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Original Article

Optimization of poly(3-hydroxybutyrate) extraction from *Cupriavidus necator* DSM 545 using sodium dodecyl sulfate and sodium hypochlorite

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ABSTRACT

Poly (3-hydroxybutyrate) (P(3HB)), a type of bioplastic, has been attractive as an alternative source to replace petroleum-based plastics. Generally, P(3HB) is produced from *Cupriavidus necator* DSM 545 under some nutrient-limiting and oxygen-limiting conditions. In this work, the batch production of P(3HB) was conducted in shake flask culture at 30 °C and 250 revolutions per minute. The results showed that the concentration of biomass, the P(3HB) yields from substrate and biomass, the volumetric production rate of P(3HB) and the concentration of P(3HB) were maximized at 40 hr of cultivation (5.845 ± 0.375 g/L, 0.128 ± 0.006 g/g, 0.229 ± 0.030 g/g, 0.031 ± 0.001 g/L hr and 1.335 ± 0.087 g/L, respectively). As P(3HB) is accumulated intracellularly in the bacterial cells, P(3HB) recovery is an essential process for industrial P(3HB) production. This work focused on the optimization of P(3HB) extraction using sodium dodecyl sulfate (SDS) and sodium hypochlorite (NaOCl) in an experiment designed using the Taguchi method. The results showed that the optimal conditions for the maximum P(3HB) recovery of 78.70% were 0.5% w/v SDS consecutively combined with 6% v/v NaOCl, while the concentration of sodium hypochlorite had a greater effect on P(3HB) recovery compared to SDS. The P(3HB) optimally extracted was analyzed using Fourier-transform infrared spectrophotometry and differential scanning calorimetry to investigate structural and thermal properties, which were compared to those of commercial P(3HB). The findings could be useful for scaling-up industrial P(3HB) recovery.

Introduction

Poly (3-hydroxybutyrate) (P(3HB)), one of the polyhydroxyalkanoate (PHA) family, can be produced using various bacterial strains such as *Ralstonia eutrophus* (Brandl et al., 1989), *Bacillus megaterium* (Findlay and White, 1983) and *Alcaligenes eutrophus* (Doi et al., 1988). Generally, the P(3HB) content is accumulated inside the bacterial cells as intracellular granules formed as reserved carbon and energy sources. The abundant carbon sources are influenced by limited nutrients (nitrogen or phosphorus) and especially by limiting oxygen condition which has been reported as an appropriate condition to promote high P(3HB) yields (Reddy et al., 2003). The mechanical properties of P(3HB) products are practically similar to those of traditional thermoplastics made from polypropylene (Matavulj and Molitoris, 1992; Oliveira et al., 2007). Moreover, P(3HB) can be completely degraded naturally by bacterial activity under aerobic or anaerobic conditions or both, resulting in the discharge of water and carbon dioxide, and methane and carbon dioxide, respectively, with these advantages implying that P(3HB) could be promisingly used as a biodegradable plastic to substitute petrochemical-based plastic materials, because there is no toxic effect on the environment, as well as the water and carbon dioxide derived from the P(3HB) degradation process being available for plant growth (Kalia et al., 2000; Verlinden et al., 2007).

However, the production of P(3HB) commercially still involves a high manufacturing cost compared to production using petrochemical-based plastic materials (Kahar et al., 2004; Oliveira

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et al., 2007), due to the high cost of the downstream process, particularly the extraction and recovery processing stages. Thus, developing a low-cost P(3HB) manufacturing process has attracted serious investigation. The use of inexpensive or renewable resources or both (for example, agro-industrial wastes and lignocellulosic materials) as substrates for bacterial growth and P(3HB) production are regarded as one alternative process for economical P(3HB) production (Kim, 2000; Gouda et al., 2001; Kahar et al., 2004). Clearly, the extraction and recovery processes should be considered for possible cost reduction. As reported previously, high purity of P(3HB) from bacterial cells extracted using sodium hypochlorite (NaOCl) or boiling chloroform or both is feasible (Kumar et al., 2004; Heinrich et al., 2012; Patjawit et al., 2014; Alarfaj et al., 2015), having advantages of higher purity and considerably less expense industrially (Heinrich et al., 2012). The mechanism of P(3HB) extracted using sodium hypochlorite/chloroform involves initially the cell membrane being denatured by the sodium hypochlorite concurrently with digested non-P(3HB) elements, and subsequently being dissolved in chloroform, resulting in high P(3HB) recovery and purity (Jacquel et al., 2008). Accordingly, the highest P(3HB) recovery was 91% (Hahn et al., 1994). Unfortunately, chloroform has been reported to be carcinogenic in animals (World Health Organization, 1980). Additionally, it can evaporate quickly when exposed to air, dissolves easily in water, and its breakdown products include phosgene and hydrogen chloride that is also toxic to the environment (Agency for Toxic Substances and Disease Registry, 1997). Therefore, a surfactant such as sodium dodecyl sulfate (SDS) is an alternative that is substituted for chloroform.

