A new method of recovering polyhydroxyalkanoate from *Azotobacter chroococcum*

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Abstract After polyhydroxyalkanoate (PHA) fermentation for 42 — 48 h by the *Azotobacter chroococcum* G-3, the PHA content reached more than 75% of the dry weight. Biomass was isolated from culture by centrifugation and pretreated with freezing to release PHA pellet, then was treated with 10 g/L sodium dodecyl sulfate (SDS) for 15 min to effectively solubilize lipid and

protein. Subsequently, it was further purified by digesting with 30% sodium hypochlorite (NaClO) for 3 min to remove peptidoglycan and non-PHA biomass. Finally, 98% PHA was obtained by diluting and rinsing with water, and the PHA recovered was suitable for processing.

Keywords: freezing treatment, separation, purification, polyhydroxyalkanoate (PHA).

There has been cosiderable exploration in degradable plastic in order to reduce the harmful effect of "white pollution" on the environment. PHA has been studied extensively because of its biodegradable and thermoplastic characteristics. PHA has been on the market since the 1980s, however, it cannot compete with synthetic plastic due to high cost of production, therefore, lowering the cost became the key to the application of PHA. PHA production involves fermentation and PHA recovery, the latter being more important, and accounting for 70%—80% of the whole cost. In addition, the methods of PHA recovery affect the properties of PHA significantly. Recently, PHA recovery has been studied extensively in Alcaligenes and recombinant *E.coli*[2]. The methods commonly used for PHA recovery involve solvent extraction with chloroform[3], enzyme digestion[4], sodium hypochlorite (NaClO) solution digestion[5], chloroform in combination with NaClO[6] and surfactants-sodium hypochlorite[7,8]. However, because of the difference in the cell wall of various bacteria and the conditions for PHA formation, the method of PHA recovery from various bacteria is also different. Some strains of *Azotobacter* are suitable for high yield of PHA, but as the culture method is different from others, its structure also possesses the speciality: the mature huge cells are irregular and easy to be disrupted. Nowadays, recovery of PHA from *Azotobacter* is by solvent extraction with $CHCl_3$. Although the method has the advantage of obtaining high-degree purity of PHA and recovery, the cost of recovering PHA with $CHCl_3$ is high as the amount of $CHCl_3$ needed is large, thus limiting the PHA production by *Azotobacter*. The present note describes an improved method to obtain PHA from *Azotobacter* without using solvent of $CHCL_3$.

1 Materials and methods

(i) Bacteria and culture. *Azotobacter chroococcum* G-3 was provided by this Lab. The culture medium (g/L) contained: K_2HPO_4 0.8, KH_2PO_4 0.2, $MgSO_4 \cdot 7H_2O$ 0.2, $CaCO_3$ 0.5, $FeCl_3 \cdot 7H_2O$ 0.125, $Na_2MoO_4 \cdot 2H_2O$ 0.25, peptone 1, trace element solution 1 mL, distilled water 1 000 mL, pH 7.2; the trace element solution: H_3BO_3 0.3 g, $CoCl_2 \cdot 6H_2O$ 0.2g, $ZnSO_4 \cdot 7H_2O$ 0.1 g, $MnCl_2 \cdot 4H_2O$ 30 mg, $NiCl_2 \cdot 6H_2O$ 20 mg, $CuSO_4 \cdot 5H_2O$ 10 mg, distilled water 1 000 mL. The cells were cultured in a 2-L autocontrol fermenter (Virtis Company, USA) for 42—48 h, containing 1.2 L medium with 10% inoculum and incubated at 30℃, with the pH value maintained between 6.9 and 7.2

(ii) Extraction and determination

(1) Solvent extraction with chloroform. The biomass was treated with a 60-time volume chloroform at 30℃ for 3—4 h with stirring, then water was added to keep static for 12 h, and clear polymer was separated through a funnel from the aqueous portion by filtration, then PHA was precipitated with methanol, and dried in air.

(2) Recovery of PHA by SDS treatment. 100 mL biomass (30 g/L dry weight) was added to a different-concentration SDS solution at 55℃ for a certain time, then PHA was recovered by centrifugation and rinsed with water, dried in air.

(3) Recovery of PHA by SDS-NaClO treatment. The biomass was pretreated with SDS solution, then treated with different-concentration NaClO for some time, finally PHA recovered was dried in air.

(4) Analysis of PHA purity. The purity of PHA was determined by gas chromatography[9].

(5) Process performance test. The PHA granules were shaped by a hot press shaper of plastic at a temperature of (175±5)℃ and a pressure of (100±5) kg/cm^2 for 3 min. Then, preheated for 3 min, the granules were made in dumbbell's shape to test the tensile strain and elongation at rupture by a German LMTI250 type stretcher at room temperature, and identify their process performance according to the standard of GB 8528-82.

