHB polymeric chains, improves the biopolymer properties such as impact resistance and flexibility[5,6]. For example, poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) or poly(3HB-*co*-3HV) possesses much better thermal properties, flexibility, and toughness than PHB. Thus, the production of copolymers of 3-hydroxybutyrate (3HB) and 3HV units, i.e., poly(3HB-*co*-3HV), can solve issues of thermal instability and stiffness associated

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with PHB. Introduction of 3HV monomers in the biopolymer also reduces its crystallinity, glass transition temperature, and melting temperature[6].

However, PHAs copolymers are still not able to substitute the synthetic polymers on the industrial scale because of the high cost of media or substrate used, high downstream recovery cost, as well as utilization of various toxic solvents during the extraction process. The high substrate cost could be minimized by using inexpensive renewable feedstock, such as crude glycerol, cane molasses, cheese whey, palm oil, and industrial wastewater. For example, crude glycerol, wheat hydrolysate, and sunflower meal have been extensively used for the synthesis of PHAs[2,7]. The second impediment in the cost-effective production of PHAs is the downstream recovery and purification step. Several researchers have investigated that 50 % of the total process cost is attributed to the extraction of PHAs[8–10]. Therefore, it is crucial to reduce the downstream recovery cost in order to replace synthetic plastics. There is an urgent need to devise more efficient and sustainable recovery systems for the economical production of PHAs from cheaper substrates. The downstream processes directly influence the quality and properties of the extracted polymer[11]. Thus, the extraction system must be designed in a way so that the molecular weight and material properties of the polymer remain unaffected.

The majority of PHAs recovery methods exploit chlorinated solvents like chloroform and 1,2-dichloromethane for extraction. These halogenated solvents are capable of removing the lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria during polymer extraction, which helps to improve the polymer quality for biomedical applications[8]. The endotoxins (LPS) generally co-purify with PHAs during extraction and have antigenic properties resulting in immunogenic response[12]. Therefore, removing undesirable endotoxins from PHAs is another important concern in the biomedical field that needs to be addressed. Gram-positive bacteria, such as *Bacillus* sp., have also been exploited for the production of LPS-free polymer, but there are reports suggesting that these bacteria also have antigenic properties because of the presence of teichoic acid and lipoglycans layer[4,12]. Another major disadvantage of employing these gram-positive bacteria is slower growth rate, and low PHAs concentrations and content.

The halogenated solvents used in the recovery of PHAs are toxic and volatile, which directly affects the environment and human health[13]. Besides, it has been observed that the recovered polymer solution is more viscous, and separation of cell residues becomes difficult if solution contains more than 5 % (w/v) PHAs. Therefore, this system requires huge quantities of solvents to dissolve the polymer, which results in high production cost. Recently, some non-toxic and recyclable solvents have also been explored[14–16]. However, the research on developing an environmentally friendly solvent extraction method with high PHAs yield and purity is still limited. In fact, there is scanty information in literature about the effect of recovery and purification methods on the biopolymer properties.

In the present investigation, the main objective was to evaluate the efficacy of various non-toxic solvents, such as 1,2-propylene carbonate, butyl acetate, and ethyl acetate in the extraction of poly(3HB-*co*-3HV) from *Cupriavidus necator*. Efficacy of an environmentally safe surfactant, such as linear alkylbenzene sulfonic acid (LAS), was also evaluated at different cultivation conditions. The capability of these agents for poly(3HB-*co*-3HV) extraction was estimated in the form of polymer recovery yield and purity. The effect of these extraction agents was also analyzed on the copolymer material properties. The effect of different alkalis in removing undesirable endotoxins that purify with the polymer was also studied in order to avoid usage of halogenated solvents, and to increase PHA's applicability in the biomedical field. The chloroform extraction method was used as reference method for comparing efficiency of each recovery strategy.

Experimental

Microorganism

Cupriavidus necator DSM 545 was procured from The Leibniz Institute DSMZ, Germany. The culture was maintained on agar slants at 4 °C and subcultured monthly. The strain was also preserved in glycerol stocks at –70 °C.

Growth medium and inoculum preparation

C. necator was grown in an optimized medium containing 10 g L^{-1} glucose, 3.59 g L^{-1} Na_2HPO_4, 2 g L^{-1} $(NH_4)_2SO_4$, 1.5 g L^{-1} KH_2PO_4, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.05 g L^{-1} NH_4Fe(III)citrate, and 1 mL trace element solution SL617. The waste glycerol was used as carbon source in place of glucose for poly(3HB-*co*-3HV) production and extraction[2]. The waste glycerol was procured from Jatropha-based biodiesel production plant at the Indian Institute of Technology Delhi. All medium constituents, except trace element solution, were sterilized by autoclaving. The trace element solution was sterilized by filtration using 0.2 μ size filter and then mixed with the other components. The pH of the medium was adjusted to 6.8

using 2 N NaOH/HCl. Inoculum development method was similar to that reported in our earlier study[2].

Poly(3HB-*co*-3HV) production on waste glycerol

For poly(3HB-*co*-3HV) production, 100 mL basal medium was taken in 500-mL shake flasks and inoculated with 5 % (v/v) microbial culture. The medium pH was adjusted to 6.8 using 2 N NaOH/HCl. The flasks were incubated in a rotary shaker at 200 rpm and 30 ºC for 48 h. The culture broth was harvested from the shake flasks at the end for biomass analysis and extraction of poly(3HB-*co*-3HV).

The cultivation of *C. necator* was also performed in a 5-L stirred tank reactor (BioSpin-05A, Bio-Age Equipments, India) using 3.5 L optimized medium containing waste glycerol. The bioreactor was inoculated with 5 % (v/v) culture from shake flask. Agitation was carried out by Rushton turbine-impellers with six flat blades. The temperature was maintained at 30 ºC, and medium pH was maintained at 6.8 by automatic addition of 2 N NaOH/HCl. Air was sparged through a glass metered needle valve rotameter using a ring-type sparger. Samples were collected regularly at intervals of 6 h for the analysis of biomass, residual waste glycerol, and poly(3HB-*co*-3HV) content. Bioreactor cultivation was performed in duplicate and average values reported.

