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Short communication

Efficient production of (*R*)-3-hydroxybutyric acid by *Pseudomonas* sp. DS1001a and its extracellular poly(3-hydroxybutyrate) depolymerase

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ABSTRACT

We demonstrate a novel strategy to produce (*R*)-3-hydroxybutyric acid [(*R*)-3-HB] using *Pseudomonas* sp. DS1001a or its extracellular poly (3-hydroxybutyrate) (PHB) depolymerase. While approximately 77.8% PHB was degraded by *Pseudomonas* sp. DS1001a after 12 h of cultivation, only 1.555 g/l of 3-HB monomer was produced because of assimilation by the strain. About 8.58 g/l (*R*)-3-HB was obtained after 8 h of incubation using extracellular PHB depolymerase from *Pseudomonas* sp. DS1001a. The optimal temperature and pH of PHB depolymerase were 50 °C and 8, respectively. $CaCO_3$ increased (*R*)-3-HB yields to 23.97 g/l by stabilizing the pH of the reaction system. High yields of the (*R*)-3-HB product and the low cost of the medium and $CaCO_3$ indicate the feasibility of bioconversion of low-value PHB waste or industrial leftovers into high-value (*R*)-3-HB using extracellular PHB depolymerase.

1. Introduction

Poly(3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules synthesized and accumulated during unbalanced growth by many bacteria [1]. Over 150 types of alkanoic acids hydroxylated at the 3-, 4-, 5- or 6-position in the (*R*)-configuration can be incorporated into PHAs [2,3]. Among the (*R*)-hydroxyalkanoic acids, (*R*)-3-hydroxybutyric acid [(*R*)-3-HB] is considered important and widely used in industrial and biomedical applications. Studies have shown that (*R*)-3-HB exhibit antimicrobial, insecticidal, and antiviral activities; and serve as chiral building blocks for synthesis of fine chemicals including antibiotics, vitamins, and pheromones [4–9].

Several researchers have attempted to produce (*R*)-3-HB. However, methods to prepare pure chiral (*R*)-3-HB are not economically feasible. Most chemical synthesis and chemical digestion methods to produce this compound are inadequate for scaled-up production because of the resulting low enantiomeric purity, use of large amounts of organic solvents, and low production efficiency attributed to complex processes [10,11]. Microbial bioconversion to produce the (*R*)-3-HB monomer in PHB-producing bacteria with intracellular depolymerase has been used to synthesize (*R*)-3-HB directly. However, the depolymerized product (*R*)-3-HB is further metabolized to acetoacetate by (*R*)-3-HB dehydrogenase, resulting in low yields and production difficulty [12]. Various recombinant *Escherichia coli* systems harboring the heterologous PHB synthesis and degradation pathways have been established to prevent cells from further utilizing hydroxycarboxylic acid; however, the (*R*)-3-HB obtained often ranges from less than 1 g/l to over 10 g/l [13–16].

In this study, we propose a simple and efficient strategy to produce (*R*)-3-HB by in vitro depolymerization of PHB. *Pseudomonas* sp. DS1001a and its PHB depolymerase were proven to efficiently convert PHB to the (*R*)-3-HB monomer.

2. Materials and methods

2.1. Materials

PHB powder with an average molecular weight of 7.31×10^5 was obtained from the Institute of Microbiology, Chinese Academy of Sciences, PR China. Standard (*R*)-3-HB monomer was purchased from Sigma. All chemicals used were of analytical grade unless stated otherwise.

2.2. Bacterial strain and growth medium

Pseudomonas sp. DS1001a (CGMCC 8638) was isolated and mutated in Microbiology Laboratory of Northeast Normal University (Changchun, China). The basal medium contained 2 g/l PHB, 1.5 g/l NH_4Cl, 14.5 g/l $Na_2HPO_4\ 12H_2O$, 4.8 g/l KH_2PO_4, 0.7 g/l $MgSO_4\ 7H_2O$, and 0.005 g/l $CaCl_2\ 2H_2O$.

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2.3. (R)-3-HB monomer assay

The degradation products were analyzed by tandem quadrupole mass spectrometry (Quattro Premier XE, Waters, USA) using the instrument parameters described by Li et al. [17]. Configuration analysis was performed through high-performance liquid chromatography (HPLC; Hitachi L2000, Japan) according to the method of Gao et al. with (*R*)-3-HB monomer as the standard [18]. (*R*)-3-HB was quantitatively analyzed by using a β-hydroxybutyric acid detection kit (Nanjing Jiancheng Institute of Biological Engineering, China).

2.4. Preparation of the crude PHB depolymerase

For the production of extracellular PHB depolymerase, *Pseudomonas* sp. DS1001a was grown in culture medium and incubated on a rotary shaker at 30 °C for 30 h. The fermented liquid was centrifuged at 4 °C, 12000 × *g* for 20 min, and the supernatant was collected as crude enzyme solution.

2.5. PHB depolymerase activity assay

A stable suspension of PHB (1.5 g/l) was used as the substrate for PHB depolymerase activity assay. The reaction system contained 3 ml of PHB substrate and 1 ml of supernatant incubated at 40 °C for 10 min unless indicated otherwise. The decrease in turbidity of the emulsions was measured at 650 nm, and one unit of PHB depolymerase activity was defined as a 0.001 OD decrease in absorbance per minute under the described assay conditions.

