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An Environmentally Friendly and Efficient Method for Extraction of PHB Biopolymer with Non-Halogenated Solvents

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The present study developed an efficient and environmentally friendly method for recovering polyhydroxybutyrate (PHB) from *Cupriavidus necator*. Several non-halogenated solvents were tested and it was found that butyl acetate and ethyl acetate are powerful solvents for the biopolymer. Testing was performed to examine the effects of temperature (25°C until temperature below solvent boiling points) and heating incubation time (0–60 min) on the two solvents. Butyl acetate had a higher recovery level (96%) and product purity (up to 99%) than ethyl acetate at 103°C and a heating incubation time of 30 min. Under these conditions, PHB recorded the highest molecular weight of 1.4×10^6 compared with the standard procedure (*i.e.*, recovery using chloroform). The proposed strategy showed that butyl acetate is a good alternative to halogenated solvents such as chloroform for recovery of PHB.

Keywords: Butyl acetate, central composite design, *Cupriavidus necator*, design expert, ethyl acetate, response surface methodology

Introduction

Polyhydroxyalkanoates (PHAs) are thermoplastic biopolymers that accumulate in a wide range of microorganisms under stress conditions [8, 13]. In recent years, PHAs have attracted much attention owing to their biodegradability and biocompatibility properties. They can be substituted for conventional plastics (such as polypropylene and polyethylene), which could decrease petroleum consumption and diminish the environmental impact of plastic waste [18]. Depending on their composition and molecular weight, these polymers have applications in agriculture, medicine, pharmaceuticals, and packaging [7, 28].

The most widely used microbial PHA for industrial applications is polyhydroxybutyrate (PHB) [4]. The main drawback to the commercial production and application of PHB in industry is its comparatively high cost compared with conventional plastics. The recovery of PHB granules from bacterial cytoplasm significantly increases total processing costs [15]. Efficient, economical, and environmentally friendly extraction of PHB from cells is required for its cost-effective industrial production. Several studies have proposed various recovery techniques that improve the yield and purity of extraction and reduce manufacturing costs. The most common techniques in these published articles are classified as chemical, physical, and enzymatic methods, as independent extraction systems or a combination of such systems [3, 5]. Among these procedures, solvent extraction is a common industrial method of recovering PHB that features high efficiency, low degradation of the biopolymer, and elimination of endotoxins from the recovered biopolymer [19, 24].

Currently, most PHB solvent extraction strategies are based on halogenated organic solvents (*e.g.*, chloroform), which are expensive, environmentally unfriendly, and may cause degradation of the biopolymer [11]. Thus, a simple, practical, efficient, and cost-effective system of PHB recovery using non-halogenated solvents should be developed; however, halogen-free solvent-based techniques require improvement of the parameters that influence the overall process to make it suitable on an industrial scale. Determining the solubility of PHB in different organic solvents is important in order to choose the most suitable one for recovery of the biopolymer from microorganisms. Ethyl acetate and butyl acetate are non-toxic solvents that have been shown to be good non-halogenated solvents for

amorphous PHB (nascent PHB in cytoplasm) [22, 25]. One of the major expenses of producing PHB is its extraction from biomass.

This study proposes a new method for extraction of biopolymer PHB from the wet biomass of *Cupriavidus necator* using non-toxic solvents. It is based on the method described for halogenated solvents (chloroform recovery system) [11] and the feasibility of scaling up those processes. Response surface methodology (RSM) was applied as an experimental strategy for modeling, comparing the effects of two solvents and optimizing time and temperature in order to enhance PHB recovery yield. Batch cultivation of *C. necator* ATCC 17699 was employed to reach a recovery yield comparable to standard methods (*e.g.*, recovery by chloroform). The temperature used was below the boiling points of the solvents to ensure the ability of this method to be scaled up in a safe manner. The effects of all parameters on the process and biopolymer properties were demonstrated for yield, purity, and molecular weight.

Materials and Methods

Biopolymer (PHB) Production

C. necator ATCC 17699 was obtained from the Persian Type Culture Collection for the production of PHB. For inoculum preparation, the cells were grown on LB medium for 10 h at 30°C on a shaker at 200 rpm.