Poly(3HB-*co*-3HV) extraction by 1,2-propylene carbonate and chloroform

Poly(3HB-*co*-3HV) extraction from *C. necator* was carried out according to the protocol by Fiorese *et al.*[18] with some modifications. The effect of incubation time (10–50 min) and temperature (80–120 °C) on copolymer purity and yield was studied. About 2 % of PHAs/1,2-propylene carbonate mixture was taken in screw cap bottles and heated at different temperatures of 80, 100, and 120 °C. Suspension was kept at these temperatures for different durations: 10, 30, and 50 min. The hot solution was then filtered with 0.45 μm Whatman filter paper to remove the impurities. The filtrate was precipitated with two volumes of acetone and kept at ambient temperature for 4 h. The precipitated polymer was then recovered by centrifugation, washed with acetone, and dried at 70 ºC in hot air oven. The poly(3HB-*co*-3HV) extraction protocol by chloroform was similar as mentioned in earlier study[2]. All extraction experiments were performed in duplicate and average values reported for reproducibility.

Poly(3HB-*co*-3HV) extraction by non-halogenated solvents

The effect of several non-halogenated solvents, i.e., butyl acetate, ethyl acetate, and isoamyl alcohol was evaluated for the extraction of poly(3HB-*co*-3HV). About 2 g of dried cells, containing 1.4 g of copolymer, were taken in screw-capped borosil bottles. In each bottle, 70 mL solvent was added to prepare 2 % copolymer mixtures. The poly(3HB-*co*-3HV) was extracted by incubating mixtures at different temperatures, i.e., 60 °C, 80 °C, and 100 °C for 2 h, and mixtures were shaken at regular intervals. Copolymer was precipitated with two volumes of n-heptane and centrifuged at 5000 g. The pellet was washed with n-heptane and dried at 70 °C. All extraction experiments were performed in duplicate and average values reported.

Poly(3HB-*co*-3HV) extraction by linear alkylbenzene sulfonic acid

Linear alkylbenzene sulfonic acid (LAS) is an anionic synthetic surfactant, which is biodegradable and environmentally safe[19]. Therefore, LAS was investigated for the extraction of poly(3HB-*co*-3HV) from *C. necator*. Biomass was treated with 5 % w/v detergent solution (cell: detergent ratio of 1:0.5) for 2 h at 60 °C. The effect of temperature (40, 60, 80 °C), and detergent solution pH (3.0, 5.0, 7.0, 9.0) was studied to check whether varying these parameters would affect recovery yield and purity. All experiments were performed in duplicate and average values reported.

Analytical procedures

The samples were collected and centrifuged at 9,000 g for 10 min. The cell pellet was dried at 70 °C until constant weight was achieved; it was then used for poly(3HB-*co*-3HV) analysis. Poly(3HB-*co*-3HV) content was estimated by gas chromatography (GC) technique[20,21]. Poly(3HB-*co*-3HV) recovery yield was estimated by a gravimetric technique[22], wherein the copolymer recovered after each extraction step was dried and dry weight calculated. The recovery yield (%) was estimated as:

$$\text{Recovery yield } (Y) = \frac{Dw_{\text{PHA}}}{Dw_{\text{cell}}} \cdot 100$$

where Dw_{PHA} (g) is the dry weight of the copolymer obtained from dry weight of cell Dw_{cell} (g).

The purity of all samples was calculated by GC technique[20,21]. The quantification of poly(3HB-*co*-3HV) copolymer by GC has been explained in detail in our previous work[2]. The purity (%) of polymer was estimated as:

$$\text{Purity } (P) = \frac{Cw_{\text{PHA}}}{Tw_{\text{PHA}}} \cdot 100$$

where Cw_{PHA} is weight of the copolymer as calculated by GC, and Tw_{PHA} is the total weight of sample used for GC quantification.

Poly(3HB-*co*-3HV) characterization

Structural characterization of poly(3HB-*co*-3HV) was done by Fourier Transform Infrared (FTIR) spectroscopy[2]. Molecular weight of the polymer was estimated by gel permeation chromatography using a Shodex column and a refractive index (RI) detector. Chloroform was used as mobile phase and polystyrene was used for standard preparation. The melting temperature was estimated by Differential Scanning Calorimetry (DSC 821^e, Mettler Toledo) technique as described in literature[23]. Thermogravimetric analysis (TGA 851^e, Mettler Toledo) was used to analyze the degradation temperature (T_d) of poly(3HB-*co*-3HV) copolymer. The polymer was placed in an aluminum pan and treated at a heating rate of 10 °C min^{-1} from 30 °C to 400 °C.

Removal of endotoxins from poly(3HB-*co*-3HV) copolymer

The effect of different alkalis, such as NaOH, NH_4OH, and KOH treatment methods was studied for the removal of endotoxins from poly(3HB-*co*-3HV) copolymer extracted by 1,2-propylene carbonate. Endotoxin levels (endotoxin units per gram of PHAs) were analyzed by Limulus Amoebocyte Lysate (LAL) gel-clot assay[24] using Pierce LAL Chromogenic Endotoxin Quantitation Kit (ThermoFisher Scientific, USA). To remove the endotoxins, the effects of digestion time (1.0, 1.5, 2.0, 3.0, 4.0, 5.0, and 6.0 h) and alkalis concentration (0.1, 0.5, 1.0, 1.5, 2, 2.5, 3, and 4 N) were examined. The polymer was repeatedly washed with acetone and dried at 70 °C. The endotoxin levels (EU g^{-1}) are reported as average values of three batch experiments.

