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EFFECTIVE RECOVERY AND PURIFICATION OF POLY(3-HYDROXYBUTYRATE) FROM A HALOPHILIC BACTERIUM BY CHEMICAL DIGESTION METHOD

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Abstract. A simple and effective process for the recovery of intracellular poly(3-hydroxybutyrate) (PHB) from a halophilic bacterial strain - *Salinivibrio* sp. M318 was developed using a chemical digestion method. The effect of temperature, chemicals (sodium hypochlorite and sodium hydroxide) and their concentration on PHB recovery was examined. It was found that sodium hydroxide was an effective chemical for the recovery of PHB from *Salinivibrio* sp. M318. High PHB recovery yield of 97% and polymer purity of 99% were obtained when 50 g/L of bacterial cells were incubated in NaOH solution at the concentration of 0.075 M for 1 h at 50^{o}C. It is expected that this simple method can be of interest for other PHA production processes.

Keywords: NaOCl, NaOH, *Salinivibrio* sp. M318, poly(3-hydroxybutyrate), recovery.

1. Introduction

Polyhydroxyalkanoates (PHA) is a group of biopolymers produced by many microorganisms as carbon and energy reserve granules, usually when grown under condition of nutrient limitation (e.g. N, O, P, Mg or S) and in the presence of excess and suitable carbon source [1]. PHA can be a potential replacement for petrochemical-based plastics due to its thermoplastic, elastomeric, biodegradable and biocompatible properties. Of the large family of PHA, poly(3-hydroxybutyrate) (PHB) is a typical homopolymer synthesized by most of the PHA producing bacteria. PHB has similar thermal and some mechanical properties compared to isotactic polypropylene. PHA and PHB have been used to make various products for packaging, agricultural or medical applications [2, 3].

Nevertheless, PHA and PHB have not become a marketable success due to their expensive production cost compared with petroleum-based plastics such as polyethylene and polypropylene. The PHA production cost depends on bacterial strain, fermentation and recovery processes, and also the substrate utilization [4]. To minimization of the

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PHA production cost, all of their factors need to be considered. Recently, many researchers have been tried to reduce the production cost of PHA by the development of better bacterial strains which can produce high cell density and accumulate high PHA content; using cheap carbon substrates such as plant oil, crude glycerol, agricultural residues for PHA production; and developing effective fermentation/recovery processes [4-8]. PHA is an intracellular product, thus, the methods for its recovery focus either on its solubilisation or on the solubilisation of the non-polymer cellular materials (NPCM). Using organic solvent such as chloroform for PHA solubilisation is the most common method. This method resulted in high PHA purity. However, chloroform is a volatile solvent and hazardous to the environment. Using chemicals such as sodium hypochlorite and sodium hydroxide for NPCM digestion is also a popular method. This method is less toxic but can also give high PHA purity [9].

Recently, we have isolated a halophilic bacterial strain - *Salinivibrio* sp. M318 from fermenting shrimp paste collected from Nam Dinh province. The isolated strain was able to synthesized high PHB content using waste fish oil and glycerol as carbon sources (unpublished data). In this study, a simple procedure for recovering PHB from isolated bacterial cells was developed using chemical digestion method. The combination effect of chemical and temperature was also investigated.

2. Content

2.1. Materials and methods

**** Bacterial strain, maintenance and PHB production***

The bacterial strain *Salinivibrio* sp. M318 was isolated from fermenting shrimp paste collected from Hai Hau district, Nam Dinh province. *Salinivibrio* sp. M318 was maintained at 4^{o}C on solid LB (Luria-Bertani) medium containing (g/L): tryptone, 10; yeast extract, 5; NaCl, 30, pH = 7.0.

For PHB production, the bacterial strain was cultivated in 250-mL flasks containing 50 mL of modified HM (medium for halophile) medium (g/L): glycerol, 15; waste fish oil, 15; $MgSO_4.7H_2O$, 0.5; $KH_2PO_4.2H_2O$, 0.5; $CaCl_2.2H_2O$, 0.01; $FeSO_4.7H_2O$, 0.01; fish sauce, 10; NaCl, 20. The pH of the medium was adjusted to 6.5. The cultures were incubated at 30^{o}C with rotary shaking at 180 rpm. The bacterial cells were then harvested after 48 h of cultivation by centrifugation and washed one time with distilled water. *Salinivibrio* sp. M318 containing 56% PHB was produced and bacterial cell solution with the concentration of 50 g/L was prepared and used for this study.

