Optimization of Culture Conditions to Improve PHB Production of Methane-Utilizing Mixed Culture

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Keywords: Poly-β-hydroxybutyrate (PHB); Methane-utilizing mixed culture; Methanotrophic bacteria; High cell density

Abstract. Poly-β-hydroxybutyrate (PHB) can be produced by various species of bacteria as carbon and energy storage materials. This biopolyester has attracted increasing attentions as biodegradable plastics not only for its similar material properties to conventional plastics but also for its biodegradable properties. PHB has thermoplastic, can be used for food packaging film bag, packaging lining layer for drinks and fresh food tray, etc. Furthermore, PHB has lower oxygen and moisture permeability, which is benefit for food storage and transportation, so that it has a broad application prospect in the field of food packaging. In this peper, Methane-utilizing mixed culture with a high PHB storage capacity was gained from the biogas soil at DA Qing oil field as enriching source after domestication with the approach of the aerobic dynamic feeding (ADF，feast-famine condition). The community structure of Methane-utilizing mixed culture and the catalytic performance of the heterotrophs and methanotrophic bacteria isolated from the mixed culture were investigated. By adjusting the medium concentration of several important nutrients. Optimized medium suitable for cell growth in the first stage and second stage of cellular accumulation of PHB were obtained. By optimizing the supply of carbon sources and the composition of the culture medium, high cell density and yield of PHB can be obtained.

Introduction

Commercial production of PHB is currently based on pure culture processes employing either natural PHB producers or genetically modified bacteria. Pure culture processes use generally pure sterile substrates and axenic reactors, leading to high production costs and thus relatively expensive products，which have prevented the use of this material on a real industrial scale，especially in the field of food packaging.

Methane-utilizing mixed culture grows with the methane and has the ability to accumulate PHB in an open condition. Recent studies have reported in this area [1, 2]. This bacteria can use in an open condition with cheap methane as a carbon source and achieve continuous production of PHB .This bacteria can reduce production costs and bring certain economic benefits. Methane oxidizing bacteria in the mixed culture is dominant bacteria, which can accumulate PHB [3, 4].

Factors affecting cell growth and PHB accumulation include growth environment of carbon, nitrogen, nutrients needed for growth, the accumulation of the growth inhibitor in fermentation process [5, 6]. These factors directly or indirectly affect catabolism and assimilation process of PHB synthetic cells. Therefore, in this paper，methane oxidation mixed culture with higher PHB storage capacity was optimized by culture medium to further improve the growth in the first phase and the PHB production in second phase. In this paper, some important nutrients for cell growth and PHB accumulation has been optimized.

Experimental Material and Experimental Method

Strain and Medium Composition. Methane-utilizing mixed culture is obtained at methane-rich area of Daqing Oilfield in Heilongjiang Province from the soil by methane as sole carbon source.

The experiments were carried out in a mineral salt medium, and the medium constituents were with the following compositions (g/L):

A: NH_4Cl(0.5)，KH_2PO_4(0.4)，K_2HPO_4(0.49)，$CaCl_2 \cdot 2H_2O$ (0.024)，$MgSO_4 \cdot 7H_2O$(0.3)，NaCl(0.3)，KNO_3(1.6)

B: $CuSO_4 \cdot 5H_2O$ (4×10^{-3})，$FeSO_4 \cdot 7H_2O$(4×10^{-3})，$MnSO_4 \cdot 5H_2O$(4×10^{-4})，$ZnSO_4$(3.4×10^{-4})，$NaMoO_4 \cdot 2H_2O$(2.4×10^{-4}).

Culture Method. 250mL shake flask fitted 100mL sterilized inorganic salt medium. Under open conditions (culture are exposed naturally environment). 10% of bacteria was inoculated; Culturing with 30 ℃constant temperature air bath and 180rpm; The culture atmosphere, $V_{(methane)}$: $V_{(air)}$ = 1: 1; the culture time: 96-144h; changing gas for the cultured bacteria every 24h.

