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RECOVERY OF POLY(3-HYDROXYBUTYRATE) FROM *Yangia* sp. ND199 BY SIMPLE DIGESTION WITH SODIUM HYPOCHLORITE

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ABSTRACT

Polyhydroxyalkanoate (PHA) is a biodegradable polymer synthesized intracellularly by many microorganisms. After extraction from the cells, they possess the common features of non-toxic, biocompatible, biodegradable and recyclable. These features render them highly competitive with polypropylene or other petroleum-derived plastics, especially in medicine. The recovery of PHA from bacterial cells is the key step of PHA production process. Many methods have been used for PHA recovery; these include solvent extraction, enzymatic hydrolysis, and chemical digestion. In this study, the chemical digestion method was used for recovery of poly(3-hydroxybutyrate) (PHB) from *Yangia* sp. ND199. Among various chemical tested (NaOH, HCl, KOH, NaOCl and SDS), NaOCl was found as an efficient chemical for PHB recovery PHB from *Yangia* sp. ND199. The optimal recovery condition was a ratio of 1:1 (v/v, solution containing 100 g/l bacterial cells and solution containing 6 % NaOCl), at 30 °C for 1 h. Under such conditions, a purity of 99 % and a recovery yield of 94 % were obtained. This purification method is simple and can be developed and used for pilot scale.

Keywords: NaOCl, polyhydroxyalkanoate, poly(3-hydroxybutyrate), recovery, halophilic bacteria, *Yangia* sp. ND199.

1. INTRODUCTION

Mankind has become highly dependent on fossil resources for its need for energy, chemicals and materials. However, fossil resources will sooner or later come to an end and also that they are found only in some regions of the world has led to a global interest in finding alternative sources that are renewable and easily accessible. Another problem that motivates a shift from fossil resources is the negative environmental impact of the processes and products in terms of greenhouse gas emissions, global warming and climate change. Fossil plastics are among the most environmentally damaging products used in enormous amounts. It is well known that these materials are not biologically degradable, causing an increasing solid waste stream with negative environmental effects. After use, about forty percent of plastics produced are discarded into landfills and several hundred thousands of tones of plastics are discarded

each year into marine environments, and cause threat to marine diversity [1]. In order to overcome the problem of pollution caused by non-degradable plastics, there is considerable interest in the development of biodegradable polymers such as polylactic acid (PLA) or polyhydroxyalkanoates (PHA) [2].

PHA is a biodegradable polymer that accumulates intracellularly as carbon and energy storage material in many microorganisms, usually when grown under the limitation of a nutrient such as oxygen, nitrogen, phosphate, sulphur, or magnesium and in the presence of excess carbon [3, 4]. PHA exists as discrete granules, with about 5 to 13 granules per cell and with diameters of 0.2 to 0.5 μm [5]. After extraction from the cells, PHAs possess the common features of non-toxic, biocompatible, biodegradable and recyclable thermoplastics. The main applications of PHAs include replacing petrochemical polymers currently in use for packaging and coating, as well as disposable items such as razors, utensils, diapers, feminine hygiene products, and cosmetic containers such as shampoo bottles and cups. PHAs are also useful as stereoregular compounds that can serve as chiral precursors for the chemical synthesis of optically active compounds. Such compounds are particularly used as biodegradable carriers for long-term dosage of drugs, medicines, hormones, insecticides and herbicides. They are also widely employed as bone plates, osteosynthetic materials, surgical sutures, vascular grafts and heart valves [1, 6].

The recovery of bacterial polyhydroxyalkanoate is the key step of the PHA production process. There are many methods having been used for PHA recovery: solvent extraction method, the use of chemicals or enzymes to digest non-PHA cellular materials (NPCM) [7, 8]. In Vietnam there is still no research focusing on the recovery PHA in lab or pilot scale. The purpose of this study was to develop a simple digestion method for PHA recovery from *Yangia* sp. ND199. The halophilic bacterium strain *Yangia* sp. ND199 was isolated from soil sample collected from mangrove forest in Giaothuy district, Namdinh province [9]. This strain was able to accumulate poly(3-hydroxybutyrate) (PHB) up to 70 % of cell dry weight.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial strain

The bacterial strain *Yangia* sp. ND199 was isolated from mangrove soil sample collected from Giaothuy district, Namdinh province.

