Bioresource Technology 102 (2011) 6766–6768

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Short Communication

Production of (*R*)-3-hydroxybutyric acid by fermentation and bioconversion processes with *Azohydromonas lata*

Charles U. Ugwu *, Yutaka Tokiwa, Toshio Ichiba

Okinawa Industrial Technology Center, Suzaki 12-2, Uruma, Okinawa 904-2234, Japan

ARTICLE INFO

Article history:
Received 29 January 2011
Received in revised form 23 March 2011
Accepted 23 March 2011
Available online 27 March 2011

Keywords:
Azohydromonas lata
Bioconversion
Fermentation
(*R*)-3-hydroxybutyric acid
UV mutant

ABSTRACT

Feasibility of producing (*R*)-3-hydroxybutyric acid ((*R*)-3-HB) using wild type *Azohydromonas lata* and its mutants (derived by UV mutation) was investigated. *A. lata* mutant (M5) produced 780 mg/l in the culture broth when sucrose was used as the carbon source. M5 was further studied in terms of its specificity with various bioconversion substrates for production of (*R*)-3-HB. (*R*)-3-HB concentration produced in the culture broth by M5 mutant was 2.7-fold higher than that of the wild type strain when sucrose (3% w/v) and (*R,S*)-1,3-butanediol (3% v/v) were used as carbon source and bioconversion substrate, respectively. Bioconversion of resting cells (M5) with glucose (1% v/w), ethylacetoacetate (2% v/v), and (*R,S*)-1,3-butanediol (3% v/v), resulted in (*R*)-3-HB concentrations of 6.5 g/l, 7.3 g/l and 8.7 g/l, respectively.

1. Introduction

Poly[(*R*)-3-hydroxybutyrate] (PHB) is a biodegradable polymer, which has been known to have good material properties that are comparable to those of the polypropylene. Microbial production of PHB and its copolymers have been extensively investigated (Doi, 1990; Sankhla et al., 2010; Hafuka et al., 2011). (*R*)-3-hydroxybutyric acid ((*R*)-3-HB), a monomer of PHB, has recently attracted much interest given its vital functions in humans (Kashiwaya et al., 2000; Massieu et al., 2003) and as a chiral compound for synthesis of fine chemicals (Gao et al., 2002; Shiraki et al., 2006; Ugwu et al., 2008). (*R*)-3-HB can be produced either by chemical synthesis (Chiba and Nakai, 1985; Seebach et al., 1992) or biotechnological process (Gao et al., 2002; Tokiwa and Ugwu, 2007). Biotechnological approach would not only ensure a clean process "environmentally friendly" for production of (*R*)-3-HB, but would also help in reducing the production cost of the monomer. Previous approaches to fermentative production of (*R*)-3-HB using some microbial species have been reported (Nath et al., 2005; Shiraki et al., 2006; Ugwu et al., 2008). In another study, it was reported that *Azohydromonas lata*, a well known PHB-producing strain, was exploited for production of (*R*)-3-HB (Lee et al., 1999). However, of all the mutation types, UV radiation is one of the most "environmentally friendly" techniques. Moreover, UV radiation is the only naturally occurring mutation, which played major role during the evolution of life on earth (Ugwu et al., 2008). In this study, we carried out UV mutation using a wild type *A. lata* in order to obtain unique mutant strains that can easily produce (*R*)-3-HB. In order to increase the yields of (*R*)-3-HB, both microbial fermentation and bioconversion processes were employed.

2. Methods

Wild type strain of *Azohydromonas lata* JCM 20724 (*A. lata*) and mutants were grown in basal medium, which contained the following components (per liter): 250 mg yeast extract, 200 mg $MgSO_4 \cdot 7H_2O$, 100 mg NaCl, 20 mg $CaCl_2 \cdot 2H_2O$, 10 mg $FeSO_4 \cdot H_2O$, 0.5 mg $Na_2MoO_4 \cdot 2H_2O$, 0.5 mg $Na_2WO_4 \cdot 2H_2O$, 0.5 mg $MnSO_4$, 1600 mg K_2HPO_4, 200 mg KH_2PO_4 and 1000 mg $(NH_4)_2 \cdot SO_4$, 30 g sucrose or glucose. In the case of cultures used for bioconversion reactions, 10 g/l polypeptone was included in the basal medium. Various concentrations of bioconversion substrates were tested. The pH was maintained at 7.0.