SDS has been reported to be effective in P(3HB) extraction (Ramsay et al., 1990; Chen et al., 1999). As the bacterial cell membrane is efficiently broken by SDS, the P(3HB) accumulates without further destruction while dissolving impurities (Kim et al., 2003). Importantly, SDS is readily biodegradable under aerobic and anaerobic conditions, and all carbons in the molecule are derived from a plant source rather than a non-renewable source (Bondi et al., 2015). Sodium hypochlorite is not bioaccumulative, rapidly degraded and does not persist in the environment (Pişkin and Türkün, 1995). Mostly, SDS has been combined favorably with sodium hypochlorite to produce high recovery and purity of P(3HB) extracted from *Azotobacter chroococcum* G-3, *Alcaligenes eutrophus* (Choi and Lee, 1997; Dong and Sun, 2000; Ramsay et al., 1990b), which lowered the P(3HB) production cost. Furthermore, P(3HB) digested from *Cupriavidus necator* (formerly known as *Wautersia eutropha*, *Ralstonia eutropha* and *Alcaligenes eutrophus* (Kunasundari and Sudesh, 2011)) with surfactant-hypochlorite involved a lower cost than with hypochlorite/chloroform (Jacquel et al., 2008).

As the production cost of P(3HB) relies on optimal extraction, the P(3HB) from *C. necator* DSM 545 extracted using SDS/sodium hypochlorite is quite limited in practice. The present work mainly focused on optimizing P(3HB) extraction from *C. necator* DSM 545 using a combination of SDS and sodium hypochlorite. Therefore, orthogonal arrays of Taguchi method, that is DOE, which is highly recommended for the cost reduction process (Idris et al., 2002; Hanley et al., 2011; Ansari and Goodarznia, 2012), was used for varying the concentration of two factors (SDS/sodium hypochlorite). The experiments were statistically minimized and the observations characterized using analysis of variance to determine the optimal conditions for maximum P(3HB) recovery and purity.

Materials and methods

Bacterial strains and inoculums preparation

Cupriavidus necator DSM 545—formerly known as *Wautersia eutropha*, *Ralstonia eutropha*, *Alcaligenes eutrophus* or *Hydrogenomonas eutropha* (Ienczak et al., 2013) —was prepared as an inoculum for P(3HB) production. The *C. necator* DSM 545 was grown on nutrient agar (NA) at 30 °C for 24–48 hr. Two loops of *C. necator* DSM 545 cells were then inoculated into 5 mL of nutrient broth (NB) and incubated aerobically on a rotary shaker at 250 rpm and 30 °C for 18 hr. Later, the obtainable inoculum was scaled up by transferring into a 250 mL Erlenmeyer flask, containing 45 mL of sterilized seed medium, and was incubated on a rotary shaker at 250 rpm and 30 °C for 24 hr. The composition of seed medium contained the chemical elements as follows (per L): glucose 10 g, $(NH_4)_2SO_4$ 1 g, KH_2PO_4 1.5 g, $Na_2HPO_4 \cdot 12H_2O$ 9 g, $MgSO_4 \cdot 7H_2O$ 0.2 g and trace element solution 1 mL; the solution was adjusted to pH 6.8. The trace element solution was composed as follows (per L): $FeSO_4 \cdot 7H_2O$ 10 g, $ZnSO_4 \cdot 7H_2O$ 2.25 g, $CuSO_4 \cdot 5H_2O$ 1 g, $MnSO_4 \cdot 5H_2O$ 0.5 g, $CaCl_2 \cdot 2H_2O$ 2 g, $Na_2B_4O_7 \cdot 10H_2O$ 0.23 g, $(NH_4)_6Mo_7O_{24}$ 0.1 g and 36% HCl 10 mL.

Poly(3-hydroxybutyrate) production in shake flask culture

C. necator DSM 545 was cultivated in the medium as described by Sirisansaneeyakul and Mahasubpaiboon (2003). Briefly, 10% volume per volume (v/v) of inoculum (25 mL) was inoculated into a 500 L Erlenmeyer flask containing 225 mL of sterile P(3HB) production medium. The composition of P(3HB) production medium contained the chemical elements as follows (per L): glucose 10 g, $(NH_4)_2SO_4$ 1.2 g, KH_2PO_4 13.3 g, $MgSO_4 \cdot 7H_2O$ 1.2 g, citric acid 1.7 g, trace element solution 10 mL; the solution was adjusted to pH 6.8. The culture with an initial working volume of 250 mL was cultivated on a rotary shaker at 250 rpm and 30 °C for 48 hr. The culture broths were taken for analyses every 4 hr. The experiments were conducted in duplicate.

