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En route to economical eco-friendly solvent system in enhancing sustainable recovery of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) copolymer

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Separation of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] from bacterial cell matter is a critical step in the downstream process with respect to material quality and eco-balance as P(3HB-co-4HB) is widely used for biomedical applications. Therefore, an efficient and eco-based extraction of P(3HB-co-4HB) using a combination of NaOH and Lysol in digesting the non-polymeric cell material (NPCM) digestion is developed. The NaOH and Lysol show synergistic influence on the copolymer extraction at a high purity and recovery of 97 and 98 wt% respectively. The optimized cell digestion method was found applicable to a vast batch of cells containing copolymers from various 4HB monomer compositions. At the largest extraction volume of 100 L, P(3HB-co-4HB) with a purity of 89 wt% was extracted with a maximum recovery of 90 wt%. The method developed has also eliminated the cell pretreatment step. The extraction method developed in this research has not only produced an economic and efficient copolymer recovery but has also retained the copolymer quality, in term of its molecular weight and thermal properties. It demonstrates a practical and promising downstream processing method in recovering the copolymer effectively from the bacterial biomass.

Keywords: Large-scale extraction / Lysol / Non-polymeric cell material (NPCM) digestion / Polyhydroxyalkanoates (PHAs) / P(3HB-co-4HB)

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1 Introduction

The P(3HB-co-4HB) is a short-chain-length polyhydroxyalkanoate (*scl*-PHA) which is like other PHAs, being produced under nutrient limited condition but with an excess carbon sources. It can be produced by various types of bacterial species such as *Alcaligenes latus*, *Comamonas acidovorans*, *Cupriavidus necator* and *Hydrogenophaga pseudoflava*, with the addition of 4HB precursors such as γ -butyrolactone, 1,4-butanediol, 1,6-hexanediol and 1,8-octanediol [1–4]. This 4HB-incorporated thermoplastic has been reported to be a promising biomaterial, either in

bio-pharmaceutical application as the excipient in drugs [5] or as wound dressing in medical application [6].

Being a potential biomaterial, the recovery process of P(3HB-co-4HB) is of critical concern. Generally, there are three approaches being used to isolate PHAs, namely solvent extraction, chemical digestion and selective enzymolysis. Among all, solvent extraction method is widely used since polymer with high purity can be recovered. In this method, PHA is dissolved either in organic solvents such as chloroform, 1,2-dichloroethane, combined with other chemicals such as methanol, ethanol, acetone, hexane or cyclic carbonate [7–10]. Although PHA with a high purity is extracted, solvent extraction method causes adverse effect on human's health and environment. Most of the solvents used are highly toxic. According to ICH Harmonized Tripartite Guideline for residual solvents, chloroform is categorized as one of Class 2 solvents. It is a non-genotoxic animal carcinogen but a possible causative agent of other irreversible toxicity such as neurotoxicity or teratogenicity [11]. Therefore, its usage should be limited in pharmaceutical processes. In this study, we develop a simple, economical and eco-based

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Abbreviations: NaOH, Sodium hydroxide; NPCM, Non-polymeric cell material digestion; PHA, Polyhydroxyalkanoate; P(3HB-co-4HB), Poly(3-hydroxybutyrate-co-4-hydroxybutyrate); RSM, Response surface methodology

NPCM method to recover the biodegradable P(3HB-*co*-4HB) from *Cupriavidus* sp. USMAA1020. The digestion of NPCM was investigated using a combination of NaOH and Lysol. The main aim of the study is to obtain the optimal purity and recovery yield of P(3HB-*co*-4HB) without disregarding the vision to minimize the digestion agent consumption, which consequently reduces the overall PHA downstream processing cost.