2.1. Screening of the mutants

A. lata was grown at 30 °C and 150 rpm for 18 h in 500 ml Erlenmeyer flask which contained 100 ml of basal medium (working volume). 10 ml of culture broth was suspended in sterile glass plate and then placed 6 cm under the UV radiation lamp (15 W at 253 nm). UV-irradiated cultures were plated out and incubated at 30 °C for 48 h to allow the survivors to produce some colonies. Selected colonies were then grown in culture broth to allow them

* Corresponding author. Tel.: +81 98 929 0111; fax: +81 98 929 0115.
E-mail address: ugutyaru@pref.okinawa.lg.jp (C.U. Ugwu).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.03.073

produce (*R*)-3-HB. Aliquot of cells were stored in 10% v/v glycerol, and maintained at −80 °C as stock cultures.

2.2. Preparation of resting cells and reaction mixtures for production of (*R*)-3-HB

Resting cells were obtained from a 66 h-culture after removing the supernatants and washing with 0.85% w/v NaCl by centrifugation at 10,000 rpm for 15 min. Resting cells (which corresponded to 50 mg dry weight) were then dispersed in a 5 ml-phosphate buffer solution (0.2 M) that contained various concentrations of bioconversion substrates. The reaction mixtures were incubated at 30 °C and 150 rpm for 6–40 h.

2.3. Analytical methods

The concentration of PHB was determined gravimetrically as previously described (Ugwu et al., 2008). Thus, 1 g of lyophilized cells was extracted with 50 ml chloroform in a reflux condenser under heating in an oil bath (75 °C) for 8 h. Cell debris were removed by filtration and the chloroform/PHB solution was then precipitated with ethanol (volume of ethanol was 4 times that of the chloroform). PHB was recovered from the solvents (i.e., chloroform/ethanol mixture) by filtration followed by drying to a constant weight. (*R*)-3-HB was quantified with HPLC and (*R*)-3-HB analytical kit (Boehringer Mannheim, Germany). HPLC analyses were performed on Waters instrument equipped with Aminex HPX-87H column (300 mm by 7.8 mm; Bio-Rad Laboratories, Hercules, CA) and refractive index detectors under the following conditions: mobile phase, 5 mM sulfuric acid in water; flow rate, 0.6 ml min^{-1}; injection volume, 20 μl; and temperature, 40 °C.

3. Results and discussion

UV mutation was strategically used in this study to obtain mutants of *A. lata* that can produce (*R*)-3-HB. Fig. 1 shows time course of the survivors (viable cells). Most cells (about 99.99%) were killed by UV radiation in 12 min. Colonies of the survivors generated (at 12 min of UV irradiation) were isolated. Although 500 colonies were tested, only six of them that could produce (*R*)-3-HB in the culture broth were selected. Concentrations of (*R*)-3-HB obtained with M5, M85, M27, M7, M109, and M108 mutants were 551 mg/l, 541 mg/l, 385 mg/l, 364 mg/l, 260 mg/l, and 156 mg/l, respectively. M5 was selected for further studies since it produced the highest concentration of (*R*)-3-HB (551 mg/l) and had wider substrate specificity compared to other mutants. When M5 was grown in the basal medium that contained sucrose as the carbon source, (*R*)-3-HB concentration attained was 780 mg/l.

Table 1 shows the effects of various carbon substrates (i.e., as sole carbon sources) on the production of (*R*)-3-HB. (*R*)-3-HB yield from substrate was defined as the concentration of (*R*)-3-HB per concentration of substrates (in percentage). Sucrose gave the highest concentration (780 mg/l) and yields of (*R*)-3-HB from substrates (2.60%) compared to other carbon sources. On the other hand, (*R,S*)-1,3-butanediol gave the lowest yield of (*R*)-3-HB. A comparative evaluation of yields of (*R*)-3-HB from cell dry weight showed that sucrose and molasses gave relatively high values. It should be noted that when either (*R,S*)-1,3-butanediol or ethylacetoacetate was used as the sole carbon in the medium, cell concentrations were relatively low, resulting in low yields of (*R*)-3-HB.

