Enhance Vitamin B₁₂ Production by Online CO₂ Concentration Control Optimization in 120 m³ Fermentation

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Abstract
Great amounts of carbon dioxide generated by Pseudomonas denitrificans during high aerobic vitamin B₁₂ fermentation, while the influence of CO₂ concentration on vitamin B₁₂ production remains unclear. In this paper, we present parallel experiments to investigate various levels of inlet CO₂ fractions on the physiological metabolism of P. denitrificans in laboratory scale fermentation. The results demonstrated that the oxygen transfer rate, cell growth and glucose consumption were inhibited with CO₂ fraction elevated from 0.03% to 8.86 ± 0.24%, while the most exciting results showed that the specific vitamin B₁₂ production rate and the yield to glucose were greatly stimulated when dissolved CO₂ increased to 8.86 ± 0.24%. Therefore, the optimal exhausted CO₂ fraction control strategy in 120 m³ fermenter was established. With the exhaust CO₂ concentration was well controlled at 7.5 ± 0.25% on-line, vitamin B₁₂ production greatly improved to 223.7 ± 3.7 mg/L, which was 11.2% higher than that of control. This strategy was proved to be significant necessary and effective for successfully scale up optimization in industrial vitamin B₁₂ fermentation.

Keywords: Dissolved carbon dioxide; Pseudomonas denitrificans; Vitamin B₁₂; Fermentation; Scale up; Optimization

Introduction
Vitamin B₁₂ is an important growth factor and has many applications in medicine and nutrition. Because of its increasing need in the world, vitamin B₁₂ has received much more attention recently. Most of the vitamin B₁₂ was produced by the culture of aerobic bacterium P. denitrificans in industrial production [1]. Due to the actively respiring characters of P. denitrificans, the poorly ventilated systems, high back pressure and hydrostatic pressure in large-scale fermenters, high dissolved CO₂ concentration were always encountered in industrial process. As for the significant influence of dissolved CO₂ on the growth and metabolism of many microorganisms [1], it must be taken into consideration in large-scale industrial fermentation.

The effects of CO₂ on growth and product formation in submerged cultures have been investigated in various microorganisms, and the controversy results were always encountered [2,3]. Researches of Gill and Lacoursiere had revealed that cell growth and metabolism of Pseudomonas fluorescens and Escherichia coli could be stimulated at low dissolved CO₂ level not more than 100 mm Hg pressure or 5% of Gill and Lacoursiere had revealed that cell growth and metabolism of P. denitrificans in laboratory scale fermentation. The results demonstrated that the oxygen transfer rate, cell growth and glucose consumption were inhibited with CO₂ fraction elevated from 0.03% to 8.86 ± 0.24%, while the most exciting results showed that the specific vitamin B₁₂ production rate and the yield to glucose were greatly stimulated when dissolved CO₂ increased to 8.86 ± 0.24%. Therefore, the optimal exhausted CO₂ fraction control strategy in 120 m³ fermenter was established. With the exhaust CO₂ concentration was well controlled at 7.5 ± 0.25% on-line, vitamin B₁₂ production greatly improved to 223.7 ± 3.7 mg/L, which was 11.2% higher than that of control. This strategy was proved to be significant necessary and effective for successfully scale up optimization in industrial vitamin B₁₂ fermentation.

Not clearly understood of the effect of CO₂ on metabolism of vitamin B₁₂ biosynthesis.

Therefore, the aim of present study was to evaluate the effect of CO₂ on production and metabolism of vitamin B₁₂ produced by P. denitrificans. Furthermore, an optimal industrial fermentation strategy based on online and CO₂ controlling strategy was successfully applied to enhance industrial vitamin B₁₂ production.

Materials and Methods
Microorganisms and medium

The industrial strain P. denitrificans used in this study was donated by Huarong pharmacy corporation (Shijiazhuang, China). The cultivations were carried out at a temperature of 32°C and agitation speed of 260 rpm. The seed medium was composed of (g/L): sucrose 40, corn steep liquor 20, betaine 5, (NH₄)₂SO₄ 1, KH₂PO₄ 0.75, COCl₂ 0.075, MgO 0.02, MgO 0.3, 6-dimethyl-benzimidazole (DMBI) 0.01, ZnSO₄ 7H₂O 0.01, CaCO₃ 1.5, the pH was adjusted to 7.2-7.4 by NaOH.

