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Production of Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) from Cottonseed Oil and Valeric Acid in Batch Culture of *Ralstonia* sp. Strain JC-64

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Abstract

A *Ralstonia* sp. strain JC-64 that is capable of accumulating poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P[3HB-*co*-3HV]) from cottonseed oil and valeric acid was isolated. By using a high limiting-nitrogen (HLN) mineral medium as the medium for the second stage of the fermentation process and by adding the two carbon sources at different times, a range of copolymers with 12–62 mol% of 3HV were produced from a series of HLN mineral mediums containing different compositions of cottonseed oil and valeric acid by *Ralstonia* sp. JC-64. The melting temperature (T_m) of polyhydroxybutyrate from cottonseed oil was 174°C and that of P(3HB-*co*-3HV) with the highest 3HV-mol fraction (62%) was 81°C.

Index Entries: Cottonseed oil; high limiting-nitrogen mineral medium; polyhydroxybutyrate; poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate); *Ralstonia* sp. JC-64.

Introduction

Polyhydroxyalkanoates (PHAs) are biodegradable polyesters that are synthesized and accumulated as a carbon or energy storage material intracellularly during unbalanced growth by a large variety of bacteria *(1)*. Currently, more than 80 hydroxyalkanoates have been detected as

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constituents of PHAs *(2)*, and more than 300 different microorganisms are known to synthesize and intracellularly accumulate PHAs *(3)*.

Polyhydroxybutyrate (PHB) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate (P[3HB-*co*-3HV]) are the most common members of the PHA family. They have attracted much attention as bioplastics. Both have been commercially produced, and many strategies for the production, such as the use of inexpensive material as carbon sources *(4–10)*, improved methods for isolating polymers *(11–13)*, and the isolation of new bacterial strains for biosyntheses *(14)* have been developed. Also, PHA synthesis genes have been cloned, and recombinant strains have been obtained *(15–18)*.

The biosynthesis of PHA from plant oil, a renewable and inexpensive agricultural coproduct, has been extensively researched. *Pseudomonas* sp. produced PHA consisting of medium- and long chain–length 3-hydroxy alkanoate units from long-chain fatty acid (castor oil and euphorbia oil) and tallow *(7,8)*, and *Aeromonas caviae* produced P(3HB-*co*-3HH) from olive oil *(6)*. *Pseudomonas* sp. 61-3 synthesized polyester blends from olive oil or corn oil and palm oil *(9)*. *Alcaligenes eutrophus* and its recombinant synthesized PHB and P(3HB-3HH$_X$) from olive oil, corn oil, and palm oil *(10)*. In spite of the great need for P(3HB-*co*-3HV) as a bioplastic, to our knowledge, there has been no report on the production of P(3HB-*co*-3HV) from plant oil.

In the present study, we investigated the production of P(3HB-*co*-3HV) from cottonseed oil and valeric acid by *Ralstonia* sp. JC-64 isolated from paddy soil. When a high limiting-nitrogen (HLN) mineral medium was used as the medium of the second-stage fermentation process and the two carbon sources were added at different fermentation times, P(3HB-*co*-3HV) containing a series of 3HV-mol fractions was produced.

Materials and Methods

Strain

The soil sample, from paddy soil in Tianjin, China, was diluted and spread on agar plates containing yeast extract, meat extract, polypeptone, and ammonium sulfate (YMPA). The YMPA agar medium (pH 7.0, 1 L) contained 10 g of yeast extract, 10 g of polypeptone, 5 g of meat extract, 5 g of $(NH_4)_2SO_4$ and 20 g of agar. The plate was incubated at 30°C for 2 d. Some colonies that appeared on the plate were screened for their PHA production by the Schlegel et al. *(19)* method. In this method, colonies in nitrogen-poor agar (0.005% ammonium chloride) were stained by sudan-black B to distinguish bacteria containing PHB. *Ralstonia* sp. JC-64, thus obtained, was used in this study. The strain was maintained in 15 (v/v)% glycerol at –80°C.

Medium and Culture Conditions of PHB Synthesis

A one-step fermentation process was used. Cells were grown in a reciprocal shaker (150 rpm) at 30°C for 72 h under aerobic conditions on a

reciprocal shaker in 500-mL Sakaguchi flasks with 100 mL of medium. The medium, containing 1% (v/v) cottonseed oil and 0.05% (w/v) NH_4Cl (NH_4Cl [w/v]:cottonseed oil [v/v] = 1:20), was a limiting-nitrogen mineral medium as described by Fukui and Doi *(10)*.

