한국환경분석학회지 제7권 (제3호) 173~178, 2004
J. of the Korea Society for Environmental Analysis

Poly-β-hydroxybutyrate Production from Waste Oils by *Burkholderia cepacia*

Kang Hye Sun, Yoo Yung Sun[1] and Park Doo Hyun†

Department of Biological Engineering, Seokyeong University, Jungneung-dong, Sungbuk-gu, Seoul 136-704, Korea
[1]Department of Applied Chemistry, Seokyeong University, Jungneung-dong, Sungbuk-gu, Seoul 136-704, Korea

Burkholderia cepacia grew on defined medium containing petroleum (diesel oil) or vegetable oil (soybean oil) as sole carbon source and accumulated polyhydroxybutyrate(PHB) in cytoplasmic region. PHB productivity by *B. cepacia* in the defined medium with petroleum and vegetable was higher than that in glucose and acetate. The electrochemical reducing power activated the PHB production but oxidizing power did not inhibit the PHB production. From these results, we found a possibility that waste petroleum and used vegetable oil may be a proper carbon source for PHB production instead of glucose, and electrochemical energy can be applied to the PHB production.

Key words: Poly hydroxy butyrate, *Burkholderia* sp. waste vegetable oil

1. Introduction

Poly-β-hydroxybutyrate (PHB) and other polyhydroxyalkanoates (PHAs) are accumulated by many bacteria as an energy and carbon reserve material[1)]. While the enzymology and the genetics of PHA biosynthesis have been extensively studied for a number of organisms and are now well understood, less is know about the production of PHB from waste oils by *Burkholderia cepacia* that can grow on aromatic hydrocarbon and its derivatives[2-5)]. *B. cepacia* can oxidize aromatic and aliphatic hydrocarbon by Mono- or dioxygenase to fatty acid, and metabolize the fatty acid by TCA cycle[6-8)]. So far, phasis appear to be present in all PHA-synthesizing bacteria, and even though they generally are not conserved in sequence and seem to be species specific, they are believed to fulfill the same function, binding to PHA granules and promoting PHA synthesis, in a manner still poorly understood[9)]. Some bacteria, which can metabolize the C_1 compounds, such as even methylotrophic bacteria have been reported to produce PHB. *Methylobacterium extorquens* AM1 accumulates PHB and forms PHB granules like other PHB-producing bacteria[10-11)]. PHB content in *M. extorquens* AM1 cells varies depending on growth substrate [12)]. We isolates a bacterium strain that can grow in defined medium containing petroleum oil or vegetable oil as a sole carbon source and tested to produce PHB in different growth condition. In this paper, we optimized the PHB productivity under various growth condition, and developed the PHB-producing system in bioelectrochemical system from waste oils such as petroleum and soybean oil.

2. Materials and Methods

2.1. Organism and cultivation

A petroleum-degrading bacterium was isolated from soil of paddy field, and identified by 16S rDNA sequencing homology. The bacterium was grown in the defined medium containing 10g/L diesel oil (or soybean oil), 20 mM phosphate buffer (pH 7.0), 3 g/L NH_4Cl, and 3 ml/L trace mineral stock solution which contained 0.01 g/L $MnSO_4$, 0.01 g/L $MgSO_4$, 0.01 g/L $CaCl_2$, 0.002 g/L

†To whom correspondence should be addressed.

$NiCl_2$, 0.002 g/L $CoCl_2$, 0.002 g/L $SeSO_4$, 0.002 g/L WSO_4, 0.002 g/L $ZnSO_4$, 0.002 g/L $Al_2(SO_4)_3$, 0.0001 g/L $TiCl_3$, and 0.002 g/L $MoSO_4$. KH_2PO_4 was added to the medium after autoclave, and pH was adjusted to 7.0. Culture was incubated at 30°C with vigorous shaking (200 strokes). 10 g/L glucose or 20 g/L acetate was used as substitute carbon source for waste oil to compare PHB production in different carbon sources.

2.2. Chemicals

All chemicals used in all tests were purchased from Sigma & Aldrich or same grade supplier.

2.3. Measurement of bacterial growth

Bacterial growth was turbidimetrically measured by spectrophotometer at 660 nm at intervals of 24 hr.