The fermentation medium lab scale and industrial scale was composed of (g/L): glucose 80, corn steep liquor 45, betaine 14, (NH₄)₂SO₄ 1, KH₂PO₄ 0.75, COCl₂ 6H₂O 0.075, MgO 0.5, DMBI 0.05, ZnSO₄ 7H₂O 0.08, CaCO₃ 1, pH 7.2-7.4. Feed media (FM) for the fed-batch fermentation were as follows (g/L), FM-1: Glucose 200, DMBI 0.15, COCl₂ 6H₂O 0.15 and FM-2: betaine 30, COCl₂ 6H₂O 0.3, DMBI 0.3.

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Determination of dry cell weight (DCW) and residual sugar concentration

For determination of biomass concentration, 10 ml ferment broth was centrifuged at 4,000 g for 10 min. After washing twice with distilled water, the cell precipitate was dried to a constant weight at 105°C. The residual sugar concentration was measured by anthrone method [15].

Determination of Pyruvate Carboxylase and intracellular $\delta$-aminolevulinic acid ($\delta$-ALA)

$\delta$-ALA is a highly complicated molecular structure [16,17] that is biosynthesized from eight molecules of $\delta$-aminolevulinic acid ($\delta$-ALA). This $\delta$-ALA precursor can be generated by either of two pathways (Figure 1): In the C4 pathway, $\delta$-ALA is made from glycine and succinyl CoA by the action of ALA synthase [1]. The $\delta$-ALA pathway (Figure 1): In the C4 pathway, $\delta$-ALA is made from glycine and succinyl CoA by the action of ALA synthase [1]. The $\delta$-ALA precursor can be generated by either of two pathways (Figure 1): In the C4 pathway, $\delta$-ALA is made from glycine and succinyl CoA by the action of ALA synthase [1]. The $\delta$-ALA precursor can be generated by either of two pathways (Figure 1): In the C4 pathway, $\delta$-ALA is made from glycine and succinyl CoA by the action of ALA synthase [1].

Measurement of organic acids and amino acids

For the analysis of the extracellular organic acids and amino acids in metabolism, the HPLC systems (Agilent 1100, USA) were equipped with an Aqua Sep C18 (250 x 4.6 mm, 5 μm, ES, USA) and Zorbax Eclipse AAA column (150 x 4.6 mm, 5 μm, Agilent, USA) respectively, the determination conditions were the same to that of previously reported [14].

Quantification of vitamin B$_12$ in the broth

The vitamin B$_12$ concentration was determined by HPLC. Broth samples (25 ml), into which 2.5 ml of 8% NaNO$_3$ and 2.5 ml of glacial acetic acid were added, were boiled for 30 min. Then the upper aqueous phase was measured using HPLC system equipped with C$_8$ column (4.6 mm, 25 cm, 5 μm) and UV detector (361 nm). 5% of acetonitrile was used as the mobile phase with flow rate of 1.0 ml min$^{-1}$ at 25°C combined with 95% of 0.25 M sodium acetate anhydrous, the pH was adjusted to 3.6 by acetic acid.

Fermentation process design

Firstly, fed-batch fermentation was carried out in a 50 L turbine-agitated bioreactor (Shanghai Guoqiang Inc., China) with 30 L working volume. The cultivation conditions were same to that of previous report. When the cultivation turned into higher vitamin B$_12$ biosynthesis phase, 2.5 L broth was incubated into four parallel 5 L bioreactors respectively (Shanghai Guoqiang Inc., China) for further investigation. Fermentations were performed at 32°C, stirrer speed was 450 rpm, and the pressure in the tar was all kept at 0.1 MP. The inlet gas stream was consisted of a defined mixture of air and pure carbon dioxide with the aeration rate at 1 vvm. The mixture was prepared by thermal mass flow controllers (Mass-Trak, SIERRA, Netherlands). In addition to the control process where air was the input gas, processes were also performed with 3.32 ± 0.12%, 8.86 ± 0.24%, and 13.84 ± 0.27% CO$_2$ addition (v/v air) in the inlet gas mixture. Meanwhile, the dissolved CO$_2$ concentration in fermentation was determined with using dissolved carbon dioxide concentration electrode (Mettler).

