Annals of Microbiology, **56 (3)** 257-260 (2006)

Synthesis of poly-hydroxyalkanoates from activated sludge under various oxidation-reduction potentials

Wenfeng HU[1], Yujie WANG[2]*, Fenglin HUA[2], Hong CHUA[2], Shirleyngai SIN[2], Hoifu YU[3]

[1]College of Food Science, South China Agricultural University, Guangzhou 510642; [2]Department of Civil and Structural Engineering, The Hong Kong Polytechnic University; [3]State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, China

Received 7 March 2006 / Accepted 13 June 2006

Abstract - The production of poly-hydroxyalkanoates (PHA) from the activated sludge subjected to conditions with various oxidation-reduction potentials (ORPs) was investigated. By controlling the dissolved oxygen concentration in the cultural media, the ORP were kept at preset levels of -20, -10, 0, and +10 mV. With glucose as the dedicated carbon source, we have demonstrated a correlating relationship with the ORP's in the culture media to the PHA accumulation rate, the PHA production-yield, cell growth rate, glucose uptakes and 3-hydroxybutyrate to 3-hydroxyvalerate (HB/HV) mole ratios in the PHA copolymers. The highest PHA production yield of 0.26 g/g with HB/HV mole ratio of 8.03 was achieved at +10 mV ORP. We concluded that oxygen plays an important role in PHA accumulation and HB/HV mole ratio activated sludge-to-copolymer PHA conversion process.

Key words: activated sludge; monomeric mole ratio; oxidation-reduction potential; poly-hydroxyalkanoates.

INTRODUCTION

Bio-polymers, poly-hydroxyalkanoates (PHA), have emerged in recent years as environment-friendly biodegradable-plastic raw materials that possess physical and mechanical properties comparable to those of the conventional plastics (Chua *et al.*, 1997a). However, widespread application of PHA is hampered by high cost of production from genetically engineered plants and pure microorganism cultures, which involve processes requiring stringent sterilization procedures and culture conditions (Takabatake *et al.*, 2002). Recent attempts to substantially lower the production cost have been made possible by employing newly developed techniques to produce PHA from the co-cultures of excess activated sludge in municipal sewage and industrial wastewater treatment plants (Serafim *et al.*, 2002). The identified issues that have aroused immense research interests include generating PHA from oil-laden wastes in municipal activated sludge (Takabatake *et al.*, 2000), accumulation of PHA from various selected and enhanced bacterial strains in activated sludge (Chua *et al.*, 1998; Liu *et al.*, 2002), optimisation of process conditions for PHA accumulation in activated sludge (Hanada *et al.*, 2002), and selection of carbon sources and culture medium compositions to produce distinct physical and mechanical properties of PHA copolymers (Yu *et al.*, 1997). However, in-depth investigation and published results on critical process control parameters that form the basis for process scale up, fine-tuning, optimisation and full-scale implementation are not available.

In this paper, we reported that the PHA production yields and rates under various dissolved oxygen concentrations in the culture media. The oxidation-reduction potential (ORP) is an index known to be linearly correlated with the logarithm of dissolved oxygen concentration (Peddie *et al.*, 1990). The on-line measurement and control of ORP's in the culture media were used as the controlled parameters in order for more precise and sensitive control of the dissolved oxygen levels, especially in the lower concentration range for PHA production. It also provided real-time monitoring of microbial activities and organic substrate degradation.

MATERIALS AND METHODS

Bioreactor system and operation. Activated sludge was collected from the outlet of the excess activated sludge (EAS) thickening process in a large-scale communal municipal sewage treatment plant (Taipo STW) in Hong Kong. EAS (7.5 g, dry weight) was inoculated into a 3-litre bioreactor and then topped up to 3 l of the cultural medium. The cultural medium consisted of 4 g/l of glucose and 0.058 g/l of NH_4Cl, resulting in a C:N mass ratio of 96 which was previously determined to provide the highest overall polymer production yield Chua *et al.* (1997b), the medium was further supplemented with micronutrients and growth factor with the following formulations (in mg/l): KH_2PO_4, 3.7; $MgSO_4 \cdot 7H_2O$, 20.0; $FeCl_3$, 28.4; $MnCl_2 \cdot 2H_2O$, 0.3; $Al_2(SO_4)_3 \cdot 18H_2O$, 2.2; $CaCl_2$, 40.0; $CoCl_2 \cdot 6H_2O$, 80.0; $NaSiO_3 \cdot 5H_2O$, 4.0; H_3BO_3, 4.0; $ZnSO_4 \cdot 7H_2O$, 2.0; $CuSO_4 \cdot 5H_2O$, 2.0; $(NH_4)_2MoO_4$, 2.0;

*Corresponding author. Phone: 852-27666072;
Fax: 852-23346389; E-mail: yujie923@yahoo.com.cn

thiamine hydrogen chloride, 8.0. The operative conditions were: batch culture under oxygen-limited condition, excess of carbon source forcing the microflora to produce PHAs, 350 rpm of stirring speed, automatically controlling temperature at 30 ± 1 °C and average pH of 6.8 respectively for 24 or 48 h fermentation.