2.1.2. Medium for PHB production

The strain *Yangia* sp. ND199 was grown on MA (medium for PHA production) medium containing (g/l): NaCl, 45; $MgSO_4.7H_2O$, 0.85; $CaCl_2.2H_2O$, 0.09; KCl, 0.5; KBr, 0.06; KH_2PO_4, 0.55; yeast extract, 1.5; fructose, 25; pH 7.0.

2.2. Methods

2.2.1. PHB production by Yangia sp. NĐ199

The cultivation for PHB production was conducted in 250 ml Erlenmeyer flask containing 50 ml of MA medium. The cultures were incubated at 32 °C with rotary shaking at 180 rpm.

After 48 h of cultivation, bacterial cells were collected by centrifugation and washed twice times with distilled water.

2.2.2. PHB recovery by simple digestion with chemicals

The chemical digestion method for PHA recovery from *Yangia* sp. ND199 was performed as reported previously [10]. The chemicals including 0.2N NaOH, 0.2N HCl, 0.2N KOH, 8 % (w/v) NaOCl, 5 % (w/v) sodium dodecyl sulfate (SDS) were used for NPCM digestion. The treatment was carried out in 2 ml microtubes containing 0.5 ml of bacterial cells (100 g dry cell per litter) and 0.5 ml of chemicals or distilled water. The reaction was conducted at 30 °C for 1h followed by centrifugation at 13 000 X g for 10 min. The harvested solid sample containing PHB was washed twice with distilled water. The recovered PHB was dried at 105 °C for 24 h. PHB quantification was performed according to the method of Law and Slepecky [11]. The recovery and the purity of PHB were calculated based on the results of PHB analysis.

2.2.3. Digestion of NPCM with different NaOCl concentrations

Different NaOCl concentrations (0; 1 %; 2 %; 3 %; 4 %; 5 %; 6% and 8 %, w/v) were tested for their ability to digest NPCM for the recovery of PHB from *Yangia* sp. ND199. The reaction was carried out as described above. Optimum NaOCl concentration for PHB recovery was determined based on the results of PHB analysis.

2.2.4. PHB recovery using NaOCl at different temperatures

The mixture of 0.5 ml of bacterial cells (100 g dry cell per litter) and 0.5 ml of 6 % NaOCl was incubated at different temperatures (30 °C, 50 °C and 80 °C) for 1 h. The procedure was carried out as described above. Optimum temperature for PHB recovery was determined base on the results of PHB analysis.

2.2.5. PHB analytical procedure

PHA concentration was determined according to the method of Law and Slepecky [11]. For this, about 10 mg of freeze-dried cells was mixed with 10 ml of 98 % (v/v) sulphuric acid. The mixture was incubated at 100 °C for 1.5 h to obtaine crotonic acid. After cooling to room temperature, the mixture was dilluted with sulphuric acid and quantified by measuring absorbance at 235 nm. Pure PHB was used as a standard for calibration.

2.2.6 Determination of purity and recovery yield

The purity of PHB is defined as the percentage of the ratio of the amount of PHB to the total dry matter 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.

3. RESULTS AND DISCUSSION

3.1. Effect of various chemicals on PHB recovery

After cultivation on MA medium for 48 h, bacterial cells were collected by centrifugation, the result of PHB analysis shown that about 71 % PHB was accumulated in the bacterial cells. PHB-containing cells were then fixed and observed under transmission electron microscopy (TEM). Figure 1A showed that the bacterial cells were full filled by PHB inclusions with only one or two big PHB granules. With high content of PHB in the cells, envelop surrourd the *Yangia* sp. ND199 cells become a thin layer, as can be seen from Figure 1B the layer was about 19 nm or less. That make the *Yangia* sp. ND199 cells become fragile and it will be easier to purify. Therefore we chosen a simple method for digestion of NPCM and recovery of PHB from *Yangia* sp. ND199 cells.