Fermentation in 120 m$^3$ fermenter

Three-stage fermentation was performed in 150 L, 9 m$^3$, and 120 m$^3$ fermenters gradually, involving two stages of seed growth and one stage of fed-batch vitamin B$_12$ fermentation. The primary seed, secondary seed and large-scale fermentation were cultivated with the methods as previous reported [14]. The geometry and dimensions of 120 m$^3$ fermenter tanks and agitators were given in Table 1.

The inlet and exhaust gas ingredients were analyzed by a mass spectrometer (MAX300-LG, Extrel). Oxygen Uptake Rate (OUR) and Carbon dioxide Evolution Rate (CER) was calculated and collected.
online Oxygen Uptake Rate (OUR), and were controlled by adjusting agitation and aeration conditions.

**Carbon dioxide control strategy model in 120 m³ fermenter**

The culture medium always enriched with CO₂ due to the high evolution rate of growing cells during fermentation process [20], especially in industrial scale fermenters with high hydrostatic pressures, high cell density or high pressure [21,22]. Previous researches revealed that *P. denitrificans* has high affinity to oxygen, with the dissolved oxygen concentration maintained nearly zero, therefore the oxygen transfer rate (OTR) was equal to OUR during the steady fermentation process. The OTR was determined by the agitation, aeration and the pressure, so the equation was used to create their relationship. Meanwhile, the active respiration make the CO₂ levels rise rapidly in large scale fermenters. In order to implement online control the oxygen supply levels and exhausted carbon dioxide concentration, we establish a response model about the OTR and exhausted CO₂ concentration related to aeration and agitation

\[
\text{OUR} = \text{OTR} = m \times F^\alpha \times P^\beta \times R^\gamma \quad \text{(Eq.1)}
\]

Where \( m \) =correction coefficient; \( \alpha, \beta, \gamma \) =response coefficient of flow, agitation and pressure relate to oxygen transfer rate; OUR= oxygen uptake rate (mmol L/h); OTR= oxygen transfer rate (mmol L/h); \( F \) =air flow (L/min); \( P \) =pressure (MPa); \( R \) =agitator speed (rpm).

Moreover, the relation between oxygen uptake rate and carbon dioxide evolution rate has presented by RQ which shown in Equation 2. In addition, equation 3 shows online determination formula of carbon dioxide evolution rate.

\[
RQ = \frac{\text{CER}}{\text{OUR}} \quad \text{(Eq.2)}
\]

\[
\text{CER} = \frac{F \times \left( E_{CO_2} - 0.03 \right)}{V} \frac{22.4}{298} \quad \text{(Eq.3)}
\]

**Results and Discussion**

**Effect of CO₂ on the dynamic of vitamin B₁₂ fermentation**

For investigating the effects of dissolved CO₂ levels on vitamin B₁₂ biosynthesis, the stationary cell growth broth in 50 L fermenter was transferred to the four 5 L reactors with 2.5 L working volume simultaneously. The fermentations were performed with operating conditions held constant to ensure that dissolved carbon dioxide was the only major variable. Samples were taken individually for analysis of biomass, glucose, inter-metabolites, and vitamin B₁₂ concentrations; and the data points represent mean values from duplicate experiments. A gas-mixing unit was used to give input CO₂ concentration in the influent gas of 0.03%, 3.32 ± 0.12%, 8.86 ± 0.24%, and 13.84 ± 0.27% respectively.

The time course of cell growth and pH under the four different CO₂
addition were illustrated in Figure 2. During those four conditions, the dissolved oxygen concentration were all maintained at nearly zero levels, while the cell growth haven’t any effect when the CO₂ addition elevated from 0.01% to 8.86 ± 0.24%; However, with influent CO₂ concentration increased to 13.84 ± 0.27%, the cell growth rate was greatly inhibited and biomass concentration decreased to 30.7 g/L after 48h cultivation, which was 8.9% lower than that of control (33.68 g/L) (Figure 2a). These might be caused by the respiration inhibition of CO₂ on biological membranes and cytoplasmic enzymes [23], and which may also interact with lipids of the cell membrane, the higher CO₂ concentration would greatly inhibit cell growth [1,9]. It could be clear seen from Figure 2b that pH had hardly any different to the control when CO₂ addition increased to 8.86 ± 0.24%, while the pH was remarkably decreased when the influent CO₂ elevated to 13.84 ± 0.27%.