2 Materials and methods

2.1 Production of PHA-containing biomass

The local isolate *Cupriavidus* sp. USMAA1020 (DSM 19416) was used in this study [12]. Biomass containing P(3HB-*co*-13 mol% 4HB) was obtained from fed-batch fermentation in 30 L bioreactor (Sartorius Biostat C plus, Germany) using oleic acid and γ -butyrolactone as carbon sources. Precultured cells (0.1 g/L) were transferred into 20 L of mineral salts medium (MSM) as formulated according to [1]. The fermentation process was carried out at 30°C using the agitation of 250 rpm, aeration of 1 vvm. DO-stat fed-batch fermentation strategy was employed whereby the carbon and nitrogen source were fed upon the increase of DO value. Two batches of biomass with similar P(3HB-*co*-4HB) content of 70 wt% but different 4HB monomer composition (5 mol% and 35 mol%) were obtained from batch fermentation using substrate mixtures as reported earlier [4].

2.2 P(3HB-*co*-4HB) freeze-dried cells

After cultivation, the cells were harvested by centrifugation at 10 000 rpm for 10 min at 10°C using Avanti® J-E Centrifuge (Beckman Coulter, USA) with JA-10 rotor. The centrifuged cells were washed with distilled water and kept in storage bottle which was then frozen at −20°C. The frozen cells were then freeze-dried using Labconco FreeZone freeze dry system (Labconco Corp., USA) for 1–3 days to obtain the lyophilized cells.

2.3 P(3HB-*co*-4HB) recovery using chloroform extraction

Lyophilized cells containing the P(3HB-*co*-4HB) copolymers were stirred in chloroform (1 g: 200 mL) for 24 hours. The solution was filtered using Whatman filter paper to separate cell debris. The chloroform containing the dissolved copolymer was concentrated using rotary evaporator (Eyela, Japan) to approximately 15 mL and poured dropwise into 150 mL of rapidly stirred chilled methanol. Precipitated copolymers were recovered by filtration using 0.45 μ m PTFE membrane filter and air dried at room temperature.

2.4 P(3HB-*co*-4HB) recovery using various surfactant and NaOH

The recovery of P(3HB-*co*-4HB) was carried out using NaOH and surfactants at varying parameters. This involves the

Figure 1. Effect of different surfactants and concentration on P(3HB-*co*-13 mol% 4HB) (A) recovery and (B) purity.

Figure 2. Effect of different NaOH concentrations on recovery of P(3HB-co-13 mol% 4HB).

screening of various surfactants such as linear alkylbenzene sulfonate (LAS), Lysol, cetyltrimethylammonium bromide (CTAB), and Triton X-100 at various concentration (0–10 % v/v); concentration of NaOH (0–1.0 M); digestion time (0–6 h); combination effect of the chosen surfactant and NaOH; and effect of various digestion routes extraction to copolymer purification. The screening was carried out in small-scale volume. Approximately 10 g/L were weighed and transferred into a conical flask containing 25 mL distilled water and incubated for 3 h at 250 rpm, with or without pretreatment step of NaCl (8 g/L). This is followed by copolymer extraction step with the addition of surfactants, NaOH at various concentrations, incubated for an hour at the agitation of 250 rpm. The solution was centrifuged at $12\,000 \times g$ for 15 min and washed with distilled water. For purification step, the pellets were polished with 20% ethanol for 3 h at 250 rpm. Upon polishing, samples were centrifuged again at $12\,000 \times g$, washed for three times and freeze-dried. The extracted P(3HB-co-4HB) was evaluated by calculating the purity and recovery using the following equation:

$$\%Purity = \frac{pha(g)}{biomass(g)} \times 100 [w/w\%] \quad (1)$$

$$\%Recovery = \frac{pha(g)}{\sum pha(g)} \times 100 [w/w\%] \quad (2)$$

$$\%NPCM = 100\% - \%Purity \quad (3)$$

2.5 Optimization and large scale recovery of P(3HB-co-4HB)

The optimization of NPCM digestion to extract P(3HB-co-4HB) was carried out with the aid of response surface method (RSM), using Design-Expert® software 7.1.6 (Stat-Ease Inc.). Three parameters with their respective ranges were chosen for the optimization: concentration of NaOH (0.05–0.2 M), concentration of Lysol (1–3 % v/v) and incubation period (0.5–1 h). A total of 20 experimental runs were carried out. The purity of the extracted copolymers was recorded for statistical evaluation. The verification of the optimized model of P(3HB-co-4HB) extraction was

done by performing and comparing the recovery performance before and after optimization.

