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ARTICLE

Activities of Key Enzymes in the Biosynthesis of Poly-3-Hydroxybutyrate by *Methylosinus trichosporium* IMV3011

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Abstract: The activities of the key enzymes involved in the intracellular poly-3-hydroxybutyrate (PHB) synthesis of *Methylosinus trichosporium* IMV3011 were studied under various cultivation conditions. The enzymes were methane monooxygenase (MMO), β-ketothiolase, acetoacetyl-CoA reductase, PHB synthetase, and PHB depolymerase. Each enzyme had a unique catalytic mechanism. MMO activity decreased continuously with PHB production, but PHB at a high concentration was beneficial for maintaining MMO activity because more NADH was released by PHB depolymerization. The important reaction for entering the PHB cycle was catalyzed by β-ketothiolase. The monomer of β-hydroxybutyrate was synthesized by the catalysis of β-ketothiolase and acetoacetyl-CoA reductase. PHB synthetase played an important role in the PHB synthesis routes. The activity of PHB synthetase increased with PHB production. The changes of enzyme activities involved in PHB synthetase and PHB depolymerase occurred together, which indicated that the polymerization and depolymerization of intracellular PHB occurred simultaneously. The molecular weight of PHB was determined mainly by the combined actions of PHB synthetase and PHB depolymerase. Some important intermediates in the tricarboxylic acid cycle were helpful for PHB production because they increased related enzyme activities in the PHB cycle.

Key words: enzyme activity; biocatalysis; poly-3-hydroxybutyrate; methanotrophic bacteria

Microorganisms known as methanotrophic bacteria can utilize methane as carbon source and energy source for growth. They play important roles in the carbon cycle and have potential applications in the production of chemicals such as epoxypropane, methanol, and some higher alcohols by their nonspecific methane monooxygenase (MMO) enzyme systems [1–3]. With sufficient nutrients, methane is oxidized via methanol, formaldehyde, and formate to carbon dioxide by these microorganisms [4]. The first reaction of the methane oxidation pathway is catalysis by MMO, which utilizes two reductions to split the O–O bonds of oxygen. One of the oxygen atoms is reduced to H_2O and the other is incorporated into methane to form methanol. Methanol is oxidized via formaldehyde and formate to carbon dioxide by methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase.

Poly-3-hydroxybutyrate (PHB), a naturally occurring biopolymer with complete biodegradability and biocompatibility, is accumulated as an intracellular carbon and energy storage material by methanotrophic bacteria under the growth conditions of nutrient deficiency [5–9]. The methanotrophic bacteria are classified as Type I and Type II based on the differences in membrane structure and assimilation pathways. PHB is accumulated by methanotrophic bacteria by two possible pathways for carbon assimilation: the ribulose monophosphate pathway (RMP) and the serine pathway. Type II bacteria are the most effective PHB producing organisms by their using of the serine pathway [6]. Low cost methane or methanol as carbon

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sources can be exploited by methanotrophic bacteria to develop a cost effective PHB production process.

The cell metabolism is triggered to change from the synthesis of the organism to the synthesis of PHB by different metabolic pathways of central metabolites such as acetyl-CoA and its cofactors [10]. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle under the growth conditions of nutrient sufficiency, and the cells grow continuously and the carbon source will be finally converted to carbon dioxide. However, the biosynthesis routes of PHB monomers will compete with the TCA cycle in the complete metabolism of the microorganism. Acetyl-CoA is not involved in the TCA cycle but it is utilized in the process of PHB synthesis under the growth conditions of nutrient deficiency.

The synthesis of PHB is a complex multistage process in which each stage is catalyzed by a specific enzyme. It has been proposed that the cyclic intracellular process of PHB synthesis and PHB degradation of methanotrophic bacteria are those of *Azospirillum brasilense* in a similar model [11]. In the PHB cycle of the methanotrophic bacteria, the key enzymes that catalyze PHB synthesis are β-ketothiolase, acetoacetyl-CoA reductase, and PHB synthetase. The β-ketothiolase catalyzes the conversion of two acetyl-CoA molecules into acetoacetyl-CoA, and it plays a crucial role in triggering polymer synthesis [12,13]. The rate of PHB synthesis is determined by the second enzyme, acetoacetyl-CoA reductase [14]. PHB synthetase is the third important enzyme and it determines the molecular mass, dispersivity, and yield of PHB [15]. The formation of the polymer is a complex process that is determined not only by the synthesis branch of the PHB cycle but also by the depolymerizing branch. The enzymes in the depolymerizing branch regulate the molecular mass of PHB and its final yield. Intracellular PHB polymer is degraded to monomers by PHB depolymerase, and the monomers are converted into acetoacetate by 3-hydroxybutyrate dehydrogenase. Acetoacetate is transferred to CoA, and the acetoacetyl-CoA produced serves as the substrate for β-ketothiolase with which acetoacetyl-CoA is converted to acetyl-CoA.