The Effect of Different Nutrients on Cell Growth and PHB Accumulation. The media components of first stage adapted to cell growth (nutritional balance stage) was optimized. After culturing the cells in the first stage 120h optimized medium, transferring the above-mentioned one component into a medium of different concentrations for the second phase of culturing. Examining production of PHB to select the appropriate concentration increased second phase (phase nutrition restricted) intracellular accumulation of PHB. In order to accurately investigate influence on cell growth and PHB accumulation ability of each of the components, using the approach of keeping the concentration of other components constant and treating only the concentration of the components examined changes.

Extraction of PHB. The appropriate amount of surfactant (betaine), chelates (EDTA disodium salt) and cells (maintained surfactant (betaine): chelates (EDTA disodium salt): dry cell weight was 0.12: 0.08: 1), was dissolved in 50mL of deionized water. PH adjustment 5mol / L of NaOH. Under the conditions of pH = 13 at 50 °C at a rotational speed of 1200rpm were treated cells broken 20min. The ionized water and washed with acetone each spent time in the oven temperature at 70 °C drying sediment obtained by centrifugation.

Determination of Cell PHB with Concentrated Sulfuric Acid. PHB with concentrated sulfuric acid upon heating can be quantitatively converted to crotonic acid. Crotonic acid at 235nm with a maximum absorption peak. By UV spectrophotometer measure OD_{235} value determines the amount of PHB. The extracted PHB dried and concentrated sulfuric acid. 100 °C water bath heated reaction 10min. Cooled to room temperature.

Results and Discussion

The Effect of Nitrogen on Cell Growth and PHB Accumulation. Nitrogen is a constituent substance or metabolites of microbial cells nitrogen source [7]. Limiting nitrogen source and increasing the ratio of carbon and nitrogen induce into the PHB cycle acetyl CoA key enzyme —β-one thiolase activity increased, and less induced into the TCA cycle acetyl-CoA citrate synthase activity high.

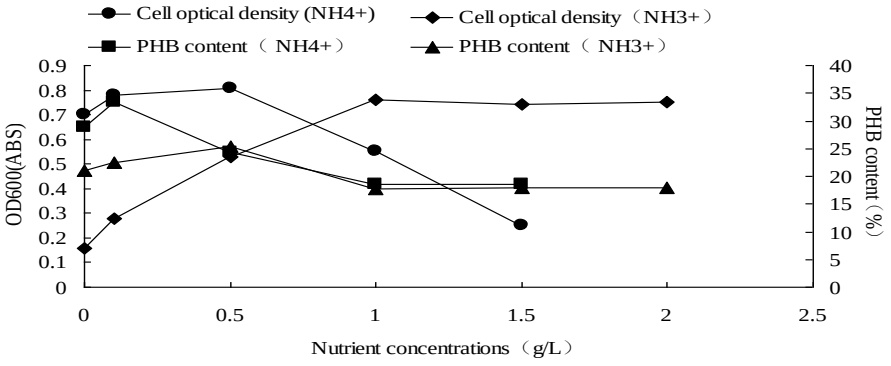

Figure 1. Effects of NH4+ and NO3- on biomass and PHB accumulation（n=3）

This figure demonstrates that the effect of nitrogen on methane oxidizing bacteria mixed cell growth and PHB accumulation of significant results showed that high concentrations of NH_4^+on growth and PHB accumulation of cells are inhibited, probably due to NH_4^+ with the same active competition MMO of methane site, high NH4 + will inhibit methane absorption rate. Reported in the literature [8, 9], as NH_4^+ oxidation product obtained in the first hydroxyethyl ammonium (NH_2OH) when concentrations as low as 0.1 mM when MMO has reversible inhibitory effects and easy by the hydroxyl ammonium produced also by NO_2^- inhibition of NADH and formate dehydrogenase catalyzes the production of cell growth and PHB accumulation have a negative impact. The figure shows when the NH_4Cl concentration of less than 0.3g / L, OD_{600} has large value. Indicating that a large amount of NH_4^+ methane oxidizing bacteria inhibit oxidation of methane, but a small amount of NH_4^+ can stimulate the growth of methane-oxidizing bacteria.