2 Result and discussion

(i) The effect of pretreatment on the structure of cell wall. The biomass containing 75% PHA

in dry weight was obtained by fermentation of *A.chroococcum* G-3. As different pretreatment of the biomass had an obvious effect on subsequently recovering PHA, we used three different methods to pretreat the biomass. Pretreatment (1): the biomass was directly dried at 90℃ to constant weight without any pretreatment when the fermentation was over, then stored at room temperature; pretreatment (2): the biomass was treated at 100℃ for 1 min then rapidly cooled to 55℃ before drying at 90℃ to steady weight, then stored at room temperature; pretreatment (3): the biomass was treated at 100℃ for 1 min and rapidly cooled to 55℃ followed by freezing treatment, then stored at −18 ℃. In fact, the effect of these three pretreatments on breaking the wall of *A.chroococcum* G-3 was different, which was manifested by the following experiments. The biomass was first pretreated according to the three different methods, then each was dissolved in 10 g/L SDS solution at 55℃ for a fixed time. The result was shown by the solution value of optical density at 600 nm (A_{600}) as demonstrated in fig. 1.

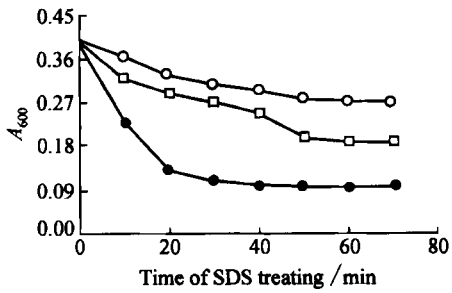

Fig. 1. Effect of different pretreatment methods on the optical density of SDS solution at 600 nm. ○, Pretreatment (1); □, pretreatment (2); ●, pretreatment (3).

The optical density values at 600 nm from the three pretreatments were obviously different, and the A_{600} of solution pretreated with freezing was evidently lower than the others, which was due to the fact that the water in cells formed ice crystal particles by freezing treatment, causing the cells to swell to disruption[10]. Moreover, cells in disruption made it easy for the lipid bilayers of the cell membrane to be completely digested by the solution of SDS and SDS to combine with protein to form SDS-protein complex which was easy to be dissolved in water[11]. Thus, the solution became clear in a short time by freezing pretreatment and solution A_{600} was low; while the other two pretreatments only made SDS alter the permeability of cell walls so that the A_{600} of solution was higher. Therefore, freezing pretreatmen facilitated to isolation and purification of PHA.

(ii) Effect of SDS concentration and the time of treating on the purity of PHA. In this experiment, the relation of PHA purity between both the concentration of SDS and the time of SDS treating was observed. By reacting 30 g/L biomass pretreated by freezing with different concentrations of SDS at 55℃ or for different times of treating at a certain concentration, the change in PHA purity was observed. The result is shown in fig. 2.

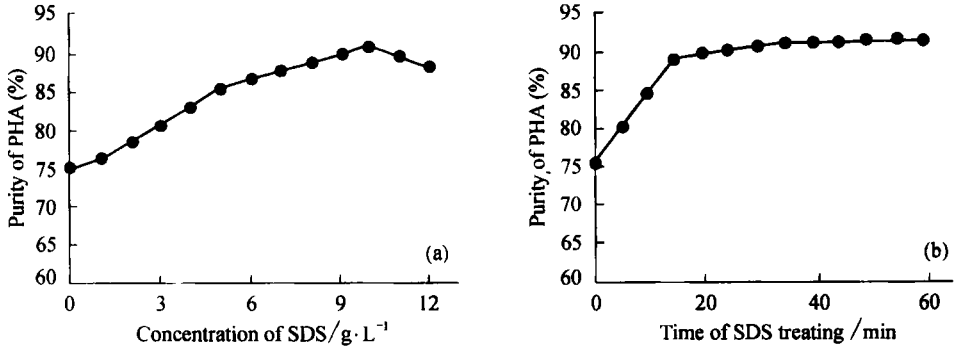

Fig. 2. Effect of SDS concentration and treatment time on purity of PHA.

At low concentration of 1 g/L SDS, a purity of 75% was achieved. Further improvement in purity of PHA was attained as the concentration of SDS was increased, and when the SDS concentration was 10 g/L, a higher purity of 90% was achieved. While the SDS concentration was higher than 10 g/L, the purity of PHA did not increase but decreased (fig. 2(a)). It was found that PHA recovered by treating with high SDS concentration was dissolved in chloroform covered with a layer of white gel, which was

identified as SDS remainder, and the excessive SDS combined with peptidoglycan and other macromolecule debris attached to the hydrophobic surface of PHA granules, which decreased the purity of PHA. Thus it was important to control the SDS concentration to improve the purity of PHA.