Analytical methods

Cell growth was analyzed by measuring both the optical density of the culture broth at 650 nm using a spectrophotometer (UV-1201, UV-VIS Spectrophotometer; Shimadzu; Kyoto, Japan) and the dry cell weight. Culture broth samples were collected using centrifugation at 4,800 revolutions per minute (rpm; 1,742 × *g*) for 15 min, washed twice with distilled water and dried at 105 °C for 24 hr to obtain the constant dry cell weight. The remaining supernatant after centrifugation was used to measure the consumption of glucose using the DNS method (Saqib and Whitney, 2011) and the amount of ammonium sulfate using phenol-hypochlorite (Weatherburn, 1967). For P(3HB) contents measurement, the recovered biomass from centrifugation was washed twice with distilled water to remove residual culture medium and thereafter was used for analysis of the P(3HB) contents produced from *C. necator* DSM 545 according to Law and Slepecky (1960, 1961). Briefly, the biomass was resuspended in 10–13% v/v of sodium hypochlorite solution and then incubated at 37 °C for 1 hr to break the cell membrane. The intracellular lipid granules were separated and washed with water, acetone, and ethanol, respectively, using centrifugation at 4,800 rpm for 15 min. Later, the dissolved polymer was extracted by incubating in boiling chloroform for 2 min. The mixture solution (dissolved polymer in chloroform) was filtered and the filtrate was then used for determining the amount of P(3HB) by adding 10 mL of concentrated sulfuric acid into the filtered P(3HB) solution and heating to 100 °C for 10 min. Then, the mixture solution was cooled at room temperature (25 °C). Absorbance of the P(3HB) content at 235 nm was measured using the spectrophotometer, with sulfuric acid solution as a blank solution. The resulting P(3HB) content (measured in grams per liter) was calculated from the P(3HB) standard curve. The kinetic parameters of the fermentation ($Y_{X/S}$, $Y_{P/S}$, Q_S and Q_P)

were calculated according to Sirisansaneeyakul et al. (2013). The P(3HB) yield coefficient from biomass ($Y_{P/X}$) was calculated as the mass of P(3HB) obtained per unit dry cell weight (Grothe and Chisti, 2000). Measurements were made in duplicate.

Poly(3-hydroxybutyrate) extraction using sodium dodecyl sulfate and sodium hypochlorite

The Taguchi method was used for experimental design. The effects of P(3HB) extraction consecutively by sodium dodecyl sulfate (SDS) and sodium hypochlorite (NaOCl) focused on two factors, that is the concentration of SDS (0.5%, 1.0% and 1.5% w/v) and concentration of sodium hypochlorite (2%, 4% and 6% v/v), and the two-factor, three-level columns in an L_9 (3^2) orthogonal array for investigation was designed using the Qualitek-4 software (Nutek Inc.; Bloomfield Hills, MI, USA), as shown in Table 1. Cell pretreatment by heat shock is required before P(3HB) extraction. This involved 150 mL of broth samples (cells included) being first heated at 100 °C for 1 hr and then maintained at 55 °C for 30 min before being cooled at 4 °C for 30 min. Thereafter, the pretreated cells were frozen at −20 °C for 48 hr before P(3HB) extraction. A sample of 60 mL of pretreated cells was homogeneously mixed in 60 mL of each SDS concentration (0.5%, 1.0% and 1.5% w/v) and then incubated on a rotary shaker at 150 rpm and 55 °C for 30 min. After the incubation, the SDS solutions were completely removed using centrifugation at 11,000 rpm (7,792 × *g*) for 10 min. The precipitated biomasses were suspended into 60 mL of each sodium hypochlorite concentration (2%, 4% and 6% v/v) and incubated on a rotary shaker at 250 rpm and 30 °C for 30 min to extract intracellular P(3HB). The precipitated biomass was removed using centrifugation at 6,200 rpm (1,292 × *g*) for 10 min and then the supernatant was dried at 55 °C for 12 hr to obtain dried P(3HB) crude powder (Kshirsagar et al., 2013). The purity of the recovered P(3HB) was determined using high-performance liquid chromatography (HPLC) as described by Sandström et al. (2015). Briefly, an Aminex HPX-87H ion exchange column (Bio-Rad; Hercules, CA, USA) was monitored at 65 °C with 5 mM H_2SO_4 as the mobile phase. The flow rate of the mobile phase was 0.6 mL/min. The percentage of purity of P(3HB) extracted from each condition was calculated using Equation (1). Finally, the percentage of P(3HB) recovery was calculated based on the purity of the total recovered P(3HB), as shown in Equation (2) (Tripathi and Srivastava, 2015):

$$\%\text{P(3HB)purity} = \frac{\text{Amount of P(3HB)after recovery}(g)}{\text{Amount of total dry matter after recovery}(g)} \times 100 \quad (1)$$

$$\%\text{P(3HB)recovery} = \frac{\text{Amount of P(3HB)recovered}(g)}{\text{Total amount of P(3HB) in the cell }(g)} \times 100 \quad (2)$$

Table 1
Experimental design of variable parameters for poly (3-hydroxybutyrate) extraction using sodium dodecyl sulfate (SDS) and sodium hypochlorite.

Symbol	Factor (parameter)	Unit	Factor level		
			1	2	3
A	SDS concentration	% w/v	0.5	1.0	1.5
B	Sodium hypochlorite concentration	% v/v	2	4	6

w/v = weight per volume; v/v = volume per volume.