2.6. Effects of temperature and pH on PHB depolymerase activity

The PHB depolymerase activity of the crude enzyme was assayed under standard conditions with varying temperatures (40 °C–85 °C) and pH (50 mM citrate buffer at pH 3–6, 50 mM phosphate buffer at pH 6–8, and 50 mM glycine–NaOH buffer at pH 8–10) to determine the optimal parameters. To determine the thermostability and pH stability of the enzyme, the crude enzyme was maintained at 40, 50, or 60 °C under different time intervals (0.5–8 h) or at 4 °C and pH 3–10 for 24 h. Residual activities were assayed under standard conditions.

2.7. Effects of $CaCO_3$ on (R)-3-HB production

The conversion reaction was conducted with 50 mg of PHB in 2 ml of crude enzyme, and different amounts of $CaCO_3$ (10, 20, 30, 40, or 50 g/l). The ratios of $CaCO_3$ to PHB were 0.4:1, 0.8:1, 1.2:1, 1.6:1, and 2:1. After 8 h of incubation under optimal conditions, (*R*)-3-HB production and the reaction pH were assayed to investigate the effects of $CaCO_3$.

3. Results and discussion

3.1. Bioconversion of PHB into (R)-3-HB by Pseudomonas sp. DS1001a

The PHB-degrading ability of *Pseudomonas* sp. DS1001a was confirmed by the formation of a clear hydrolytic zone on agar plates containing PHB powder, and degradation products generated in the culture supernatant were analyzed by MS. The HB monomer with a peak at *m*/*z* 103, rather than the dimer or trimer, was detected, thereby suggesting that the main product of PHB degradation is HB monomer (Fig. 1a). The liquid sample was ultra filtered and acidified to facilitate HPLC analysis with (*R*)-3-HB monomer as the standard. Fig. 1b shows that the sample and (*R*)-3-HB standard exhibit the same retention times, indicating that the monomer product of PHB degradation is (*R*)-3-HB.

(*R*)-3-HB production in liquid culture was measured as a function of time, and results showed a maximum yield of (*R*)-3-HB at 12–18 h (Fig. 2). (*R*)-3-HB yields decreased with increasing fermentation time because of the assimilation and metabolism of the strain. The production conditions of the (*R*)-3-HB monomer were optimized by using the RSM method (data not shown), and the optimal culture medium constituents (w/v) were determined to include 2 g/l PHB, 0.5 g/l NH_4Cl, 10.1 g/l $Na_2HPO_4\,12H_2O$, 3.9 g/l KH_2PO_4, 0.7 g/l $MgSO_4\,7H_2O$, and 0.005 g/l $CaCl_2\,2H_2O$. The culture medium without complex nitrogen sources or inducers showed the feasibility of low-cost production of (*R*)-3-HB. (*R*)-3-HB monomer yield obtained under optimized conditions after 12 h of fermentation was 1.555 g/l, and the percentage of PHB conversion was 77.8%.

3.2. Bioconversion of PHB into (R)-3-HB by PHB depolymerase

Monomer production using crude enzyme is an attractive alternative considering the assimilation of (*R*)-3-HB by the strain. Hydrolysis products generated by the crude depolymerase of *Pseudomonas* sp. DS1001a were analyzed by MS. The (*R*)-3-HB monomer with a peak at *m*/*z* 103, rather than the dimer, trimer, or other oligomers, was detected in the water-soluble product. The HPLC chromatograms of the monomer product and (*R*)-3-HB standard matched perfectly (Fig. 1c and d).

3-HB monomer bioconversion from PHB by crude PHB depolymerase was conducted at 50 °C with 25 g/l PHB powder. After incubation for 2 h, 3.08 g/l (*R*)-3-HB monomer was detected in the supernatant. Higher yields of (*R*)-3-HB were obtained with increasing incubation time. (*R*)-3-HB production and the weight loss of the PHB powder after incubation for 8 h were measured, and results showed 8.58 g/l (*R*)-3-HB production and 30% of PHB powder degradation. These values indicate that nearly all of the degraded powder was converted into (*R*)-3-HB monomer. However, a decrease in productivity was observed after 6 h of incubation, which may be attributed to the reduced activity of PHB depolymerase resulting from the decreased pH of the reaction mixture (Fig. 4b). Replacement of fresh PHB depolymerase efficiently recovered bioconversion. After about three to four cycles of medium change, the PHB powder was completely degraded into water-dissolved substances. The effective degradation of PHB and high yield of the (*R*)-3-HB degradation product observed in this work suggest that crude PHB depolymerase can recycle PHB by enzymatic degradation into the desired monomer.

3.3. Factors influencing of bioconversion by PHB depolymerase

3.3.1. Effect of temperature on PHB depolymerase

Temperature is an important factor affecting the yield of a product by influencing the enzymatic activity. The PHB-degrading activity of the depolymerase was assayed from 40 °C to 85 °C, and maximal enzymatic activity was observed at 50–65 °C (Fig. 3a). In the thermostability test, the crude depolymerase remained stable at temperatures of up to 50 °C for 8 h, but decreased by 100% after 2 h at 60 °C (Fig. 3b). Considering the optimum temperature and thermostability of the enzyme, 50 °C was selected as the optimal temperature for PHB bioconversions into (*R*)-3-HB by PHB depolymerase.

3.3.2. Effect of pH on PHB depolymerase

Figs. c and 3 d respectively show the optimal reaction pH and pH stability of crude PHB depolymerase from *Pseudomonas* sp. DS1001a. PHB depolymerase activity increased as pH increased