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Improved vitamin B_{12} production by step-wise reduction of oxygen uptake rate under dissolved oxygen limiting level during fermentation process

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ABSTRACT

Effects of different oxygen transfer rates (OTR) on the cell growth and vitamin B_{12} biosynthesis of *Pseudomonas denitrificans* were first investigated under dissolved oxygen limiting conditions. The results demonstrated that high OTR accelerated cell growth and initial vitamin B_{12} biosynthesis rate, while lower OTR was critical for higher productivity in the late fermentation process. The oxygen uptake rates (OUR) corresponded well with OTR. Based on the metabolic intermediate analysis, a step-wise OUR control strategy was proposed. The strategy was successfully implemented in scale-up to an industrial fermenter (120,000 l). A stable maximum vitamin B_{12} production of 208 ± 2.5 mg/l was achieved, which was increased by 17.3% compared with the control. Furthermore, the glucose consumption coefficient to vitamin B_{12} was 34.4% lower than that of the control. An efficient and economical fermentation process based on OUR criterion was established for industrial vitamin B_{12} fermentation by *P. denitrificans*.

1. Introduction

Vitamin B_{12} has numerous applications in medicine and nutrition industries, and has been widely used for the treatment of pernicious anemia and peripheral neuritis (Hunik, 2002). Recently, vitamin B_{12} has received much attention because of the increasing need in the world market. It can be produced by cultures of the anaerobic bacteria *Propionibacterium shermanii* or *Propionibacterium freudenreichii* etc. as well as the aerobic bacteria *Pseudomonas denitrificans*. *P. denitrificans* has been successfully and exclusively used in industrial companies for its rapid growth and high productivity in industrial production (Martins et al., 2002). Several decades of efforts have been made to improve vitamin B_{12}-producing strains. Some have tried random mutagenesis and genetic engineering (Bykhovsky et al., 1998), while others have attempted to optimize the effects of betaine, Zn^{2+}, Co^{2+}, DMBI and other potential precursors on vitamin B_{12} biosynthesis (Li et al., 2008b,c; Roman et al., 2001). However, there were still a large fluctuation of yields and glucose consumption existed in the current industrial fermentation, and large amounts of the industrial production data witnessed the major impact of the oxygen uptake rate (affected by the oxygen supply levels) of the microorganisms on vitamin B_{12} production. To date, little information is available on controlling oxygen supply conditions to form a more cost-effective and stable vitamin B_{12} production scenario by *P. denitrificans* in large-scale fermentation.

It is well known that dissolved oxygen tension (DOT) plays a significant role on aerobic fermentation. DOT affects the cell growth and the biosynthesis of the product in numerous microorganisms by influencing metabolic pathways (Li et al., 2008a; Yegneswaran et al., 2008). Many researchers have proved that high dissolved oxygen tension was necessary for aerobic microorganisms to effectively re-oxidize NAD(P)H or FADH in order to generate ATP for metabolism (Huang et al., 2006). Oxygen supply profiles have also been reported as one of the crucial factors in optimal regulating metabolic distribution or activities in many fermentation processes such as organic acid (Hua and Shimizu, 1999), amino acids (Xu et al., 2009) and polysaccharide productions (Tang and Zhong, 2003) etc. Nevertheless, an excessive oxygen supply would cause a decrease of productivity because of the resulting losses of substrate by direct oxidation, dramatically elevating the costs of production for industrial fermentation. Therefore, the successful industrial fermentation process means a higher productivity, as well as a cost-effective way of production.

Oxygen transfer rate was a significant factor in the scale-up of the industrial fermentation process (Demirtas et al., 2003; Pollard et al., 2007). With the development of sensor technologies for DOT,

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the scale-up method related to oxygen transfer was established by controlling the DOT at a suitable level, which had been used more and more popular in the fermentation process (Yegneswaran et al., 2008). However, DOT was influenced by the oxygen transfer rate in the fermentation broth and the oxygen uptake rate of microorganisms as well (Bandaiphet and Prasertsan, 2006), it could hardly reflect the oxygen supply capacity when fermentation was conducted under DOT limiting conditions (García et al., 2000), which was always the case in large-scale fermentation with high aerobic microorganisms or in high cell density cultures. This phenomenon always occurred during industrial vitamin B_{12} fermentation by *P. denitrificans*. So DOT could not be used as an available on-line parameter for guiding the scale-up and development of a cost-effective fermentation process. Unlike DOT, OUR is one of the typical physiological parameters of microorganisms (Zou et al., 2009), and it changes with the oxygen transfer rate, especially when the fermentation occurs under dissolved oxygen limiting conditions (Sebastià et al., 2005). Therefore, OUR was used for assessing metabolic fluxes, and also employed as on-line controlling parameter in order to optimize the fermentation process (Feng et al., 2006).

In the present work, the fermentation characteristics of *P. denitrificans* were investigated in a 50 l fermenter with a multi-parameter monitoring system (Zhang et al., 2004). Research efforts focused on determining the effects of different oxygen supply strategies on vitamin B_{12} production. Based on the results of the metabolic process analysis, an optimal oxygen supply strategy was proposed and successfully performed for enhancing vitamin B_{12} production. Furthermore, a scale-up method using OUR as a scale-up parameter was successfully applied to scale-up from laboratory scale (50 l) to industrial scale (120,000 l).

2. Methods

2.1. Strain and medium

The industrial strain *P. denitrificans* was used for the production of vitamin B_{12}. This strain was donated by Huarong pharmacy corporation (Shijiazhuang, China).