Poly(3-hydroxybutyrate) characterization

The conformational characterization of the obtained P(3HB) extracted using the combination of SDS and sodium hypochlorite was determined using a Fourier-transform infrared spectrophotometry (FTIR; Bruker Tensor 27; Bruker; Bellerica, MA, USA). The infrared spectrum in the range 400–4000 cm^{-1} was assigned for scanning. Commercial P(3HB) (Sigma-Aldrich Co.; Darmstadt, Germany) was provided for comparison. The thermal properties of P(3HB), that is the melting temperature (T_m) and crystallization temperature (T_c) were also estimated using differential scanning calorimetry (DSC; Mettler Toledo DSC1 Star^e System; Mettler Toledo; Columbus, OH, USA). Analyses were performed using P(3HB) crude powder (3–10 mg) with the following temperature program according to Rodrigues et al. (2005). Briefly, heating from −50 to 200 °C (10 °C/min), cooling from 200 to −50 °C (10 °C/min) and a second heating from −50 to 200 °C (10 °C/min), compared to the commercial P(3HB). A probability value of $p < 0.1$ was chosen to evaluate statistical significance and confidence intervals.

Results and discussion

Poly(3-hydroxybutyrate) production in shake flask culture

The batch fermentation profiles consisted of glucose and ammonium sulfate consumption and an intracellular accumulation of P(3HB), produced from *Cupriavidus necator* DSM 545 in shake flask culture, conducted under specified conditions reported previously (Patjawit et al., 2014) and are summarized in Fig. 1. During the first 20 hr of cultivation, both glucose and ammonium sulfate were gradually consumed as carbon and nitrogen sources, respectively, to prompt cell growth, whilst the P(3HB) contents were initially produced and accumulated in the cells. After 20 hr of cultivation, as the ammonium sulfate was exhausted, the excess glucose rapidly reduced and was used for further cell growth and P(3HB) production. The cell concentration moderately increased approaching a stationary growth phase after 32 hr of cultivation. P(3HB) clearly increased in the medium with exhausted ammonium sulfate and the excess carbon source. Finally, the maximum concentration of P(3HB) (1.335 ± 0.087 g/L) was produced at 40 hr of cultivation, as thereafter glucose was not further consumed (Fig. 1).

Under sufficient nutrient conditions for bacterial growth, glucose was mainly used as the carbon and energy source for cell growth and maintenance. On the other hand, when the growth was imbalanced under nutrient-limited conditions, excess glucose could be transformed into an intermediate substance (acetyl coenzyme A), via the glycolysis pathway, to provide a P(3HB) synthesis pathway (Liu et al., 1996). The batch fermentation kinetics of the bacterial biomass and P(3HB) produced from *C. necator* DSM 545 in a shake flask are summarized in Table 2. The highest concentrations of biomass and P(3HB) were 5.845 ± 0.375 g/L and 1.335 ± 0.087 g/L, respectively. At 40 hr of cultivation together with a P(3HB) content of 22.937 ± 2.953 wt%, defined as the ratio of P(3HB) concentration to dry cell concentration according to Wang et al. (2013). The yields of P(3HB) ($Y_{P/S}$) and biomass ($Y_{X/S}$) from substrate were 0.128 ± 0.006 g/g and 0.537 ± 0.082 g/g, respectively, indicating the glucose consumption favored cell growth rather than P(3HB) production. Consequently, the P(3HB) yield from biomass ($Y_{P/X}$), the volumetric rates of P(3HB) production (Q_P) and substrate consumption (Q_S) were 0.229 ± 0.030 g/g, 0.031 ± 0.001 g/L hr and 0.243 ± 0.029 g/L hr, respectively. Then, the bacterial cells collected from the shake flask culture were substantially used as feedstock for an optimization of P(3HB) extraction with SDS and sodium hypochlorite using the Taguchi approach.

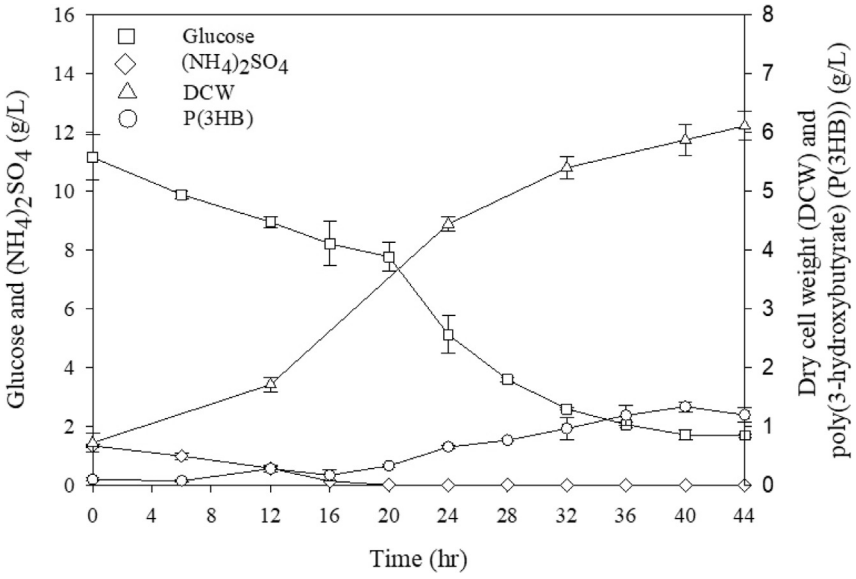

Fig. 1. Batch culture of *Cupriavidus necator* DSM 545 in shake flask using poly (3-hydroxybutyrate) (P(3HB)) production medium (Adapted from Patjawit et al. (2014)).