**** Recovery and purification***

The eppendorf tubes containing 1 ml of bacterial cells were centrifuged at 13 000 rpm for 5 min, the supernatant was then removed. One milliliter of NaOCl with different concentrations (0, 1, 2, 3, 4, 5, 6, or 7%, w/v) or 1 mL of NaOH with different molar concentrations (0, 0.05, 0.075, 0.1, 0.125, 0.15, 0.175, or 0.2 M) was added to each eppendorf tube. The tubes were then vortexed and incubated at different temperatures of 30, 50, and 70^{o}C. The reaction was carried out for 1 h followed by centrifugation at 15 000 rpm for 10 min. The pellet containing PHB was washed twice

with distilled water and freeze-dried for further analysis. All experiments were carried out in triplicate.

**** Analysis of PHB***

Polymer content (weight percent, wt%) in freeze-dried pellet was determined by gas chromatography (GC) analysis. GC samples were prepared according to the method described by Huijberts *et al.* [10], methyl ester was analysed by using HP5890-II system (Hewlett Packard CO, USA) equipped with capillary HP-5 column. Pure PHB (Sigma) was used as a standard for calibration.

**** Determination of purity and recovery yield***

The purity of PHB is defined as the percentage of the amount of PHB to the total dry mass after recovery. The recovery yield is defined as the percentage of the amount of PHB recovered from the total amount of PHB in the cell.

2.2. Results and discussion

2.2.1. Effect of NaOCl concentration and temperature

The effect of NaOCl concentration and temperature on the extraction of PHB from *Salinivibrio* sp. M318 was investigated. As can be seen from Figure 1A, the PHB recovery yield of more than 90% was obtained at all three tested temperatures and at NaOCl concentrations of 0% to 6%. It was slightly decreased when NaOCl concentration was increased, and only about 85% of PHB was recovered at higher NaOCl concentration of 7%. Overall, the PHB recovery yield obtained at temperature of 30^{o}C and 50^{o}C was not significantly different and higher than that obtained at temperature of 70^{o}C. Figure 1A showed that higher PHB purity was obtained at 30^{o}C and 50^{o}C, and there was no significant difference between these two temperatures. The PHB purity was increased when NaOCl concentration was increased and reached maximum value at NaOCl concentrations of 3% to 5%. The highest PHB purity of about 90% was obtained at 30^{o}C and NaOCl concentration of 4% to 5% or at 50^{o}C and NaOCl concentration of 3% to 4%. However, the PHB purity obtained here is still low, further purification step or another method need to be carried to get higher polymer purity.

The results obtained in this study agreed with previous studies reported by Berger *et al.* [11] and Thuoc *et al.* [12]. The polymer purity was increased when NaOCl concentration increased. When either the temperature or the NaOCl concentration was increased, the rate of both biomass digestion and PHA degradation increased, resulted in low polymer recovery yield.

It is interesting to note that PHB purity increased from 56% to 67% after 1 h of incubation in water. It means that 25% of non-PHB cellular material was removed by treating with water. It is due to the weakness of the cell membrane of the halophilic bacteria when they are exposed to hypotonic environment. Under the low concentration of salts the bacterial cells lyse, releasing all the cell materials into the medium and some of them can be removed by centrifugation at low speed [13]. It is a property of halophilic bacteria, which makes it possible to develop a purification process in order to obtain high yields and purity using low and less toxic chemical.

