The Journal of Microbiology, March 2001, p.79-82
Vol. 39, No. 1

NOTE

Effect of Levulinic Acid on the Production of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) by *Ralstonia eutropha* KHB-8862

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(Received February 26, 2001 / Accepted March 13, 2001)

The influence of levulinic acid (LA) on the production of copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV) by *Ralstonia eutropha* was investigated. Addition of LA into the culture medium greatly increased the molar fraction of 3HV in the copolyester, indicating that LA can be utilized as a precursor of 3HV. In shake flask culture, the 3HV content in the copolyester increased from 7 to 75 mol% by adding 0.5 to 4.0 g/L LA to the medium containing fructose syrup as a main carbon source. A maximal copolyester concentration of 3.6 g/L (69% of dry cell weight) was achieved with a 3HV content of 40 mol% in a jar fermentor culture containing 4.0 g/L of LA. When LA (total concentration, 4 g/L) was added repeatedly into a fermentor culture to maintain its concentration at a low level, the copolyester content and the 3HV yield from LA reached up to 85% of dry cell weight and 5.0 g/g, respectively, which were significantly higher than those when the same concentration of the LA was supplied all at once. The present results indicated that LA is more effective than propionate or valerate as a cosubstrate for the production of copolyesters with varying molar fractions of 3HV by *R. eutropha*.

Key words: Copolyester, levulinic acid, polyhydroxyalkanoate, *Ralstonia eutropha*

Poly-β-hydroxyalkanoates (PHAs), a family of polyesters produced by a variety of microorganisms, have been the focus of extensive research because of their potential application as biodegradable and biocompatible thermoplastics (17). In particular, a copolyester consisting of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), poly(3HB-*co*-3HV), has been of the greatest commercial interest since this polyester exhibits a considerable range of thermomechanical properties, which depend on its 3HV content (1). Production of poly(3HB-*co*-3HV) is usually achieved by providing bacteria with a cosubstrate, such as propionate and valerate, along with a main carbon source (3, 15, 19-20). It is known that propionate is utilized to form 3-ketoacyl-CoA via propionyl-CoA which is condensed with acetyl-CoA by 3-ketothiolase (7). Although the 3HV fraction in copolyester increases as the concentration of the cosubstrate in a culture medium increases, the maximum content of 3HV in the copolyester is generally low due to the toxic effect of the cosubstrates at relatively low concentrations (3, 6, 20). Copolyesters with a high 3HV content are not necessarily more useful individually, but alteration of 3HV content in poly(3HB-*co*-3HV) is desirable from an industrial viewpoint because it may offer the opportunity for the production of different thermoplastics having various degrees of flexibility and toughness (5, 7).

Levulinic acid (LA) is a 4-ketopentanoic acid which is well known as a typical inhibitor of the synthesis of tetrapyrrols such as porphyrin, heme and vitamin B_{12} analogues (22). Recently LA has been found to accumulate in cells of *Alcaligenes* sp. SH-69 grown on glucose and can be utilized by the cells as a precursor of 3HV (9). Thus, the capability of this organism to produce poly(3HB-*co*-3HV) from single unrelated carbon sources, such as glucose, sucrose, and sorbitol (13, 21), may be attributable to metabolic pathways in which catabolic metabolites of LA are converted to precursors (propionyl-CoA and/or 3-hydroxyvaleryl-CoA) of 3HV (9). However, the effects of LA on the production of poly(3HB-*co*-3HV) by other PHA-producing microorganisms have not been investigated. The current work describes the effect of LA addition on the biosynthesis of PHA by *Ralstonia eutropha*

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(formerly *Alcaligenes eutrophus*) and aims at developing a fermentation process to achieve a high yield of poly (3HB-*co*-3HV) with higher 3HV mol%.

The microorganism used was *R. eutropha* KHB-8862 (12). Cultivation of stock cultures was the same as described previously (11). The organism was grown on a basal medium in which the concentration of different cosubstrates was changed to evaluate their effect on growth and PHA biosynthesis. Each liter of basal medium contained 20 g fructose syrup, 0.2 g $(NH_4)_2SO_4$, 4.6 g $Na_2HPO_4{\cdot}12H_2O$, 1.5 g KH_2PO_4, 0.6 g K_2HPO_4, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.03 g $CaCl_2{\cdot}2H_2O$, 0.02 g $FeSO_4{\cdot}7H_2O$, 3.0 g yeast extract, and 4 ml trace-element solution. The trace element solution contained 0.2 g $ZnSO_4{\cdot}7H_2O$, 0.06 g $MnCl_2{\cdot}4H_2O$, 0.6 g H_3BO_4, 0.4 g $CoCl_2{\cdot}6H_2O$, 0.02 g $CuSO_4{\cdot}4H_2O$, 0.04 g $NiCl_2{\cdot}6H_2O$, and 0.06 g $NaMoO_4{\cdot}2H_2O$ per liter of 0.5 N HCl.