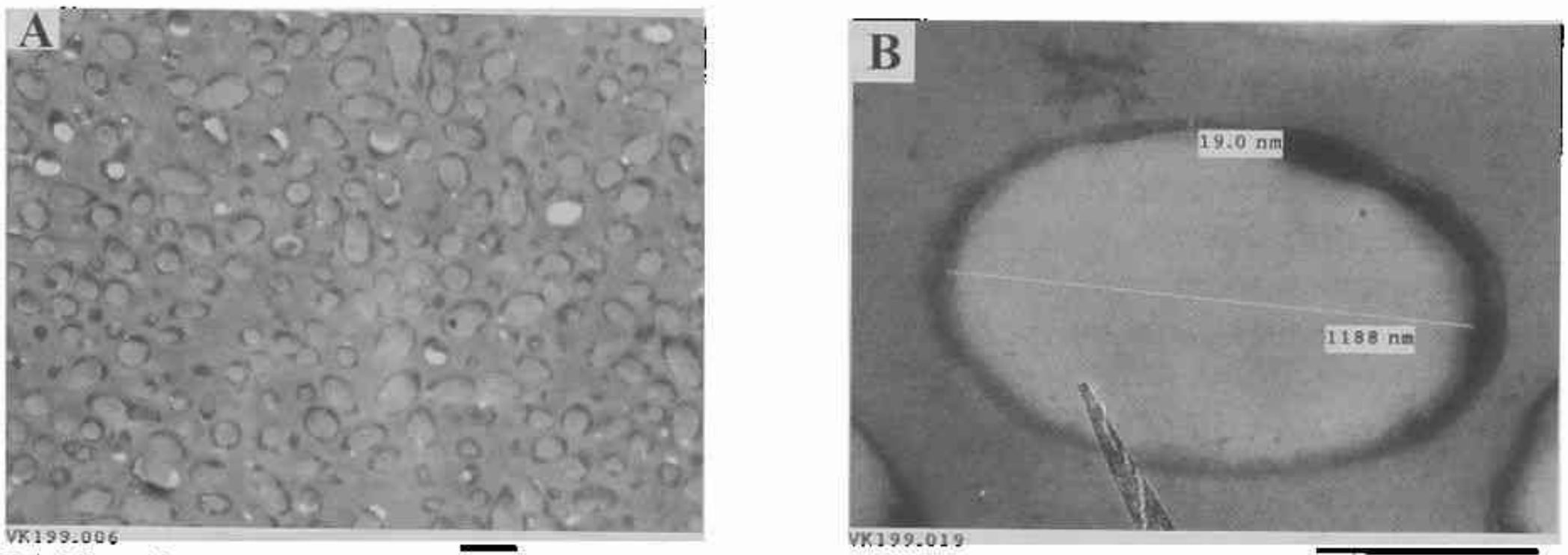

Figure 1. Transmission electron micrograph (TEM) showing (A) PHB granules and (B) bacterial cell envelope.