Results and discussion

Poly(3HB-*co*-3HV) production on waste glycerol

Batch cultivation kinetics of *C. necator* demonstrated accumulation of total biomass dry weight of 6.76 g L^{-1} and PHAs concentration of 4.84 g L^{-1} in the shake flask (Data not shown)[2]. At the end of flask cultivation, total PHAs content was 72 % of CDW and productivity was 0.12 g L^{-1} h^{-1}. The content or molar fraction of 3HV unit in PHAs was found to be 24.6 mol%. Upon gas chromatography analysis, the synthesized PHAs polymer was characterized as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) copolymer, which is a highly thermostable copolymer. Thus, *C. necator* showed the ability to utilize waste glycerol as carbon source and synthesized copolymer poly(3HB-*co*-3HV). Batch kinetics of *C. necator* was studied later on under controlled environmental conditions in a bioreactor with respect to biomass and poly(3HB-*co*-3HV) production. The culture started growing exponentially and PHA accumulation started during growth phase. In bioreactor cultivation, a maximum biomass dry weight of 10.46 g L^{-1} and PHA concentration of 7.32 g L^{-1} was obtained, and PHAs content of 69 % of CDW was accumulated in 40 h. During fermentation, 18.8 g L^{-1} glycerol was consumed from its initial concentration of 20 g L^{-1}, and 1.21 g L^{-1} glycerol was left unconverted in the broth. Microbial cultivation exhibited a maximum PHAs productivity and PHAs yield of 0.183 g L^{-1} h^{-1} and 0.38 g of PHAs per g of glycerol, respectively.

Poly(3HB-*co*-3HV) extraction by chloroform

Poly(3HB-*co*-3HV) copolymers are barely soluble in classical polymer solvents and are completely soluble in toxic halogenated solvents such as chloroform. The chloroform extraction method is used at lab-scale only, but with limited success at pilot-plant and large scale level[8]. Moreover, this method requires large amounts of solvent (about 20 units) for the recovery of 1 unit of polymer because of high viscosity of extracted polymer and presence of cell debris in the solution. These huge quantities of solvents ultimately lead to high production costs.

In the present work, chloroform method was used as a benchmark for comparing the efficacy of other extraction methods. The effect of solvent on the extracted polymer was evaluated in terms of PHAs yield, purity, and molecular weight. Chloroform method extracted almost 95 % of the poly(3HB-*co*-3HV) present in the *C. necator* cells with a total recovery yield of 95 % and purity of 97 % (Figs. 1a to c). The extracted polymer demonstrated a molecular weight (M_w) of 150.5 kDa and polydispersity index (PDI) of 2.9. The M_w and the PDI values of the polymer are most significant parameters in determining their suitability for a particular application. These observations show that the poly(3HB-*co*-3HV) copolymer extracted by chloroform exhibits a good molecular weight with relatively lower dispersity. Previously, almost similar findings were reported by Fiorese *et al.*[18] using propylene carbonate solvent for extraction with PHB yield of 96 % and purity of 95 %.

Extraction of poly(3HB-*co*-3HV) by 1,2-propylene carbonate

To overcome problems associated with chloroform, extraction of poly(3HB-*co*-3HV) from the *C. necator* was thus assessed by 1,2-propylene carbonate (1,2-PC), a recyclable solvent[25,26]. 1,2-PC can be reused for various purification cycles because of its high boiling point of 240 °C, and low evaporation rate[18]. The influence of varying temperatures from 80 to 120 °C and incubation periods from 10 to 50 min was studied to find out the optimized conditions for the complete extraction (Figs. 1a to c). The effectiveness of the method after each incubation time was evaluated by the recovery yield and purity obtained.

The poly(3HB-*co*-3HV) recovery was incomplete at 80 and 100 °C, and this was mainly due to low temperatures used during extraction, as shown by the low recovery yield of 45 to 68 %. At shorter incubation time of 10 min, extraction was not complete even at 120 °C, resulting in lower yield of 70 %. On increasing the incubation time from 10 to 30 min at 100 °C, PHAs yield was improved to 70 % (shown in Fig. 1b). Further increase in the incubation period (30 to 50 min) demonstrated negligible improvement in recovery yield (76 %), and the polymer purity was enhanced from 62 to 80 %. Possibly, longer incubation time (50 min) could have resulted in an increase in the solubilization of impurities. The highest extraction efficiency was achieved at optimized conditions of 120 °C temperature and incubation period of 30 min. The polymer recovery yield and purity at these optimized conditions were 90 % and 95 %, respectively. This could be due to high solubility of poly(3HB-*co*-3HV) in 1,2-PC at 120 °C temperature. These results were in accordance with Fiorese *et al.*[18], who demonstrated that PHB extraction by 1,2-PC was complete at temperatures above 120 °C.

Poly(3HB-*co*-3HV) extraction by non-halogenated solvents

The poly(3HB-*co*-3HV) extraction was also performed by non-halogenated solvents like ethyl acetate, butyl acetate, and isoamyl alcohol at different temperatures, i.e., 60 °C, 80 °C, and 100 °C. Higher polymer concentrations inside the cells would yield more viscous PHAs solutions[8], which are difficult to handle during downstream recovery. Therefore, lower concentration (2–5 %) of PHA/solvent mixture were selected for testing the efficacy of different solvents. The copolymer to solvent ratio of 2 % (w/v) was used for all extractions. Among all solvents, isoamyl alcohol was observed to be unsuitable for the recovery of poly(3HB-*co*-3HV) from cells.