The composition of the basal mineral salt medium (MSM) used in this study was as follows: $(NH_4)_2SO_4$ 2.0 g/l, KH_2PO_4 1.54 g/l, $MgSO_4{\cdot}7H_2O$ 1.2 g/l, citric acid 1.7 g/l, and trace metal solution 10 ml/l. The trace element stock solution was composed of $ZnSO_4{\cdot}7H_2O$ 2.25 mg/l, $FeSO_4{\cdot}7H_2O$ 10 mg/l, $CaCl_2{\cdot}2H_2O$) 2 mg/l, $Na_2B_4O_7{\cdot}7H_2O$) 0.23 mg/l, $(NH_4)_6Mo_7O_{24}$ 0.1 mg/l, $CuSO_4{\cdot}5H_2O$) 1 mg/l, $MnSO_4{\cdot}5H_2O$) 0.6 mg/l, and HCl 35% 10 ml/l [14]. Fructose was used as the carbon source at a concentration of 20 g/l for production media and inoculum development. The initial pH was adjusted to 7.0.

Testing Solvents for PHB Recovery

To test selected solvents for PHB recovery efficiency, PHB with a purity of 100% was used as the starting material. Equal volumes of the non-halogenated solvents ethanol, ethyl acetate, butyl acetate, methanol, and propanol were added to PHB in sealed test tubes to obtain 7% PHB solutions. The PHB was dissolved by heating at 100°C on a heat block for 60 min. After incubation, each solution was filtered through a membrane filter. Equal aliquots of the samples were transferred into tubes. The tubes were incubated at temperatures below the boiling point of their corresponding solvent until dry. The samples were further dried until they reached a constant weight.

PHB Recovery with Chloroform (Standard Sample)

This method was used as the standard recovery technique for comparison [27]. One gram of cell powder was treated with dispersions of 50 ml of chloroform and 50 ml of hypochlorite 10% solution. After treatments at 37°C for 1 h, the dispersion was centrifuged. Three separate phases were obtained. The upper phase was hypochlorite solution, the middle phase contained undisrupted cells, and the bottom phase was chloroform containing PHB. First, the hypochlorite phase was removed, and then the chloroform phase was obtained by filtration. After filtration, PHB was recovered by precipitation with acetone.

PHB Recovery with Non-Halogenated Solvent

Approximately 1 g of wet biomass was suspended in 100 ml of solvent (ethyl acetate or butyl acetate) and transferred into a tube immersed in a thermostatic water bath while stirring. The suspensions were stirred and heated at different times and temperatures (13 experiments were designed and formulated for these two factors using the Design Expert software). Time and temperature were selected because they have a significant effect on solvent extraction, according to the literature [11, 22]. Temperature and incubation time range (the length of time the suspension is heated) for the variables were selected according to other reports and the boiling points of these two solvents. The temperature was set below the boiling point in order to maintain process safety at the industrial scale. Once the suspension reached the set temperature, it was maintained at that temperature to test different incubation times. Later, the suspension was kept at room temperature for 24 h and centrifuged under appropriate conditions. The supernatant was collected and precipitated with acetone. The precipitated biopolymer was washed with distilled water. Finally, the biopolymer was dried at room temperature.

Experimental Design

Statistical experimental design is a powerful and useful tool, which can explain interactions between different variables and decrease the number of experiments [17]. The most popular choices are Central Composite Design (CCD), along with RSM. RSM is a statistical technique for designing experiments, assessing the relative importance of several variables, and determining the optimum conditions for desired response values [2, 21].

In this study, a two-factor CCD was obtained using Design Expert 7.0 software (State-Ease Inc., Minneapolis, MN, USA). The variables used in this design were extraction temperature and heating incubation time (the length of time the suspension is heated), with each being represented at two levels, high (+1) and low (-1). The complete design consisted of 13 experiments (Table 2). In each experiment, the yield percentage was calculated and each trial was performed in duplicates. The measured responses were PHB amount (g/l) and yield (% of PHB weight).