Meanwhile, when the biomass was treated with 10 g/L SDS for 15 min, a purity of 90% was obtained; however, when the time of exposure to SDS was prolonged, the purity of PHA did not increase but remained at about 90%. Therefore, the optimal SDS concentration and the time of SDS treating of recovering PHA respectively were 10 g/L and 15 min.

(iii) Effect of NaClO concentration and the time of treating on the purity of PHA. Although recovery of PHA with SDS treatment was highly effective in removing lipid and protein of the disrupted cell, the peptidoglycan and other debris were still not removed. However, these debris not only affect the PHA purity, but also have a great effect on the tensile strain of PHA products rendering them discolored during processing as thermoplastic. So it needs further purification which can be achieved with NaClO treatment, as low NaClO concentration was effective in digesting non-PHA biomass. Furthermore, it had little undesirable effect on PHA granules. The effect of the different NaClO concentration and the time of NaClO treating on the purity of PHA are shown in fig. 3.

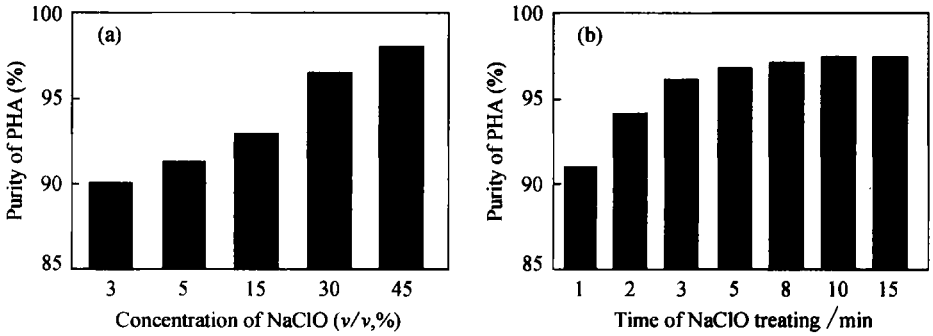

Fig. 3. Effect of NaClO concentration and treatment time on purity of PHA.

NaClO of lower concentration in a short time had poor effect on degrading non-PHA biomass (fig. 3(a)). At the concentration of 30% NaClO, the purity of PHA was 96.5%, and further improvements in purity were attained as the concentration of NaClO increased, but it resulted in severe degradation of PHA, rendering it poor in mobility and difficult to be processed as a thermoplastic. The time of NaClO treating also had a significant effect on the purity of PHA (fig. 3(b)). It is found that when biomass was treated with 30% NaClO for 3 min, the purity of PHA reached 96.5%, and did not degrade the polymer; however, the time of treating by NaClO longer than 3 min began to degrade polymers. Moreover, when the time of treating increased to 15 min, the degradation of PHA became more severe, while the improvement in purity of PHA was small. Thus, it was also important to control the time of NaClO treating during the PHA recovery. Good results were achieved by diluting the concentration of NaClO with 10-time volume water after treating with NaClO for 3 min; meanwhile, the SDS remainder and hydrophilic degradation product were removed by dilution and rinsing, the purity of PHA was improved and a PHA of 98% purity was obtained for desirable process performance.

3 Conclusion

Pretreatment with freezing broke up cells releasing both the PHA granules and cell contents to be easily digested by SDS and NaClO in a short time. 30 g/L biomass was treated by 10 g/L SDS at 55℃ for 15 min, then exposed to 30% NaClO for 3 min at 30℃, and diluted immediately with water to end the function of NaClO. Finally a purity of 98% was obtained and the PHA was suitable for processing as a thermoplastic, and the PHA recovery was 86.6%.

The advantages of the combined treatment are that it is simpler, that PHA granules obtained from *Azotobacter* are lower in cost than with other traditional techniques such as solvent extraction or enzyme treatment, and that industrialized production of PHA from *Azotobacter* will be possible. Although Ramsay et al. had reported recovering PHA from *Alcaligenes eutrophus* with

surfactant-hypochlorite, the ratio of surfactant to biomass was 1 : 1, which means an increase in cost. Furthermore, the molecular weight of PHA recovered by Ramsay was about 60% of the original MW, hence another advantage of this method——the pretreatment with freezing enhances the function of SDS and NaClO, and greatly decrease the cost of PHA recovery. In addition, dilution with water was effective in controlling the function of NaClO and limiting NaClO degradation on PHA, and the final PHA product obtaind by this method was suitable for processing as a thermoplastic.

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