2.4. Analysis of PHB concentration

PHB concentration was measured by Law and Slepeckys method[13]. The bacterial cells were centrifuged in polypropylene centrifuge tubes, which had been previously washed thoroughly with ethanol and hot chloroform to remove plasticizers. The cell paste was resuspended in a volume of commercial sodium hypochlorite solution (Clorox) equal to the original volume of medium. After 1 hr at 37°C the lipid granules were centrifuged, washed with water, and then washed with acetone and alcohol. Finally, the polymer was dissolved by extraction with three small portions of boiling chloroform, the chloroform solution was filtered, and the filtrate was used for PHB assay. For the spectrophotometric assay of PHB, a sample containing 5 to 50 mg PHB in chloroform is transferred to a clean test tube. The chloroform is evaporated and 10 mL of concentrated H_2SO_4 are added, the tube is capped with a glass marble and heated for 10 min at 100°C in a water bath. The solution is cooled, and, after thorough mixing, a sample is transferred to a silica cuvette and the absorbance at 235 nm is measured against a sulfuric acid blank. The amount of crotonic acid is calculated from the molar extinction coefficient, which is 1.55×10^4[4,14].

2.5. Measurement of lipase activity

Lipase activity was determined by fatty acid produced from enzyme-degrading soybean oil by resting cell which was harvested from 4-days old culture. Biomass of resting cell was adjusted to 13.5 as optical density at 660 nm. The fatty acid concentration was determined by alkaline titration of sample containing phenolphthalein.

2.6. Effects of nitrogen and phosphorus on PHB production

The extra nitrogen and phosphorus except nitrogen and phosphorus contained in defined medium were added to the bacterial culture. 0.5 g/L Yeast extract or 0.5 g/L Peptone was used as organic nitrogen, and 3 g/L ammonium chloride was used as inorganic nitrogen sources. 20 mM phosphate buffer (pH 7.0) was used as phosphorus source. Bacterial production of PHB in the culture containing extra nitrogen and phosphorus was compared with that in the culture.

2.7. Electrochemical reactor

An electrochemical reactor was designed to be a two-compartment system of which cathode compartment was separated from anode compartment by porcelain membrane (5 mm thickness) and the working volume of each compartment was adjusted to 350 ml. The graphite felt electrode modified with neutral red (NR) and plain graphite electrode was used as cathode and anode, respectively. 20 mM phosphate buffer (pH 7.0) containing 100 mM glucose or 100mM neutral red was used as basal reaction mixture (catholyte), and 200mM potassium phosphate buffer (pH 7.0) was used as anolyte. Before starting reaction, 2 volt of DC electricity was supplied and N_2 was sparged into the reaction mixture to remove O_2 and reduce reaction mixture for 20 min. The reaction was started by inoculation of bacterial cell.

3. Results and Discussion

3.1. Growth of *B. cepacia* in different carbon sources

As shown in Fig. 1, growth of *B. cepacia* was highest in glycerol and lowest in butanol. The bacterial growth

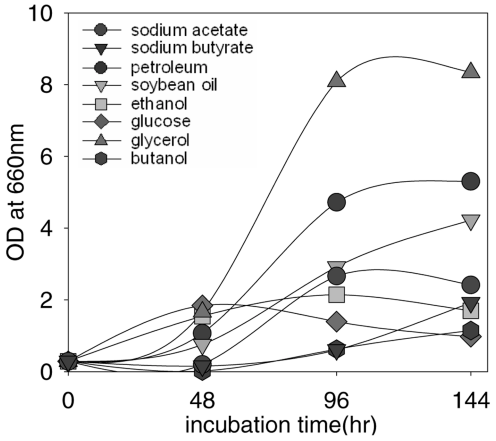

Fig. 1. Growth of *Burkholderia cepacia* in defined medium containing different carbon sources. The bacterial culture was incubated at 30°C with vigorous shaking (200 strokes). Each 100 mM of carbon source was added to defined medium except petroleum and soybean oil. Each 10 g/L of petroleum and soybean oil was added because the molar concentration of petroleum and soybean oil cannot be calculated.

was higher in petroleum and soybean oil than in glucose or acetate, which is very different phenomenon from the results we expected. The bacterial growth is proportional to the metabolic activity and free energy production of bacterial cell growing with specific substrate. From these results we suggested that *B. cepacia* has different membrane transport system to uptake carbon source from other soil bacteria, and can produce more energy from petroleum or soybean oil than sugars or organic acids. Generally, most bacteria isolated from soil can metabolize glucose and organic acids, which are the metabolic intermediates of glycolysis. This property of *B. cepacia* is useful for recycle of waste oils such as petroleum and soybean oil.