To study the effect of 4HB monomer composition on P(3HB-co-4HB) recovery performance, the optimized condition was applied in two different fermentation batches. The recovery process was conducted at the end of fermentation process in 5 L benchtop bioreactor (Sartorius Biostat B plus, Germany), with an agitation speed of 800 rpm. The digested cells were then centrifuged, washed and polished using 20% ethanol for 3 h in the same bioreactor. The recovery was evaluated by determining the percentage of purity, percentage of recovery and molecular weight of recovered polymers. Similar procedures were applied to the large scale recovery of P(3HB-co-4HB) in 100 L fermentation. The recovery performance was evaluated by comparing the purity, recovery, molecular weight, thermal and mechanical properties of the conventional chloroform extracted copolymer.

Figure 3. Recovery of P(3HB-co-13 mol% 4HB) using NaOH (0.1 M) with Lysol (5%, v/v). Recovery by chloroform was used as a control.

Figure 4. The recovery routes of P(3HB-co-13 mol% 4HB) by NaOH and Lysol.

Figure 5. Effect of different digestion routes on the extracted PHA. Condition 0: Initial cell sample containing 45.64 ± 0.62 wt% P(3HB-co-13 mol% 4HB). Condition 1: Treated with water. Condition 2: Pre-treated with NaCl and extracted with 0.1 M NaOH. Condition 3: Pre-treated with NaCl and extracted with 5% Lysol. Condition 4: Extracted with 0.1 M NaOH then with 5% Lysol. Condition 5: Extracted with 5% Lysol then with 0.1 M NaOH. Condition 6: Pre-treated with NaCl, then extracted with 0.1 M NaOH then with 5% Lysol. Condition 7: Pre-treated with NaCl then extracted with 5% Lysol then with 0.1 M NaOH. Condition 8: Extracted with 0.1 M NaOH and 5% Lysol. Condition 9: Pre-treated with NaCl then extracted with 0.1 M NaOH and 5% Lysol. The highest significant average values are denoted with (A) or (a) and the lower average values symbolized by the following alphabet. Bar that was labeled with the same alphabet showed no significant difference at $p = 0.05$ by Tukey test (HSD).

Table 1. Optimization of P(3HB-co-13 mol% 4HB) recovery NPCM digestion method

Run	X_1 : Concentration of NaOH (M)	X_2 : Lysol (v/v %)	X_3 : Digestion Time (h)	Purity (wt%)	
				Actual value	Predicted value
3	0.05	1.00	0.50	81.2	75.38
4	0.20	1.00	0.50	88.23	88.76
14	0.05	3.00	0.50	84.04	83.96
11	0.20	3.00	0.50	91.66	91.16
5	0.05	1.00	1.00	74.46	74.60
1	0.20	1.00	1.00	90.72	90.44
7	0.05	3.00	1.00	85.77	84.88
8	0.20	3.00	1.00	89.08	94.54
18	-0.00	2.00	0.75	67.45	71.23
10	0.25	2.00	0.75	93.88	90.61
13	0.13	0.32	0.75	77.78	80.84
16	0.13	3.68	0.75	94.06	91.51
20	0.13	2.00	0.33	85.74	89.06
9	0.13	2.00	1.17	94.06	91.25
17	0.13	2.00	0.75	89.25	90.43
12	0.13	2.00	0.75	92.69	90.43
15	0.13	2.00	0.75	91.95	90.43
6	0.13	2.00	0.75	84.49	90.43
2	0.13	2.00	0.75	92.74	90.43
19	0.13	2.00	0.75	91.54	90.43

2.6 Analytical procedures

The PHA content and polymer composition of lyophilized cells were determined using gas chromatography (Shimadzu auto-injector GC-2010, Kyoto, Japan) analyses. Approximately 15 mg of the freeze-dried cells were subjected to methanolysis in the presence of methanol and sulfuric acid [85%:15% (v/v)]. The reaction mixture was incubated at 100°C for 3 h to convert monomer units to their corresponding methyl esters. Distilled water (1 mL) was added to the cooled mixture and vortexed to enhance phase separation. The lower organic phase contain-

ing the reaction products was separated, dried over anhydrous Na_2SO_4 , and analysed by GC.