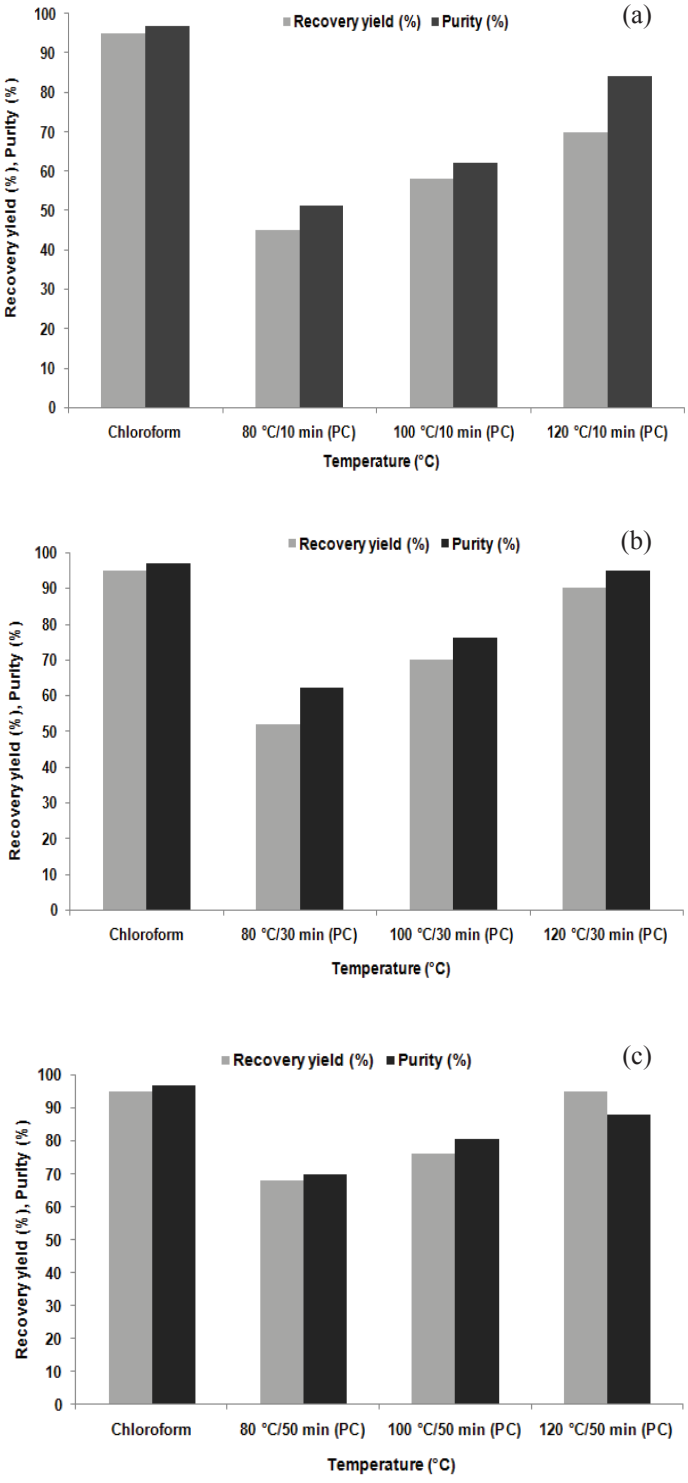

Fig. 1 – *Effect of different temperatures on purity and recovery yield (%) of PHAs obtained by extraction with chloroform and 1,2-propylene carbonate (1,2-PC) at a) 10 min incubation period; b) 30 min incubation period; c) 50 min incubation period*

The poly(3HB-*co*-3HV) recovery using ethyl acetate demonstrated a high recovery yield of 94 % and 96 % at 80 °C and 100 °C, respectively (Figs. 2a, b). At lower temperature of 60 °C, the recovery

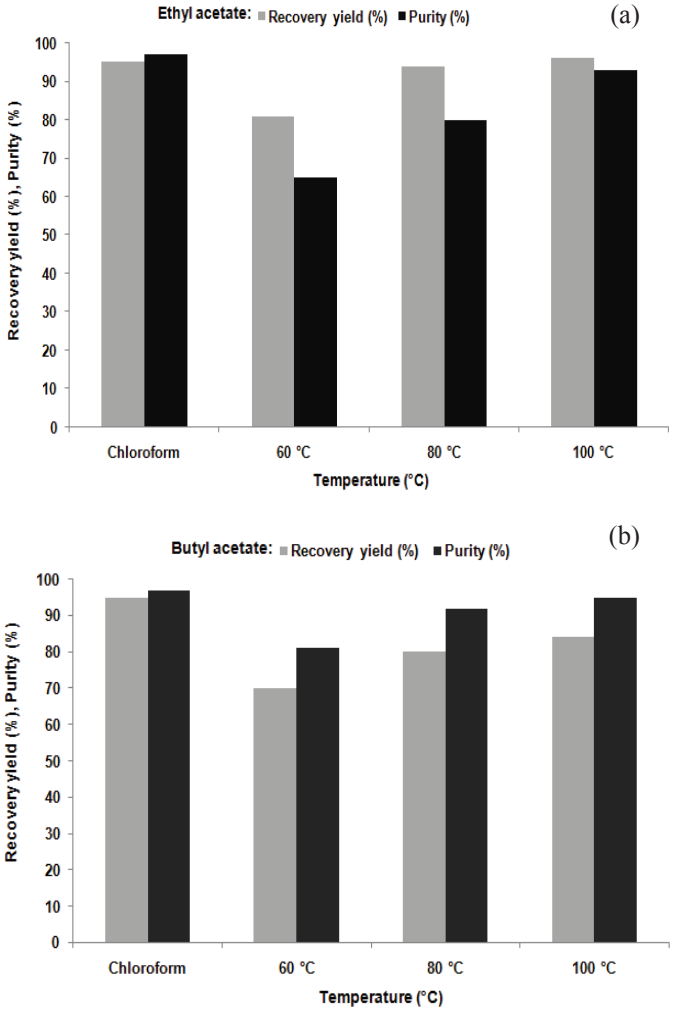

Fig. 2 – *Effect of different temperatures on purity and recovery yield (%) of PHAs obtained by extraction with a) ethyl acetate; b) butyl acetate*

yield decreased to 80 to 83 %. The polymer purity also decreased from 93 % to 65 % as the temperature decreased from 100 °C to 60 °C. Ethyl acetate demonstrated both high yields (~96 %) and high polymer purities (≤ 93 %) at 100 °C. These results indicate that the extraction efficiency had improved as the temperature increased. In case of butyl acetate, the recovery yield was low (~84 %) even at higher temperature of 100 °C. The recovery yield was further decreased to 70 % as temperature was lowered to 60 °C. However, purity decreased from 95 % to 81 % with the decrease in temperature from 100 to 60 °C. At 100 °C, butyl acetate treatment exhibited a maximum recovery yield of 84 % and purity of 95 %. Ethyl acetate exhibited better capacity to recover poly(3HB-*co*-3HV) from *C. necator* than butyl acetate.