Table 2
Fermentation kinetics of biomass and poly (3-hydroxybutyrate) (P(3HB)) production from *Cupriavidus necator* DSM 545 in shake flask culture.

Kinetic parameter	Unit	
Percentage of glucose consumption	%	85.362 ± 2.051
Concentration of biomass (C_X)	g/L	5.845 ± 0.375
Concentration of P(3HB) (C_P)	g/L	1.335 ± 0.087
Biomass yield from substrate ($Y_{X/S}$)	g/g	0.537 ± 0.082
P(3HB) yield from substrate ($Y_{P/S}$)	g/g	0.128 ± 0.006
P(3HB) yield from biomass ($Y_{P/X}$)	g/g	0.229 ± 0.030
Volumetric rate of substrate consumption (Q_S)	g/L hr	0.243 ± 0.029
Volumetric rate of P(3HB) production (Q_P)	g/L hr	0.031 ± 0.001

Values are shown as mean ± SE.

Poly(3-hydroxybutyrate) extraction with sodium dodecyl sulfate and sodium hypochlorite optimized using the Taguchi method

The L_9 orthogonal array, resulting in nine experimental conditions from two factors at three different levels—SDS (0.5%, 1% and 1.5% w/v) as factor A and sodium hypochlorite (2%, 4% and 6% v/v) as factor B, as shown in Table 1)—were carried out in triplicate. The results, as P(3HB) recovery (%), are summarized in Table 3. The P(3HB) recovery was analyzed accordingly with "bigger is better" quality characteristics using the Qualitek-4 software and the statistical results and S/N ratio provided in Table 3 for use in describing the effect of each of the two factors at the different levels and the optimal P(3HB) extraction.

The percentages of P(3HB) recovery were significantly affected by the two factors at the three levels (Table 4), indicating that the P(3HB) recovery depended on the extractants, especially sodium hypochlorite (factor B). As the sodium hypochlorite increased from 4% to 6% v/v, the P(3HB) recovery increased from 60.06% to 73.42%, whereas the S/N ratio increased from 35.50 to 37.28 (Table 4). On the other hand, the SDS increased from 0.5% to 1.5% w/v, with little effect on the P(3HB) recovery, with comparable values between 71.07% and 70.32% for the S/N ratios 36.96 and 36.80, respectively, as shown in Table 4. However, the main effect of the combined two factors was clearly from sodium hypochlorite (factor B, 63.99%) playing a more important role than SDS (factor A, 36.01%). As a result, the sodium hypochlorite (factor B) at 6% v/v (level 3) combined with the SDS (factor A) minimized at 0.5% w/v (level 1) was

Table 3
L_9 array and the results of poly (3-hydroxybutyrate) (P(3HB)) recovery using sodium dodecyl sulfate (SDS) and sodium hypochlorite extraction.

Run	A (% w/v)	B (% v/v)	P(3HB) recovery (%)	SD	MSD	S/N
1	0.5	2	74.31	5.05	0.00018	37.38
2	0.5	4	60.31	2.45	0.00028	35.59
3	0.5	6	78.58	1.74	0.00016	37.90
4	1.0	2	62.18	2.35	0.00026	35.86
5	1.0	4	61.58	4.25	0.00027	35.74
6	1.0	6	66.91	5.05	0.00023	36.46
7	1.5	2	77.89	5.22	0.00017	37.79
8	1.5	4	58.30	7.66	0.00031	35.15
9	1.5	6	74.76	2.64	0.00018	37.46
Average			68.31	4.04	0.00022	36.59

A = SDS concentration as % weight per volume (w/v); B = sodium hypochlorite (NaOCl) concentration as % volume per volume (v/v); MSD = mean squared deviation; S/N = signal to noise ratio.

the optimal synergistic combination to increase the P(3HB) recovery (Fig. 2). Therefore, sodium hypochlorite showed promising potential, efficiently digesting protein/non- P(3HB) and P(3HB) from the biomass resulting in high P(3HB) purity in the range 83.29–91.50%. As the P(3HB) granules extracted using only sodium hypochlorite are quite vulnerable to alkaline saponification and rapidly decomposed (Jacquel et al., 2008), the cells disrupted with SDS (factor A) and consecutively extracted with sodium

Table 4
Analysis of the main effect of factors based on (a) signal to noise ratio (S/N ratio) and (b) average.