Medium and Culture Conditions of P(3HB-co-3HV) Synthesis

A two-step fermentation process was used. Cells were grown in YMPA medium in a reciprocal shaker (150 rpm) at 30°C for 24 h, harvested, and transferred to an HLN mineral medium containing cottonseed oil, NH_4Cl (NH_4Cl [w/v]:cottonseed oil [v/v] = 1:80), and other components as already mentioned. After the cells were cultivated in the reciprocal shaker (150 rpm) at 30°C for a given time, valeric acid (pH 7.0) was added and the cells were cultivated for a further 48 h.

Isolation and Analysis of PHB and P(3HB-co-3HV)

The grown cells were harvested, washed with hexane and then distilled water, and lyophilized. The polymer and copolymer accumulating in the cells were extracted with hot chloroform in a Soxhlet apparatus and purified by reprecipitation with methanol. ^1H and ^{13}C nuclear magnetic resonance (NMR) analyses were carried out using a Nihondenshi JNM A400 (400 MHz) spectrometer. Differential scanning calorimetry (DSC) was carried out using a Rigaku DSC-8230B system equipped with a cooling accessory. The melting temperature was determined from the DSC endotherm.

Lipase Activity of Ralstonia *sp. Strain JC-64*

Lipase activities of the supernatant of *Ralstonia* sp. strain JC-64 in culture and fermentation solution were assayed at 30°C by the method of Kurooka et al. *(20)*; 2,3-Dimercaptopropan-1-ol tributyroate was synthesized by treating 2,3-dimercaptopropan-1-ol with butyric anhydride *(21)* and used as a substrate. 5,5'-Dithiobis(2-nitrobenzoic acid) as a chromogenic reagent and sodium dodecyl sulfate as a lipase activator were used. The substrate was hydrolyzed by lipase, and the absorbance of 5-thio-2-nitrobenzoate was measured at 412 nm.

Results and Discussion

Identification of Ralstonia *sp. Strain JC-64*

Morphologic and taxonomic characteristics of the isolated *Ralstonia* sp. strain JC-64 were investigated by the established method of Krieg and Holt *(22)*. Table 1 summarizes the results. The isolated strain was an aerobic, non-spore-forming, Gram-negative, motile, and short rod–shaped bacterium. Since *A. eutrophus* has been renamed *Ralstonia eutropha* by the International Union of Microbiological Societies *(23)*, it was designated as *Ralstonia* sp. JC-64.

Table 1
Morphologic and Taxonomic Properties of *Ralstonia* sp. JC-64

Properties	Results
Morphology	
Form	rods
Size (μm)	0.5–0.8 × 1.5–2.4
Gram reaction	–
Presence of spore	–
Motility	+
Cultural characteristics	
Colony shape: circular convex	
Optimum temperature	30–38°C
Optimum pH	6.5–7.2
Growth on	
MacConkey agar	–
SS agar	–
Starch agar	–
DNase agar	–
Biochemical properties	
β-Galactosidase	–
β-Glucosidase	–
Arginine dihydrolase	+
Lysine decarboxylase	–
Ornithine decarboxylase	–
Tryptophan deaminase	–
Urease	+
Gelatinase	+
Cytochrome oxidase	+
Catalase	+
Physiological properties	
Production of H_2S	–
Production of indole	–
Voges-Proskauer test	+
Nitrate reduction to nitrite	+
Nitrate reduction	+
Fermentation/oxidation of	
Glucose, mannitol, arabinose	–
Sorbitol, rhamnose, melibiose	–
Amygdalin, inositol	–
Utilization of	
D-Glucose, D-fructose	+
Rhamnose, L-arabinose	+
Sucrose, D-mannose	+
D-Ribose, valerate	+
Acetate, DL-lactate	+
Propionate, gluconate	+
Malonate, itaconate	+
Sorbitol, glycerol	+
Inositol, mannitol	+
N-Acetyl-glucosamine	+
Tween-85	+
L-Fucose, ethanol	–
Cellulose, glycogen	–
D-Melibiose, citrate	–
Salicin, methanol	–