Table 1
Extracellular production of (*R*)-3-HB with M5 mutant using various carbon substrates.

Substrates	Conc. (g/l)	DCW (g/l)	(*R*)-3-HB yield from substrates (%)	(*R*)-3-HB/DCW (g/g)
Sucrose	30	6.30	2.60	0.12
Glucose	30	5.70	1.84	0.09
Molasses	76 (10% w/v)	6.00	0.96	0.12
Ethylacetoacetate	20	3.30	1.14	0.07
(*R,S*)-1,3-butanediol	30	2.80	0.62	0.07

Cells were grown for 96 h in 500 ml flasks that contained 100 ml of basal medium and various carbon substrates.

In order to increase the production of (*R*)-3-HB, a two-step-process was employed (Fig. 2). In the first step, the cell concentration was increased by growing the cells with glucose or sucrose for 48 h, followed by addition of 3% v/v (*R,S*)-1,3-butanediol and subsequent continuation of the culture for another 48 h. Cell concentrations of M5 and wild type *A. lata* were 4.0 g/l and 3.8 g/l, respectively. (*R*)-3-HB concentrations of M5 was 200 mg/l whereas no production of (*R*)-3-HB by the wild type *A. lata* was observed. Addition of 3% v/v (*R,S*)-1,3-butanediol resulted in final concentrations of (*R*)-3-HB at 0.998 g/l and 1.14 g/l in the culture broth for glucose and sucrose, respectively. In the case of wild type *A. lata*, (*R*)-3-HB concentrations of 0.3 g/l and 0.42 g/l were attained with glucose and sucrose, respectively.

To understand whether the specificity of these bioconversion substrates were limited to ethylacetoacetate and (*R,S*)-1,3-butanediol or not, various carbon sources (e.g., sugars) were also tested. Preliminary investigation on the effects of various soluble sugars

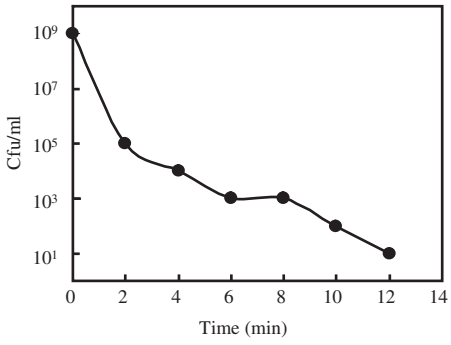

Fig. 1. Time course of the survivors (viable cells) during the UV mutation.

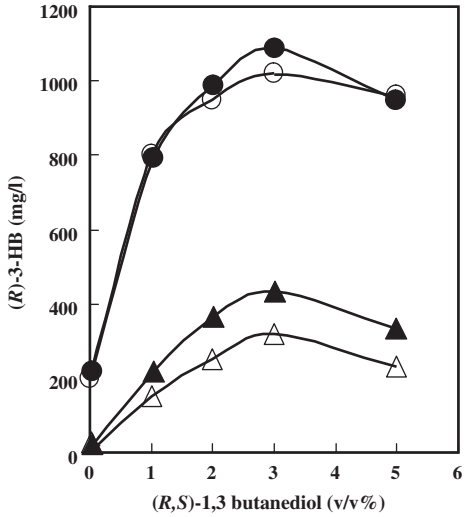

Fig. 2. Production of (*R*)-3-HB in a two-stage process using either glucose or sucrose with (*R,S*)-1,3-butanediol. (*R*)-3-HB produced by M5 mutant when glucose (open circle) and sucrose (closed circle) were used. (*R*)-3-HB produced by wild *A. lata* using glucose (open triangle) and sucrose (closed triangle).

