The effect of sodium hypochlorite concentration on extraction of poly-β-hydroxy-butyrate (PHB) produced from soil bacteria Burkholderia sp B37

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Abstract. Burkholderia sp., a soil bacterium, is a poly-hydroxy-butyrate (PHB) producer. A study reported that sodium hypochlorite solution (NaOCl) is very effective and immediate to lysis the cell wall of bacteria. However, no data available on the effect of NaOCl concentration on extraction of PHB produced from Burkholderia sp. B37. We therefore aimed to evaluate the effect of NaOCl concentrations (2.5, 5, and 12.5 % v/v) on the extractability of produced PHB from Burkholderia sp. B37. The PHB production was carried out in the Ramsay’s minimal medium. The PHB extracted were purified and identified by Gas-Chromatography-Mass Spectrometry (GC-MS) then further evaluated for its physicochemical characteristics including surface morphology, functional group properties by Fourier transform infra-red (FTIR) spectroscopy and thermal characteristic. The results showed that NaOCl facilitates the extraction of produced PHB by Burkholderia sp. B37. The concentration of NaOCl influenced on the extractability of PHB and the surface morphology of purified PHB obviously displayed cleaner surface area when 12.5 % v/v of NaOCl was applied. The FTIR and thermal analysis showed no change detected on the functional group properties and full degradation of purified PHB. However, more works are needed to optimize the yield of PHB recovered.

Keywords: PHB, Burkholderia sp B37, sodium hypochlorite

1. Introduction
Plastic materials have taken place in our daily lifestyle due to the benefits of its properties including durability and resistance [1,2]. In the last decade, plastic derived from petrochemical is the main contributor in environmental problem because it requires long time for being degraded [3–5]. In addition, alternative plastic called oxoplastic, which is easily destroyed, is still leaving microplastic as residues [1]. Concerning to global environment and waste management, there is a great interest to develop biodegradable plastic without leaving any residue with similar characteristics of petrochemical derived plastics including polypropylene and polyethylene [3,6–10].

Recently, poly-β-hydroxy-butyrate (PHB), a poly-hydroxyl-alkanoate (PHA) polymer [11–15], has received great attention as emerging “bioplastic” due to its biological origin and biodegradability characteristics [3,6,12,16,17]. PHB has similar physical properties and molecular structure to...
petrochemical derived polymer [18,19]. Moreover, recent studies have proved the potency of PHB and its future prospect as medical devices, packaging film, carrier of bioactive ingredients, containers, as well as tissue engineering including bone replacement [20–24].

PHB is accumulated by bacteria intracellularly as a carbon or energy storage [15,25–33]. Burkholderia sp. has been noticed as PHB producer [28,34,35]. We reported previously that Burkholderia sp. B37 accumulates large amounts of PHB (52.9 % w/w) [28]. However, the use of biologically produced PHB and its commercial exploitation are limited owing to its recovery problem from microorganism. Different approaches have been applied for the recovery of PHB from the cells, including solvent extraction and aqueous enzymatic digestion. However, these methods require additional digestion as well as solvent extraction steps for the removing of cell residues and escalating the product purity, rendering the PHB recovery cost become expensive.

A differential digestion employing sodium hypochlorite (NaOCl) has been reported as an effective and efficient method for disruption of Bacillus sp. and Alcaligenes autrophus cell walls [36–38]. In addition, dispersion of NaOCl and chloroform could facilitate the improvement of PHB extraction in Alcaligenes autrophus [38]. We, therefore, hypothesized that NaOCl can facilitate the extraction of PHB from Burkholderia sp. B37 in similar manner. This paper emphasizes the effects of NaOCl concentration to facilitate the extraction of PHB from Burkholderia sp. B37. In addition, physicochemical characteristics and percentage of PHB recovered were also evaluated.

