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Improved Vitamin B_{12} Fermentation Process by Adding Rotenone to Regulate the Metabolism of *Pseudomonas denitrificans*

Xin Cheng · Wei Chen · Wei-fu Peng · Kun-Tai Li

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Abstract Our previous research had revealed that the dissolved oxygen limitation was more favorable for vitamin B_{12} fermentation, due to its inducement to the increased glycolytic flux in *Pseudomonas denitrificans*. In this paper, a novel strategy was implemented to further investigate the metabolic characteristics of *P. denitrificans* under different oxygen supply levels, by exogenously adding rotenone (a respiratory chain inhibitor interfering with the oxygen consumption) to the fermentation broths. Compared to the fermentation process without rotenone treatment, it was observed that 5 mg/L rotenone treatment could significantly strengthen the glycolytic flux of *P. denitrificans* via activating the key glycolytic enzymes (phosphofructokinase and pyruvate kinase), resulting in the accelerated generations of anterior precursors (glutamate and 5-aminolevulinic acid) for vitamin B_{12} biosynthesis. Although 5 mg/L rotenone treatment had a negative effect on cell growth of *P. denitrificans*, the vitamin B_{12} yield was increased from 48.28±0.62 mg/L to 54.70±0.45 mg/L, which further proved that an increased glycolytic flux in *P. denitrificans* was a consequence of higher vitamin B_{12} production.

Keywords *Pseudomonas denitrificans* · Rotenone · Metabolic process · Vitamin B_{12}

Introduction

Vitamin B_{12} is used to describe the complex compounds of cobalt corrinoid family, which was first detected as "the anti-pernicious anaemia factor" in 1926 [1]. The physiologically active forms of vitamin B_{12} include adenosylcobalamin and methylcobalamin, and they are the essential cofactors for methionine synthase and (R)-methylmalonyl-CoA mutase in animals and humans [2].

Due to the chemical synthesis processes being highly complicated and costly, some industrial microorganisms have been successfully developed to the commercial vitamin B_{12} production, such as *Pseudomonas denitrificans*, *Propionibacterium freudenreichii*, *Propionibacterium shermanii*, and so on [3]. In recent years, more and more researches have

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been focused on how to improve the vitamin B_{12} productivity by *P. denitrificans*, including the optimization of fermentation processes and the manipulation of metabolic engineering [4]. For example, our previous studies have detailedly performed the optimization and scale up of the industrial vitamin B_{12} fermentation by *P. denitrificans*, like the control strategies of dissolved oxygen (DO) [5], pH [6], feeding [7], and medium components [8].

There are two different routes for vitamin B_{12} biosynthesis in nature, namely, the aerobic (oxygen-dependent) pathway and the anaerobic (oxygen-independent) pathway. Although *P. denitrificans* is a typical microorganism owning the aerobic pathway for vitamin B_{12} biosynthesis, it had been proved that the DO concentration should be controlled at a limiting level during vitamin B_{12} biosynthetic phase [5, 9]. In terms of the effects of oxygen supply on microbial fermentation processes, a lot of literatures reported that the genes encoding the enzymes in the tricarboxylic acid (TCA) cycle and hexose monophosphate (HMP) pathway were downregulated in low oxygenation, but the glycolytic flux showed a large increase as oxygenation was reduced [10, 11]. As a hypothesis, did the increased glycolytic flux in *P. denitrificans* being a consequence of higher vitamin B_{12} production?

Rotenone is the specific inhibitor of complex I of the mitochondrial respiratory chain, which can interfere with the oxygen consumption in cell [12]. It was reported that rotenone treatment could significantly increase the glycolytic flux in some microorganisms [13, 14]. Based on this fact, this paper put forward a novel strategy to verify the inference that an increased glycolytic flux in *P. denitrificans* was beneficial to vitamin B_{12} productivity, by exogenously adding various rotenone concentrations to the fermentation broths.