and other biomass materials was done under shaking conditions. Thus with M5 mutant, (*R*)-3-HB concentrations obtained with glucose, fructose, xylose, sucrose and molasses (i.e., as bioconversion substrates) at 6 h-reaction under shaking conditions were 1.90 g/l, 1.13 g/l, 1.12 g/l, 0.20 g/l, 0.08 g/l, respectively. These values indicated that glucose gave the highest concentration of (*R*)-3-HB while sucrose and molasses gave very low values. Bioconversion potentials of these substrates can be ranked as follows; glucose > [fructose, xylose] > sucrose > molasses. There was no significant difference between fructose and xylose ($p < 0.05$). Further investigations showed that bioconversion reactions with glucose, (*R,S*)-1,3-butanediol and ethylacetoacetate were better at static compared to shaking conditions. Furthermore, the choice in the selection of the suitable concentrations of these substrates was based on the cost of the substrates. Apparently, (*R,S*)-1,3-butanediol and ethylacetoacetate are relatively cheaper compared to other substrates used in this study. Concentrations of glucose, ethylacetoacetate, and (*R,S*)-1,3-butanediol that gave the best results were 1% v/w, 2% v/v and 3% v/v, respectively. Although concentrations of these substrates were different, a rough comparison of (*R*)-3-HB obtainable with them can be made. Thus by using glucose (1% v/w), ethylacetoacetate (2% v/v), and (*R,S*)-1,3-butanediol (3% v/v), in a 40 h-reaction under static conditions, final concentrations of (*R*)-3-HB obtained were 6.5 g/l, 7.3 g/l and 8.7 g/l, respectively. An attempt to increase the concentrations of these substrates with the aim of increasing the yield of (*R*)-3-HB, resulted in poor dissolution of the mixture and inhibition of enzyme reaction which invariably led to very low production of (*R*)-3-HB. In the case of glucose as a bioconversion substrate, higher concentrations resulted in increased production of (*R*)-3-HB. However, yield with respect to the substrate was the highest with 1% w/v.

Bioconversion with (*R,S*)-1,3-butanediol and ethylacetoacetate can be explained in terms of oxidation and reduction activities, respectively. Table 2 shows the effects of some metal salts (10 mM each) on the bioconversion reaction with (*R,S*)-1,3-butanediol or ethylacetoacetate. (*R*)-3-HB values for reaction mixtures that contained (*R,S*)-1,3-butanediol or ethylacetoacetate but without metal salts served as controls (relative activity was set at 100%). $FeSO_4$ greatly enhanced the production of (*R*)-3-HB while other metal salts (Na_2SO_3, NaCl, KCl, $MgSO_4$, and $FeCl_3$) seemed to decrease its production. It was thought that $FeSO_4$ might have induced oxidative activities of (*R,S*)-1,3-butanediol resulting in increased production of (*R*)-3-HB.

In the case of ethylacetoacetate, $CaCl_2$, Na_2SO_3, and $FeCl_3$ might have induced reductive activities, resulting in increased production of (*R*)-3-HB. NaCl and KCl slightly inhibited the production of (*R*)-3-HB whereas $FeSO_4$ and $MgSO_4$ showed pronounced inhibition of (*R*)-3-HB.

Interestingly, wild type strains also produced (*R*)-3-HB with the bioconversion substrates. Given that M5 mutant could still produce PHB, it is likely that UV radiation did not inhibit the PHB metabolic pathway. Evidently, the PHB contents (i.e., concentration of PHB per dry cell weight) of a mutant (M5) and wild *A. lata* strains were found to be 40% and 42%, respectively. Generally, *A. lata* is a growth-associated PHB-producing strain, implying that it can produce PHB even during its growth phase. Previous studies with a mutant of *Cupriavidus necator*, showed that it lacked the ability to produce PHB after UV mutation (Ugwu et al., 2008).

(*R*)-3-HB concentration (8.7 g/l) obtained in this study using (*R,S*)-1,3-butanediol is comparable to about 7.3 g/l (*R*)-3-HB attained in a 100 h reaction with a recombinant *Escherichia coli* which was reported by Shiraki et al. (2006). Gao et al. (2002) reported 12 g/l of (*R*)-3-HB (a higher value than that of our study) in a fed-batch culture of a recombinant *E. coli* that used 6% w/v glucose.

Table 2
Effect of metal salts on the bioconversion reaction with ethylacetoacetate or (*R,S*)-1,3-butanediol.