The molecular weight of polyesters was analyzed by gel permeation chromatography (GPC) using a Shimadzu LC-9A system equipped with a refractive index detector (RID-10A) and a PLgel Mixed C column (Polymer Laboratories, Ltd., UK). Chloroform was used as the eluent at a flow rate of 1.0 mL/min. The sample concentrations and injection volumes were 1.0 mg/mL and 10 μL respectively. The operating temperature was maintained at 35°C. Polystyrene standards with a low polydispersity were used to construct a

Table 2. Analysis of variance (ANOVA) of P(3HB-co-13 mol% 4HB) recovery process

Source	Sum of squares	df	Mean square	F Value	> p-value Prob > F	
Model	804.51	9	89.39	5.09	0.0090	significant
X_1 -NaOH	453.17	1	453.17	25.80	0.0005	
X_2 -Lysol	137.41	1	137.41	7.82	0.0189	
X_3 -Digestion Time	5.79	1	5.79	0.33	0.5785	
X_1X_2	19.10	1	19.10	1.09	0.3216	
X_1X_3	3.03	1	3.03	0.17	0.6869	not sig.
X_2X_3	1.45	1	1.45	0.082	0.7801	
X_1^2	162.91	1	162.91	9.28	0.0123	
X_2^2	32.61	1	32.61	1.86	0.2029	
X_3^2	0.14	1	0.14	7.736E-003	0.9316	
Residual	175.64	10	17.56			
Lack of Fit	124.98	5	25.00	2.47	0.1721	
Cor Total	980.15	19	10.13			
Std. Dev.	4.19		R^2		0.8208	
Mean	87.04		Adj R^2		0.6595	
C.V. %	4.82		Pred R^2		-0.0798	
PRESS	1058.36		Adeq Precision		7.867	

calibration curve. Number-average molecular weight (M_n), weight-average molecular weight (M_w) and polydispersity index (M_w/M_n) were calculated using Mark-Houwink equation. The Mark-Houwink constant for polystyrene in chloroform was taken as $K = 0.001$ mL/mg and $\alpha = 0.73$, whereas those for P(3HB) in chloroform were taken as $K = 0.016$ mL/mg and $\alpha = 0.76$ [13].

The thermal properties of the recovered polymers were determined using Pyris 1 differential scanning calorimetry (DSC) (Perkin Elmer, US). Samples (5–10 mg) were encapsulated in aluminium pans and heated, quenched, reheated in the temperature range of -50 to 200°C . The heating rate was $10^\circ\text{C}/\text{min}$, whereas the cooling rate was $20^\circ\text{C}/\text{min}$. The midpoint of the heat capacity change was taken as the glass transition temperature (T_g) and the DSC endotherm were determined for the melting temperature (T_m) and the enthalpy of fusion (ΔH_m). The decomposition temperature (T_d) was determined by heating approximately 10 mg of copolymer samples in nitrogen atmosphere at a rate of $10^\circ\text{C}/\text{min}$ from 30 to 80°C , using TGA/SDTA 851 thermobalance with STARe thermal analysis software (Metler Toledo, Switzerland). The T_d was defined at temperature for 5% weight loss of the sample.

The tensile test was carried out using the Universal Testing Machine (GOTECH A1-3000 with U60 software, Taiwan). Films were cut into dumbbell shapes (4 mm with 75 mm length) using steel ASTM regulation punches. Mechanical tensile data were calculated from the stress-strain curves on an average of four samples.