2. Materials and Methods

2.1. Materials

Burkholderia sp B37 is collection of Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong-Indonesia. Organic solvent (methanol, chloroform, and acetone), glucose, (NH₄)₂SO₄, Na₂HPO₄·7H₂O, KH₂PO₄, MgSO₄·7H₂O, ferrous ammonium citrate, CaCl₂·7H₂O, MnCl₂·4H₂O, CoSO₄·7H₂O, CuCl₂·2H₂O, ZnSO₄·7H₂O, and FeSO₄·7H₂O are bought from Merck (Darmstadt, Germany) with pro analysis grade.

2.2. Methods

2.2.1. Preparation of Ramsay minimal medium

About 1 L of Ramsay’s minimal medium [29] was prepared with the following composition: 20 g of glucose, 1 g of (NH₄)₂SO₄, 6.7 g of Na₂HPO₄·7H₂O, 0.1 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 60 mg of Ferrous ammonium citrate, 10 mg of CaCl₂·7H₂O, 1 mL of micro element containing 1.98 mg of MnCl₂·4H₂O, 2.81 mg of CoSO₄·7H₂O, 0.17 mg of CuCl₂·2H₂O, 0.29 mg of ZnSO₄·7H₂O, 2.78 mg of FeSO₄·7H₂O and aquadest. The prepared Ramsay’s minimal medium was then sterilized using autoclave (121 °C, 15 min, 1 atm) and keep in room temperature before use.

2.2.2. Preparation of Burkholderia sp. B37 subculture

Burkholderia sp. B37 was inoculated on nutrient agar (NA) then incubated in controlled oven incubator (24 h, 300 °C). The fresh Burkholderia sp. B37 culture was then sub cultured in nutrient broth. About 75 mL of sub-culture medium was prepared in 250 mL Erlenmeyer conical flask. Subsequently, 3 loops of Burkholderia sp B37 were taken and inoculated in sub-culture medium. The sub-culture medium containing Burkholderia sp. B37 was then incubated in a shaker incubator (150 rpm, 30 °C, 24 h). After 24 h of cultivation, the inoculum of Burkholderia sp. B37 bacteria were harvested and used for PHB production.

2.2.3. Production of PHB

The production of PHB was carried out in a laboratory scale. About 75 mL of Ramsay minimal medium was put in 500 mL Erlenmeyer flask and was introduced with 3.75 mL of Burkholderia sp.
B37 inoculum aseptically. The mixtures were then fermented in an orbital shaking incubator (JEIO tech, Japan) (30 °C, 150 rpm, 72 h).

2.2.4. Extraction of PHB

After 72 h fermentation, the process was stopped, and the bacteria cells were harvested. The supernatant and bacteria cells were separated by centrifugation (4000 rpm, 4 °C for 15 min) (Tomy, MX-301). The supernatant was removed, and pellet cells were washed using distilled water. The collected pellet cells were then dried in a controlled oven (70 °C, 24 h). The PHB accumulated in dried pellet cells were then extracted and were purified following procedure as described in Hahn [38]. About 1 g of dried pellet cells was mixed with a solution containing 50 mL chloroform and 50 mL NaOCl (2.5; 5; and 12.5 % v/v) for the digestion. The digestion was conducted in a shaker incubator (180 rpm, RT, 24 h). The mixtures were then centrifuged (4000 rpm, 4 °C, 15 min) and resulted in three layers. The bottom layer (chloroform containing PHB) was collected then further purified using the following step: The PHB in chloroform was added with mixture of cold methanol and water (7:3, v/v) to precipitate the PHB. The precipitated PHB grains were then dried in controlled oven (40 °C). Subsequently, the dried PHB grains were rinsed with acetone to remove impurities. The purified PHB grains were then dried in controlled oven and used for further characterization.

2.2.5. Identification of PHB by Gas chromatography-Mass Spectrometry (GC-MS)

Approximately 2 mg purified PHB grains was methanolysed with 1 mL acidified methanol (in 15 % sulphuric acid) and 1 mL chloroform under heating condition at 100 °C for 140 min. The reaction mixture was washed twice with 2 mL of water. The chloroform fraction was then dehydrated with magnesium sulphate, concentrated and determined by a gas chromatography instrument (GCMS-QP2010 Ultra, Shimadzu, Tokyo, Japan). The GC-MS instrument was equipped with DB-5 column (30 mm × 0.25 mm × 0.25 μm). The temperature was maintained at 90 °C for 3 min, then increased up to 190 °C with rate of 7 °C /min and held for 5 min at this temperature; the increased up to 270 °C with a rate of 8 °C/min and held for 5 min, the injected volume was 1 μL. The identification of peaks was carried out by comparing the mass spectrum peak in the experimentally derived gas chromatogram to the WILEY and NIST library of known mass spectra associated with the instrument.