The seed medium was composed of (g/l): sucrose 30; corn steep liquor 20; KH_2PO_4, 3.5; $(NH_4)_2SO_4$, 2.5; $(NH_4)_2HPO_4$, 5.0; $MnSO_4 \cdot H_2O$ 0.5; $MgSO_4$ 1.5; betaine 4; 5,6-dimethyl-benzimidazole (DMBI) 0.0045.

The fermentation medium was composed of (g/l): glucose 80; corn steep liquor; 30; $(NH_4)_2HPO_4$ 10; KCl 0.2; $MgSO_4 \cdot 7H_2O$ 1.4; $(NH_4)_2SO_4$ 2; betaine 15; DMBI 0.0075; $ZnSO_4 \cdot 7H_2O$ 0.2; $CoCl_2 \cdot 6H_2O$ 0.15.

Feed medium (FM) for the fed-batch fermentation in laboratory scale (50 l) and industrial scale (120,000 l) were similarly composed of (g/l):

FM-1: glucose 500; $CoCl_2 \cdot 6H_2O$ 0.15; DMBI 0.15.
FM-2: betaine 39; $CoCl_2 \cdot 6H_2O$ 0.15; DMBI 0.15.

The pH of the seed medium, fermentation medium, and FM-1 were adjusted in the range of 7.2–7.4 with NaOH prior to sterilization. The pH of the FM-2 was adjusted in the range of 6.2–6.4 prior to sterilization.

2.2. Optimization of oxygen supply and oxygen uptake rate in 50 l fermenter

Seed culture was carried out in 300 ml Erlenmeyer flasks containing 100 ml of seed medium inoculated with cells from fresh slant, and cultivated at 28 °c on a rotary shaker at 260 rpm for 18 h. About 1.5 l of the harvested seed was then inoculated into the 50 l turbine-agitated bioreactor (Shanghai Guoqiang Inc., China) with a working volume of 30 l. The bioreactor was equipped with devices to monitor and control more than 17 on-line measurable parameters (Zhang et al., 2006). The aeration rate and pressure were kept at 0.8 vvm and 0.05 MP throughout the experiments. During 50–140 h of the fermentation process, the residual sugar and betaine concentrations were kept at 20–30 g (glucose)/l and 5–7 g/l by continuous feeding of feed media, respectively.

To investigate the effects of oxygen supply on vitamin B_{12} fermentation, different oxygen supply conditions were analyzed at various agitation rates: 200, 250, 300, 350, and 450 rpm. A low-drift polarographic electrode (Mettler Toledo) was employed to measure the dissolved oxygen in broth, while pH and temperature were measured by a glass pH electrode (Mettler Toledo) and a Pt100 temperature sensor, respectively. The oxygen and carbon dioxide concentrations of the inlet and exhaust gases were determined by a process mass spectrometer (MAX300-LG, Extrel). All sensors were connected to the bioreactor control system NCB-1040 and real-time data collection software for analysis (NCB, Shanghai, China).

2.3. Fermentation in 120,000 l fermenter

Three-stage fermentation was performed in 150, 9000, and 120,000 l fermenters gradually, involving two stages of seed growth and one stage of vitamin B_{12} production. The ingredients of primary and secondary seed medium were the same. First stage seed culture was carried out in a 150 l fermenter containing 100 l sterile medium with inoculum from five fresh slants. Cultivation was carried out at 28 °C for 40 h with an aeration rate at 0.5 vvm and agitation speed at 180 rpm. Then the primary seed culture (80–90 l) was inoculated into a 9000 l secondary fermenter with 5000 l of seed medium. It was cultivated at 28 °C with stirrer speed at 130 rpm and aeration rate at 0.4 vvm for 30 h. Fermentation was performed in a 120,000 l fermenter equipped with 3-bladed propeller impellers, a temperature probe, pH probe (Mettler Toledo) and dissolved oxygen probe (Mettler Toledo). The secondary seed culture (about 5000 l) was inoculated into a large fermenter containing 75,000 l of fermentation medium. Fermentation was controlled at 32 °C, and finished at 168 h. When the total sugar in the broth dropped to 35 g/l, feeding of glucose (FM-1) began and maintained 20–30 g glucose/l during the whole fermentation process. In addition, the betaine concentrations in the broths were controlled at 5–7 g/l by continuous feeding of betaine (FM-2) till 140 h of the fermentation. The inlet and exhaust gas ingredients were analyzed by a mass spectrometer (MAX300-LG, Extrel). The OUR was calculated and collected online (Zhang et al., 2006). The oxygen transfer rate was adjusted by changing agitation rates and aeration rates so as to keep the OUR at the desired level.

2.4. Determination of oxygen uptake rate and carbon dioxide evolution rate

OUR and CER (carbon dioxide evolution rate) were determined from gas analysis. The inlet flow rate was measured, as were the mass fractions of oxygen and carbon dioxide in the inlet and the exhaust gases. By using these quantities and a nitrogen (inert) balance, OUR and CER were calculated from the following balance Eqs. (1) and (2) (Bodizs et al., 2007; Santos et al., 2006):

$$\mathrm{OUR} = \frac{F_{\mathrm{in}}}{V}\left[C_{\mathrm{O_2 in}} - \frac{C_{\mathrm{inert\ in}} \cdot C_{\mathrm{O_2 in}}}{1-(C_{\mathrm{O_2 out}} + C_{\mathrm{CO_2 out}})}\right] \cdot \frac{273}{273+t_{\mathrm{in}}} \cdot P_{\mathrm{in}} \cdot \frac{1}{1+h} \times 10^{-5} \quad (1)$$