3.2. Lipase activity of *B. cepacia*

The lipase activity is an indicator to estimate the activity of bacterial cell capable of growing in vegetable oil. The bacterial cell growing in vegetable oil may produce extra-cellular enzyme for degradation of insoluble polymer in water. As shown in Fig. 1, the growth of *B. cepacia* was highest in glycerol, from which we extracted one possibility that the isolate may easily uptake glycerol produced from enzymatic hydrolysate of vegetable oil.

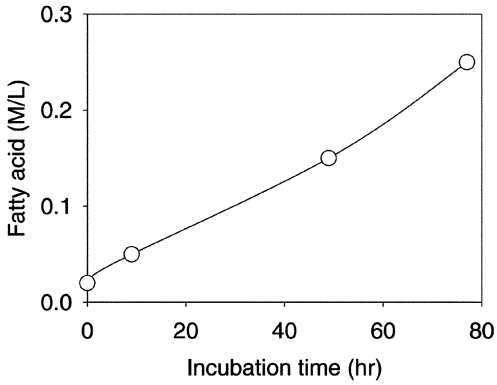

Fig. 2. Production of fatty acid from vegetable oil by extra-cellular lipase produced from *Burkholderia cepacia*. The resting cell harvested from 4 days-old culture of *B. cepacia* was used as the biocatalyst.

Fig. 2 shows that the concentration of fatty acid produced from vegetable oil by lipase. It shows that *B. cepacia* is proper physiological property for growing in vegetable oil, and the vegetable oil can be a proper carbon source for PHB synthesis by *B. cepacia*. Average molecular weight of vegetable oil is about 900-1000 dalton, which is 5-6 times of glucose. The vegetable oil is one of unsaturated lipids (triglyceride) and is about 6-8 times higher than glucose in the energy potential calculated with redox balance because H/O balance of vegetable oil is 12 times higher than glucose.

3.3. PHB productivity in different substrates

On the basis of growth of *B. cepacia* in petroleum and vegetable oil, we measured PHB produced by *B. cepacia* from petroleum and vegetable oil, and compared with the PHB produced from glucose and acetate. PHB is accumulated in cytoplasmic region of bacterial cell under the growth condition of being shortage or lack of nitrogen or phosphorus. As shown in Fig. 3, bacterial production of PHB was highest in petroleum and lowest in glucose. This is an evidence that *B. cepacia* has completely adapted to petroleum or insoluble organic polymer such as vegetable oil, and has developed transport system for petroleum and metabolic enzymes for petroleum degradation and oxidation. Generally, soil bacteria growing under both anaerobic and aerobic condition can use organic compounds such as sugars, organic acids and fatty acids as carbon source, and

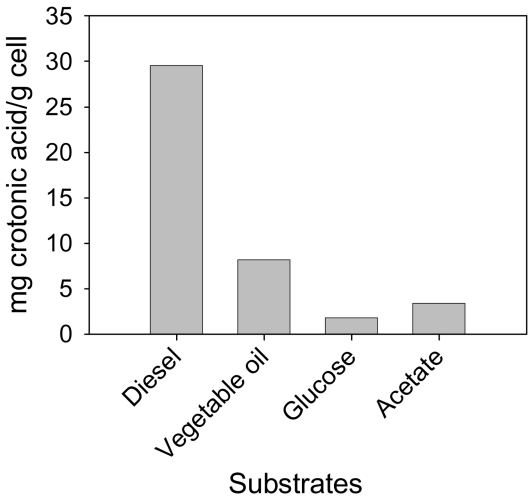

Fig. 3. Production of PHB, which was hydrolyzed to crotonic acid by sulfuric acid for analysis, by *Burkholderia cepacia*. Isolate was cultivated in defined medium containing different carbon source at 30°C for 72 hr.

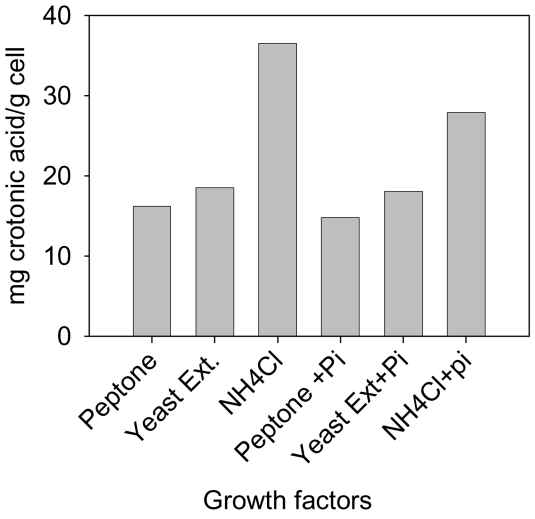

Fig. 4. Production of PHB, which was hydrolyzed to crotonic acid by sulfuric acid for analysis, by *Burkholderia cepacia* in defined medium with different growth factors such as organic nitrogen, inorganic nitrogen or phosphate. Isolate was cultivated in defined medium containing extra nitrogen and phosphorus source at 30°C for 72 hr.

