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RECOVERY OF POLYHYDROXYALKANOATES (PHAs) FROM BACTERIAL CELLS USING ENZYMATIC PROCESS

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Abstract

Polyhydroxyalkanoates (PHAs) are intracellular material accumulated by several bacteria. Commercial production of PHAs faces the issue of high production cost especially substrate cost and recovery/separation cost. An alternative to reduce the production cost is to use enzyme and or chemical to recover PHAs from bacterial cells. Recovery of PHAs from bacterial cells was done using enzyme, chemical, and a mixture of enzyme and chemical. Enzyme (s) and or chemical(s) were added into culture broth to disrupt cells after adjusting pH and temperature of the culture broth. Treatment by adding enzyme or chemical only into culture broth showed a low level of PHAs recovered from bacterial cells. Treatment by adding a mixture of enzymes and chemicals showed the best result among 22 examined combinations, i.e. a mixture of EDTA, lisozyme, papain enzyme, and SDS. This combination gave a PHA recovery of 65 % w/w.

Keyword: *culture broth; enzymatic process; recovery of PHAs*

Introduction

Polyhydroxyalkanoates (PHAs) from bacteria have drawn much attention as biodegradable plastic to substitute synthetic polymer in numerous applications (Lee, 1996; Byrom, 1994). PHAs possess material properties similar to those of synthetic polymers. However, production cost of PHAs is much higher than those of synthetic polymers, particularly cost for substrate in fermentation process and recovery of PHAs from bacterial cells. Much effort has been devoted to reduce microbial production cost of PHAs such as to increase PHA productivity by using recombinant cells, to use agricultural substrate (carbon sources) and to develop an efficient fermentation process. However, there is no a decisive method for solving the cost problem (Hori et al., 2002; Wall et al., 2001).

PHA recovery from bacterial cells takes almost 40 % of PHA production cost. Therefore an alternative to develop PHA recovery will contribute a significant effect in reducing PHA production cost. A number of processes have been developed to recover PHAs including (Kessler et al., 2001; Hazenberg and Wiltholt, 1997):

(1) The use of chlorinated solvent such as chloroform and methylene chloride.
(2) Digestion methods to dissolve all cell materials apart from PHAs using inorganic chemical such as alkaline sodium hypochlorite.
(3) The use of enzymes and detergent to rupture cells and dissolve all cell materials apart from PHAs.

Among them, enzymatic process has been adopted for industrial recovery PHAs. In the commercial Zeneca (previously Biopol) recovery process, bacterial cells are ruptured by thermal treatment and the resultant of cell debris is treated with enzymatic coctail and a surfactant to dissolve all cell components apart from PHAs (Koning et al., 1997). The use enzyme gives some advantages compare than that of chlorinated solvents or other chemical because chlorinated solvents apart from being expensive, these are regarded as pollutants. The digestion method by sodium hypochlorite causes severe reduction of PHAs molecular weight.

Polyhydroxyalkanoates (PHAs) are intracellular products, which are accumulated by numerous bacteria. Since bacterial cell consists of almost 50 % of cell dry weight, disruption of cell walls followed by solubilization of all cell materials apart from PHAs is an alternative to recover PHAs from bacterial cells. Rupturing, disrupting, and solubilizing the cell materials may be done by heating, followed by an enzyme and a surfactant treatment such as protease and SDS, respectively. This treatment is possible since most of dry cell components consist of protein and most of cells component could be solubilized in water by SDS.

Papain enzyme and bromelin enzyme are protease enzymes. These enzymes are well known as protease and are used as meat tenderizer. This advantage leads these enzymes as potential enzyme used for disruption of bacterial cell walls. Addition of SDS is important as solubilizing agent of bacterial cell components apart from PHAs.

The aims of this research are: (1) to study proteolytic activity of papain and bromelin enzyme (2) to study the capability of some enzymes and a mixture of enzyme and chemical to disrupt cell walls in PHA recovery process from bacterial cells, including the use of SDS.