These results demonstrated that these non-halogenated solvents are a potential alternative to toxic halogenated solvents for the extraction of PHA in an eco-friendly manner along with high recovery yields. Moreover, these solvents systems are recyclable and reusable due to the substantial differences in the boiling temperature of extraction solvent and precipitant, which helps in reducing the downstream recovery cost[14]. Utilization of non-halogenated solvents will also minimize the toxic effects on both the worker and the environment. In literature, various non-halogenated solvents have been reported for extraction, but only a few have been investigated in detail. For example, Koller *et al.*[15] developed a new method for the extraction of short-chain-length poly(hydroxyalkanoates) from microbial biomass using "anti-solvent" acetone at elevated temperature and pressure in a closed system. This new strategy reported polymer purity of 98.4 % and extraction yield of 91.6 %, and was much faster than well-established chloroform extraction method. In a recent report, Jiang *et al.*[27] investigated the use of two non-chlorinated solvents, i.e., cyclohexanone and γ-butyrolactone, for the extraction of poly(3-hydroxybutyrate). The use of these extraction solvents at 120 ºC for 3 min resulted in recovery of 95 % of the PHB from the cells with a similar purity to that extracted by chloroform.

Poly(3HB-*co*-3HV) extraction by linear alkylbenzene sulfonic acid (LAS)

LAS is a biodegradable and environment-friendly anionic surfactant. It could be degraded easily under aerobic conditions[28,29]. Extraction of PHAs with LAS could potentially reduce or eradicate the usage of toxic organic solvents, which will help in developing a benign PHAs production technology. A small quantity of LAS could degrade non-PHAs material better than any other surfactant, facilitating the easy separation of PHAs from non-PHA materials[19].

Efficacy of LAS extraction process was studied at different temperatures and pH ranges under an incubation time of 2 h, and these optimization strategies were successful in improving the recovery yields. At lower temperature values (40 °C and 60 °C), polymer recovery yields and purities were very low (Figs. 3a to c). Upon increasing the temperature to 80 °C, the recovery yield of the polymer was enhanced. LAS gave a higher yield of 84 % at 80 °C, and the strategy used was easy and environmentally safe. Moreover, the purity of the poly(3HB-*co*-3HV) copolymer was observed to be around 90 %, which was lower than other methods. A pH of 5.0 was observed to be optimal for LAS-mediated extraction of PHAs with recovery yield of 84 % and purity of 90 % at 80 °C. However, recovery yield and purity decreased to 64 % and 77 %, respectively, with the increase in pH from 5 to 9. Decreasing the pH from 5 to 3 lowered the purity and recovery

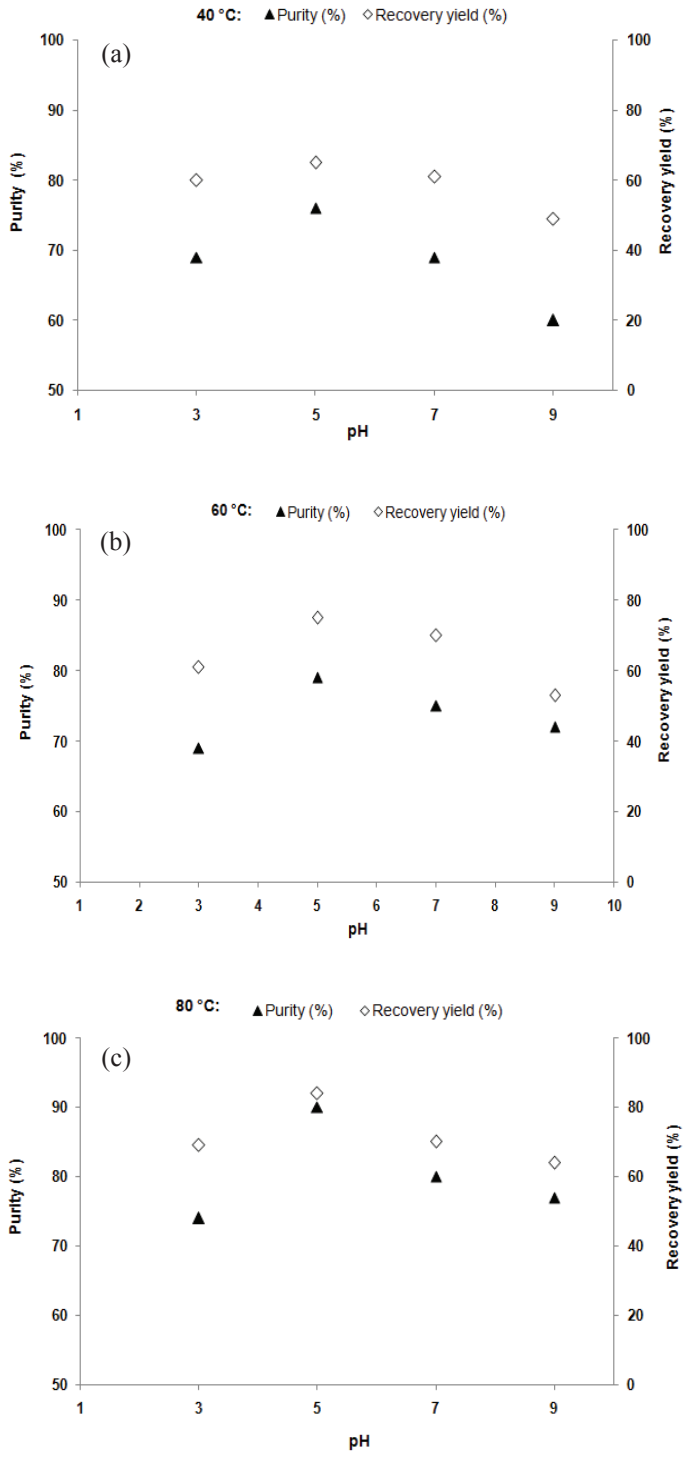

Fig. 3 – *Effect of different pH on purity and recovery yield (%) with LAS-99 extraction at a) 40 °C incubation temperature; b) 60 °C incubation temperature; c) 80 °C incubation temperature*

yields. The purity and recovery yield were decreased to 76 % and 65 %, respectively, with the decrease in temperature from 80 to 40 °C at pH 5. Thus, temperature and pH played a greater role in the high recovery separation of PHA from cellular materials via detergent or surfactant. Yang *et al.*[19] reported similar observation using LAS for the extraction of poly(3HB-*co*-3HV) from *Ralstonia eutropha* H16, with a recovery yield of 87 % and a purity of 86 %. However, their report did not conduct the analysis of molecular mass and thermal properties of PHAs, which could directly influence the quality and properties of the extracted polymer.