absolutely require nitrogen and phosphorus which can be either inorganic or organic forms. From these informations, we are suggesting a possibility that PHB production by *B. cepacia* may be affected by nitrogen or phosphorus.

3.4. Effect of nitrogen or phosphorus on PHB production

Under the growth condition with sufficient nitrogen or phosphorus, PHB synthesis by bacteria has been reported to stopped because energy metabolism and biosynthetic metabolism have to be activated [15]. As shown in Fig. 4, PHB production by *B. cepacia* was negatively influenced by the organic nitrogen and inorganic phosphorus. This means that *B. cepacia* do not accumulate the storage in cytoplasmic region under growth condition with sufficient nitrogen and phosphorus. Two pathways for PHB synthesis are known in bacteria. In *Ralstonia eutropha*, *Methylobacterium extorquens*, *Zoogloea ramigera*, and A*zotobacter beijerinckii* PHB is synthesized from acetyl coenzyme A and PHB synthase. In *Rhodospirillum rubrum* and *Methylobacterium rhodoezianum* PHB sythesis is catalyzed by NADH-linked acetoacetyl-CoA reductase and PHB synthase[16-18]. These serve a clue that extra reducing power coupled to reduction of $NAD(P)^+$ can activate or increase PHB productivity. We tested the effect of electrochemical reducing power on the PHB productivity.

3.5. Effect of extra reducing or oxidizing power on PHB production

The electrochemical reduction power can be transferred from electrode to bacterial cytoplasm by electron mediator. We used neutral red as an electron mediator, which catalyzes NAD^+ reduction to NADH without enzyme catalyst[19-22]. As shown in Fig. 5 and 6, PHB production was more increased in cathode compartment in the electrochemical reactor with NR modified-cathode or soluble NR. These show that the NADH/NAD^+ balance in more increased by extra NADH produced by extra reducing power, by which the enzymes functioning in the pathway for PHB synthesis can be activated. In most biosynthetic pathway, NADH and ATP function as reducing power and free energy for enzyme action, respectively. The higher balance of $NADH/NAD^+$ and ATP/ADP in the cytoplasmic region can induce activation of biosynthetic metabolism. The electrochemical energy can be converted to biological

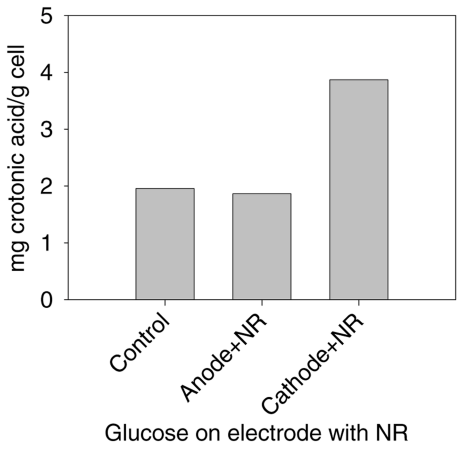

Fig. 5. Production of PHB, which was hydrolyzed to crotonic acid by sulfuric acid for analysis, by *Burkholderia cepacia* in different bioelectrochemical reactors without electrode, with anode (oxidized condition) and cathode (reduced condition). 100 μM neutral red was added to the bioelectrochemical reactor as an electron mediator.

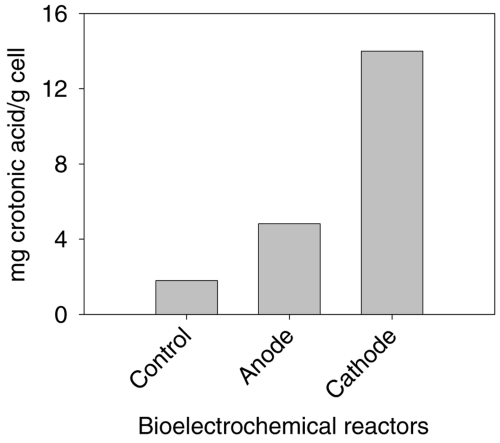

Fig. 6. Production of PHB, which was hydrolyzed to crotonic acid by sulfuric acid for analysis, by *Burkholderia cepacia* in different bioelectrochemical reactors without electrode, with anode (oxidized condition) and cathode (reduced condition). The electrode was modified with neutral red.