Relative activity (%)		
Metal salts	Ethylacetoacetate	(*R,S*)-1,3-butanediol
No addition	100	100
$CaCl_2$	169	69
Na_2SO_3	135	21
$FeSO_4$	81	138
NaCl	97	54
KCl	99	70
$FeCl_3$	148	40
$MgSO_4$	92	62

4. Conclusion

It seemed that the UV mutagen did not inhibit the PHB production in *A. lata* mutant (M5) and this could be the reason why mutants could produce both PHB and (*R*)-3-HB. Production of (*R*)-3-HB can be carried out in two steps; (1) accumulation of cells during the cultivation stage, and (2) bioconversion of the resting cells with suitable substrates. Sucrose, molasses and glucose can be used for growing the cells while ethylacetoacetate, (*R,S*)-1,3-butanediol and glucose would serve as bioconversion substrates. Production of (*R*)-3-HB by bioconversion process seems to be a very promising method for production of high yields of (*R*)-3-HB. It is anticipated that much higher values could be obtained when our production system is successfully scaled up and operated under fed-batch conditions.

Acknowledgement

The authors acknowledge the support of Okinawa prefecture for funding this work under "Development of technology for production of useful materials using industrial by-products".

References

Chiba, T., Nakai, T., 1985. A synthetic approach to (+)-thienamycin from ethylene (*R*)-3-hydroxybutanoate. A new entry to (3R, 4R)-3-[(*R*)-I-hyrdoxyethylene]-4-acetoxy-2-azetidinone. Chem. Lett. 651–654.

Doi, Y., 1990. Microbial Polyesters. VCH publishers, New York.

Gao, H.J., Wu, Q., Chen, G.Q., 2002. Enhanced production of D-(−)-3-hydroxybutyric acid by recombinant *E. coli*. FEMS Microbiol. Lett. 213, 59–65.

Hafuka, A., Sakaida, K., Satoh, H., Takahashi, M., Watanabe, Y., Okabe, S., 2011. Effect of feeding regimens on polyhydroxybutyrate production from food wastes by *C. necator*. Bioresour. Technol. 102, 3551–3553.

Kashiwaya, Y., Takeshima, T., Mori, N., Nakashima, K., Clarke, K., Veech, R.L., 2000. D-beta-hydroxybutyrate protects neurons in models of Alzeimer's and Parkinson's disease. Proc. Natl. Acad. Sci. USA 97, 5440–5444.

Lee, S.Y., Lee, Y., Wang, F., 1999. Chiral compounds from bacterial polyesters: sugars to plastics to fine chemicals. Biotechnol. Bioeng. 65, 363–368.

Massieu, L., Haces, M.L., Montiel, T., Hernandez-Fonseca, K., 2003. Acetoacetate protects hippocampal neurons against glutamate-mediated neuronal damage during glycolysis inhibition. Neuroscience 120, 365–378.

Nath, A., Bhat, S., Devle, J., Desai, A.J., 2005. Enhanced production of 3-hydroxybutyric acid (3-HB) by in vivo depolymerization of polyhydroxybutyric acid in 3-HB dehydrogenase mutants of Methylobacterium sp. ZP24. Ann. Microbiol. 55, 107–111.

Sankhla, I.S., Bhati, R., Singh, A.K., Mallick, N., 2010. Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) co-polymer production from a local isolate, *Brevibacillus invocatus* MTCC 9039. Bioresour. Technol. 101, 1947–1953.

Seebach, D., Beck, A.K., Breitschuh, R., Job, K., 1992. Direct degradation of the biopolymer poly (*R*)-3-hydroxybutyric acid to (*R*)-3-hydroxybutanoic acid and its methyl ester. Org. Synth. 71, 39–47.

Shiraki, M., Endo, T., Saito, T., 2006. Fermentative production of (*R*)-(−)-3-hydroxybutyrate using 3-hydroxybutyrate dehydrogenase null mutant of *Ralstonia eutropha* and recombinant *E. coli*. J. Biosci. Bioeng. 102, 529–534.

Tokiwa, Y., Ugwu, C.U., 2007. Biotechnological production of (*R*)-3-hydroxybutyric acid monomer. J. Biotechnol. 132, 264–272.

Ugwu, C.U., Tokiwa, Y., Aoyagi, H., Uchiyama, H., Tanaka, H., 2008. UV mutagenesis of *C. necator* for extracellular production of (*R*)-3-hydroxybutyric acid. J. Appl. Microbiol. 105, 236–242.