The bioreactor was equipped with an on-line ORP meter, ORP-linked compressed air pump, central controller and computer data-logging system (Fig. 1). It can be seen from the diagram, two gas containers, oxygen and nitrogen, were standing by there and linked with the ORP monitoring and controlling system. They were used for the ORP regulation. If the ORP was higher than the designed level, the nitrogen gas would be purged into the cultural broth at a gas flow rate of 0.5 l/min to evaporate any residual dissolved oxygen in the liquid phase and to avoid surface oxygen transfer from the atmosphere; contrarily if the ORP was lower than the designed level, the oxygen gas would be pump into the broth at a gas flow rate of 0.5 l/min instead.

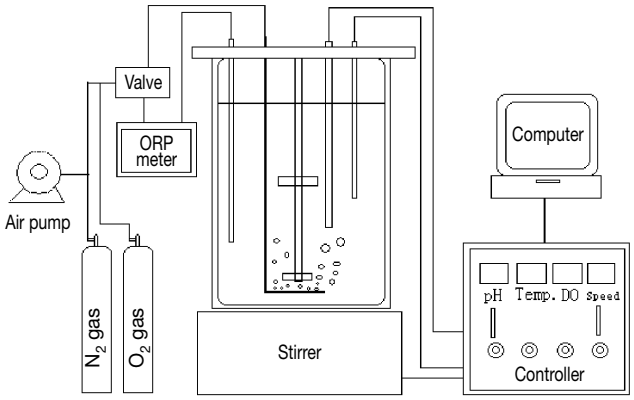

FIG. 1 – Schematic diagram of fermentation setup including magnetic stirrer; glass jar reactor; pH meter equipped with a pH sensor; ORP meter equipped with an ORP sensor; temperature and computer-controller unit.

ORP regulation and pH maintenance. By adjusting the oxygen gas-flow rate pumped into the cultural medium, the ORP was regulated at -20, -10, 0, and +10 mV. To avoid the possible fluctuations of ORP value caused by the addition of NaOH and HCl solution, the pH value of the system was instead stabilized by a phosphate buffer solution containing 1.794 g/l of sodium di-hydrogen phosphate and 12.350 g/l of di-sodium hydrogen phosphate. The initial pH was at 7.5 and it was successfully stabilized at average of 6.8 throughout the fermentation process.

PHA extraction. After fermentation, the biomass was concentrated by centrifugation at 8000 rpm for 25 min, washed twice, and freeze-dried. Then, 8 g of cells were treated with 100 ml chloroform and 100 ml of 30% sodium hypochlorite. The mixture was agitated in a shaker at 200 rpm, 30 °C for 150 min. After the treatment, the dispersion was centrifuged at 4000 rpm for 10 min. The bottom phase was the chloroform layer containing PHB. First the hypochlorite phase was removed with a pipette, the chloroform phase was obtained by filtration and concentrated by distillation, and then PHB material was precipitated by mixing methanol with the concentrated chloroform.

PHA analysis and microbial identification. The cultural broth was periodically sampled and analysed for dry weight, total organic carbon (TOC) and pH. The dry weight of EAS was measured by weighing the suspended solid while TOC analysis was carried out by using Shimadzu TOC-500 A equipped with an ASI-5000 A auto sampler. PHA content and beta-hydroxybutyrate (3-HB) to beta-hydroxyvalerate (3-HV) ratios were detected by gas chromatography. The techniques used to extract PHA from the EAS and identify the microbial genus are both described in Yu *et al*. (1999).

RESULTS AND DISCUSSION

PHA accumulation under various ORP values

The initial PHA content in EAS was found to be about 2.5% of the dry cell weight. The maximum PHA accumulation in the EAS of 8.34% of cell dry weight was achieved when ORP was maintained at +10 mV. PHA content in the EAS declined to 5.33% when ORP was kept at 0 mV. The minimum PHA accumulation was 3.93% when ORP was kept at −20 mV. The results indicated that PHA accumulation rate increased with the ORP levels (Fig. 2). On the other hand, the polymer production yield ($Y_{P/S}$) under different ORP was also analysed (Fig. 3). $Y_{P/S}$ (g PHA/g carbon) was calculated as the polymer accumulated divided by TOC consumed. The maximum polymer production yield of 0.26 was achieved by keeping the ORP level at +10 mV. When the ORP level was decreased to 0 mV, the $Y_{P/S}$ declined to 0.24. Further reduction of ORP (-10 and -20 mV) resulted in 0.16 and 0.07 of $Y_{P/S}$ respectively.