The effect of influent CO₂ on vitamin B₁₂ production rate, glucose consumption rate and specific carbon dioxide evolution rate were summarized in Table 2. Results revealed the inhibitory effects of CO₂ concentration on the specific glucose consumption rate (SGCR). Little decrease of SGCR was appeared with CO₂ addition increased to 3.32 ± 0.12%; However, the SGCR were dramatically decreased to 0.14 and 0.10 mmol/gDCW/h when the influent CO₂ increased to 8.86 ± 0.24% and 13.84 ± 0.27% respectively, which were 19.2% and 46.7% lower than that of control (0.18 mmol/gDCW/h).

Just different from cell growth and SGCR, vitamin B₁₂ productions were greatly stimulated when influent CO₂ increased from 0.03% to 8.86 ± 0.24% (Figure 2c). The highest specific vitamin B₁₂ production rate reached to 69.4 μg/gDCW/h under 8.86 ± 0.24% CO₂ addition, 53.5% higher than that of control (45.2 μg/gDCW/h). While vitamin B₁₂ productions and specific production rate were all significantly inhibited when the CO₂ concentration raised to 13.84 ± 0.27%. Analysis of vitamin B₁₂ yields to the glucose consumption (Yᵦᵡ) (Figure 2d) showed that the maximum yield of 480.5 μg/mmol (vitamin B₁₂/glucose consumption) were obtained when the influent CO₂ reached 8.86 ± 0.24%, while 13.84 ± 0.27% CO₂ addition led the yields dramatically decreased to lowest levels of 248.3 μg/mmol. Exhaust gas analysis showed that specific...
carbon dioxide evolution rate (SCER) of *P. denitrificans* decreased with elevated CO2 concentration in input gas. The statistics of the percent of glucose used for CO2 generation were decreased from 94 ± 2.1% to 85 ± 1.5% with influent CO2 increased from 0.03% to 8.86 ± 0.24%; while under the highest CO2 concentration of 13.84 ± 0.27%, nearly 93.3 ± 1.3% of the glucose consumption was seriously transformed to CO2. In conclusion, maintained an appropriate input CO2 concentration around 8.86 ± 0.24% was positive for the economical vitamin B12 production.

Previous researches on the effects of elevated CO2 on the industrial producing strains of bacteria, yeasts, and fungi [1, 9] revealed that the inhibitory action of product biosynthesis was probably caused by the decreased substrate consumption with elevated CO2 concentration. In our research, however, glucose consumption decreased with the increased influent CO2 concentration, while the yields of vitamin B12 based on glucose consumption increased greatly with the elevated influent CO2 concentration. These results indicated that there must exist abnormal metabolism characters of *P. denitrificans* under the various influent CO2 concentrations. The reasons were further investigated by organic acid and amino acid analysis of the central metabolism.

**Effect of CO2 on the inter-metabolites in fermentation**

The researches on vitamin B12 biosynthesis had demonstrated that the concentrations of the precursor of δ-aminolevulinic acid (δ-ALA) played an important role on vitamin B12 biosynthesis [16]. δ-ALA could be synthesized from C4-pathway (from glycosine and succinyl-CoA by δ-ALA synthesis) or C5-pathway (from glutamate by glutamyl-tRNA reductase) associated with central metabolism [24]. The dynamic processes of extracellular organic acids concentration under different CO2 levels were shown in Figures 3 and 4.

When influent CO2 increased from 0.03% to 8.86 ± 0.24%, α-ketoglutarate and succinate concentrations greatly increased, which were actually accelerated the biosynthesis of glutamate, and the other organic acids such as pyruvate, acetate, lactate and citrate hadn’t any different. However, the acetate and pyruvate were accumulated much higher when influent CO2 reached 13.84 ± 0.27%. These were probably caused by the inhibition of respiration under high CO2 concentration, and the resulted high NADH accumulation in the cell really repressed the activity of pyruvate dehydrogenase and TCA cycle, and then attributed to the accumulation of acetate and pyruvate.

The dynamic analysis on the concentrations of extracellular amino acids (Figure 3) showed that glutamate and glycine synthesis rate was significantly enhanced with elevated CO2 concentration. Under 8.86 ± 0.24% CO2 addition, glutamate and glycine concentration reached 1430.2 mg/L and 2716.4 mg/L, respectively, which were 55% and 122% higher than that of control. Threonine reached highest synthesis rate when inlet CO2 increased to 3.32 ± 0.12%. These metabolites accumulation could be accelerate the precursor’s biosynthesis for vitamin B12 synthesis.