One approach to study the metabolism theory of PHB is to determine the activities of the key enzymes involved in cell growth and polymer production. In some previous studies on PHB producing microorganisms, all the enzymes of the synthesis branch of the PHB cycle were referred to as constitutive proteins and their activities were practically constant under the conditions of nutrient deficiency [16–18]. But obvious changes in the activities of the key enzymes in the PHB cycle were observed in some other reports [19,20]. However, data on PHB metabolism dynamics by methanotrophic bacteria are very limited. The purpose of this work is to get the activities of the key enzymes involved in bacterial growth and the PHB synthesis process of *Methylosinus trichosporium* IMV3011. The key enzymes are MMO, β-ketothiolase, acetoacetyl-CoA reductase, PHB synthetase, and PHB depolymerase. This study takes into account the effects of enzyme activities on PHB production under various cultivation conditions and will be helpful for getting a high yield of PHB with an appropriate molecular mass in the fermentation of methanotrophic bacteria.

1 Experimental

1.1 Microorganism and cell cultivation

The methanotrophic bacteria used was *Methylosinus trichosporium* IMV3011, which was obtained from the Russia Institute of Microbiology and Virology. The experiments were carried out in a mineral salt medium of the following compositions (g/L): KH_2PO_4 (0.4), K_2HPO_4 (0.4), Na_2HPO_4 (0.74), $CaCl_2$ (0.024), $MgSO_4·7H_2O$ (0.3), NH_4Cl (0.5), NaCl (0.3), KNO_3 (1.6), EDTA (0.01), $MnSO_4·5H_2O$ (5.2×10^{-4}), $ZnSO_4·7H_2O$ (2.4×10^{-4}), $NaMoO_4·2H_2O$ (2.88×10^{-4}), $CuSO_4·5H_2O$ (4×10^{-4}), $FeSO_4·7H_2O$ (4×10^{-3}), and $FeCl_3$ (1×10^{-3}). The tests were carried out in a 5 L automatic stirred fermenter equipped with standard control units (Baoxing BIOTECH-2001, Shanghai). The initial culture media volume was 2 L, which was inoculated with 10% (v/v) bacterial seeds. The temperature was maintained at 34 °C and the pH value was maintained at 7 using 1 mol/L sodium hydroxide or 1 mol/L hydrochloric acid. The dissolved oxygen concentration was maintained at 50% saturation by manually adjusting the gas mixture (50% air + 50% methane) flow rate at a fixed agitation speed of 300 r/min. The initial concentration of methanol was controlled at 1 g/L to avoid the inhibition by methanol on cell growth. The feeding culture medium without a nitrogen source was added separately five times to make a total amount equal to 1 L during the intermittently fed batch process. 0.1% (v/v total volume of medium in the fermenter at that time) methanol was added into the reactor with each feeding. By using this feeding strategy, the initial nitrogen source was exhausted quickly and the carbon source was continuously supplied. The production of PHB in the cells was improved by the imbalance between the carbon source and nitrogen source.

1.2 PHB analysis

The PHB content was determined by gas chromatography [21]. About 40 mg of dried biomass powder was suspended in 4 ml of chloroform containing 20 mg benzoic acid, sulfuric acid, and methanol (15:85, v/v, sulfuric acid/methanol). The samples were boiled at 100 °C in an oil bath (IKA RCT-basic, GER) for 4 h. After cooling to room temperature, 4 ml of distilled water was added and the samples were shaken for 30 s. The heavier phase was analyzed directly on a Agilent 6820 gas chromatograph with a flame ionization detector (FID) and a capillary column of 0.23 mm × 30 m with a SE-54 stationary phase. High purity nitrogen gas was used as the carrier gas. The operation parameters were: column flow, 1.65 ml/min; H_2 flow,