The ORP was regulated by controlling the airflow rate purged into the bioreactor, which reflected the concentration of dissolved oxygen (DO) in the cultural broth. The reason for the increase in PHA production with the ORP levels is probably due to the supply of energy by oxidation of glucose. Under anaerobic conditions, when the supply of energy through substrate oxidation stops, the amount of energy storage polymers such as polyphosphate and glycogen is the limiting factor for PHA accumulation. When these energy sources are also used up, the PHA accumulation will be stopped. So when the ORP value is adjusted to a higher level (0 and 10 mV), which in fact increasing the concentration of DO, microorganisms in EAS can take up glucose again via oxidative degradation to get energy as well as to regenerate energy storage polymers, consequentially enhancing the PHA accumulations and polymer production yields. The microorganisms that are abundant in the anaerobic-aerobic activated sludge can accumulate not only PHA but also polyphosphate and glycogen, and can utilize polyphosphate and glycogen as the energy source under anaerobic conditions (Satoh *et al.,* 1998). At lower ORP than −20 mV, it is estimated that PHA content will decrease at lower ORP than −20 mV, whereas, the PHA content will increase at higher ORP than +10 mV. But we don't think the PHA will accumulate at higher level DO concentration, because the oxygen will result in the digestion of PHA as carbon and energy source.

Variation of HB/HV ratio of PHA co-polymer under different ORP values

According to the results of the gas chromatographic analysis, the PHA produced by the EAS was identical to the Aldrich's standard PHA sample with poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV). Hence the polymers formed by EAS

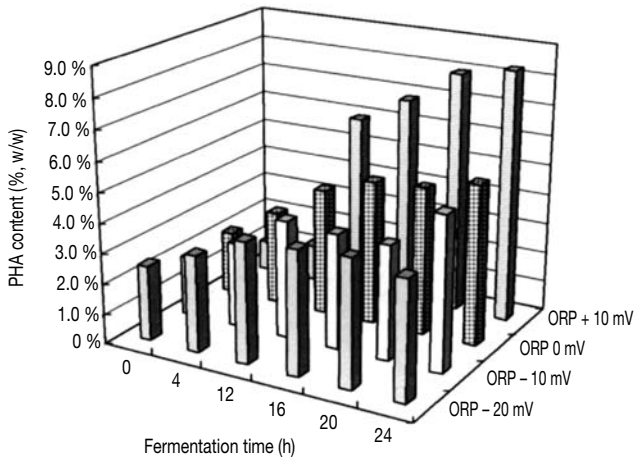

FIG. 2 – Time courses of PHA accumulation from EAS under various ORP values.

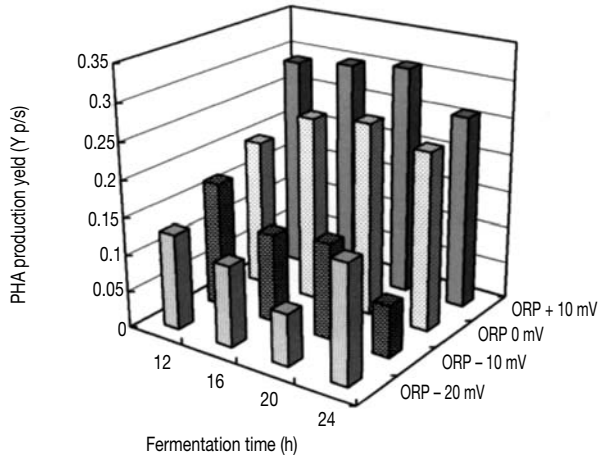

FIG. 3 – Time courses of the yields of PHA accumulation from EAS ($Y_{P/S}$) under various ORP values.

under different ORP conditions were recognized as PHBV copolymers. In addition, it was found that 3-hydroxybutyrate (3-HB) unit fraction to 3-hydroxyvalerate (3-HV) unit fraction (HB/HV) mole ratios increased with ORP values as well (Fig. 4). Higher HB/HV ratio accounts for higher brittleness of the PHA plastics as HV would affect the elasticity. The maximum HB/HV mole ratio was 8.03 (HB 88.93 g/g, HV 11.07 g/g) when the ORP level was maintained at +10 mV. The HB/HV value declined with the ORP level and it reached the minimum value of 0.275 (HB 21.60 g/g; HV 78.40 g/g) when the ORP value was kept at –20 mV (Fig. 4).