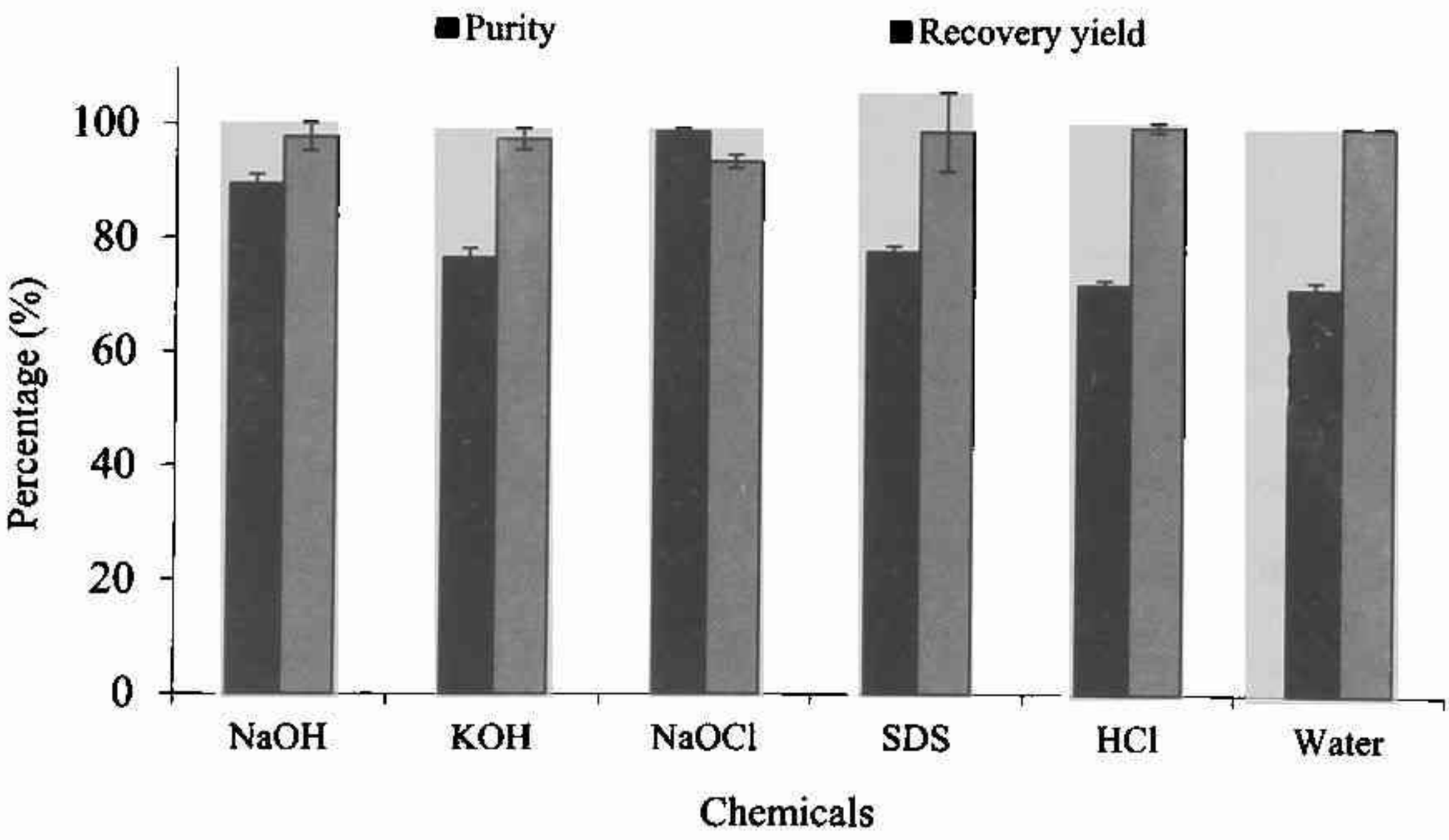

Figure 2. Effect of various chemicals on the recavery of PHB from *Yangia* sp. ND199.

Yangia sp. ND199 cells were treated with five different chemicals (NaOH, HCl, KOH, NaOCl and SDS). The results obtained after treating bacterial cells with five chemicals at 30 °C are shown in Figure 2. Among the five different chemicals tested, KOH, SDS and HCl were found to be inefficent for the purification of PHB from *Yangia* sp. ND199, resulting in the PHB purity of less than 80 %. Because of lower purity, the recovery yield obtained by using KOH, SDS or HCl was high (97.5 %, 99 % or 100 %, respectively). Again digestion with KOH, SDS or HCl resulted in low PHB purity. NaOH and NaOCl efficiently digested NPCM resulting in high PHB purity and recovery yield (equal or greater than 90 %). When the cells were treated with NaOH, PHB was recovered with the recovery yield of 97 % but with a little lower purity of about 90 %. NaOH would digest the bacterial cell wall, releasing the biological macromolecules into the aqueous solution [12]. That make the reaction solution become viscous, preventing the washing step to get purity PHB from *Yangia* sp. ND199 cells.

Among the various tested chemicals, NaOCl was efficient for the recovery of PHB with high purity of 99 %. The recovery yield obtained after treatment with NaOCl was also high (more than 90 %). NaOCl was chosen in this reseach because it can gave high purity, high recovery yield and inexpensive. A recovery method using NaOCl was found to be the most simple method that can be easier to apply for pilot scale.