Materials and Methods

Microorganism and Media

P. denitrificans, an industrial vitamin B_{12}-producing strain, was used in this study, which was maintained on agar slant containing (grams per liter) the following: sucrose, 30; peptone, 10; $(NH_4)_2SO_4$, 0.25; $(NH_4)_2HPO_4$, 1.5; $MnSO_4 \cdot H_2O$, 0.1; $ZnSO_4 \cdot 7H_2O$, 0.1; and agar, 20. The initial pH was adjusted to 7.2 with 2 M NaOH prior to sterilization.

Seed medium was composed of (grams per liter) the following: maltose, 35; peptone, 20; KH_2PO_4, 5.0; $(NH_4)_2SO_4$, 2.0; $(NH_4)_2HPO_4$, 0.8; $MnSO_4 \cdot H_2O$, 0.2; $MgSO_4 \cdot 7H_2O$, 1.5; $ZnSO_4 \cdot 7H_2O$, 0.02; $CoCl_2 \cdot 6H_2O$, 0.02; 5,6-dimethylbenzimidazole (DMBI), 0.0045; and pH 7.20–7.40.

Fermentation medium was as follows (grams per liter): maltose, 60; peptone, 25; betaine, 10; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 2.0; KH_2PO_4, 0.8; $ZnSO_4 \cdot 7H_2O$, 0.08; $CoCl_2 \cdot 6H_2O$, 0.15; DMBI, 0.08; and pH 7.20–7.40.

Fermentation in Shake Flasks

P. denitrificans was grown on agar slant (18×180 mm) at 28 °C for 48 h, and the fresh cell was washed with 10 mL of sterilized water. One milliliter of the suspended cell was then inoculated into a 250-mL Erlenmeyer flask containing 50 mL of seed medium, and the cultivation was performed at 28 °C on a rotary shaker at 180 rpm. When the optical density value (determination at 700 nm) of the seed biomass reached 9–10, the seed culture was then transferred into a 250-mL Erlenmeyer flask containing 30 mL of fermentation medium with 10 % inoculum and incubated at 28 °C on a rotary shaker at 180 rpm for 96 h.

Analytical Methods

Cell biomass was quantified with dry cell weight (DCW): Fermentation broth was centrifuged at 5,000 rpm for 10 min, and the cells were collected after washing twice with distilled water and then dried to a constant weight at 105 °C.

Vitamin B_{12} concentration in fermentation broth was determined by HPLC: Broth sample (25 mL), to which 2.5 mL of $NaNO_2$ 8 % (*w*/*v*) and 2.5 mL of glacial acetic acid were added, was boiled for 30 min; the mixture was then filtrated, and 20 μl of NaCN 10 % (*w*/*v*) was added to 1 mL of the aqueous phase; the resulting upper aqueous phase was injected into the HP1100 HPLC system (Agilent); NH_2 column (4.6 mm×250 mm, 5 μm) was used for HPLC analysis with a flow rate of 1.7 mL min^{-1} and a wavelength of 360 nm; the mobile phase was 250 mM of phosphoric acid/acetonitrile (30/70, *v*/*v*).

The pyruvic acid in fermentation broth was assayed by HP1100 HPLC system (Agilent), and the HPLC conditions were as follows: Metacarb-H plus column (300 mm×7.8 mm; Varian Inc., PaloAlto, CA); mobile phase, 0.01 M of H_2SO_4; flow rate, 0.4 mL min^{-1}; column temperature, 50 °C; injection volume, 10 μl; and wavelength, 210 nm.

The specific activities of phosphofructokinase and pyruvate kinase were assayed with the corresponding kits purchased from Nanjing Jiancheng Biological Engineering Institute.

The concentration of total sugar in the broth was determined by the dinitrosalicylic acid reagent (DNS) method.

The 5-aminolevulinic acid (ALA) concentration in fermentation broth was determined according to the literature [15].

The concentrations of glutamate in fermentation broth were assayed according to the method reported by Ebert [16].

