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Short communication

Characteristics of scale-up fermentation of mixed methane-oxidizing bacteria

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ABSTRACT

The mixed methane-oxidizing bacteria, which can keep the unique catalytic properties of methanotrophs and overcome the general limitations of slow growth and low activity of the pure methanotrophic culture, have promising applications for biotechnology. In this study, scale-up fermentation for the mixed methane-oxidizing bacteria was carried out from 5 L (working volume 3 L) to 100 L (working volume 65 L) and then to 600 L (working volume 350 L) fermenter, and the mixed bacterial concentration with 2.69 g L^{-1} dry cell weight was obtained in a 600 L fermenter after 25 h culture. The existence of the coexisting bacteria together with methanotrophs could make a positive contribution to the bacterial growth and MMO activity of the mixed culture. During the scale-up cultivation, the growth, methane-oxidizing capacity, and community of the mixed bacteria were maintained, indicating that it would be possible for large-scale preparation of the mixed methane-oxidizing bacteria.

1. Introduction

Aerobic methanotrophs, or methane-oxidizing bacteria, which are widely present in natural environments, can utilize methane as the sole carbon and energy source through a unique enzyme system of methane monooxygenase (MMO) [1]. MMO has a soluble cytoplasmic form (sMMO) and a particulate membrane-associated form (pMMO). Besides catalyzing methane to methanol, both of the enzyme forms have broad substrate specificities, especially sMMO, which can oxidize alkanes, alkenes, alicyclics, aromatics, ethers, heterocyclics and ammonia [2–4].

The unique characteristics of methanotrophs make them have significant potential applications in the fields of pollutant bioremediation, biotransformation and methane assimilation [5]. But one of the major prerequisites for the engineering applications is to prepare enough amount of methanotrophic cells with high MMO activity, especially in large scale. Jiang et al. [6] reviewed the published studies on batch and continuous cultivation of two model methanotrophic strains, *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath, using methane as the sole carbon source. When methanol was applied as the carbon source for *M. trichosporium* OB3b, Adegbola [7] achieved a high biomass density of 62 g L^{-1} in a 5 L bioreactor after 140 h cultivation. But till now, the limited methanotrophic strains, slow growth rate, complex MMO purification process, and poor enzyme stability make large-scale cultivation of methanotrophs and preparation of MMO difficult.

In addition to pure culture of methanotrophs, use of a mixed methane-oxidizing culture is another approach for biotechnological applications. A mixed methane-oxidizing culture is generally considered to be a system where methanotrophic bacteria can dominantly coexist with some other kinds of bacteria, which may improve the physiological growth conditions for methanotrophic bacteria through either removing the inhibitory metabolites (e.g. methanol) excreted by methanotrophs or providing the essential vitamins and growth supplements [8]. Mixed methane-oxidizing cultures have been used to produce single-cell protein [9] or poly-β-hydroxybutyrate (PHB) [8,10,11], and to degrade organic

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pollutions [12–15]. The methane-oxidizing cultures were enriched from agricultural soil, anaerobic sludge, digester effluent, and other methane-rich environments, or just mixed with different pure methanotrophic cultures. Ho et al. [16] provided direct evidence showing how heterotroph richness exerted a stimulation of methanotrophic activity. Generally, compared with pure culture, the mixed methane-oxidizing bacteria can show better growth and MMO activities, high culture stability and environmental suitability, and can be operated in open systems.

So far, research on cultivation of mixed methane-oxidizing bacteria is rare, and the large-scale cultivation of them is a blank. For practical applications of mixed methanotrophic bacteria, the scale-up technique and related engineering data would be important. In this study, an enrichment culture of the mixed methane-oxidizing bacteria was used for scale-up fermentation. The amplified fermentation was achieved from 5 L (working volume 3 L) to 100 L (working volume 65 L) and then to 600 L (working volume 350 L) continuous stirred tank reactor (CSTR) in a short period of time. The key question is: whether the composition and function of the community can be kept during the scale-up. Accordingly, the growth, MMO activity, methane oxidation capacity as well as community structure were monitored to estimate the effect of the scale-up process.

2. Material and methods

2.1. Mixed methane-oxidizing culture

The mixed methane-oxidizing culture was domesticated from soil near the vent-hole of Xin-Feng Coal Mine in Henan Province, China. The cultivation was carried out in a 300 mL sealed baffled flask with 50 mL NMS medium [17] containing 5 μM Cu^{2+}. The volume ratio of air to methane in the headspace of a flask was about 5:1. The flasks were cultivated on a shaker at 170 rpm and 30 °C. The gas phase was refreshed every 24 h afterwards. At first, 20 g soil was added to the NMS medium. When cultivated for 10 days, 10% (v/v) of the culture was transferred into a fresh NMS medium and a new passage started. This domestication operation was unsterilized. After 30 subculture passages, the mixed methane-oxidizing culture showed stable methane consumption capability [18], which was then used in large-scale fermentation.