Level	(a) S/N ratio		(b) Average	
	A	B	A	B
1	36.96	37.01	71.07	71.46
2	36.02	35.50	63.55	60.06
3	36.80	37.28	70.32	73.42
Minimum	36.02	35.50	63.55	60.06
Maximum	36.96	37.28	71.07	73.42
Main effect	0.94	1.78	7.52	13.36
% Main effect	34.52	65.48	36.01	63.99

A = sodium dodecyl sulfate concentration (% weight per volume); B = sodium hypochlorite (NaOCl) concentration (% volume per volume).

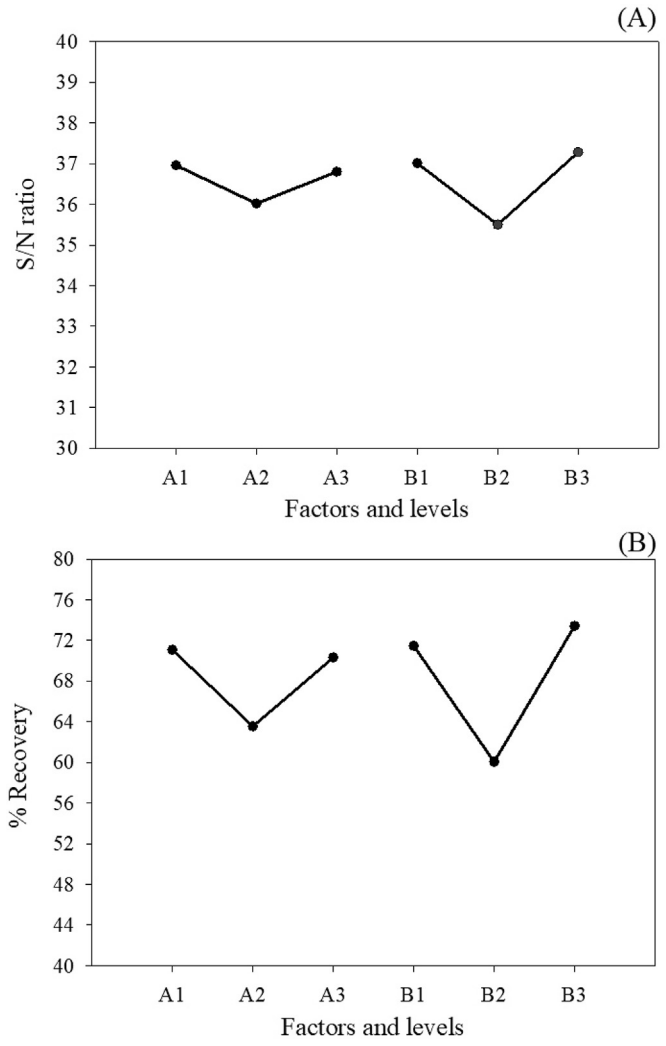

Fig. 2. Effect of sodium dodecyl sulfate (SDS) and sodium hypochlorite extraction on poly (3-hydroxybutyrate) (P(3HB)) recovery based on (A) signal to noise ratio (S/N ratio) and (B) average, where A levels are SDS concentration (% w/v) and B levels are sodium hypochlorite (NaOCl) concentration (% v/v) at each level (1–3).

hypochlorite (factor B) could effectively promote the P(3HB) extraction (Table 4). In contrast, SDS attached to the lipid bilayer membrane of cells breaking the membrane and forming micelles between the surfactant and membrane phospholipids; therefore, P(3HB) dispersed finally into the mixtures. In addition, SDS solubilized proteins and other non- P(3HB) cellular material, resulting in a higher recovery of P(3HB) (Jacquel et al., 2008). As reported previously, P(3HB) extracted with solely with the sodium hypochlorite or the higher SDS concentration did not greatly promote the recovery of P(3HB) (Hahn et al., 1994).

Analysis of variance was used to investigate the significance of each factor on the P(3HB) recovery from the SDS and sodium hypochlorite extraction, as shown in Table 5. The results demonstrated that the most significant factor (based on the S/N ratio and average value) affecting P(3HB) extraction with the highest P(3HB) recovery (%) was the sodium hypochlorite concentration (factor B), contributing Percent *P* of 51.86% and 45.42%, with confidence levels of 93.75 and 99.99 ($p < 0.1$), respectively (Table 5). The concentration of SDS had less effect on P(3HB) extraction, contributing 6.65% and 12.81%, with confidence levels of 69.83 and 98.36 ($p < 0.1$), respectively, based on the S/N ratio and an average value (Table 5). These results clearly revealed that the effect of sodium hypochlorite concentration on P(3HB) extraction was greater than the SDS concentration. The optimal conditions for P(3HB) extraction were determined from the main effect plots, as shown in Fig. 2. Only the maximized values of levels in each factor were suggested as the optimal conditions for P(3HB) extraction for the highest P(3HB) recovery (Table 4). Therefore, the maximal P(3HB) recovery from SDS and sodium hypochlorite extraction could be predicted for the optimal conditions, as shown in Equation (3):