Shake flask cultures were carried out aerobically in 500 ml Erlenmeyer flasks containing 100 ml of basal medium. Batch fermentations were conducted in a 7 L jar fermentor (Korea Fermentor Co. Ltd.) with a working volume of 4 L. The medium was inoculated with a 10% (v/v) inoculum of an overnight culture in LB medium supplemented with 0.5 g/L $(NH_4)_2SO_4$. Temperature and pH were automatically controlled at optimal values, 30°C, and 7.0, respectively. The air flow rate was 1.0 vvm and agitation speed was 360 rpm. Cell growth was monitored spectrophotometrically at 660 nm. After cultivation for a given time depending on the kinds of carbon sources, cells were harvested by centrifugation and then lyophilized. All experiments were repeated at least three times.

PHA was isolated from lyophilized cells by extraction with hot chloroform using a Soxhlet apparatus and then purified according to the method described previously (10). Dry cell weight was measured by drying the harvested cells to constant weight at 105°C. PHA content and its composition were determined by gas chromatography as described elsewhere (2), using PHA standards of which 3HB and 3HV monomer contents are known. A Hewlett-Packard 5890 gas chromatograph equipped with a flame ionization detector and a HP-1 capillary column was used for gas chromatography analysis. The oven temperature was initially maintained at 80°C for 4 min and then was raised to 230°C at a ramp of 10°C/min. The total amount of PHA was quantified by taking the sum of the amounts of 3HB and 3HV monomers detected.

R. eutropha is the most widely investigated microorganism among those producing poly(3HB-*co*-3HV) because it is easy to grow in a simple defined medium and it produces a large amount of poly(3HB-*co*-3HV) in the presence of a cosubstrate such as propionate and valerate (3, 7, 11). However, this organism is very sensitive to these short-chain-length fatty acids. In most cases the growth of *R. eutropha* was almost completely inhibited when the concentration of the cosubstrate exceeds 1.5 g/L and 3HV content in the copolyesters produced was restricted to less than 20 mol% (14, 20). In the present study, to investigate and improve the ability of *R. eutropha* to produce poly(3HB-*co*-3HV), studies on the effect of LA addition