LAS is a promising surfactant for the extraction of PHAs since relatively small amounts of detergent were sufficient to recover a high purity polymer with high yields. Yields and purities of PHAs achieved by the surfactant extraction were also strongly dependent upon the intracellular PHA content. It was observed that LAS detergent efficiently extracted high yields of PHAs from cells accumulating great amounts of PHAs.

Characterization of extracted poly(3HB-*co*-3HV) copolymer

The recovered polymers were scanned by the FTIR spectrophotometer. Figs. 4a and b show the FTIR spectrum of poly(3HB-*co*-3HV) standard purchased from Sigma and the polymer extracted from *C. necator*, respectively. Both the samples exhibited strong stretching vibration near 3440 cm^{-1}, which is due to the hydroxyl (O–H) groups present in polymer. The characteristic band for poly(3HB-*co*-3HV) was observed near 1728 cm^{-1}, which is responsible for ester carbonyl (C = O) stretching vibration due to crystalline nature of poly(3HB-*co*-3HV). In addition, the absorption peaks at 1275 cm^{-1} and 1225 cm^{-1} are due to the C–O–C stretching vibration present in sample. The characteristic peak near 2974 cm^{-1} is due to the C–H..O hydrogen bond. The other prominent crystallinity band near 1178 cm^{-1} (C–O–C) helps in distinguishing types of PHAs. The poly(3HB-*co*-3HV) sample showed this band at 1178.51 cm^{-1}. The absorption peak at 1379 cm^{-1} is responsible for the CH_3 (methyl) group vibrations. All these absorption peaks confirm that the produced PHA is poly(3HB-*co*-3HV) copolyester. Bera *et al.*[30] also showed that purified poly(3HB-*co*-3HV) samples exhibit their adsorption peaks at 1728 cm^{-1}, 1277 cm^{-1}, and 1180 cm^{-1}. The similar poly(3HB-*co*-3HV) characteristics bands have also been recorded in earlier literature reports[5,31].

The efficiency of each extraction process was assessed by the recovery yield and purity, and thermo-physical properties, mainly, molecular weight, melting point (T_m), and thermal degradation temperature (T_d). The molecular weight (M_w) and polydispersity index (PDI) of the poly(3HB-*co*-3HV) obtained after chloroform extraction was 150,500 Da and 2.9, respectively. Extraction of PHAs using other solvents reported PDI values in the range of 2.2 to 3.8 showing slight non-uniformity or heterogeneity in molecular weights of copoly-

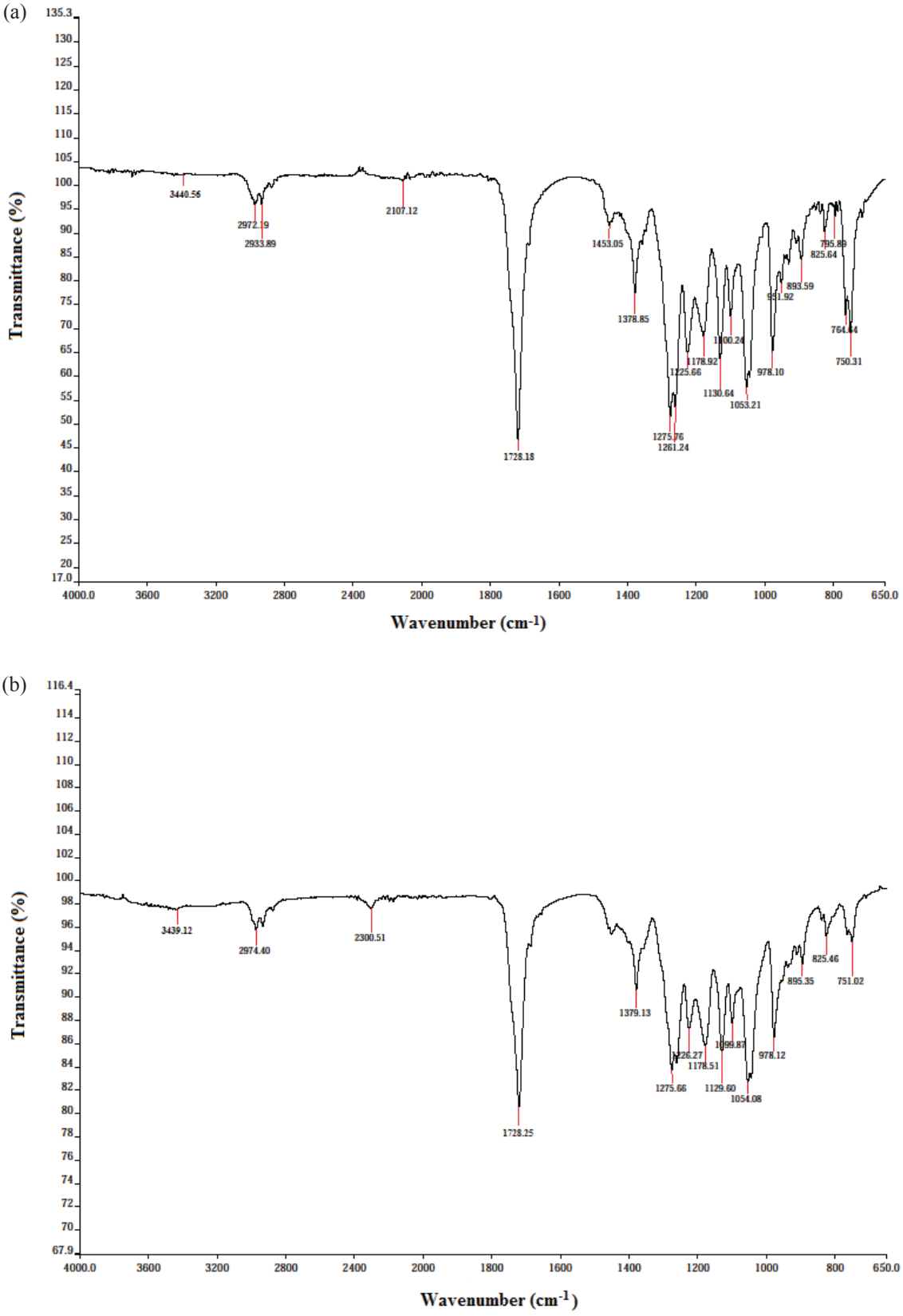

Fig. 4 – *FTIR spectra of a) standard poly(3HB-co-12 %-3HV), and b) copolymer extracted from C. necator*