2.2. Cell density and dry cell weight

Cell density was determined by measuring the absorbance at 660 nm (OD_{660}) in an ultraviolet/visible spectrometer (UV-1206, Shimadzu, Japan). The dry cell weight (DCW) was determined by centrifuging (10,000 rpm, 10 min) to collect the cells, washing twice with distilled water, and drying the cell pellet at 80 °C for 24 h. The formulas below expressed the corresponding relationship between dry cell weight and OD_{660}. Eqs. (1) and (2) were respectively suitable for flask and fermenter cultivation within proper OD_{660} ranges. The line of Eq. (2) was deviated from the coordinates zero. It might be due to the extracellular products secreted more during the scale-up cultivation.

$$DCW(g/L) = 0.5667 \times OD_{660} + 0.0143 (OD_{660}: 0.2 - 1.4; R^2 = 0.9986) \quad (1)$$

$$DCW(g/L) = 0.3924 \times OD_{660} + 1.1540 (OD_{660}: 0.5 - 6.0; R^2 = 0.9958) \quad (2)$$

2.3. MMO activity and methane oxidation capacity

The whole-cell MMO activity was determined routinely by measuring the epoxidation rate of propene [19]. The MMO activity was defined as nmole poxypropane (EPO) formed per min and per mg DCW.

The methane oxidation capacity was expressed by methane consumption rate. Methane and air were supplied at 1:5 (v/v) in a 150 mL sealed serum vial containing 10 mL culture, and then the vial was placed at 30 °C for 15 h. The control vial contained 10 mL NMS medium. The concentration of methane was measured by a gas chromatograph (GC-2010, Shimadzu, Japan) with a capillary column PLOT-Q (30 m × 0.54 mm, 40 μm) and a FID detector. Nitrogen was the carrier gas, and the temperatures of injector, column and detector were 250 °C, 200 °C, and 250 °C, respectively. The methane oxidation capacity was defined as milliliter methane oxidized per hour and per gram DCW. Considering proliferation of the bacteria, the DCW took the average of the initial value and the end value.

2.4. Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA genes

For the DGGE experiment, 2 mL culture was sampled and the total DNA was extracted by the phenol–alcohol extraction method [20]. GC-341f and 907r were used as the PCR primers. DGGE was performed on a Dcode universal mutation detection system (Bio-Rad Laboratories, Inc., CA), and the gel was 6% (w/v) polyacrylamide with a denaturant gradient from 40% to 70%. The PCR-DGGE experiment conditions were described elsewhere [21]. The diagrams of compare lane images were converted by the software of Quantity One, and dice index (C_s) was used to evaluate the similarity of bacterial community [22,23]. The calculation formula is $C_s = 2j/(a + b)$, where j represents the number of bands common to samples A and B, while a and b are the numbers of bands in sample A and B, respectively. This index ranges from 0 (no common bands) to 1 (identical band patterns).

2.5. Cultivation in 5 L fermenter

300 mL mixed methane-oxidizing culture in logarithmic growth was prepared according to 2.1, and inoculated into a 5 L fermenter (Biostat A plus, Sartorius BBI Systems GmbH, Germany) with 3 L sterilized NMS medium. The fermentation was carried out at 30 °C with a continuous supply of methane (100–120 mL min^{-1}) and air (800–1000 mL min^{-1}). The dissolved oxygen (DO) level in the medium was maintained above 5% by adjusting the agitation speed and air flow rate. The pH was kept between 6.8 and 7.0 through the automatic addition of 1.0 M HCl and 1.0 M NaOH.

2.6. Cultivation in 100 L and 600 L fermenters

The 100 L and 600 L fermenters (Fengze Manufacturing Company, China) have the same mechanical design, and Fig. 1 illustrates a schematic of them. Before fermentation, the fermenters containing NMS medium were sterilized by heating to 70 °C and kept for 1 h. The cultivation temperature was 30 °C, and the pH was kept between 6.8 and 7.0 through the automatic addition of 5.0 M HCl and 5.0 M NaOH. In the 100 L fermenter, the NMS medium volume was 65 L and the agitation speed was 200–300 rpm; the methane flow was 0.10–0.15 $m^3\ h^{-1}$ and the air flow was 1.0–1.4 $m^3\ h^{-1}$. In the 600 L fermenter, the NMS medium volume was 350 L and the agitation speed was 200–260 rpm; the methane flow was 0.56–1.0 $m^3\ h^{-1}$ and the air flow was 6.0–9.0 $m^3\ h^{-1}$.

The culture harvested at the early stationary phase from the 5 L fermenter was used as the inoculum for the 100 L fermentation, and that obtained from the 100 L fermenter was inoculated into the 600 L fermenter for further cultivation.