δ-ALA is the critical precursors of vitamin B12 biosynthesis, the time course of δ-ALA concentration under different levels of inlet CO2
were shown in Figure 5a, experiment results demonstrated the positive
effects of moderate CO₂ concentration exerted to the δ-ALA synthesis.
In bioprocess sparged with 8.86 ± 0.24% CO₂ addition, the δ-ALA
concentration reached the top point at 26.4 ± 1.2 mg/gDCW, which
was 40.4% higher compared to that of control (18.6 ± 0.9 mg/gDCW).
However, the δ-ALA biosynthesis was seriously inhibited with 13.84 ±
0.27% CO₂ addition, the final δ-ALA concentration was lowest as 13.02
± 0.5 mg/gDCW, 30% lower than that of control.

Experiment results showed that the biosynthesis of glycine, succinic
acid, and glutamate were elevated with the increased influent CO₂
concentration from 0.03% to 8.86 ± 0.24%, which actually augmented
δ-ALA generation for higher vitamin B₁₂ biosynthesis.

Effect of CO₂ on pyruvate carboxylase activity

Previous researches have illustrated that anaplerotic reaction
catalysed by pyruvate carboxylase was mostly existed for replenishing
the TCA cycle in Pseudomonas spp [25]. In this work, the dynamic
analyses of specific pyruvate carboxylase activity in different input CO₂
addition were shown in Figure 5b. In the process sparged with 8.86 ±
0.24% CO₂, the maximal specific activity reached 37.2 U/gDCW, which
was 35% higher than that of control (27.5 U/gDCW).

Tricarboxylic acid (TCA) cycle is one of the main central pathways
in aerobic bacteria. It is responsible for the complete oxidation
of acetyl-CoA and provision of precursors for many TCA-related
metabolites biosynthesis. As precursors of the metabolites succinyl-
CoA and glutamate for δ-ALA biosynthesis were all generated from

The experiment results demonstrated that elevated levels of CO₂
concentration had significant effect on vitamin B₁₂ fermentation. When
the influent CO₂ concentration increased from 0.03% to 8.86 ± 0.24%,
the specific vitamin B₁₂ production rate and the yields of vitamin B₁₂
to glucose were stimulated. However, cell growth, glucose utilization,
specific vitamin B₁₂ production rate, and the yields of vitamin B₁₂
to glucose were all greatly inhibited when CO₂ addition up to 13.84 ±
0.27%. Therefore, the influences of CO₂ on industrial vitamin B₁₂
production have to take into consideration.

Industrial fermentation strategy based on online exhaust CO₂ controlling

Exhaust CO₂ concentration controlling strategy: With the
process parameters determined under various agitation speed, air flow,
and pressure obtained from 120 m³ fermentation tank are shown in
Table 3, we obtained relevant respond coefficient through regression
analysis of these parameters between OTR and Process parameters
determined (Eq.1). Then, oxygen transfer rate and oxygen uptake rate
could be calculated according to these parameters (Eq.4).

\[ \text{OUR} = \frac{\text{OTR}}{0.95 \pm 0.04} \times \text{CER} \times \text{RQ} \times \text{ OUR } \approx \text{ OTR } = 1.0074 \times \text{F}^{0.3729} \times \text{R}^{0.4635} \times \text{P}^{0.2472} \]  

(Eq.4)

The carbon dioxide fraction in exhausted gas was calculated from a
mutual relation between RQ, CER, and OUR (Eq. 2,3,5)

\[ \text{ECO}_2 = RQ \times F^{-0.56271} \times P^{0.46455} \times V^{2.257} + 0.03 \]  

(Eq.5)

During the vitamin B₁₂ fermentation process, glucose was used
as the main carbon substrate, the respiration quotient (RQ) of this
strain was always maintained at 1.03 ± 0.02 in microorganism growth
period and RQ was calculated as 0.95 ± 0.04 in vitamin B₁₂ synthesis
period. Then OUR and exhaust CO₂ concentration from 120 m³
fermentation tank could be obtained by adjusting agitator speed and
air flow according to the created model above (Figure 6).