Can be seen from the figure, the growth of the mixed methane-oxidizing bacteria OD_{600} value increases with increasing concentration of KNO_3, OD_{600} value and high content of PHB respectively 0.688 and 19.62% when KNO3 concentration of 0.5g / L. Continued to increase KNO3 concentration, OD_{600} value continues to increase, but PHB content decreased. KNO3 don't have inhibitory effect on cell growth. Mixed methane-oxidizing bacteria more appropriates of carbon, because of the mixed methane-oxidizing bacteria in the methane-oxidizing bacteria dominant strains are strictly aerobic bacteria, the need for a high redox potential. KNO_3 compared NH_4Cl having a relatively high redox potential [10] so that KNO3 is a role in promoting cell growth while limiting nitrogen high nitrogen ratio (C / N) conducive to the accumulation of PHB.

The Effect of Phosphorus on Cell Growth and PHB Accumulation. KH_2PO_4 and K_2HPO_4 medium not only provide a microbial cell growth needed source of phosphorus, but also the microorganism to provide a buffer system to maintain the balance of the medium and other mineral elements and stable environment conducive to effective microorganisms for nutrients uses. However, high concentrations of phosphorus inhibit the oxidation of methanol to formaldehyde dehydrogenase catalyzed methanol. Studies have shown that phosphorus concentrations exceeding 40 mM inhibit the cell growth [11].

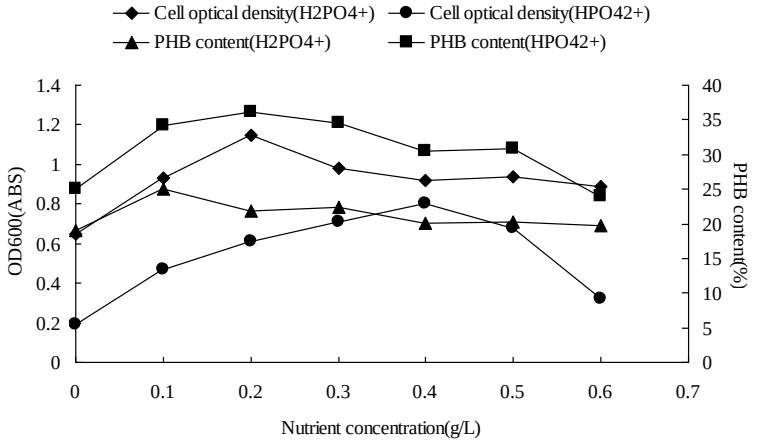

Figure 2. Effects of H2PO4+ and HPO42+ on biomass and PHB accumulation（n=3）

Data can also be seen from the graph, an important source of phosphorus nutrients as cell growth, one of the cell growths and PHB accumulations have the same effect as a nitrogen source. Within a certain range, when KH_2PO_4 is a relatively high concentration so that cell growth can provide positive momentum and low concentrations of KH_2PO_4 is conducive to the synthesis of PHB. The study found that low concentrations of HPO_4^{2-} did not promote the accumulation of PHB, also on cell growth is also negative because HPO_4^{2-} in the medium accounted for a larger proportion. The concentration changes a great impact on the environment pH. Concentration is too low to make the medium pH acidic. Cells are not well adapted to survive the acidic environment resulting in decreased cell growth. To some extent, reducing the accumulation of PHB.

The Effect of Magnesium on cell Growth and PHB Accumulation. Mg^{2+} is activator of certain enzymes such as hexokinase, isocitrate dehydrogenase, carboxylase and nitrogenase enzyme. Mg^{2+} also play a role stabilizing ribosomes, cell membranes and nucleic acids.