PHB and P(3HB-co-3HV) from Cottonseed Oil and Valeric Acid

Table 2 summarizes the production of PHB and P(3HB-*co*-3HV). Run 1 shows the results of PHB accumulation in *Ralstonia* sp. JC-64 at 30°C in a medium (1 L) containing 10.0 mL of cottonseed oil. *Ralstonia* sp. JC-64 gave a higher polymer content (77%). Runs 2–5 show the results of copolymers from cottonseed oil and valeric acid. In this experiment, cottonseed oil-to-valeric acid weight ratios in the medium were respectively adjusted to 80:20, 60:40, 40:60, and 20:80. The initial total amount of substrates from runs 2–5 was 10 g/L, and the initially inoculated cell weight was about 3.7 g/L. After the operation, the final weight of dried cells ranged from 4.45 to 3.91 g. Thus, an extent of weight increase of dried cells was from 0.20 to 0.75 g. The copolyester content ranged from 49 to 71%. It had been reported that the weight of dried cells was not changed in the nitrogen-free mineral medium as in the second-stage fermentation medium *(24,25)*. However, in the HLN mineral medium, the dried cell weights increased because the medium contained NH_4Cl as the nitrogen source.

Lipase Activity and Composition of HLN Mineral Medium

In our experiment, the lipase activity of *Ralstonia* sp. JC-64 in the supernatant of the medium was lower (0.2–0.3 mU/mL) when it was grown on glucose or fructose alone. Fukui and Doi *(10)* obtained a similar result. However, the lipase activity increased to 2.7–3.9 mU/mL during the PHB accumulation phase in the presence of cottonseed oil (run 1 in Table 2). Therefore, it was thought that microbial lipase activity was induced to increase when cottonseed oil was used as the sole carbon source in the medium.

In the synthesis of P(3HB-*co*-3HV) from glucose and organic acid by the two-step fermentation process, a nitrogen-free mineral medium was used as the medium of the second stage *(24–27)*. In our study, a nitrogen-free mineral medium as the second-stage medium was also used; Table 3 presents the results (runs 3 and 4). The copolymer contents were <15%, and the lipase activities in the supernatant were <1.6–2.7 mU/mL. These results suggested that secretion of lipase was suppressed under a nitrogen-free condition. Therefore, the limiting-nitrogen mineral medium was used in the second stage of the fermentation process in this study.

Because the fermentation time of PHB production by the limiting-nitrogen mineral medium, NH_4Cl (w/v):cottonseed oil (v/v) = 1:20, was 72 h, the limiting-nitrogen mineral mediums containing different ratios of NH_4Cl (w/v): cottonseed oil (v/v) (=1:40, 1:50, 1:60, 1:70, 1:80, and 1:90) were used to find a medium that would shorten the fermentation period and maintain high lipase activity. The limiting-nitrogen mineral medium with a ratio of 1:80 gave higher lipase activities, and the fermentation period of the second stage became 50–54 h (runs 2–5 in Table 2) and gave higher copolymer contents. We called this medium an HLN mineral medium in this study.

Table 2
Production of PHB and P(3HB-*co*-3HV) by *Ralstonia* sp. JC-64 from Cottonseed Oil and Valeric Acid at 30°C (pH 7.0)

Run[a]	Carbon source (g)		Cell dry wt (g)	Polyester content (wt%)	Composition (NMRmol%)		Lipase activity in supernatant (mU/mL)	T_m (°C)
	Cottonseed oil	Valeric acid			3HB	3HV		
1	9.17 (10 mL)	—	3.70	77	100	—	2.7–3.9	174
2	8.00 (8.7 mL)	2.00	4.45	71	88	12	2.7–3.7	153
3	6.00 (6.5 mL)	4.00	4.10	60	69	31	2.7–3.7	125
4	4.00 (4.4 mL)	6.00	4.06	58	56	44	2.7–3.7	92
5	2.00 (2.2 mL)	8.00	3.91	49	38	62	2.7–3.7	81

[a]Run 1 was the one-step fermentation process with the limiting-nitrogen medium (NH_4Cl [w/v]:cottonseed oil [v/v] = 1:20) (1.0 L). Cells were cultivated for 72 h. Runs 2–5 were the two-step fermentation process by the HLN medium (NH_4Cl [w/v]:cottonseed oil [v/v] = 1:80) (1.0 L) as the second-stage fermentation medium. The cells from the first stage were collected and cultivated in the HLN medium containing cottonseed oil. Valeric acid (pH 7.0) was added to the HLN medium, and the cells were cultivated for a further 48 h. Time of addition of valeric acid from the beginning of the second-stage fermentation: run 2 (6 h), run 3 (5 h), run 4 (3 h), run 5 (2 h).