Scale-up fermentation based on exhaust CO₂ concentration control:
Based on the results obtained from 5 L vitamin B₁₂ fermentation,
the optimal controlling strategies based on exhausted

<table>
<thead>
<tr>
<th>F(L/min)</th>
<th>R(rpm)</th>
<th>P(MPa)</th>
<th>OUR (mmol/L/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1263.1</td>
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<td>0.031</td>
<td>47.38</td>
</tr>
<tr>
<td>929.9</td>
<td>78</td>
<td>0.034</td>
<td>46.66</td>
</tr>
<tr>
<td>406.4</td>
<td>50</td>
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<td>24.73</td>
</tr>
<tr>
<td>600.5</td>
<td>90</td>
<td>0.041</td>
<td>39.10</td>
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<td>850.4</td>
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<td>0.032</td>
<td>37.51</td>
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<td>110</td>
<td>0.040</td>
<td>63.2</td>
</tr>
<tr>
<td>1224.5</td>
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<td>46.07</td>
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<tr>
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<tr>
<td>1121.8</td>
<td>78</td>
<td>0.027</td>
<td>43.82</td>
</tr>
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a calculated from the obtained equation 2 with the corresponding process parameters

Table 3: The determined and predicted OUR values at various aeration, agitation and pressure levels in the 120 m³ bioreactor.


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CO₂ concentration were implemented for vitamin B₁₂ fermentation in 120 m³ fermenters. The exhausted CO₂ concentration was controlled at 7.5 ± 0.25% during the early fermentation phase. The maximum vitamin B₁₂ production reached 223.7 ± 3.7 mg/L at 190 h, which was 11.2% higher than that of without CO₂ concentration controlled (201.4 ± 2.9 mg/L). It can be observed that higher production would be realized when exhausted CO₂ concentration increased greatly to 9.1 ± 0.15%, cell growth rate was lower than that of optimal strategy, especially when it elevated to 12.0 ± 0.24% after 75 h cultivation, the vitamin B₁₂ production rate was gravely inhibited (Figure 7c).

Time course of OUR, dry cell weight, vitamin B₁₂ production, and exhausted CO₂ concentration were shown in Figure 7. By control the aeration and agitation throughout the fermentation process, the changes of OUR were maintained at similar levels in fermentation processes (Figure 7b), the optimal strategy by stepwise reduction of oxygen uptake rate under dissolved oxygen limiting level during fermentation process was used for oxygen supply levels control [11]. Meanwhile, the exhausted CO₂ concentration changed greatly and maintained at the suitable levels with the adjustment of agitation and aeration conditions. In the contrast conditions, the exhaust CO₂ concentration increased greatly to 9.1 ± 0.15%, cell growth rate was lower than that of optimal strategy, especially when it elevated to 12.0 ± 0.24% after 75 h cultivation, the vitamin B₁₂ production rate was gravely inhibited (Figure 7c).

The results presented demonstrated that cell growth and vitamin B₁₂ production were greatly stimulated by controlling a lower CO₂ concentration of 6.0 ± 0.24% during the early fermentation phase. The highest vitamin B₁₂ production rate was achieved when exhausted CO₂ concentration were controlled at 7.5 ± 0.25%, The maximum vitamin B₁₂ production reached 223.7 ± 3.7 mg/L at 190 h, which was 11.2% higher than that of without CO₂ concentration controlled (201.4 ± 2.9 mg/L). It can be observed that higher production would be realized through simultaneously regulate the appropriate OUR and CO₂ concentration levels are more beneficial to vitamin B₁₂ synthesis.

Conclusion

CO₂ was produced in aerobic and anaerobic fermentations through decarboxylation reactions in a number of metabolic pathways, and hence has an influence on the performance of microbial cultivations [1,9]. Especially in industrial large bioreactors with high total pressure, poorly ventilated system, and inefficiently aerated fermenters. In this work, we investigated the effects of dissolved CO₂ concentration on vitamin B₁₂ fermentation of P. denitrificans, established the correlation of exhausted CO₂ concentration and the activity of the microbial metabolism by inter-scale observation and data association. The novel and optimal fermentation controlling strategy based on OUR and exhausted CO₂ concentration was successfully applied in industrial scale up of vitamin B₁₂ fermentation.
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