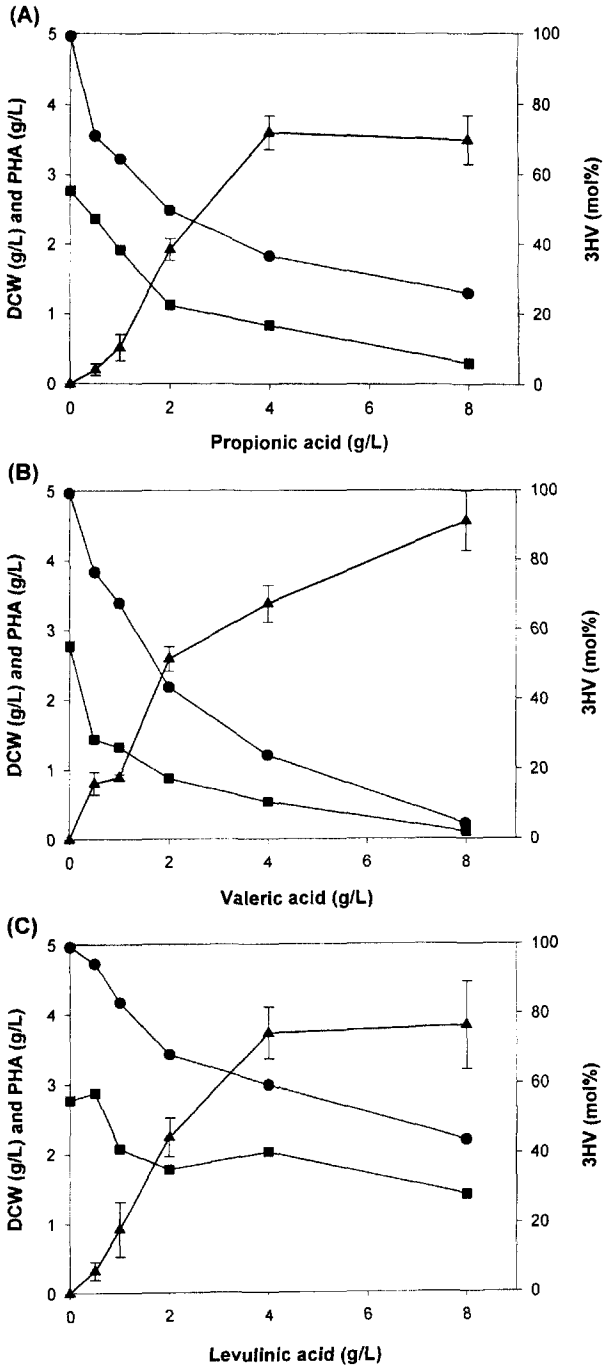

Fig. 1. Effects of various concentrations of propionate (A), valerate (B), and levulinic acid (C) on cell growth, PHA content and molar fraction of 3HV in the copolyesters synthesized by *R. eutropha* in three independent shake flask cultures. Symbols: ●, dry cell weight (DCW, g/L); ■, PHA content (g/L); ▲, molar fraction of 3HV (mol%).

on the accumulation of poly(3HB-*co*-3HV) and its composition were conducted and compared with those for propionate and valerate addition. Fig. 1 shows the results of shake flask experiments which were carried out using basal media containing various concentrations of the cosubstrates. All the polyesters obtained from these experiments were copolyesters of 3HB and 3HV, indicating the utilization of LA as well as propionate and valerate by *R. eutropha* as a precursor of 3HV. The molar fractions of 3HV in the copolyesters produced significantly rose with increasing concentrations of the cosubstrates from 0.5 to 4.0 g/L. The final molar fractions of 3HV at propionate, valerate, and LA concentrations of 4.0 g/L were approximately 72, 67, and 75 mol%, respectively. Meanwhile, cell growth and accumulation of poly(3HB-*co*-3HV) varied with the cosubstrates used. Although all the cosubstrates showed the inhibitory effects on both cell growth and PHA production, the media supplemented with LA gave higher cell growth and PHA accumulation than those supplemented with the same concentration of either propionate or valerate. At an LA concentration of 2.0 g/L, the dry cell weight (DCW) and poly(3HB-*co*-3HV) content reached 3.4 g/L and 52%DCW, respectively, which compared with 2.3-2.5 g/L and 39-44%DCW, respectively, obtained by addition of the same concentration of propionate or valerate. The differences in the inhibitory effects between LA and other cosubstrates were relatively more distinct as the concentration of cosubstrates added increased. These results indicate that LA is a better cosubstrate for cell growth as well as for poly(3HB-*co*-3HV) production than the conventionally used cosubstrates such as propionate and valerate.

Similar experiments were conducted in a jar fermentor using 4.0 g/L of LA as a cosubstrate. As shown in Fig. 2A, the 3HV mol% reached a maximum at 9 h (53 mol%), before the most active PHA biosynthesis occurred. Thereafter the 3HV mol% declined as synthesis of 3HB rather than 3HV continued, so that the final 3HV content was 40 mol% after 24 h of cultivation. The decrease in the final 3HV mol%, compared with the result (75 mol%) obtained in the shake flask culture, was primarily due to the difference in the ratio of total cell mass to LA concentration, considering that DCW obtained was about 3.0 g/L in the flask culture and 5.9 g/L in the fermentor culture. In this fermentor culture, the yield of 3HV from LA ($Y_{3HV/LA}$) was 0.38 g/g. In batch fermentation cultures of *R. eutropha* using 4.0 g/L of propionate or valerate, the maximal concentrations of cell mass and poly(3HB-*co*-3HV) produced at the end of 24 h cultivation amounted to 3.3-3.7 g/L and 1.8-2.1 g/L, respectively (data not shown).