$$Y_{opt} = T + (A_1 - T) + (B_3 - T) \quad (3)$$

where Y_{opt} is the expected maximal P(3HB) recovery (%), T is the average of summation, A_1 is the maximal P(3HB) recovery from 0.5% w/v SDS (level 1) and B_3 is the maximal P(3HB) recovery from 6% v/v sodium hypochlorite (level 3). By calculation (Equation (3)), the optimal conditions of factor levels maximizing P(3HB) recovery are summarized in Table 6. The maximal P(3HB) recovery based on the S/N ratio and average value were estimated to be 76.20% and 76.17%, respectively (Table 6). The experiment conducted under the optimal conditions attained 78.70%, which was impressively close in agreement with the prediction (Table 6). The optimal conditions of 0.5% w/v SDS combined with 6% v/v sodium hypochlorite were also very consistent with the observations (Table 3), as the higher sodium hypochlorite (factor B) and the lower SDS concentration (factor A) were definitely favorable. Thus, sodium hypochlorite increased to 6% v/v and/or 0.5% w/v SDS resulted in higher P(3HB) recovery with high purity (approximately 90%) are recommended for extraction using SDS and sodium hypochlorite.

As previously reported, poly-3-hydroxyalkanoic acid (PHA) with 97% purity was recovered from *A. eutrophus* DSM 545 with 1% w/v SDS and hypochlorite (pH 10) extraction (Ramsay et al., 1990a). High purity PHA was recovered from *A. chroococcum* G-3 at 98% and 86.6%, extracted with 10 g/L SDS and 30% sodium hypochlorite, respectively (Dong and Sun, 2000). In the present work, the combination of SDS and sodium hypochlorite were minimized

Table 5
Analysis of variance based on a signal to noise ratio (S/N ratio) and average values of Taguchi statistics.

Factor	DOF (f)	Sum of Squares. (S)	Variance (V)	*F*–ratio (F)	Pure Sum (S′)	Percent *P* (%)	Confidence
S/N ratio							
SDS	2	1.51	0.76	1.64	0.59	6.65	69.83
Sodium hypochlorite	2	5.53	2.77	6.00	4.61	51.86	93.75
Other/Error	4	1.84	0.46			41.48	
Total	8	8.89				100.00	
Average							
SDS	2	308.40	154.20	4.98	246.55	12.81	98.36
Sodium hypochlorite	2	936.24	468.12	15.14	874.38	45.42	99.99
Other/Error	22	680.42	30.93			41.77	
Total	26	1,925.07				100.00	

DOF = degree of freedom; S/N = signal to noise ratio; SDS = sodium dodecyl sulfate.

Table 6
Optimum conditions and performance for poly (3-hydroxybutyrate) (P(3HB)) recovery derived from factors analysis based on a signal to noise ratio (S/N ratio) and average using the Taguchi approach.

No.	Factor	Level	Level description	Expected P(3HB) recovery (%)	
				S/N ratio	average
1	SDS concentration (% w/v)	1	0.5	76.20*	76.17**
2	Sodium hypochlorite concentration (% v/v)	3	6		
P(3HB) recovery experimental results for optimum conditions (%)				78.70	

SDS = sodium dodecyl sulfate.
* and ** confidence intervals of expected result of P(3HB) recovery (%) based on S/N ratio and average are ± 8.32 and ± 3.86, respectively.

impressively to 0.5% w/v and 6% v/v, respectively with reasonable yield and purity. The less SDS and sodium hypochlorite used, the greater the potential cost reduction and the more reduced the environmental concerns, especially regarding the wastewater treatment cost, to remove the residual chemicals from the process (Kunasundari and Sudesh, 2011).

Characterization of poly (3-hydroxybutyrate) extracted using sodium dodecyl sulfate and sodium hypochlorite

The crude P(3HB) powder from the SDS/sodium hypochlorite extraction was a turbid white color. However, no color differences were noticed among the P(3HB) samples taken from the nine treatments. The conformational characterization of the obtained P(3HB) crude powder was then analyzed using FTIR. The FTIR spectra of the P(3HB) extracted from the combined SDS/sodium hypochlorite are shown in Fig. 3, compared to commercial P(3HB) and P(3HB) samples previously extracted from chloroform/sodium hypochlorite (Patjawit et al., 2014). The spectral region of the P(3HB) from SDS/sodium hypochlorite was quite similar to the previous work (Patjawit et al., 2014), that is the spectral region at 1721 cm^{-1} was attributed to the crystallinity of the carbonyl group (C=O), while the spectral region between 1050 cm^{-1} and 1150 cm^{-1} characterized the C–O stretching bands (Kansiz et al., 2000). These findings precisely confirmed the desired purity of P(3HB), which was extracted using SDS and sodium hypochlorite.