Table 3
Production of P(3HB-*co*-3HV) by *Ralstonia* sp. JC-64 from Cottonseed Oil and Valeric Acid at 30°C (pH 7.0)

Run[a]	Carbon source (g)		Cell dry wt (g)	Polyester content (wt%)	Composition (NMRmol%)		Lipase activity in supernatant (mU/mL)
	Cottonseed oil	Valeric acid			3HB	3HV	
1	8.00	2.00	3.90	21	86	14	2.0–3.1
2	6.00	4.00	3.88	23	68	32	2.0–3.1
3	8.00	2.00	3.70	15	86	14	1.6–2.7
4	6.00	4.00	3.70	13	70	30	1.6–2.7

[a]For runs 1 and 2, cells from the first stage were collected and cultivated in the HLN mineral medium (NH_4Cl [w/v]:cottonseed oil [v/v] = 1:80) (1.0 L) for 48 h. Cottonseed oil and valeric acid were added to the HLN mineral medium simultaneously. For runs 3 and 4, cells from the first stage were collected and cultivated in the nitrogen-free mineral medium (1.0 L) containing the cottonseed oil and valeric acid for 48 h.

Addition of Carbon Source

In the HLN mineral medium containing both cottonseed oil and valeric acid, the latter suppressed secretion of lipase by the cells. As shown in Table 3 (runs 1 and 2), the copolymer contents were <23%, and the lipase activity in the supernatant was <2.0–3.1 mU/mL. To avoid such an unfavorable effect of valeric acid, the two carbon sources were added to the HLN mineral medium at different times. First, when the HLN mineral medium was prepared, the cottonseed oil was added. Second, valeric acid was added at pH 7.0 after the cells were cultivated for a proper time in the HLN mineral medium. Therefore, the beginning time for PHB accumulation from cottonseed oil must be decided to determine the time for adding valeric acid.

We investigated the relationship between the beginning time of PHB accumulation in the cells and the concentration of cottonseed oil in the HLN mineral medium. The cells from the first stage were transferred to the HLN mineral medium (NH_4Cl [w/v]:cottonseed oil [v/v] = 1:80) containing 2.0, 4.0, 6.0, and 8.0 g/L cottonseed oil, respectively. By detecting PHB accumulation in preliminary experiments, suitable times for the addition of valeric acid were determined. When cottonseed oil was 8.0 g/L in the HLN mineral medium, a very small amount of PHB accumulation in the cells was detectable at 7 h but not yet at 6 h from the beginning of the second-stage fermentation. From this result, we concluded that valeric acid should be added after 6 h from the beginning of the second-stage fermentation when 8.0 g/L of cottonseed oil was used (run 2 in Table 2). The suitable times of the addition of valeric acid for other conditions (runs 3–5 in Table 2) were also determined the same way. By adopting this procedure, the lipase activities of the fermentation solution were kept at a relatively high level (2.7–3.7 mU/mL) as well as during PHB production in cells.

Effect of Valeric Acid on 3HV Fraction in Copolymer

Table 2 also shows the effect of valeric acid concentration in the HLN mineral medium on the 3HV-mol fraction of P(3HB-*co*-3HV). According to ^{1}H NMR spectroscopy, as the concentration of valeric acid increased, the 3HV-mol fraction in the copolymer increased. Thus, the 3HV-mol fraction depended on the concentration of valeric acid in the HLN mineral medium. This indicated that when P(3HB-*co*-3HV) was accumulated from cottonseed oil and valeric acid by *Ralstonia* sp. JC-64, the composition of the copolymers could be controlled by changing the concentration of valeric acid. On the other hand, the product was also P(3HB-*co*-3HV) when the cottonseed oil (8.0 g/L) and propionic acid (2.0 g/L) were used as the carbon sources by *Ralstonia* sp. JC-64. However, the 3HV-mol fraction (4.5%) in the copolymer was lower than those of copolymer from the cottonseed oil and valeric acid (12%).

Melting Temperature of Polymer and Copolymer

Table 2 also shows the T_m of PHB and P(3HB-*co*-3HV). T_m decreased from 174 to 81°C with an increase in the 3HV-mol fraction from 0 to 62%, suggesting that the degree of crystallinity decreases with an increase in the 3HV-mol fraction. This result confirmed that the mechanical properties of the copolymers can be controlled by changing the concentration of valeric acid in the HLN mineral medium *(24)*.