3 Results and discussion

3.1 Effect of surfactants and concentrations on P(3HB-co-4HB) recovery

Thus far, PHA extraction using NPCM digestion are employed on lyophilized cell with high PHA content such as 75 wt% [14], 80 wt% [15] and 87 wt% [16]. This condition will assist in the overall PHA recovery performance since there is only a small percentage of NPCM needed to be digested. In other words, this method could be less effective when it is used to extract PHA from the cells with lower PHA content (higher NPCM). In this research, the lyophilized cells containing low PHA content of 50 wt% P(3HB-co-13 mol% 4HB) were used in the screening of various parameters on the extraction using NPCM digestion method. Figure 1 shows the recovery and purity of P(3HB-co-13 mol% 4HB) using different surfactants and concentrations. Based on the results, Lysol was the most effective surfactant in digesting the NPCM. The effectiveness of surfactants can be arranged in the following order: Lysol > CTAB > Triton X-100 > LAS. Among all the surfactants tested, the highest recovery and purity was obtained by the use of Lysol at the concentration of 5% (v/v). Lysol demonstrated an outstanding effect as a sole digestion agent. At a concentration of 5% (v/v), the extracted copolymer has the highest purity and recovery of 85 and 95 wt%. This indicates that Lysol digested 35 wt% of the cells. This is the first study reporting the use of Lysol to recover PHA.

Figure 6. The 3D response surface model of purity based on the influence of variation of (A) Lysol concentration and NaOH concentration at digestion time of 0.75 h, (B) digestion time and NaOH concentration at 2% Lysol and (C) digestion time and Lysol concentration at 0.13 M NaOH.

Table 3. Purity of P(3HB-*co*-13 mol% 4HB) before optimization, after optimization and validation of the optimized condition

Condition	X ₁ : Concentration of lysol (v/v%)	X ₂ : Concentration of NaOH (M)	X ₃ : Digestion time (h)	Purity (wt%)
Without optimization	5	0.1	1	97
Optimized	2	0.15	1.14	93
Validation of optimization	2	0.15	1.14	94

Table 4. Molecular weight of P(3HB-*co*-4HB) by extraction using chloroform and cell digestion method

Copolymers	Optimized cell digestion				Chloroform extraction	
	Purity (wt%)	Recovery (wt%)	Molecular weight, kDa (M_w)	PDI (M_w/M_n)	Molecular weight, kDa (M_w)	PDI (M_w/M_n)
P(3HB- <i>co</i> -5 mol% 4HB)	89 ± 1	90 ± 1	380 ± 0.1	2.9 ± 0.1	490 ± 0.2	2.5 ± 0.6
P(3HB- <i>co</i> -35 mol% 4HB)	89 ± 1	87 ± 1	770 ± 0.0	3.0 ± 0.1	830 ± 0.1	3.2 ± 0.6

The effectiveness of Lysol as a strong digestion agent could be explained by the presence of p-chlorochresol, a phenolic compound. This active ingredient inactivates the intra cytoplasm enzymes on the cell membranes by forming unstable complexes [17]. The lipophilic molecules were trapped by the phospholipid on the membranes which subsequently alter the cell wall permeability of the bacterial cell.

3.2 Synergistic effect of NaOH and Lysol on P(3HB-*co*-4HB) recovery via various digestion routes

Sodium hydroxide (NaOH) exhibits a strong capability to digest the NPCM and release PHA. This is due to the presence of OH[−] ions which saponify the lipids components in the membrane which lead to severe structural destruction [17]. In this study, NaOH was chosen as it is not only the most superior digestion agent, but it is also beneficial to the overall extraction process in terms of price, reduced toxicity and the ease of handling. Prior to this, the effect of NaOH concentrations on the extraction of P(3HB-*co*-13 mol% 4HB) was carried out (Fig. 2). Based on the results, it can be deduced that an increase in NaOH concentration decreases the percentage of recovery. At a NaOH concentration of higher than 0.1 M, the copolymer started to degrade. This could be due to hydrolyzes of PHA into water soluble monomer in high concentration of NaOH as previously reported [18]. Therefore, extraction using 0.1 M NaOH was chosen for further analysis as this concentration resulted in the highest recovery and purity of 92 and 74 wt% respectively.