Table 1 – *Physical and thermal properties of poly(3HB-co-3HV) using different extraction methods and its comparison with literature reports*

Extraction method	Production strain	Yield (%)	Purity (%)	M_w* (kDa)	PDI#	T_m (°C)	$T_{d,max}$ (°C)	3HV (%)	Reference
Chloroform	*Cupriavidus necator* DSM 545	95	97	150.50	2.9	156	292	24.6	Present study
1,2-Propylene carbonate		90	95	138.60	2.2	157	283		
Ethyl acetate		96	93	125.00	3.1	152	272		
Butyl acetate		84	95	109.50	3.4	155	279		
LAS		84	90	118.60	3.8	149	273		
Acetone	*Haloferax mediterranei*	91.6	98.4	1032	1.5	152.8	232	21.81	15
Cyclohexanone‡	*Cupriavidus necator* H16	95	97.2	211	2.36	–	–	–	27
1,2-Dichloroethane	Wastewater sludge	–	–	13.8	3.2	153.3	283.3	–	36
Dichloromethane	*Burkholderia cepacia* ATCC 17759	–	–	115	1.9	168.5	243.5	11.4	37
Chloroform	*Haloferax mediterranei*	75	–	253.00	2.7	138.8	280	10	38
Chloroform	*Pseudomonas oleovorans* NRRL B-14682	80	–	511	1.97	99	–	27	39
1,2–Dichloroethane	*Ralstonia eutropha* H16	81.2	–	–	–	150.2	315	16	40

*M_w = Weight average molecular weight; #PDI = Polydispersity index; T_m = Melting temperature; $T_{d,max}$ = Maximum thermal degradation temperature; ‡PHB extraction only; Dash symbol means values not mentioned

mers (Table 1). Extraction with 1,2-PC demonstrated a PDI value of 2.2 with a molecular weight of 138,600 Da. These results indicate that the 1,2-PC method can produce biopolymers with relatively lower dispersity, and that the molecular weight was comparable to the chloroform method. Butyl acetate and LAS showed molecular weight values of 109,500 Da and 118,600 Da, respectively, with high PDI values in comparison to chloroform. Overall, the low molecular weights in all cases could be due to the utilization of crude glycerol for copolymers production. Previous reports have also suggested that glycerol serves as a chain termination agent during polymerization resulting in a decrease in M_w[2,31–34]. Madden *et al.*[33] showed that glycerol binds covalently at the carboxyl end of the chain resulting in premature chain termination and low M_w.

The DSC thermogram reported melting temperatures (T_m) in the range of 149 to 157 °C for poly(3HB-*co*-3HV) using different extraction methods (Table 1). DSC analysis showed that melting temperatures were not significantly affected by the extraction method used. The DSC thermogram showed that the melting temperature of chloroform-extracted poly(3HB-*co*-3HV) copolymer was 156 °C. In contrast, 1,2-PC and butyl acetate exhibited melting temperatures of 157 °C and 155 °C, respectively. Even so, melting temperatures of ethyl acetate- and LAS-treated polymers were comparable to chloroform extraction. DSC endotherm of poly(3HB-*co*-3HV) showed two thermal peaks at 142 °C and 156 °C (Fig. 5). The two peaks were observed because of melting-recrystallization-remelting phenomenon during successive heating of poly(3HB-*co*-3HV). This phenomenon of two melting peaks of poly(3HB-*co*-3HV) was also observed by Gunaratne and Shanks[35] and Alsafadi and Mashaqbeh[5]. Both melting temperatures were lower than the melting point of the homopolymer poly(3-hydroxybutyrate), i.e., 175 °C. This highlights that the introduction of 3HV unit into PHB decreases the melting point of polymer and improves its elasticity, which ultimately increases its industrial usefulness. Similar results have also been reported by Cha *et al.*[36] under different environmental conditions for PHAs production with melting temperatures in the range of 146 to 153 °C.

TGA analysis showed the differences in maximum thermal degradation temperatures ($T_{d,max}$) of poly(3HB-*co*-3HV) ranging from 272 to 292 °C using different extraction methods. Highest thermal degradation temperatures of 292 °C and 283 °C were obtained for poly(3HB-*co*-3HV) from chloroform and 1,2-PC, respectively, which demonstrated that these copolymers were more thermally stable (Table 1). Butyl acetate, ethyl acetate, and LAS exhibited almost similar $T_{d,max}$ values within the range of 272 to 279 °C. Various other researchers have