Conclusion

Ralstonia sp. strain JC-64 is a new member of the family of PHA-producing bacteria. By using an HLN mineral medium as the medium for the second stage of the fermentation process and by adding two carbon sources at different times of the second-stage fermentation process, a wide range of copolymers with 12–62 mol% of 3HV was produced from a series of HLN mineral mediums containing different ratios of cottonseed oil and valeric acid by *Ralstonia* sp. JC-64. The melting temperature of PHB from cottonseed oil was 174°C and that of P(3HB-*co*-3HV) with the highest 3HV-mol fraction (62%) was 81°C.

References

1. Lee, S. Y. (1996), *Tibtech* **14,** 431–438.
2. Lee, S. Y. (1996), *Biotechnol. Bioeng.* **49,** 1–14.
3. Lee, S. Y., Choi, J. I., and Wong, H. H. (1999), *Int. J. Biol. Macromol.* **25,** 31–36.
4. Eggink, G., van der Wal, H., Huijberts, G. N. M., and Waard de, P. (1993), *Ind. Crops Prod.* **1,** 157–163.
5. Shimamura, E. and Kasuya, K. (1994), *Macromolecules* **27,** 878–880.
6. Doi, Y., Kitamura, S., and Abe, H. (1995), *Macromolecules* **28,** 4822–4828.
7. Eggink, G., Waard de, P., and Huijberts, G. N. M. (1995), *Can. J. Microbiol.* **41(Suppl. 1),** 14–21.
8. Cromwick, A.-M., Foglia, T., and Lenz, R. W. (1996), *Appl. Microbiol. Biotechnol.* **46,** 464–469.
9. Kato, M., Fukui, T., and Doi, Y. (1996), *Bull. Chem. Soc. Jpn.* **69,** 515–520.
10. Fukui, T. and Doi, Y. (1998), *Appl. Microbiol. Biotechnol.* **49,** 333–336.
11. Choi, J. I., and Lee, S. Y. (1999), *Biotechnol. Bioeng.* **62(5),** 546–553.
12. Hrabak, O. (1992), *FEMS Microbiol. Rev.* **103,** 251–256.
13. Lee, S. Y., Choi, J. I., Han, K., and Song, J. Y. (1999), *Appl. Environ. Microbiol.* **65(6),** 2762–2764.
14. Kim, G. J., Yun, K. Y., Bae, K. S., and Rhee, Y. H. (1992), *Biotechnol. Lett.* **14(1),** 27–32.
15. Slater, S. C., Voige, W. H., and Dennis, D. E. (1988), *J. Bacteriol.* **170,** 4431–4436.
16. Yim, K. S., Lee, S. Y., and Chang, H. N. (1996), *Biotechnol. Bioeng.* **49,** 495–503.
17. Slater, S., Gallaher, T., and Dennis, D. (1992), *Appl. Environ. Microbiol.* **58,** 1089–1094.
18. Hustede, E., Steinbüchel, A., and Schlegel, H. G. (1992), *FEMS Microbiol. Lett.* **94,** 285–290.
19. Schlegel, H. G., Lafferty, R., and Krauss, I. (1970), *Arch. Microbiol.* **71,** 283–294.
20. Kurooka, S., Okamoto, S., and Hashimoto, M. (1977), *J. Biochem.* **81,** 361–369.
21. Doyle, F. P., Holland, D. O., Mansford, K. R. L., Nayler, J. H. C., and Queen, A. (1960), *J. Chem. Soc.* **56,** 2660–2665.
22. Krieg, N. R. and Holt, J. G. (1984), *Bergey's Manual of Systematic Bacteriology*, vol 1, Williams Wilkins, Baltimore.

23. International Union of Microbiological Societies. (1996), *Int. J. System. Bacteriol.* **46(2),** 625, 626.
24. Chua, H., Yu, P. H. F., and Lo, W. (1998), *Appl. Biochem. Biotechnol.* **70–72,** 929–935.
25. Doi, Y., Tamaki, A., Kunioka, M., and Soga, K. (1988), *Appl. Microbiol. Biotechnol.* **28,** 330–334.
26. Byrom, D. (1992), *FEMS Microbiol. Rev.* **103,** 247–250.
27. Ishihara, Y., Shimizu, H., and Shioya, S. (1996), *J. Ferment. Bioeng.* **81(5),** 422–428.