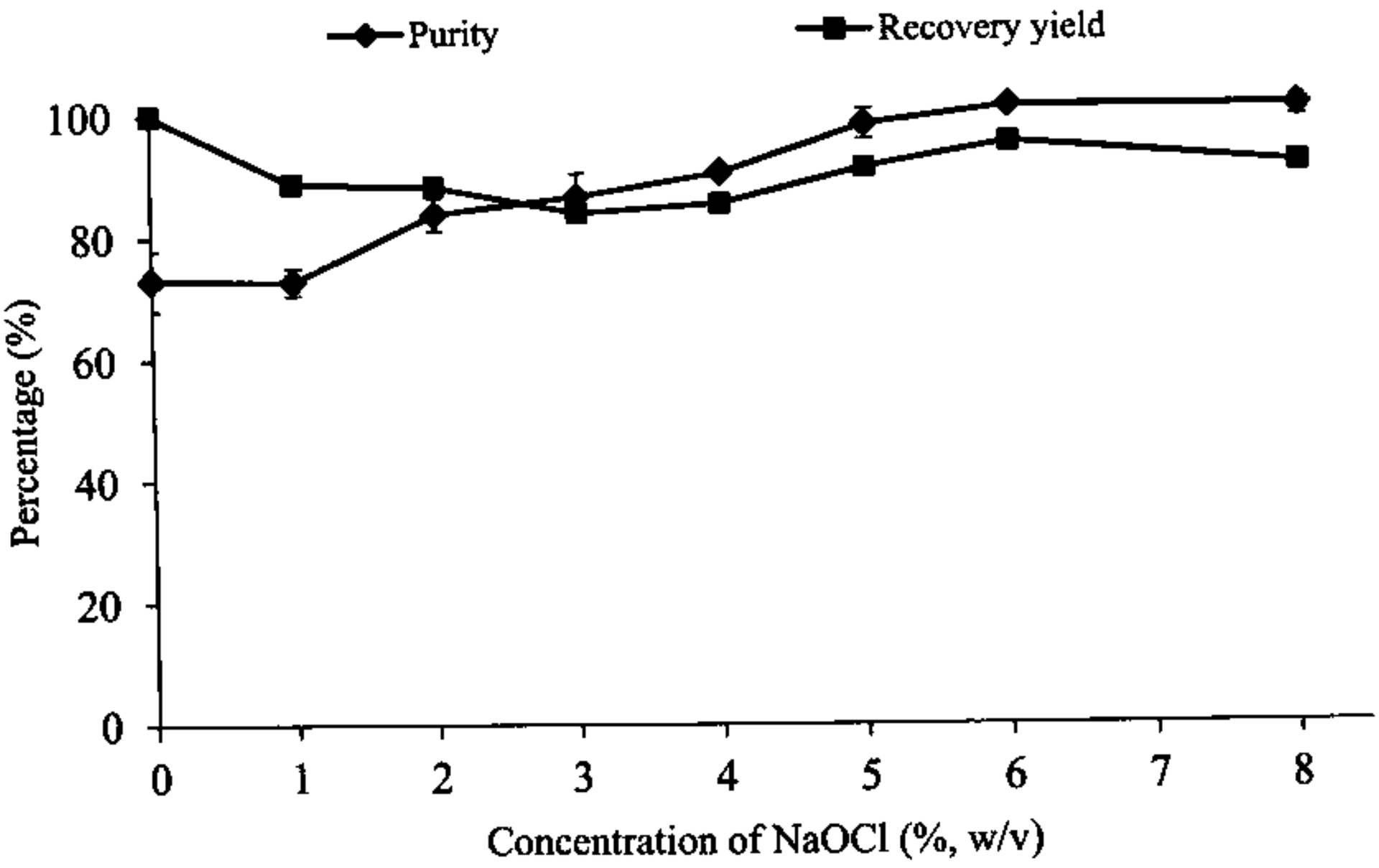

Figure 3. Effect of different NaOCl concentrations on the recovery of PHB from *Yangia*sp. ND199.

3.2. Effect of NaOCl concentration

The treatment experiments using different concentrations of NaOCl were carried out to find the optimal alkali concentration for the recovery of PHB from *Yangia* sp. ND199 cells with high purity and recovery yield. As shown in Figure 3, at low concentration of NaOCl (0 and 1 %, w/v) the bacteria cells were not digested, resulting in low PHB purity of 76 %. The digestion of NPCM was started at 2 % NaOCl. The PHB purity increased from 83 % to 90 and nearly 99% with increasing NaOCl concentration from 2 % to 4 and 6 %, respectively. Further increase in NaOCl from 6 to 8 % showed no significant difference in PHA purity but polymer

recovery yield decreased from 94 to 91 %. Anis and co-workers [12] had also found that purity of PHA increased and recovery yield of PHB decreased when the concentration of chemical increased. The decrease in PHB recovery yield could be due to the degradation of the polymers occurs because the high concentration of NaOCl would not only digest the NPCM but also digest the polymers into water soluble monomers.