To reduce the toxic effect of LA on cell growth, repeated addition of LA was applied to a fermentor culture (Fig. 2B). When LA was added four times to a total concentration of 4.0 g/L, cell mass and poly(3HB-*co*-3HV) accumulation were significantly enhanced up to 8.7

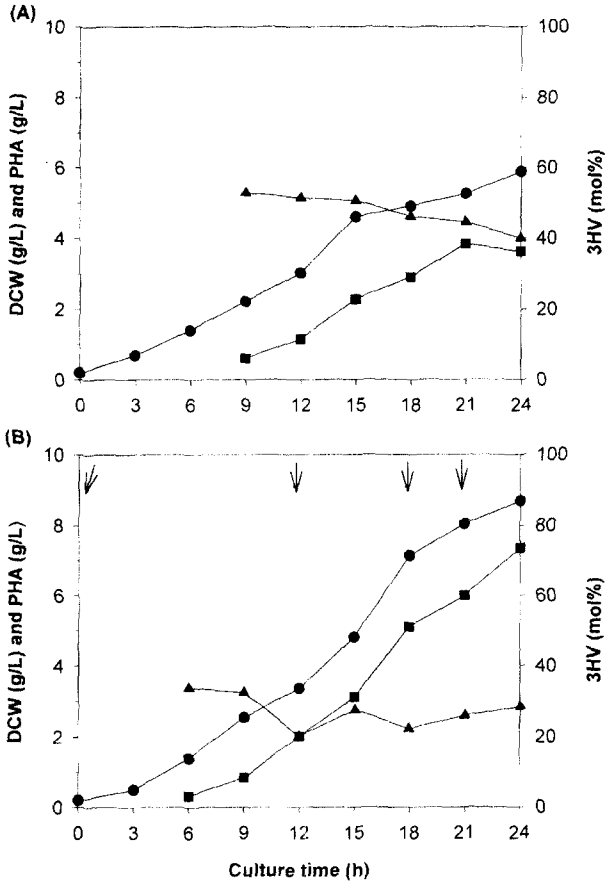

Fig. 2. Time courses of cell growth and PHA accumulation during the cultivation of *R. eutropha* in a fermentor with a basal medium containing levulinic acid. (A) Levulinic acid (4.0 g/L) was added once at the start of fermentation, (B) Levulinic acid was added four times to a total concentration of 4.0 g/L. Symbols: ●, dry cell weight (DCW, g/L); ■, PHA content (g/L); ▲, molar fraction of 3HV (mol%). Arrows indicate levulinic acid addition time.

g/L and 85%DCW, respectively. Compared with the fermentation experiment in which LA was added once at the beginning of culture, the concentrations of cell mass and PHA increased by 147% and 203%, respectively. However, in the case of repeated addition of LA, the final 3HV mol% in the copolyester decreased to 28 mol%, which was due to the remarkable increase of cell and PHA content during the cultivation. From these results it is apparent that the intermittent supplement of a fixed amount of LA to maintain its concentration at a low level is effective for cell growth and the production of poly(3HB-*co*-3HV). The present result can be compared with data of Lee *et al.* (15) who reported 3HV contents of 15-26 mol% with propionate (4 g/L) addition in two-stage fermentor cultures of *R. eutropha* using various concentrations of glucose as the main carbon source.

Comparing with *Alcaligenes* sp. SH-69 which showed the stimulation of cell growth as well as PHA accumulation at a relatively low level (0.5 g/L) of LA addition

(9), the stimulatory effect on the cell growth of *R. eutropha* was not observed in this study even at a low level of LA addition. However, the present results indicate that LA is a more effective cosubstrate than propionate and valerate for the production of poly(3HB-*co*-3HV) by *R. eutropha* and that LA concentration in the medium could be an effective means to control the molar fraction of 3HV in the copolyesters. In a fermentor culture with repeated addition of LA, the $Y_{3HV/LA}$ value reached up to 0.50 g/g, which was at a relatively high level compared to the reported 3HV yields from propionate ($Y_{3HV/propionate}$ = 0.06-0.13 g/g) in *R. eutropha* (8, 18). However, it needs to be increased considerably to make the copolyester commercially more attractive since the 3HV yield from a cosubstrate has a significant effect on the total production cost of poly(3HB-*co*-3HV) and the production cost increases linearly with the increase in 3HV mol% in the copolyester (4). Recent studies showed that many parameters favour the conversion of propionate to 3HV (6, 15-16, 18). Similarly, the 3HV yield from LA is expected to be enhanced by regulating the various fermentation parameters, although the metabolic pathways leading to 3HV synthesis from LA have not been elucidated so far. Further study is in progress to optimize the culture conditions for the production of poly(3HB-*co*-3HV) with high productivity and high 3HV yield by fed-batch fermentation of *R. eutropha* using LA as the cosubstrate.

Acknowledgment

This work was supported by the Brain Korea 21 Project.

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