Results and Discussion

Effects of Rotenone on Cell Growth and Vitamin B_{12} Biosynthesis

To investigate the effects of rotenone on cell growth and vitamin B_{12} biosynthesis of *P. denitrificans*, various concentrations of rotenone (0, 2.5, 5, 10, and 20 mg/L) were

Table 1 Effects of five various rotenone concentrations on cell growth and vitamin B_{12} production by *Pseudomonas denitrificans* in shake-flask fermentation

Rotenone concentration (mg/L)	DCW (g/L)				Vitamin B_{12} (mg/L)			
	n_1	n_2	n_3	Mean±SD[a]	n_1	n_2	n_3	Mean±SD[b]
0	22.53	22.67	23.06	22.75±0.27^{A}	47.62	48.37	48.85	48.28±0.62^{C}
2.5	22.46	22.54	22.83	22.61±0.19^{A}	49.53	50.12	50.81	50.15±0.64^{B}
5	20.65	21.09	21.77	21.17±0.56^{B}	54.21	54.77	55.11	54.70±0.45^{A}
10	18.86	18.87	19.44	19.06±0.33^{C}	45.09	45.54	45.92	45.52±0.42^{D}
20	16.40	16.62	16.63	16.55±0.13^{D}	40.67	41.01	42.18	41.29±0.79^{E}

n_1, n_2, and n_3 meant the three independent determinations of DCW and vitamin B_{12}

[a] Multiple comparisons for DCW were done by least significant difference (LSD) test at 1 % level

[b] Multiple comparisons for vitamin B_{12} production were done by LSD test at 1 % level

Uppercase letters were tested by least singnificat difference (LSD)

exogenously added to the culture broths at the pre-exponential growth phase (12 h of fermentation), respectively. After 96 h of shake-flask cultivation, the final DCW and vitamin B_{12} production were determined, and the results were listed in Table 1.

With the increased rotenone concentration addition to the fermentation broths, the final DCW presented a significant descent trend, which demonstrated that rotenone treatment had a negative effect on the cell growth of *P. denitrificans*. As mentioned above, rotenone can interfere with the oxygen consumption in cell, which would result in a decreased ATP supply for cell growth [17]. Therefore, more rotenone addition to *P. denitrificans* fermentation broth would cause more severe inhibition to cell growth.

Unlike cell growth, low concentrations of rotenone (2.5 and 5 mg L^{-1}) had a positive promotion to the biosynthesis of vitamin B_{12} (Table 1). When 2.5 and 5 mg/L rotenone were added to the fermentation broths, the vitamin B_{12} yield reached 50.15±0.64 and 54.70± 0.45 mg/L, which were both higher than that obtained without rotenone treatment (48.28± 0.62 mg/L). However, once the addition concentrations of rotenone were further increased to