Statistical models were developed for PHB yield and content. They were also validated using the analysis of variance (ANOVA).

Table 1. Properties of solvents that can be used for PHB recovery process.

Solvent	Density (g/cm^3)	Boiling point (°C)	Viscosity (cP)	Water solubility (g/100 ml)	Safety
Water	1	100	1	-	Safe
Ethyl acetate	0.89	77	0.42	8.3	Low toxicity
Butyl acetate	0.88	126	0.68	6.8	Medium toxicity
Ethanol	0.78	78	0.001	Infinite	Low toxicity
Chloroform	1.48	61	0.54	0.8	Toxic
Methanol	0.79	64	0.5	Infinite	Low toxicity
Propanol	0.80	98	1.9	Infinite	Low toxicity

Treatment effects upon both responses were then assessed by RSM, and the optimized condition was chosen to obtain an optimized recovery procedure. Three-dimensional (3D) plots were also generated to understand the interaction of the two factors and then used to find the optimized conditions affecting the response (PHB recovery yield).

Biopolymer (PHB) Characterization

Cell dry weight determination. Determination of cell dry weight (CDW) was done by weighing the dry cell mass obtained as follows. The samples were centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was resuspended in distilled water (10 ml) and centrifuged again for washing. The washed cells were dried at 90°C for 24 h in a hot air oven. The drying was repeated until a constant weight was obtained [12].

Analytical methods for detection of PHB. In order to evaluate PHB quantity and purity, a methanolysis procedure was used according to the method described by Martino *et al*. [20]. Commercial PHB (Sigma-Aldrich) was used as a standard. PHB content (% (w/w)) was defined as a percentage of CDW. The percentage of recovered PHB was calculated regarding a known amount of PHB in the biomass, based on the purity of the total mass of samples recovered. Purity (%) was calculated as follows: purity = (mass of polymer / mass of sample) × 100, where mass of polymer was quantified by GC. The recovery yield percentage (Y%) was expressed as follows: Y = [mass PHA recovered (g) × purity (%) / cell mass (g) × PHB content (%)].

Biopolymer molecular weight. The biopolymers obtained with each procedure were analyzed with a viscometer in order to calculate the average molecular weight. Intrinsic viscosities of solutions were measured using a Ubbelohde dilution viscometer. All the measurements were done in chloroform at room temperature. The solution concentration of each sample was adjusted to set the relative viscosity of solutions in the range of 1.1–1.8. The molecular weight of PHB was calculated according to following equation [1]: $[\eta] = 0.0119 \times 10^{-4} M_v^{0.78}$, where [η] is the intrinsic viscosity of PHB in chloroform.

Results and Discussion

Solvent Selection

The less toxic non-halogenated solvents (ethanol, methanol,

Table 2. PHB recovery yield (%) and purity (%) recovered by ethyl acetate from wet biomass.

Experiment No.	Temperature (°C)	Time (min)	Recovered PHB (Average) (g/l)	Purity (%)	Yield (%)
1	21	30	1.65	85 ± 2	78 ± 4
2	25	0	1.7	97 ± 3	82 ± 3
3	25	60	1.84	86 ± 3	87 ± 3
4	35	0	1.79	98 ± 2	84 ± 3
5	35	30	1.73	98 ± 1	82 ± 2
6	35	30	1.74	98 ± 1	82 ± 1
7	35	30	1.74	99 ± 1	82 ± 3
8	35	30	0.8	98 ± 1	38 ± 1
9	35	30	1.77	97 ± 2	82 ± 1
10	35	72	1.48	99 ± 1	70 ± 2
11	45	0	0.7	99 ± 1	28 ± 3
12	45	60	0.6	99 ± 1	4.7 ± 1
13	49	30	0.1	99 ± 1	33 ± 2
Standard	-	-	2.2	99 ± 1	96 ± 1

propanol, ethyl acetate, and butyl acetate) were chosen on the basis of the study by Terada and Marchessault [26], which described them as good alternative solvents to chloroform for PHB recovery. The physical properties of the solvents are shown in Table 1. As can be seen, the density of all solvents is <1 (*i.e.*, water density), which permits easy removal of the PHB solution after extraction.