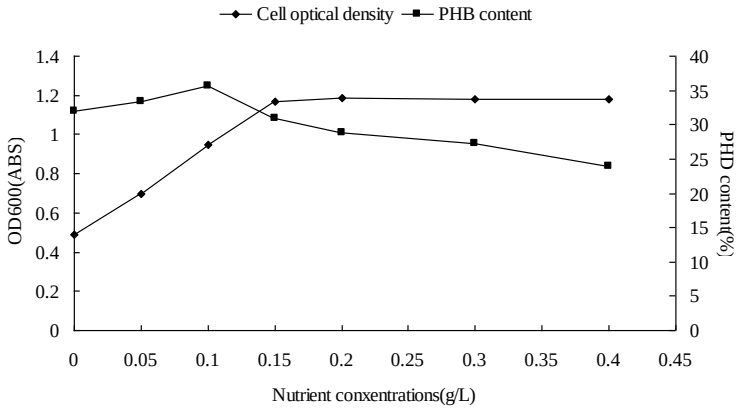

Figure 3. Effects of Mg2+ on biomass and PHB accumulation（n=3）

K.-D. Wendlandt et al. [12, 13] reported Mg^{2+} as nutrients at low and high concentrations respectively played the same role with nitrogen. Data on the map prove this conclusion. It is reported that the Methylosinus trichosporium OB3b by controlling the concentration of Mg^{2+} to product PHB. PHB content can reach 40% and for methane oxidation mixed bacteria, can also be used to improve the regulation of Mg^{2+} content of PHB. When the concentration of Mg^{2+} in 0.15-0.25g / L range, cell growth favorable. In 0.05-0.15g / L range, cells accumulate PHB advantageous. Mg^{2+} concentration regulation for improving the yield of PHB also has an important role.

The Effect of Copper and Iron on Cell Growth and PHB Accumulation.

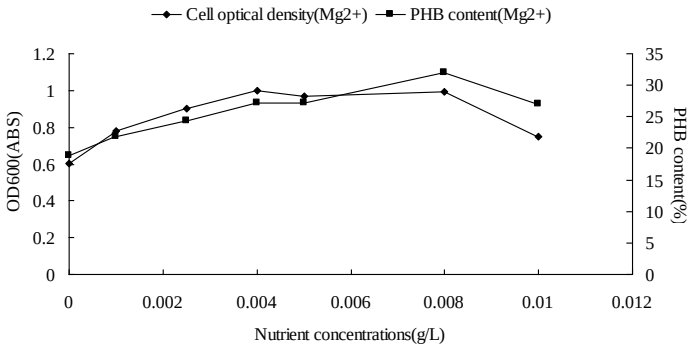

Figure 4. Effects of Cu2+ on biomass and PHB accumulation（n=3）

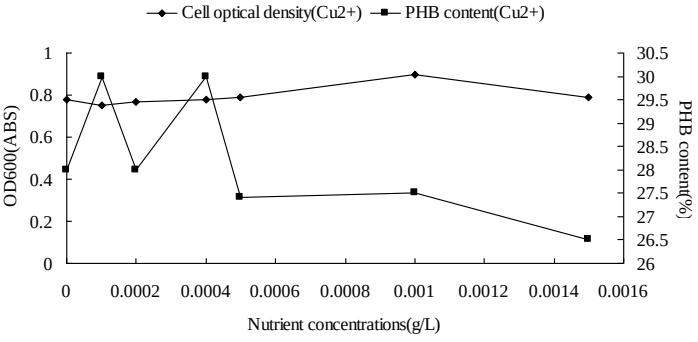

Figure 5. Effects of Fe3+ on biomass and PHB accumulation（n=3）

As can be seen from the results in Fig, It was found Cu^{2+} concentration is about 0.008g / L to maintain optimal cell growth and metabolism and accumulation of PHB. High concentrations of Fe^{3+} ($>4\times10^{-4}$ g / L) cells accumulate PHB has a certain extent. There was the highest accumulation of PHB when concentration was 2.0 × 10-4g / L. MMO is characterized enzymes of methane oxidizing bacteria Methane reaction. Soluble methane monooxygenase (sMMO) contains a hydroxylase (sMMOH). sMMOH is a dimer composed of three subunits ($\alpha\ \beta\ \gamma$)$_2$. Each monomer has a dual-core Fe active site. Located hydroxylase α subunit, which is the active site of the methane oxidation. Therefore, the presence of Fe can stimulate play sMMO activity [14].

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