3.3. Effect of digestion temperature

The effect of the digestion temperature on the purity and recovery yield of PHB was investigated by digesting *Yangia* sp. ND199 cells with 6 % NaOCl (ratio 1:1, v/v) for 1 h at three different temperatures (30 °C, 50 °C and 80 °C). The results are shown in Figure 4. The purity of PHB was not changed (nearly 100 %) when the temperature was increased. However, higher digestion temperature resulted in a lower recovery yield of PHB. Maximum recovery yield of 94% was obtained at digestion temperature of 30 °C, the yield was decreased to 79% at 50 °C and only 50% at 80 °C. Previous studies have been reported that at a higher temperature, the digestion reactions were accelerated, which cause both cell disruption and PHA degradation to speed up [10, 12]. For that reason, the recovery yield of polymer was decreased. Temperature of 30 °C was found to be most effective in this study (gave high PHB purity and recovery yield). In addition, less energy for heating was required during the recovery process at treatment temperature of 30 °C, particularly in tropical countries such as Vietnam.

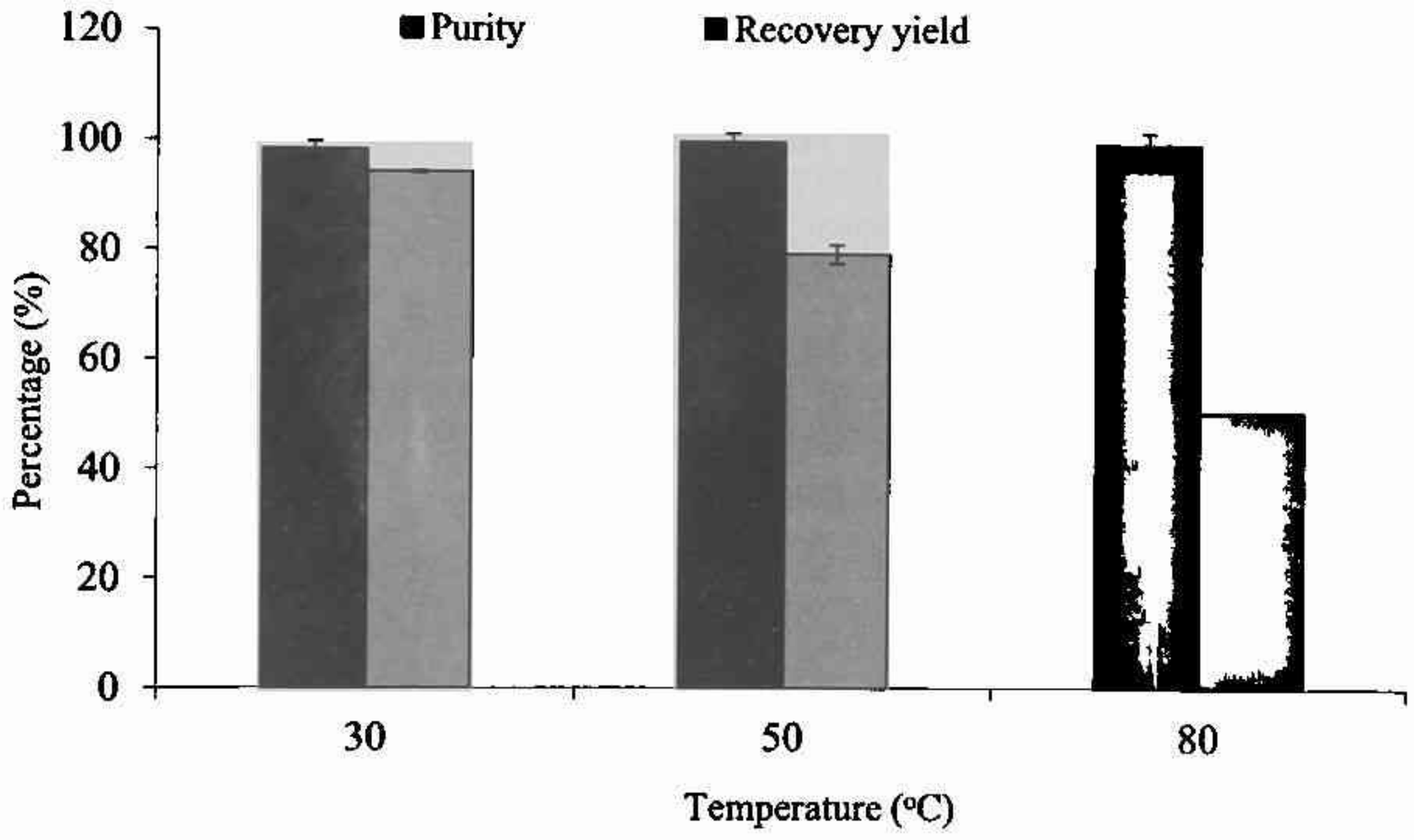

Figure 4. Effect of different temperatures on the recovery of PHB from *Yangia* sp. ND199.

The purified polymer samples after treatment with 6 % NaOCl at 30 °C for 1 h were then observed under transmission electron micrograph (TEM). The Figure 5 shown that the NPCM was removed and PHB granules with clean surface were obtained. The PHB granules were ranged from 0.3 to 1.2 μm in diameter and they retained a typical shape as it had exist in the cells.

The results obtained here are comparable to that of the highest reported so far for other bacterial strains. Hahn and co-workers have reported that PHB purity of 97 % and recovery yield of 91 % were obtained when *Alcaligenes eutrophus* cells was digested with NaOCl [14]. In another report, NaOCl was also used for the digestion of *Alcaligenes eutrophus* DSM545 cells and PHB purity of 95 % was obtained [15].