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Materials and Methods

Pseudomonas putida was cultivated in 3-liter fermentor to accumulate PHAs using basal salt medium containing saponified palm oil as a carbon source. At the end of fermentation, culture broth was heated at 100 °C for 10 minutes to inactivate depolymerase enzyme. After that, pH and temperature of culture broth were adjusted according to the optimum condition for each enzyme used. Several experiments were done to get information of optimum condition for papain and bromelin enzyme.

To disrupt cells, enzyme or chemical were added into culture broth. When a mixture of enzyme and chemical was applied, enzyme was first added into culture broth followed by chemical treatment after an interval of 30 minutes. After that the mixture were agitated for 15 minutes by a magnetic stirrer. The cell debris and dissolved cell were separated by centrifugation at 5,000 rpm for 15 minutes. PHAs as the bottom layer was than washed with water to remove impurities from insoluble cell components. The amount of PHAs recovered by this treatment is called PHAs recovered by enzymatic process.

As control for this recovery process, instead of addition enzyme and or chemical, the culture broth was centrifuged to obtain cells and then the cells were dried at 60 °C over night. PHAs inside the cells were then extracted with chloroform for 4 hours in a shoxlet extractor. The extract was then concentrated by evaporation on a rotary evaporator. The concentrate containing PHAs was then dropped into a 10-fold volume of 96 % methanol. Finally, precipitated PHAs were dried on air. Percentage of PHAs amount obtained from enzymatic process divided by PHAs amount obtained from chloroform extraction was called as percent of PHAs recovered.

Results and Discussion

Poly(hydroxyalkanoates) (PHAs) are intracellular storage material accumulated by many Pseudomonas sp. which typically contain PHAs up to 65 wt % of cell dry weight (Sudesh et al., 2004). The main target of the recovery process is to disrupt and solubilize all cell components apart from PHAs using enzymatic and or chemical process.

Growing cell to accumulate PHAs

Pseudomonas putida was grown to accumulate PHAs using saponified crude palm oil as a carbon source. The time course of cell growth is shown in Figure 1. Base on this figure, fermentation to accumulate PHAs inside the cell could be terminated between 42-48 hours of cultivation since at this range time cells reached a maximum concentration of 3.4 g/l, correspond the highest PHA content inside the cells.

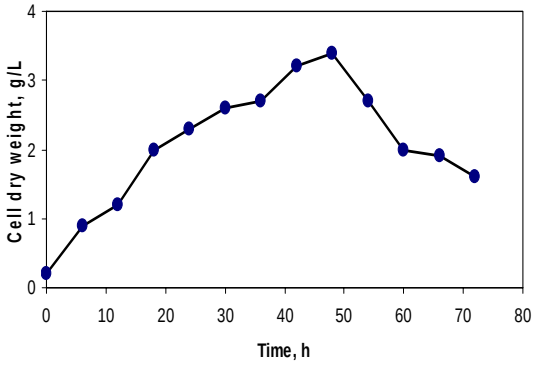

Figure 1 Time course of cell growth using basal salt medium containing saponified crude palm oil as a carbon source

Separation of cells from culture broth

Since PHAs are intracellular product, after terminating fermentation process for production cell containing PHAs, the separation of cells from culture was conducted by physical and chemical process to reduce the volume of culture broth or to obtain cells with out supernatant. Centrifugation, addition of carboxymethyl cellulose (CMC), and alum into culture broth were done, ranging from 5,000 rpm – 10,000 rpm, 0,25 – 1,5 % w/volume, and 0,25 % - 1, 5 % weight /volume, respectively.

The results show that centrifugation at 10,000 rpm for 10 minutes gave highest obtained cells. Addition of CMC into culture broth at 0.75 % w/v gave the highest obtained cells but the cells were not separated. By using alum, addition at 0.75 % w/v also gave the highest obtained cells and the cells were well separated. Cells obtained by centrifugation, addition of CMC, and addition of alum were 3.2 g/l, 2.4 g/l, and 10.6 g/l, respectively as shown in Figure 2.