Figure 3 shows the P(3HB-*co*-13 mol% 4HB) extraction via NPCM digestion using a combination of 0.1 M NaOH with 5% (v/v) Lysol. The copolymer recovery using chloroform extraction was used as control for comparison. Interestingly, this combination shows a synergistic effect in digesting the NPCM, which resulted in P(3HB-*co*-13 mol% 4HB) with high purity and recovery of 97 and 98 wt% respectively. Further investigations

were carried out to identify the route with most efficient P(3HB-*co*-4HB) recovery and purity. This is done by manipulating the cell pretreatment and digestion step of the extraction process as shown in Fig. 4. The effect of the different digestion routes on the extracted PHA is shown in Fig. 5. Interestingly, it was found that route 8 using 0.1 M NaOH with 5% (v/v) Lysol, without any cell pretreatment using NaCl has resulted in the highest purity (98 wt%) and recovery (100 wt%). The elimination of NaCl in the recovery process would help in overall recovery operation and cost as compared to previous studies which essentially needed an additional step of cell pretreatment by chemicals such as NaCl, H₂O₂, and SDS to enhance the recovery performance [19–21].

3.3 Optimization of P(3HB-*co*-4HB) recovery using RSM

Table 1 shows the RSM based optimization of P(3HB-*co*-4HB) recovery, considering the factors such as the Lysol concentration, NaOH concentration and digestion time. The optimization is still in need although a high recovery was already achieved as reported. This would provide an understanding on the interaction effect between each parameters on the purity of recovered P(3HB-*co*-4HB). The optimization model for P(3HB-*co*-4HB) recovery using NPCM digestion by combination of Lysol

Table 5. Large scale recovery of P(3HB-*co*-8 mol% 4HB) in 70 L fermentation using cell digestion and chloroform extraction method

Properties		Optimized cell digestion	Chloroform extraction
Purity (wt%)		89 ± 1	–
Recovery (wt%)		90 ± 1	–
Thermal	T_g (°C)	−7.0 ± 0.1	−6.4 ± 0
	T_m (°C)	150.6 ± 1.3	155.5 ± 0.2
	T_d (°C)	265 ± 0	260 ± 0

Figure 7. Transmission electron micrograph of P(3HB-co-8 mol% 4HB) after cell digestion of 70 L fermentation (8000 × magnification).

and NaOH was expressed in the regression equation as shown below:

$$\begin{aligned} \text{Purity} = & 90.43 + 5.76 X_1 + 3.17 X_2 + 0.65 X_3 - 1.54 X_1 X_2 \\ & + 0.62 X_1 X_3 + 0.43 X_2 X_3 - 3.36 X_1^2 - 1.50 X_2^2 \\ & - 0.097 X_3^2 \end{aligned}$$

Both Lysol and NaOH concentration demonstrate a significant interaction towards the purity of recovered P(3HB-co-4HB). The selected quadratic model was further analysed by analysis of variance (ANOVA) to verify the significance of the model as reported by [22]. Table 2 shows that the model's *F* value of 5.09 implies that the model is significant. The *p* value indicates that the concentration of NaOH (X_1) and Lysol (X_2) have significant effect on the purity (%) of the extracted P(3HB-co-13 mol% 4HB). A negative predicted *R*-squared implies that the overall mean is a better predictor of the purity of PHA response than the current model. The model can be applied to navigate the design space.

Figure 6 shows the response surface curve plotted to determine the interaction of NaOH concentration, Lysol concentration and incubation time towards the purity of the recovered P(3HB-co-13 mol% 4HB). Figure 6(a) shows the extracted P(3HB-co-13 mol% 4HB) purity based on the variation of Lysol concentration and NaOH molarities with the digestion time fixed at 0.75 h. It demonstrates the combination effect of NaOH concentration with Lysol concentration where at any given NaOH concentration from 0.05 to 0.2 M, an increased in amount of Lysol concentration from 1 to 3% (v/v) led to higher purity of extracted P(3HB-co-13 mol% 4HB).

This could be attributed to the optimum amount of Lysol which allows to dissolve cell membrane and denatures the protein released [17]. Based on the RSM, a regression equation generated described the extracted P(3HB-co-13 mol% 4HB) in coded unit as specified in the following equation:

$$\begin{aligned} \text{Purity} = & 90.43 + 5.76 X_1 + 3.17 X_2 + 0.65 X_3 - 1.54 X_1 X_2 \\ & + 0.62 X_1 X_3 + 0.43 X_2 X_3 - 3.36 X_1^2 - 1.50 X_2^2 \\ & - 0.097 X_3^2 \end{aligned}$$

The final predicted optimal values of these three variables (NaOH (0.15 M), Lysol (2% (v/v)) and digestion time (1.14 h)

were calculated based on the equation with the predicted maximum purity (93 wt%) of P(3HB-co-13 mol% 4HB).