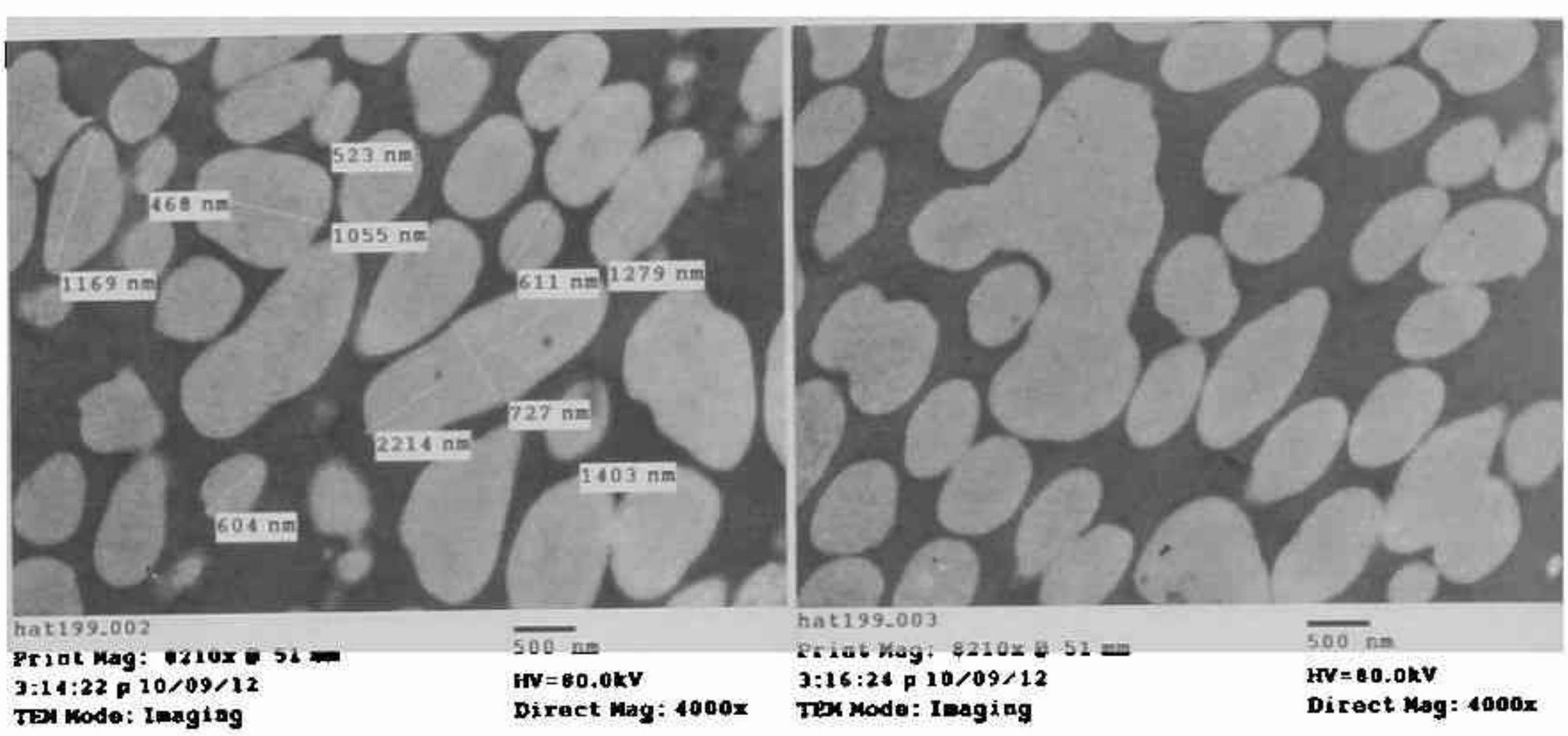

Figure 5. Transmission electron micrograph (TEM) of purified PHB granules.

4. CONCLUSION

Among various chemicals tested for their ability to digest non-PHB cellular materials, NaOCl was found to be an efficent chemical for PHB recovery from *Yangia* sp. ND199. High PHB purity of 99 % and recovery yield of 94 % were obtained after treatment bacterial cells with 6 % NaOCl (ratio 1:1, v/v) at 30 °C for 1 h. The NaOCl digestion method developed in this study for the recovery of PHB from *Yangia* sp. ND199 cells is simple, effective and economical method which can be easily applied in large scale. This is a first study for PHA recovery in Vietnam.

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TÓM TẮT

NGHIÊN CỨU SỬ DỤNG SODIUM HYPOCHLORITE ĐỂ THU HỒI POLY(3-HYDROXYBUTYRATE) TỪ CHỦNG VI KHUẨN *Yangia* sp. NĐ199

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Polyhydroxyalkanoate (PHA) là loại polymer sinh học được tích lũy trong tế bào của rất nhiều vi sinh vật. Sau khi tách chiết từ tế bào, PHA thể hiện các tính chất