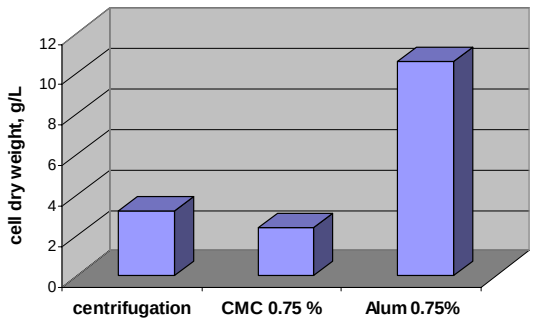

Figure 2 Separation of cells from culture broth by centrifugation, addition of CMC, and alum

Base on Figure 2, addition of alum gave the highest cell concentration but this concentration exceeding the highest cell concentration as shown in Figure 1, i.e. 3.4 g/l. It means that there was other material that precipitated together with the cells. The highest possibility was alum itself. Therefore both

addition of CMC and alum were not suitable to separate cell from culture broth.

Study of enzyme activities

Study of enzyme activity was done to get information of optimum condition with in a range of pH and temperature. It was examined that at pH of 7 and temperature of 37 °C, the activity of papain and bromelin enzyme were 0.084 unit/ml and 0.058 unit/ml, respectively. The value of 0.084 unit/ml means that 0.084 μmol tirosen was released by papain enzyme within 1 minute.

The effect of pH and temperature on proteolytic activity of papain and bromelin are shown in Figure 3. Based on this figure, the pH value of 6.5 and 7 could be applied for enzymatic recovery of PHAs using papain and bromelin enzyme, respectively. When both enzymes were used together, the pH value of 7 was selected as an operation condition for enzymatic recovery of PHAs from bacterial cells.

The effect of temperature on proteolitic activity of papain and bromelin is shown in Figure 4. At temperature of 50 °C, the curve of papain and bromelin achieved the highest activity. Therefore, temperature of 50 °C was selected as an operation condition for enzymatic recovery of PHAs.

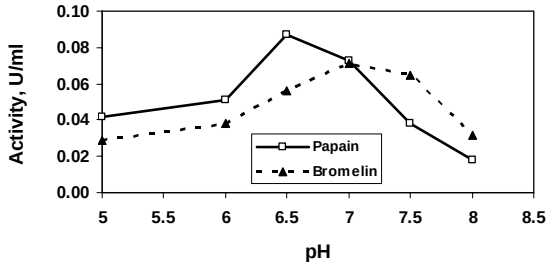

Figure 3 Proteolytic activity of enzyme at various pH

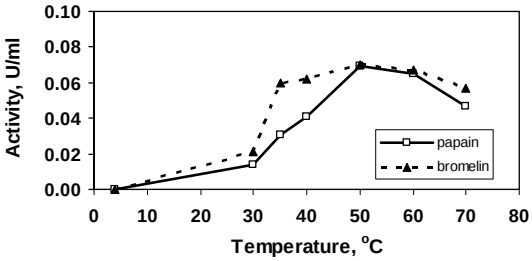

Figure 4 Proteolitic activity of enzyme at various temperatures

Disrupt cell components to recover PHAs.

To recover PHAs from bacterial cells, two methods were considered: (1) separation of cells from culture broth followed by enzymatic process and (2) direct enzymatic process inside culture broth. Separation of cells from culture broth has been done but the result was not satisfied as explained before when CMC and alum were used to obtained cells. Separation by centrifugation and filtration still give the best result compared to other methods.

Direct enzymatic process was done to recover PHA from bacterial cell using enzyme(s), chemicals, and a mixture of enzyme and chemical(s). The result is shown in Figure 5. Based on this figure, % recovery of PHA using papain was better than using bromelin. However, both these enzyme gave low level of % recovery, i.e. less than 10 %. When chemical such as NaOCl, EDTA, SDS were used individually, % recovery was less than 35 %. The best result was shown on a mixture treatment of EDTA, lisozim, papain, and SDS. Synergy of this mixture gave a highest % recovery, i.e. 65 % w/w of PHA recovered from bacterial cells. However, this result was still lower compared to that obtained by Koning and Witholt (1997) who got a PHA recovery of 95 % w/w.