2.2.6. Characterization of the surface morphology from purified PHB grains by SEM

The surface morphology of purified PHB grains was evaluated using a scanning electron microscopy (SEM; JSM IT300, JEOL, Japan) at 20 kV. Dry purified PHB grains were placed on double sided conducting adhesive carbon tapes and coated with a gold layer for 2 min by D11-29030SCTR Smart Coater. Representative SEM images were taken for each samples at 5000x magnification.

2.2.7. Characterization of functional group property from purified PHB grains by Fourier transform infrared (FTIR) spectroscopy

The presence of functional groups in the purified PHB grains were analyzed by FTIR spectroscopy Nicolet IS5 (ThermoScientific Fisher, USA). The scanning range was from 400 to 4000 cm\(^{-1}\) with 64 scans each and resolution of 2 cm\(^{-1}\).

2.2.8. Characterization of purified PHB grains by thermal gravimetry analysis (TGA)

TGA thermograms were recorded in TGA instrument (Netzsch TG 209F1 Libra). Samples weighed about 10 mg were packed in aluminium convacus pan and then heated from 40 to 400 °C with scanning rate of 10 °C per min under nitrogen atmosphere.
3. Results and Discussions

3.1. Production of PHB from Burkholderia sp. B37 in Ramsay minimal medium

PHB was successfully obtained from the *Burkholderia* sp. B37 when glucose was used as sole carbon source at 30 °C after 72 h of incubation. To confirm the presence of PHB extracted and evaluated the effect of NaOCl on extraction of PHB, the purified PHB grains was analysed using GC-MS. As seen in Figure 1, the peak of PHB presents at retention time of 2.3 min. To evaluate the effect of additional of NaOCl to facilitate the extraction of PHB from *Burkholderia* sp. B37, the percentage of recovered PHB in purified grains was calculated. The addition of NaOCl at 2.5, 5, and 12.5 % v/v resulted in 4.02, 8.83, and 14.2 % PHB recovery, respectively (Table 1). There is a linear correlation between the increment of NaOCl concentration and % recovered PHB. It seems that within the concentrations used for the extraction of PHB from *Burkholderia* sp. B37 resulted in a monotonic increase in PHB extraction. The highest PHB recovery (14.2 %) was obtained with addition of 12.5 % v/v NaOCl. Our results showed that NaOCl disrupts the cell wall of *Burkholderia* sp. B37 in a similar manner with *Alcaligenes eutrophus* as well as *Bacillus* sp. and therefore possible to facilitate the extraction of PHB [38]. We did not perform separation of PHB from *Burkholderia* sp B37 without addition of NaOCl, because it is very viscous and the removal of cell residue very difficult. Since, the optimum concentration of NaOCl for disruption of cell walls of *Burkholderia* sp. B37 has not been reached, therefore further investigation on optimization of NaOCl concentration for extraction of PHB from *Burkholderia* sp. B37 is suggested.

![Figure 1](image1.png)

**Figure 1.** GC-MS analysis of purified PHB grains produced from *Burkholderia* sp. B37

| NaOCl concentration (% v/v) | % PHB recovered |
|-----------------------------|-----------------|
| 2.5                         | 4.02            |
| 5                            | 8.83            |
| 12.5                        | 14.15           |