đặc trưng như: không độc hại, tương thích sinh học cao, phân hủy sinh học và có thể tái sử dụng. Những đặc tính này giúp cho PHA có nhiều tiềm năng thay thế các loại polymer hóa dầu đang được sử dụng hiện nay, đặc biệt là dùng trong y tế. Tách chiết là bước quan trọng trong qui trình sản xuất PHA. Rất nhiều phương pháp tách chiết đã và đang được nghiên cứu sử dụng như dùng dung môi hữu cơ, sử dụng enzyme và hóa chất. Trong nghiên cứu này, chúng tôi đã sử dụng hóa chất để tinh sạch poly(3-hydroxybutyrate) (PHB) từ chủng vi khuẩn *Yangia* sp. ND199. Trong các loại hóa chất sử dụng (NaOH, HCl, KOH, NaOCl và SDS), NaOCl là loại hóa chất cho hiệu quả tinh sạch PHB cao nhất. Điều kiện tinh sạch tối ưu là trộn lẫn dung dịch chứa 100 g/l tế bào vi khuẩn với dung dịch chứa 6 % NaOCl theo tỉ lệ 1:1, ủ ở 30 °C trong thời gian 1 giờ. Ở điều kiện này, độ tinh sạch của PHB là 99 % và hiệu suất thu hồi là 94 %. Phương pháp tách chiết đơn giản này có thể phát triển để sử dụng ở quy mô lớn hơn.

Từ khóa: NaOCl, polyhydroxyalkanoate, poly(3-hydroxybutyrate), tách chiết, *Yangia* sp.NĐ199.