PHB biopolymers remain in an organic phase separate from cell debris in the aqueous phase. This is an advantage over conventional chloroform-based recovery. PHB-chloroform solutions form the bottom phase in combination with cell debris because their density is higher than water. All the solvents are inexpensive and are less toxic than chloroform, which is hazardous to the environment.

After choosing the solvents according to their density, safety, and price, their abilities for solubility/recovery of PHB were tested. Pure commercial PHB (Sigma-Aldrich) was used to measure PHB solubility. The results are shown in Fig. 1. The recovery yield was between 60% (for ethanol) and 97% (for butyl acetate). Recovery yields of up to 97% were achieved from the 7% PHB solution using butyl acetate. Although all solvents can effectively dissolve PHB, ethyl acetate and butyl acetate showed a greater ability to dissolve PHB than the others. The higher capacity of these solvents for dissolving PHB led to their selection for PHB recovery optimization using RSM. All experiments were done from wet biomass to eliminate multiple costly and energy-consuming drying steps from the process.

Effects of Temperature and Heating Incubation Time on PHB Recovery Using Ethyl Acetate

The temperature and heating incubation time were selected for PHB recovery optimization using RSM. The lower and upper levels of these two parameters were determined on the basis of the boiling point (°C) of the solvents. The efficiency of the process at each temperature/heating incubation time was evaluated by considering the biopolymer extraction yield, purity, and PHB molecular weight. Chloroform extraction was used as the reference method for comparing the results.

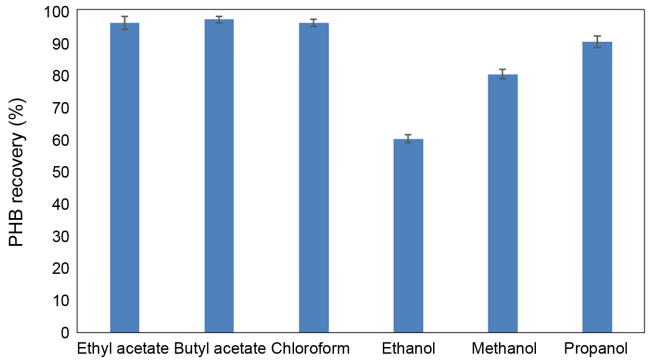

Fig. 1. PHB solubility in different solvents.
PHB solutions (7%) were made by chloroform, ethanol, propanol, methanol, ethyl acetate, and butyl acetate.

The ethyl acetate recovery experiments were conducted using a quadratic model consisting of 13 trials. The actual levels of all parameters and responses (recovery yield, purity, and recovered PHB (g/l)) are shown in Table 2. The PHB recovered in each experiment is the average of the duplicate experiments. The results were analyzed by Design Expert Software and PHB recovery (g/l) can be expressed in terms of the following equation:

$$PHB = +1.57 - 0.55\,A + 0.047\,B - 0.060\,AB - 0.36\,A^2 + 0.011\,B^2 \quad (1)$$

where A is temperature and B is incubation time.

The PHB recovery yield was 4.7% to 87%. Experiment 12 produced the minimum yield and experiment 3 produced the maximum yield. It can be seen that the maximum yield was below the recovery yield of the standard method (chloroform extraction). Table 3 shows the results of statistical analysis of the model based on ANOVA. An F-value of 6.23 confirms that the model was significant.

The model was shown to be significant and reliable at $p < 0.05$. A p-value of 0.0163 indicates that there is only a 1.63% chance of the results being produced by noise from the experiments. The p-value of temperature and heating incubation time was 0.0021 and 0.7, respectively. This shows that the effect of temperature on PHB extraction is more significant than heating incubation time. Positive values for the coefficients denote a positive effect of the parameters on the reaction. It was observed that both temperature and incubation time coefficients were negative. The lack of fit ($p = 0.96$) was not significant.

Table 3. ANOVA for the model developed for PHB recovery by ethyl acetate.