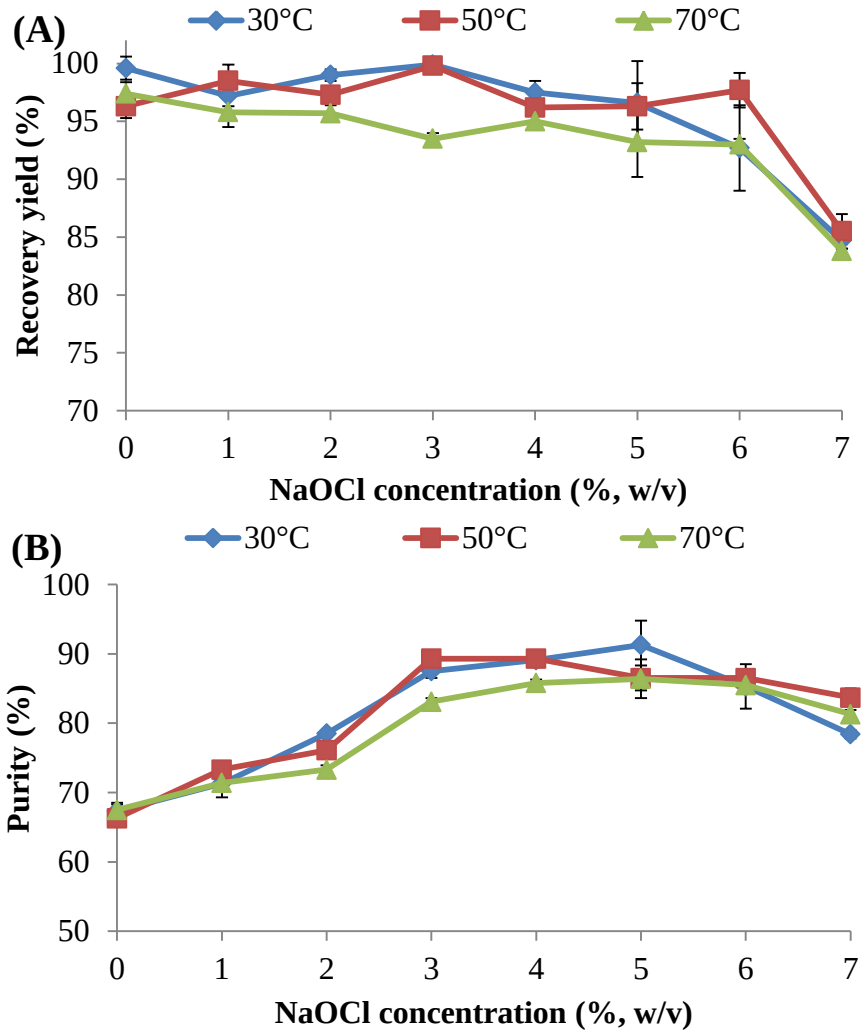

Figure 1. The effect of NaOCl concentration and temperature on PHB recovery (A) and polymer purity (B) from Salinivibrio sp. M318 cells

2.2.2. Effect of NaOH concentration and temperature

In order to find effective method for the recovery and purification of PHB from *Salinivibrio* sp. M318, sodium hydroxide was then tested in this study. The combination effect of NaOH concentration and temperature on the recovery yield is shown in Figure 2A. The recovery yield was decreased when NaOH concentration increased. At temperature of 30^{o}C or 50^{o}C, the recovery yield only depended on the concentration of NaOH, and high polymer yield of above 90% was obtained at NaOH concentration of 0.05 to 0.125 M. At the temperature of 70^{o}C, the recovery yield was dramatically decreased when the concentration of NaOH increased, and only 45% of PHB was recovered at NaOH concentration of 0.2 M.

On the other hand, high PHB purity was achieved when both NaOH concentration and temperature increased (Figure 2B). It was found that the polymer purity only increased at NaOH concentration of 0.05 to 0.075 M, further increase in NaOH concentration to above 0.075 M showed a decrease in PHB purity. At the temperature of

30°C, the PHB purity was increased when NaOH concentration increased but it cannot be higher than 86%. However, high PHB purity can be obtained at the temperature of 50°C or 70°C, maximum polymer purity of 99% and 98% were achieved at the temperature of 50°C and 70°C, respectively (Figure 2B). Figure 3 shows the freeze-dried cells before treatment and the recovered polymer after treatment with 0.075 M NaOH at 50°C. It is clear to see that the NPCM was removed and purified PHB granules were obtained.