In addition, the thermal properties of P(3HB)—the melting temperature (T_m) and crystallization temperature (T_c) —were determined using DSC, as shown in Table 7. The results showed that the P(3HB) crude powder prepared using SDS/sodium hypochlorite under the optimal conditions had values for T_m and T_c of 159.67 °C and 92.16 °C, respectively, which were consistent with the prepared P(3HB) film from chloroform/sodium hypochlorite extraction (Patjawit et al., 2014) and the commercial P(3HB). Furthermore, both extraction methods for P(3HB)—SDS/sodium hypochlorite and chloroform/hypochlorite (Patjawit et al., 2014) —resulted in an increased T_c value and a decreased T_m value. The crystallinity (X_c) of P(3HB) was calculated from the enthalpy of the polymer melting analyzed using DSC (Gunaratne and Shanks, 2005). The P(3HB) extraction using SDS/sodium hypochlorite and chloroform/hypochlorite (Patjawit et al., 2014) resulted in decreased X_c values of P(3HB) to 57.29% and 63.77%, respectively, compared with the commercial P(3HB) (X_c = 65.95%) (Table 7). This lower crystallinity of P(3HB) decreased its brittleness, making it suitable for a wider

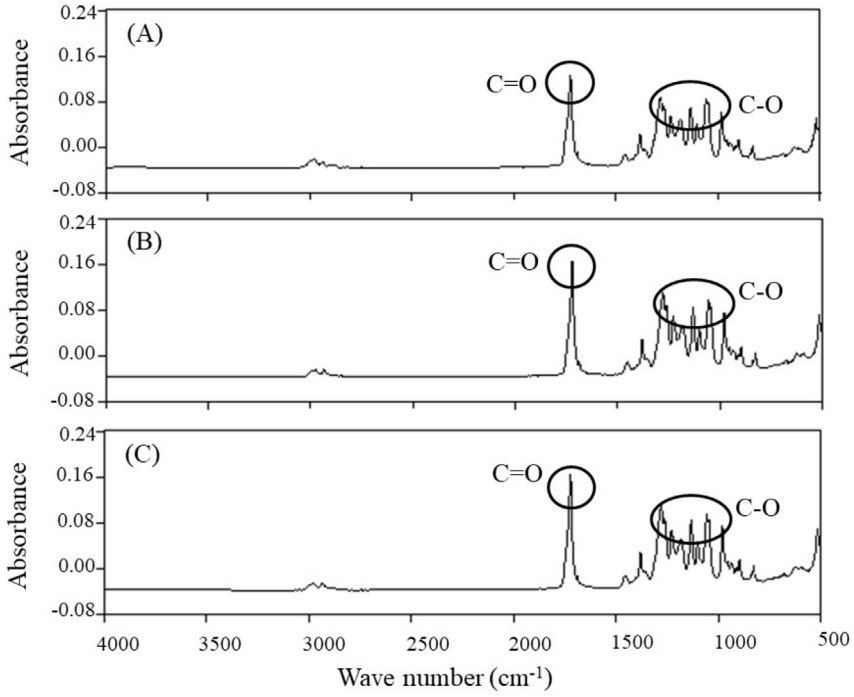

Fig. 3. Fourier-transform infrared spectra of (A) commercial poly (3-hydroxybutyrate) (P(3HB)), (B) P(3HB) crude powder from sodium dodecyl sulfate and sodium hypochlorite extraction and (C) P(3HB) film prepared from chloroform/sodium hypochlorite extraction.

Table 7
Thermal properties of commercial poly (3-hydroxybutyrate) (P(3HB)) and P(3HB) samples, analyzed using differential scanning calorimetry.

P(3HB) product/Extraction method	Thermal property (°C)		X_C (%)[a]
	T_m	T_c	
Commercial P(3HB)	173.17	84.67	65.95
Chloroform and sodium hypochlorite (Patjawit et al., 2014)	167.66	99.58	63.77
SDS and sodium hypochlorite (this study)	159.67	92.16	57.29

SDS = sodium dodecyl sulfate.
[a]Crystallinity (X_C) was calculated according to Gunaratne and Shanks (2005).

range of applications (Xu et al., 2010). These thermal properties and the crystallinity of P(3HB) products could be selectively used for suitable applications.

P(3HB) could be produced from *C. necator* DSM 545 in batch fermentation with the maximum P(3HB) concentration of 1.335 ± 0.087 g/L and P(3HB) yield from biomass of 0.229 ± 0.030 g/g. The crude P(3HB) could be recovered by the combination of SDS with sodium hypochlorite extraction, under the optimal conditions of sequential 0.5% w/v SDS and 6% v/v sodium hypochlorite, resulting in maximum P(3HB) recovery of 78.70%. However, the P(3HB) recovery was somewhat lower than that previously reported. Thus, repeated batch extraction might be required to increase the percentage recovery of P(3HB), in case one step was not indeed sufficient to extract all the P(3HB) in the bacterial cells. FTIR and DSC analysis showed the probable results for the extracted P(3HB) if applied to large-scale P(3HB) production, to provide mostly commercial P(3HB) usage. Finally, the optimal conditions from this work could be promising for scaling up P(3HB) extraction in the fermentation industry.

Conflict of interest

The authors declare no conflict of interest.

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