This phenomenon gave important hints of the significant role of oxygen in the control of PHA compositions. Oxygen alters the HB/HV ratio by posing influences to the metabolisms of the microbial communites in the EAS. The bacteria in EAS can be divided into two groups. One is the "storage" microorganisms that are able to survive in nutrient-lacking environment, this group of microorganisms possessing substrate storage capability have a strong competitive advantages in activated sludge environment, because they can quickly store substrate as storage polymer when substrate is present and utilize it when the substrate is depleted by undergoing different metabolic pathways. While another group of microorganisms does not possess this kind of storing and re-consuming ability, and they are usually less vying. The monitoring of ORP is critical to PHA compositions as the provision of oxygen is also deteminant to these microbial metabolic pathways, which in turn alternates the occurrence of aerobic and anaerobic metabolic processes. As a result of carefully adjusting the ORP value throughout the fermentation process, and hence influencing the metabolic pathways of the microbial communities, different ratio of HB/HV in the synthesized PHA could be established, and hence co-polymer with wide range of morphologies and physical properties could be obtained.

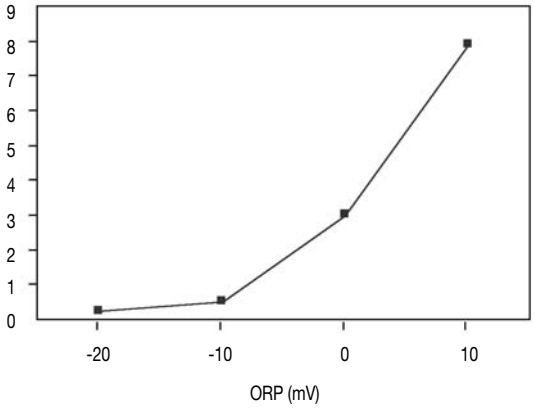

FIG. 4 – The variation of HB/HV ratio of PHA co-polymer from EAS under different ORP values.

Cell growth and glucose consumption efficiency under various ORP value

After 24 h fermentation, the net cell growth in the system was 1.236 g (12.4% of the initial cell mass), 1.692 g (15.8%), 3.498 g (36.5%), and 4.330 g (45.2%) when ORP was maintained at -20, -10, 0, and +10 mV respectively. High ORP value speeds up the oxidation rate of nutrients, resulting in faster growth rate of the microorganisms. Microaerophilic-aerobic (for instance, ORP was increased from -20 mV to +10 mV) process is more efficient than that of anaerobic process in term of biomass conversion rate. In other words, the increase of ORP, led to a high net EAS growth comparatively. This judgment could be further proved by the TOC removal efficiencies at different ORP levels, that were 43.4%, 41.9%, 57.4%, 66.2% when ORP were kept at -20, -10, 0, and +10 mV respectively. All these figures explained that when more oxygen was supplied to the cultural broth, the microorganisms consumed more carbonaceous substances for faster growth rate, finally resulting in higher PHA accumulation.

After 24 h fermentation and acclimation, it was observed under microscope with 1000 x magnification that the main bacteria in the EAS were cocci and bacilli.

The cocci were 0.9-2.0 mm in diameter, appearing in pair, tetrad or aggregates; Sudan Black staining and Gram staining positive. There were not flagella observed. Bacilli were rod shape single cells, 2.5-3.5 mm in size, Sudan Black and Gram Staining positive. Flagella were observed with motile ability. More detail morphology identification proved that

Alcaligenes spp was the main group existed in the cultural broth after 48 h cultivation for the PHA accumulation. This result agreed well with that described by Dave *et al*. (1996) and Chua *et al*. (1997a). When activated sludge was incubated under nitrogen- and phosphorus-limiting conditions, selective overgrowth of *Bacillus* spp. from 5 to 80% (cell count) was observed (Dave *et al*., 1996).

CONCLUSION

The fermentation process leading to the PHA accumulation in EAS could be monitored and controlled by adjusting the ORP values, with glucose used as the sole carbon source. The increase in the PHA content and net cell growth as well as the improved PHB/PHV ratio of the synthesized PHA, were shown in this study to be proportional to the ORP values. This study confirmed that complex design of the composition of these biological polymers with plastic physical properties as well as biodegradability could be synthetically possible at industrial scale. The final products can be competitively priced in the market because the only additional required raw material is the relatively cheap glucose as the carbon source.

Acknowledgements
The research grants from the Hong Kong Government Research Grant Council and The Hong Kong Polytechnic University that supported this work are hereby acknowledged.

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