3.2. Physicochemical characterization of purified PHB grains

As depicted in Figure 2, SEM micrographs of purified PHB grains from *Burkholderia* sp. B37 using different concentrations of NaOCl obviously displayed different appearances. The purified PHB grains treated with NaOCl at 2.5 % v/v (Figure 2a) shows the presence of cell walls mixed with PHB grains. This indicates NaOCl at 2.5 % v/v is less effective to facilitate the intracellular extraction of PHB from *Burkholderia* sp. B37. On the other hand, Figure 2b and 2c presented the effect of NaOCl at higher
concentration (5 and 12.5 % v/v). Those figures obviously showed cleaner surface area of PHB grains than that of Figure 2a. It should be noted that NaOCl does not only offer an effective and a fast cell wall disruption. NaOCl is also able to digest non-PHB cell material (NPCM) [37]. It was suggesting that the higher of NaOCl concentration, the more cell walls were disrupted and the more the NPCM were digested, hence, improving the extraction of PHB from \textit{Burkholderia} sp. B37 cell walls and producing cleaner surface area.

\textbf{Figure 2.} SEM micrographs at 5000x magnification of purified PHB grains from \textit{Burkholderia} sp. B37 treated with NaOCl at v/v concentration value of (a) 2.5 %, (b) 5 %, and (c) 12.5 %.

A study reported that employment of NaOCl may cause severe degradation of PHB. We therefore further analyzed the functional group properties of purified PHB by FTIR spectrophotometry. As depicted in Figure 3a-c, it reveals the presence of prominent peaks at wavenumbers of 1722 cm\(^{-1}\) and 1277 cm\(^{-1}\) corresponding to C=O of thioester group and aliphatic stretching of carbonyl group. Bhagowati et al. [20] reported that the FTIR spectrum of standard PHB shows peaks at wavenumber of 1725 and 1288 cm\(^{-1}\), whereas the blend PHB shifts the wavenumber of C=O to 1658 cm\(^{-1}\). The peak at 1722 cm\(^{-1}\) of purified PHB grains from \textit{Burkholderia} sp. B37 reflects to C=O in the monomer unit of PHB and this also confirms its integrity. Hence, these findings are completely in agreement with the FTIR spectrum of standard PHB. Additionally, direct comparison among the three of FTIR spectrums of purified PHB grains at various concentration of NaOCl, obviously displayed that those spectrums are completely preserved. Those results suggest that NaOCl at concentration up to 12.5 % v/v has no effect in the change of functional group properties of PHB.

Finally, the thermal characteristics of purified PHB grains extracted from \textit{Burkholderia} sp. B37 were evaluated as well as to confirm the degradability of purified PHB. As depicted, Figure 4a-c shown the thermogram of purified PHB grains from \textit{Burkholderia} sp. B37. All thermograms clearly displayed small weight loss at temperature of 45, 65 and 88°C indicating the presence of volatile compounds or water. In addition, at temperature of 293-295°C, all thermograms levelled off and left residual mass below 0 % w/w. Thus, the temperature of maximum rate of decomposition (T\(_{\text{max}}\)) of purified PHB from various NaOCl treatment are similar. The T\(_{\text{max}}\) values of the three samples of purified PHB from \textit{Burkholderia} sp. B37 are also similar with standard PHB [39]. We therefore confirmed that the employment of NaOCl has no effect on the thermal properties of purified PHB grains extracted from \textit{Burkholderia} sp. B37. More importantly, all purified PHBs can be fully degraded without leaving any residue.
Figure 3. FTIR spectrums of purified PHB grains from *Burkholderia* sp. B37 treated with NaOCl at v/v concentration value of (a) 2.5 %, (b) 5 %, and (c) 12.5 %.
Figure 4. TGA thermogram of purified PHB grains from *Burkholderia* sp. B37 treated with NaOCl at 2.5 % (a), 5 % (b) and 12.5 % (c) v/v concentration at temperature of 40 to 400 °C.
4. Conclusion

*Burkholderia* sp. B37 is a promising PHB producing bacteria. NaOCl could facilitate the intracellular extraction of PHB from *Burkholderia* sp. B37. The higher concentration of NaOCl was applied, the higher yield of PHB was recovered. NaOCl up to concentration of 12.5 % v/v did not affect physical properties of purified PHB. However, further works are needed to optimize and validate the NaOCl concentration for PHB recovery.

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