Source	Mean of square	F Value	p Value	Coefficient
Model	0.68	6.23	0.0163	
Temperature	2.46	22.63	0.0021	-0.55
Incubation time	0.014	0.13	0.72	-0.047
Residual	0.11			
Lack of fit	0.015	0.082	0.96	
Pure error	0.18			
Corr. total				

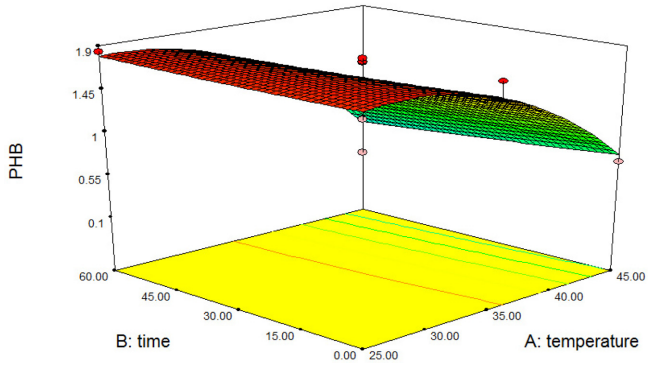

Fig. 2. 3D surface showing the interactive effect of temperature and incubation time on PHB recovery using ethyl acetate as solvent.

Table 2 indicates that maximum PHB recovery was obtained at 25°C for 60 min; a higher temperature (49°C) decreased the recovery of PHB. One reason for this could be the fact that PHB-enriched solvents must be cooled to room temperature before final centrifugation. In many cases, cooling the PHB enriched the solvent and caused formation of an unwanted gelatinous substance by the cellular debris after final centrifugation that decreased PHB recovery and had to be removed [22].

In this form, the biopolymer was recovered by compressing the gel, which produced a layer of biopolymer and a small amount of solvents. The remaining solvent can be removed from the biopolymer layer by evaporation of the solvent under vacuum and increasing the temperature to above the solvent boiling point (Kurdikar *et al*. 2000; US patent 6043063 A). This adds to the time and cost by adding condensing steps to the process. The results showed that complete recovery of PHB by ethyl acetate cannot be achieved through simple steps that are suitable on an industrial scale [22].

Fig. 2 is a 3D plot that shows the interaction strength and optimal values for the independent variables. The plot clearly shows a linear decrease in PHB recovery as the temperature increased and that time does not significantly affect recovery efficiency. No previous reports have been found on the use of ethyl acetate as a solvent for PHB extraction from wet biomass.

Riedel *et al*. [22] used dry biomass (lyophilized pellets) to study the recovery yield of PHB by ethyl acetate. They found that increasing the temperature increased the extraction yields from dry biomass. This may have occurred because no gelatinous compound will form from lyophilized dry biomass and PHB is not removed from the solvent by centrifugation. The results of the proposed method indicate that ethyl acetate is not as suitable as chloroform for PHB recovery on an industrial scale. The drying steps were eliminated to save on time and cost to the process, which was not suitable for scaling up.

Effects of Temperature and Incubation Time on PHB Recovery Using Butyl Acetate

Table 4 shows the results of the use of butyl acetate as the solvent and extraction to optimize temperature and heating incubation times (Table 4) using RSM for maximum recovery

Table 4. PHB recovery yield (%) and purity (%) recovered by butyl acetate from wet biomass.

Experiment No.	Temperature (°C)	Time (min)	Recovered PHB (Average) (g/l)	Purity (%)	Recovery (%)
1	12	30	0.9	99 ± 1	39 ± 2
2	25	0	1.12	97 ± 2	48 ± 3
3	25	60	1.01	97 ± 3	44 ± 1
4	57	0	1.70	97 ± 2	74 ± 2
5	57	30	1.72	100 ± 1	75 ± 2
6	57	30	1.73	99 ± 1	75 ± 1
7	57	30	1.72	98 ± 2	75 ± 1
8	57	30	1.73	96 ± 3	75 ± 2
9	57	30	1.71	99 ± 1	75 ± 2
10	57	72	1.87	98 ± 2	81 ± 1
11	90	0	1.80	99 ± 1	78 ± 2
12	90	60	2	98 ± 3	87 ± 1
13	103	30	2.2	98 ± 2	96 ± 1
Standard	-	-	2.2	99 ± 1	96 ± 1