reducing power by mediation of NR, and the biological reducing power can function to increase balance of $NADH/NAD^+$ and ATP/ADP. The electrochemical energy is more comfortable for application to bacterial culture than chemical reducing agents such as hydrogen and hydrogen sulfide, however, which has to be continuously added to bacterial culture and are dangerous for handling and using. And some bacteria cannot metabolize hydrogen or hydrogen sulfide, which is limiting factor for application of chemical reducing agents to the system for control of bacterial metabolism.

4. Conclusion

Producing PHB from waste polymers such as waste vegetable oil or petroleum is more useful process for both environment and waste-recycling system. We tested bacterial production of PHB from waste oils and applied the electrochemical culture system to bacterial culture. PHB productivity was highest in petroleum and more increased in cathode compartment. This serves a possibility that the expensive material, PHB can be produced from cheap materials such as waste vegetable oil.

Acknowledgments

This research was supported by a grant No. R01-2003-000-10563-0 from the Basic Research Program of the Korea Science & Engineering Foundation.

References

1. Anderson, A.J. and E.A. Dawes. 1990. *Microbiol. Rev.* **54**: 450-472.
2. Shields, M.S, M.J. regain, R.R. Gerger, R.Campbell, and C. Somerville. *Appl. Environ. Microbiol*. **61**: 1352-1356.
3. Hur, H.-G., L.M Newman, L.P. Wackett, and M.J. Sadowsky. 1997. *Appl. Environ. Microbiol*. **63**: 1606-1609.
4. Anja, E., U. Fork, R.M. Wittich, K.N. Timmis, and D.H. Peiper. 1997. *Appl. Environ. Microbiol*. **63**: 1974-1979.
5. Young, F.K., J.R. Kastner and S.W. May. *Appl. Environ. Microbiol*. **60**: 4195-4198.
6. Chang, H.K., P. Mohseni, and G.J. Zylstra. 2003. *J. Bacteriol*. **185**: 5871-5881.
7. Danganan, C.E., S. Shankar, R.W. Ye, and A.M. Charkrabarty. 1995. *Appl. Environ. Microbiol*. **61**: 4500-4504.
8. Suwa, Y., A. Wright, F. Fukimori, K.A. Nummy, R. Hausinger, W.E. Holben, and L.J. Forney. 1996. *Appl. Environ. Microbiol*. **62**: 2464-2469.
9. Chohan, S.N. and L. Copeland. 1998. *Appl. Environ.*

Microbiol. **634**: 2589-2686.
10. Föllner, C.G., W. Babel, and A. Steinbüchel. 1995. *Can. J. Microbiol.* **41**(suppl. 1):124-130.
11. Föllner, C.G., M. Madkour, F. Mayer, W. Babel, and A. Steinbüchel. 1997. *J. Basic Microbiol*. **37**:11-21.
12. Korotkova, N., and M.E. Lidstrom. 2001 *J. Bacteriol*. **183**: 1038-1046.
13. Law, J.H. and R.A. Slepecky. 1961. *J. Bacteriol*. **82**: 33-36.
14. Slepecky, r. a., and J.H. Law. 1960b. *Anal. Chem*. **32**: 1697-1699.
15. Suzuki, T., T. Yamane, and S. Shimizu. 1986. *Appl. Microbiol. Biotechnol*. **24**: 370-374.
16. Belova, L., A.P. Solotov, and .A. Trotsenko. 1997. *FEMS Microbiol. Lett*. **156**:275-279.
17. Fukui, T., M. Ito, T. Saito, and T. Kenkichi. 1987. *Biochem. Biophys. Acta.* **917**: 365-371.
18. Mothes, G. and W. Babel. 1995. *Can. J. Microbiol*. **41**: 68-72.
19. Park, D.H., and J. G. Zeikus. 1999. *J. Bacteriol*. **181**: 2403-2410.
20. Park, D.H. and J.G. Zeikus. 2002. *Appl. Microbial. Biotechnol.* **59**: 58-61.
21. Park, Doo Hyun and J. Gregory Zeikus. 2002. *Biotech. Bioengin*. **81**: 348-356.
22. Park, D.H. and J.G. Zeikus. 2000. *Appl. Environ. Microbiol*. **66**: 1292-1297.