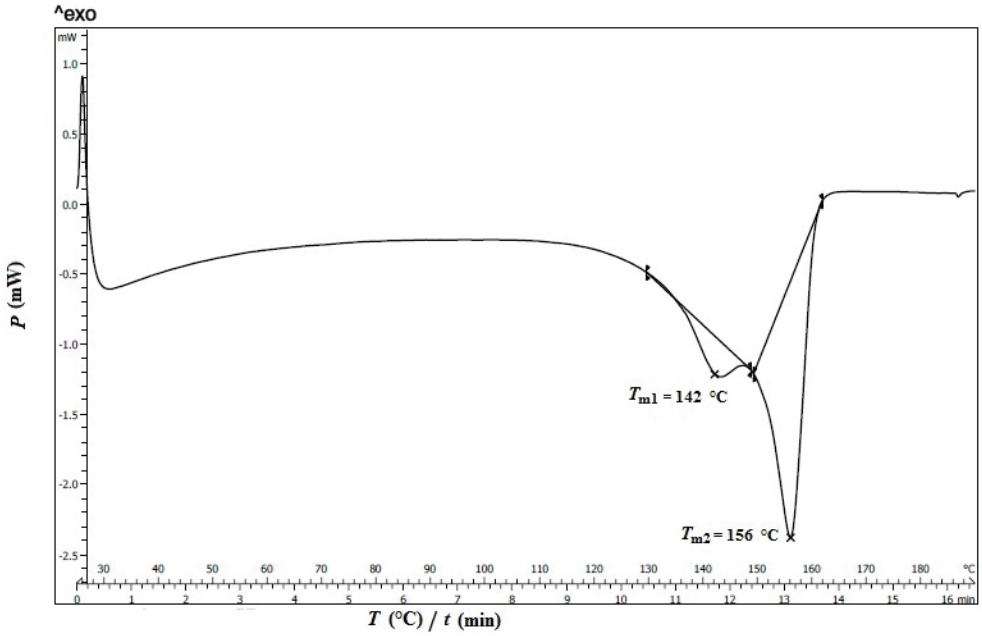

Fig. 5 – *DSC thermogram of poly(3HB-co-24.6 %-3HV) copolymer obtained by C. necator DSM 545*

also demonstrated $T_{d,max}$ values of 243.5 °C[36], 280 °C[37], and 283.3 °C[35] for poly(3HB-*co*-3HV), which were comparable to the present study. Table 1 lists the comparison of PHAs recovery yields and thermo-physical properties with the respective references[15,27,36–40]. The extraction methods affected the recovery yield and its molecular weight, but thermo-physical characteristics of the copolymers remained unaffected.

Removal of endotoxins from poly(3HB-*co*-3HV) copolymer

Among all tested alkalis, NaOH showed the best endotoxin removal efficiency. The amount of endotoxin present in PHAs before alkalis treatment was around $4 \cdot 10^7$ endotoxin unit (EU) g^{-1} of PHAs. After NaOH (0.1 N) treatment, it was reduced to 10^3 EU g^{-1} PHAs. The endotoxin removal efficiencies were drastically affected by change in digestion time and concentration. The endotoxin level could be decreased to 4.1 EU g^{-1} of PHAs when the digestion time was increased to 6 h (Fig. 6a). The purity of PHAs recovered was also increased to 95 % by increasing the digestion time. The endotoxin levels present in PHAs recovered with NaOH solutions of different concentrations are presented in Fig. 6b. Upon increasing NaOH concentration from 0.1 N to 2.0 N, there was a sudden decline in endotoxin level to 8.2 EU g^{-1} PHAs. When the NaOH concentration was further enhanced to 2.5 N, the endotoxin level present in PHAs was around 4.85 EU g^{-1} PHAs. Further increase in alkalis concentration showed no effect on endotoxin levels. According to

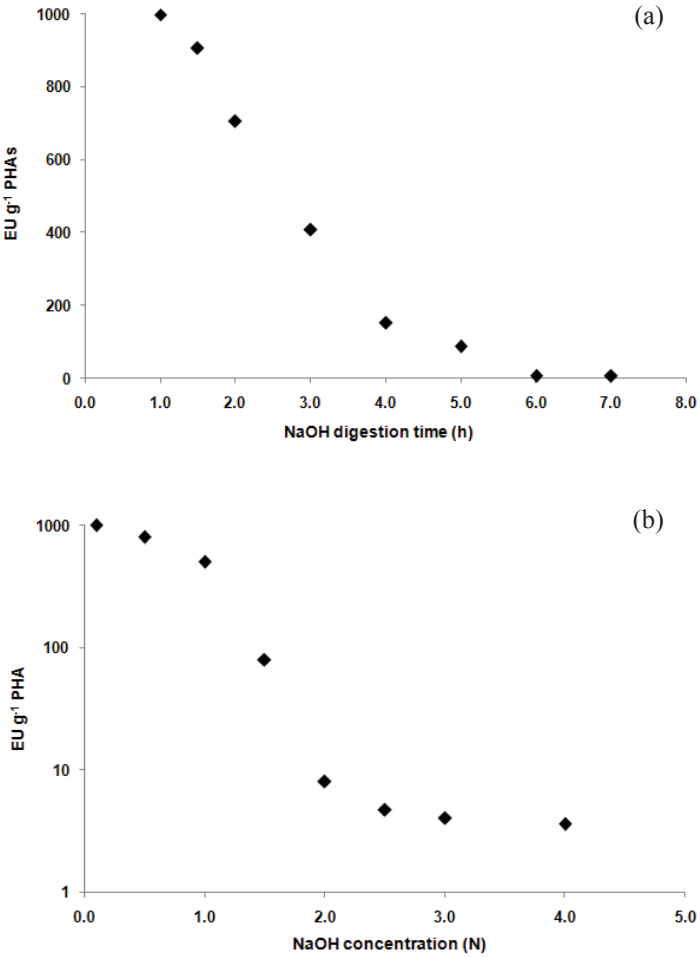

Fig. 6 – *a) Effect of NaOH digestion time (h) on endotoxin level (EU g^{-1}) present in extracted PHAs; b) Effect of different NaOH concentration (N) on endotoxin levels present in PHAs*

United States Pharmacopeia, the internationally allowed LPS limit for medical devices is 20 EU g^{-1} [41]. Thus, 2.5 N NaOH concentration and digestion time of 6 h were selected as optimized concentration for endotoxin removal. PHAs with lower endotoxin levels can be used for various biomedical applications.

Conclusion

Solvents 1,2-propylene carbonate and ethyl acetate can serve as effective PHAs recovery agents, and green alternative to highly toxic halogenated solvents. Among all solvents, highest recovery yield of 96 % and purity of 93 %, was obtained with ethyl acetate at an extraction temperature of 100 °C. Solvent 1,2-PC showed almost similar recovery yield (90 %) and purity (95 %), and resulted in high M_w PHAs of 138.6 kDa, but a low PDI value of 2.1. Recovery yield and purity on LAS and butyl acetate were slightly lower than the 1,2-propylene carbonate and ethyl acetate. The NaOH digestion method can be efficiently used at large-scale for the production of endotoxin-free PHAs for medical applications.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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