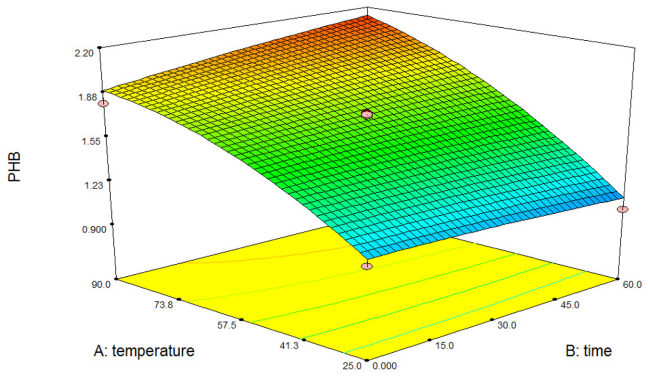

Fig. 3. 3D surface showing the interactive effect of temperature and incubation time on PHB recovery using butyl acetate as solvent.

of PHB from the *C. necator*. As stated, the maximum temperature was set below the boiling point for overall process safety and energy consumption.

The results were analyzed by Design Expert Software and PHB recovery (g/l) can be expressed in terms of the following equation:

$$PHB = +1.72 + 0.439\ A + 0.0413B + 0.0775\ AB - 0.132\ A^2 - 0.0147\ B^2 \quad (2)$$

where A is temperature and B is incubation time.

A maximum yield of 96% and a biopolymer purity of 98% were obtained using a temperature of 103°C and a contact time of 30 min. The recovery yield and purity were similar to those obtained from chloroform extraction. At low temperatures, extraction was insufficient, as evidenced by the low yields obtained (39%–48%).

Table 4 shows that as the temperature increased from 25°C to 103°C, the PHB extraction efficiency increased; increasing the temperature increased the disruption of cells and the solubility of PHB in the solvents. At a lower incubation time, extraction at 25°C was very low, resulting in a yield of 48%. An increase in heat incubation time alone from 0 to 60 min slightly increased the yield from 70% to 75%. The *p*-value indicates that this increase was not significant. Recent reports on butyl acetate recovery shows that the recovery yields increased as temperature increased [9].

Fig. 3 shows the effects of temperature/heating incubation time on PHB recovery. At each incubation time increment, a temperature increase from 25°C to 105°C resulted in a substantial increase in PHB recovery. The results of ANOVA for the model are shown in Table 5. Temperature had a significant effect on PHB recovery yield ($p < 0.05$). ANOVA

Table 5. ANOVA for the model developed for PHB recovery by butyl acetate.

Source	Mean of square	F Value	*p* Value	Coefficient
Model	0.34	31.7	0.00011	
Temperature	1.54	143	0.0001	0.439
Incubation time	0.0136	1.27	0.297	0.0413
Residual	0.0297			
Lack of fit	0.0249	356	0.0001	
Pure error	0.00122			
Corr. total				

showed that the quadratic models adequately represented the data for PHB recovery. The model was highly significant ($p < 0.0001$) for all experiments.

The coefficient of variation (CV) was 6.35, which confirms the high reproducibility of the trials. A high CV indicates that variation in the mean value is high and the model is not suitable and reliable [9]. The results of ANOVA for the independent variables indicate that solvent temperature was the most significant factor affecting PHB extraction ($p < 0.001$). The model indicates that incubation time had no significant linear effects on PHB extraction.

The results show that butyl acetate can be efficiently used as a solvent for extraction of PHB from wet biomass. Its low toxicity, safety, and cost make it a suitable alternative to highly aggressive chlorinated solvents such as chloroform. The best results were obtained at a temperature of 103°C and incubation time of 60 min for a biopolymer yield of 96% and a purity of 98%, which is comparable to the standard chloroform extraction yield. The recovery yield by butyl acetate was greater than that reported by Riedel *et al*. [22]. This could have been the result of differences in the methods of recovery. Increasing the temperature to 103°C and the heating time to 60 min increased recovery yields by 43% over the results from Riedel *et al*. [22].