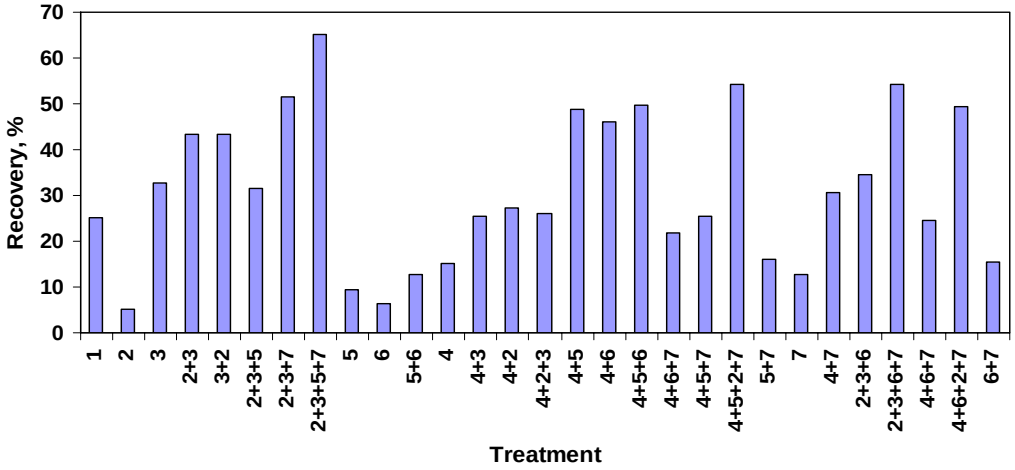

Figure 5 Percent Recovery of PHAs from bacterial cells (1) NaOCl (2) EDTA (3) Lysozyme (4) Heating (5) papain enzyme (6) Bromelin enzyme (7) SDS.

Each enzyme or chemical has its own capability to disrupt cell components. Alkaline hypochlorite (NaOCl) treatment was a good chemical in disrupting cells, but was abandoned because of possible damage to the polymer (Berger et al., 1989). Lysozyme gave a PHA recovery of 31 % w/w. When it was combined with EDTA (treatment of 2+3, Figure 5), PHA recovery was increased to more than 40 % w/w. This combination treatment was consistent with that reported by Marvin and Witholt (1987). In the presence of EDTA, lysozyme treatment was more effective. EDTA complexes divalent cations (mainly Mg^{2+} and Ca^{2+}) and extracts the lipopolysacharides from the outer membrane. This destabilizes the outer membranes which enhances the penetration of lysozyme, thus facilitating the digestion of the peptidoglycan enveloping the cell.

When papain enzyme was added in a mixture of EDTA and lysozyme (treatment of 2+3+5, Figure 5), this enzyme gave a negative effect to PHA recovery and there was a lot of aggregate remain insoluble, but when SDS was used instead of papain enzyme, PHA recovery increased to 52 % w/w (treatment of 2+3+7). On the other side, when SDS was combined with papain enzyme, PHA recovery increased from 15 % to 20 % w/w. Therefore a mixture of EDTA, lysozyme, papain, and SDS was examined. The results showed that this mixture gave the highest percent recovery (treatment 2+3+5+7, Figure 5). The presence of SDS decomposed aggregates that may consists of phospholipids, hydrophobic polypeptides, and membrane fragment. SDS also solubilized these components by incorporation in micelles (Koning and Witholt, 1997).

Conclusion

1. Cultivation time of cells reached a maximum cell concentration of 3.4 g/l at 48 h cultivation.
2. Both CMC and alum were not suitable to separate cells from culture broth.
3. The highest proteolytic activity of papain and bromelin enzyme were at pH of 6.5 and 7, respectively where both enzymes showed the highest activity at temperature of 50 °C.
4. Papain and bromelin enzyme could be used to recover PHA from bacterial cells; however the recovery was very low. Combination of chemical and enzyme treatment gave higher recovery of PHA compared to that of chemical or enzyme treatment only.
5. A mixture EDTA, lisozim, papain, and SDS gave highest result among 22 combinations examined giving a % recovery of 65 % w/w.

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