The verification of the optimization model was carried out to compare the extraction condition (Table 3). It was found out that the amount of Lysol was greatly reduced to 2% (v/v) (a reduction of 2.5-fold) with a slight increase of NaOH concentration (0.15 M) and incubation time (1.14 h). The RSM based optimization successfully predicted the optimum amount of NaOH and Lysol concentration which resulted a considerably high purity of extracted P(3HB-co-4HB).

3.4 Bioreactor scale recovery of P(3HB-co-4HB) using optimized cell digestion method

The optimized cell digestion method was applied in extracting polymer harvested in the bioreactor scale (100 L). Two batches of fermentation which resulted in a similar PHA content (70 wt%) but different 4HB monomer compositions (5 and 35 mol% 4HB) was used in this study. Table 4 shows the comparison on the purity, recovery and molecular weight of the extracted copolymer using optimized cell digestion and chloroform extraction method. Generally, 4HB monomer composition does not affect the extraction using NPCM digestion. Both recovered copolymers possessed similar purity (89 wt%) and recovery (90 and 87 wt%). According to the result, we can also conclude that there was only a slight decrease on the molecular weight of the extracted copolymers from optimized cell digestion method (380 and 770 kDa), as compared to copolymers obtained from the chloroform extraction method (490 and 830 kDa). The optimized cell digestion method was found able to retain the molecular weight of the copolymer extracted from the cells. Table 5 shows the large scale recovery of the fermentation containing P(3HB-co-4HB). A 100 L scale fermentation with biomass containing 70 wt% of P(3HB-co-8 mol% 4HB) was extracted using optimized cell digestion method at a agitation speed of 400 rpm. In order to ensure an industrial feasibility of the recovery process, the polishing steps of the digested cells were avoided in the large scale recovery to simplify the operation. Figure 7 shows the physical differences observed between the dried cells obtained

from the fermentation and the recovered copolymer using optimized cell digestion method. Despite removing the polishing step, high purity and recovery of 89 and 90 wt% of the recovered copolymer was still obtained. It is of utmost importance that the P(3HB-co-4HB) extracted should have high purity as it will be used in biomedical application.

4 Concluding remarks

This extraction route is ecofriendly and economical as it utilizes green solvents and it requires no pre-treatment step, no organic solvents, and no heating process during cell digestion. Furthermore, this method has shown to be time efficient since it took only about an hour for the cell digestion. It can also be prepared by direct centrifugation for a larger scale P(3HB-co-4HB) production and extraction. Apart from that, the purity of extracted P(3HB-co-4HB) will be more than 93% and can be recovered up to 94% considering low P(3HB-co-4HB) content in the bacterial cells. As an overall conclusion, this research finding has demonstrated a scalable, reproducible and easy-to-implement method for extracting biopolymer with high purity for biomedical applications.

Practical application

Poly(3-hydroxybutyrate-co-4-hydroxybutyrate) [P(3HB-co-4HB)] is intracellular material accumulated by several bacteria. Commercial production of P(3HB-co-4HB) faces the issue of high production and recovery/separation cost, as well as the extensive use of chloroform (1 g of dried cells stirred in 200 mL chloroform) which are detrimental to the environment. Therefore, development of alternative method for PHA recovery will contribute a significant effect in reducing both production cost and impact on environment. The present invention refers to a potential industrial-feasible process, which uses a combination of NaOH and surfactant (Lysol) to recover P(3HB-co-4HB) from bacterial biomass. The alternative solvents and amount being used are unaggressive to the environment and it allows obtaining P(3HB-co-4HB) of high purity and high molecular weight at the same time. This eco-based recovery system is efficient and safe as P(3HB-co-4HB) is widely used for biomedical applications.

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