Solvent cost is considered to be 20% of the total PHB production cost. It can be said that the use of butyl acetate can save about 14% of total PHB cost, because butyl acetate is about 30% of the price of chloroform.

Molecular Weight Determination of the Recovered PHB

Molecular weight is an important parameter that will determine the suitability of the biopolymer for a specific application. In general, biopolymers with high molecular weights are less susceptible to thermal degradation. Most processing methods use a thermal treatment that induces degradation of the biopolymer. Thus, in any application,

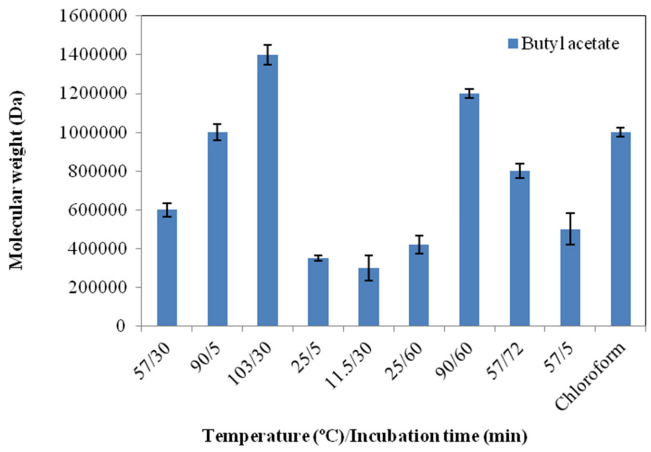

Fig. 4. Molecular weight of PHB obtained by solvent extraction with chloroform and butyl acetate at different temperatures and heat incubation times.

starting with a high molecular weight PHB, even after a significant decrease in molecular weight (Mw) during processing, it is still possible to introduce PHB with sufficient Mw to make a substance with acceptable properties.

In addition, PHB with a high molecular weight degrades more slowly than low molecular weight PHB, which results in slower degradation of the biopolymer, especially in orthopedic applications. The molecular weight of PHB extracted using chloroform (standard) was 1.0×10^6. This is similar to the results of previous reports (1.0×10^4 to 4.0×10^6) [25]; thus, PHB purified by chloroform has an appropriate molecular weight.

The effects of temperature and incubation time on the molecular weight were evaluated for each solvent extraction. The molecular weights of the PHBs obtained using ethyl acetate were lower at all temperatures and incubation times tested than those for the butyl-acetate-extracted biopolymer (data not shown). The highest values were 1.4×10^6 for butyl-acetate-extracted PHB at 130°C/30 min and 1.2×10^6 for 90°C/60 min. The PHB molecular weight was strongly influenced by temperature and duration of heat treatment during extraction [6]. Previous studies reported a decrease in PHB polymer molecular weight as temperature and heat incubation time increased. A limit exists for the increase in temperature during extraction because at temperatures above the boiling point, the solvent begins to degrade the biopolymer, which in turn decreases its molecular weight [17].

Because the temperature was set to below the boiling point, a decrease in molecular weight caused by biopolymer degradation was not observed. Elevating the extraction temperature increased the solubility of the long PHB chains. Interestingly, the biopolymers extracted with butyl acetate had higher molecular weights (3×10^5 to 1.4×10^6) compared with those extracted by ethyl acetate (3×10^5 to 1.0×10^6). These values were nearly equal and were higher than the values obtained for the standard chloroform extraction method (Fig. 4).

These biopolymer recovery results indicate that the use of wet biomass makes butyl acetate a good candidate for substitution of chloroform because it is less miscible with water, has a relatively high boiling point, and is less toxic. The recovery studies showed a higher molecular weight PHB using butyl acetate. One drawback to the use of butyl acetate is that it degrades by hydrolysis in the presence of water [2] and cannot be recycled on an industrial scale.

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