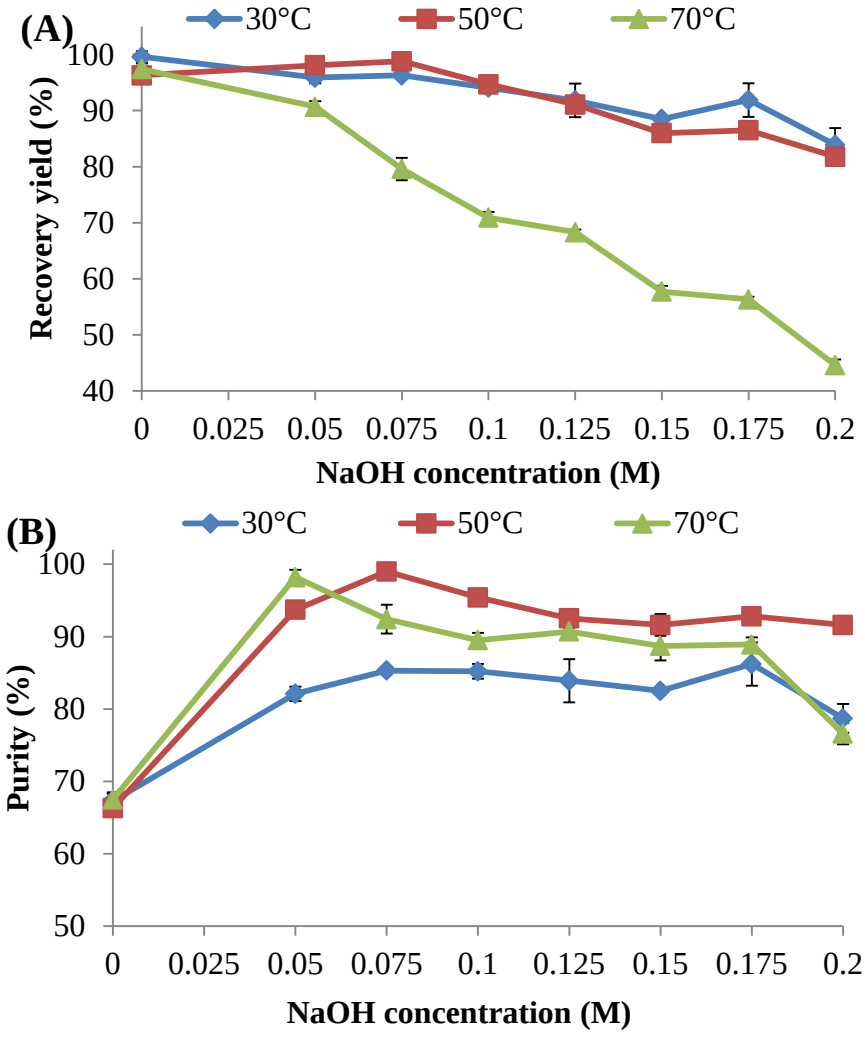

Figure 2. The effect of NaOH concentration and temperature on PHB recovery (A) and polymer purity (B) from Salinivibrio sp. M318 cells

The results obtained in this study are comparable to that of the highest reported so far for other bacterial strains (Table 1). Both polymer purity (99%) and polymer recovery (97%) obtained in this study are among the highest reported so far. The polymer purity obtained in our study is similar to that obtained by previous studies reported by Choi and Lee [14], and Jiang *et al.* [15]. However, the concentration of NaOH used in this study (0.075 M) is lower than that used in two previous studies (0.2 M) (Table 1). As mentioned in previous section, the halophilic bacterial cells lyse easily

when exposed to the low concentration of salts. For that reason, in the case of *Salinivibrio* sp. M318 only low concentration of NaOH needs to be used.

The most common method used for PHA purification is organic solvent extraction. The use of solvent leads to highly pure PHAs. However, this method is relatively costly and causes of environmental problems [9]. Recently, alkaline treatment using NaOH digestion was considered as an environmentally friendly method as compared to the solvent extraction method. The results obtained in this and previous studies showed that pure PHA with high recovery yield can be obtained by NaOH digestion method [14, 15]. In addition, this method is at low cost as compared to other chemical extraction methods.

Figure 3. Picture shows the freeze-dried cells before treatment process (A) and recovery polymer after NaOH treatment (B)

Table 1. Literature comparison of PHA purity and recovery yield

Strain	Treatment parametes	Results	References
Salinivibrio sp. M318	0.075 M NaOH, 50^{o}C, 1 h	Purity: 99%; yield: 97%	This study
E. coli	0.2 M NaOH, 1 h, 30^{o}C	Purity: 99%; yield: 92%	[14]
Mixed enriched culture (*Pseudomonas acidivorans*)	0.2 M NaOH, 0.2% SDS, 1 h, 30^{o}C	Purity: 99%; yield: 93%	[15]
Cupriavidus necator	0.1 M NaOH, 20% (v/v) ethanol, 30^{o}C, 1-3 h,	Purity: 91%; yield: 79%	[16]
Comamonas sp. EB172	0.05 M NaOH, 4^{o}C, 1 h	Purity: 89%; yield: 97%	[17]

3. Conclusions

The combination effect of chemical and temperature on PHB recovery from a halophilic bacterium *Salinivibrio* sp. M318 was studied. High PHB purity of 99% and PHB recovery yield of 97% were achieved after treatment of 50 g/L bacterial cells with 0.075 M NaOH at 50^{o}C for 1 h. The NaOH digestion method developed in this study is a simple, convenient, effective and economical procedure for PHB recovery and purification from *Salinivibrio* sp. M318. It is expected that this simple method can be of interest for other PHA production processes.

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