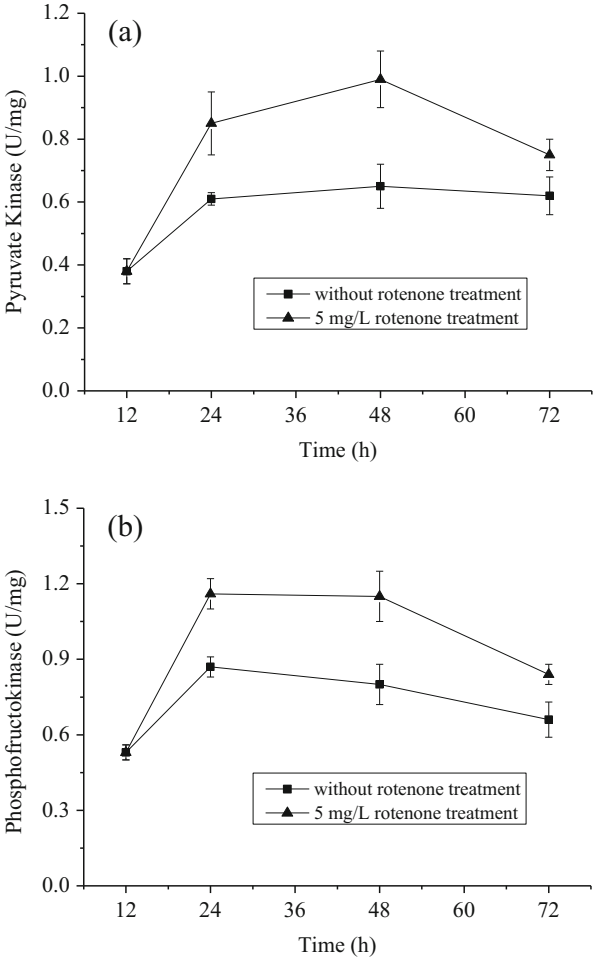

Fig. 1 Time courses of the specific activities of pyruvate kinase and phosphofructokinase under 0 and 5 mg/L rotenone treatment during *Pseudomonas denitrificans* cultivation in shake flasks

10 and 20 mg/L, the vitamin B_{12} production would present an acute decrease, due to their severe inhibition to cell growth.

Effects of Rotenone on the Glycolytic Flux in *P. denitrificans*

As described in Table 1, compared to the fermentation process without rotenone treatment, an approach of 5 mg/L rotenone addition was negative to the cell growth, but the vitamin B_{12} productivity had an obvious improvement. In order to clarify whether the improved vitamin B_{12} production was characterized by the increased glycolytic flux under rotenone treatment, the specific activities of the key glycolytic enzymes were assayed.

As shown in Fig. 1, compared to the fermentation process without rotenone treatment, 5 mg/L rotenone could obviously activate both phosphofructokinase and pyruvate kinase during the whole fermentation phases. Through further calculation, the specific activities of phosphofructokinase and pyruvate kinase were increased by 33.33 and 39.34, 43.75 and 52.31, and 27.27 and 20.97 % at 24, 48, and 72 h, respectively.

As we all know, pyruvic acid is the metabolic end product in glycolytic pathway. In order to further confirm that rotenone treatment could strengthen the glycolytic flux in *P. denitrificans*, we investigated the pyruvic acid kinetics during the fermentation processes. As described in Fig. 2, when the fermentation process was implemented with 5 mg/L rotenone treatment at 12 h, the generations of pyruvic acid were significantly higher than those obtained without rotenone treatment during 24–96 h, which was parallel to the activities kinetics of the key glycolytic enzymes.

According to the kinetics of the two key enzymes and pyruvic acid, it could be revealed that the fermentation process with 5 mg/L rotenone treatment could significantly improve the glycolytic flux in *P. denitrificans*.

Effects of Rotenone on the Generation of Anterior Precursors for Vitamin B_{12} Biosynthesis

ALA is the first precursor for vitamin B_{12} biosynthesis by *P. denitrificans*, which can be synthesized via C_5 pathway (glutamate as the substrate) [18]. Therefore, the accumulation of glutamate is crucial to ALA formation, thus further influences vitamin B_{12} productivity. In

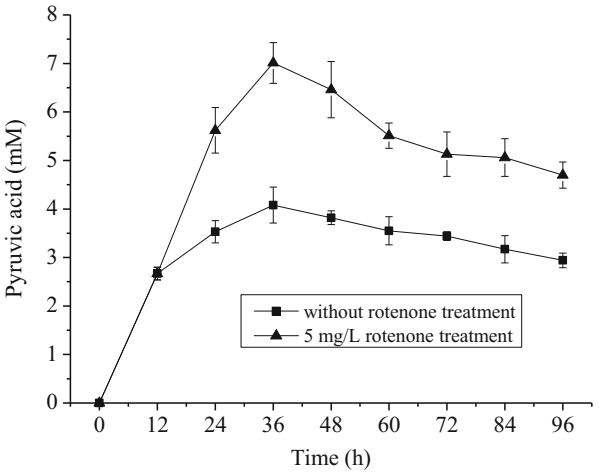

Fig. 2 Time courses of pyruvia acid concentrations under 0 and 5 mg/L rotenone treatment during *Pseudomonas denitrificans* cultivation in shake flasks

order to investigate the effects of rotenone treatment on the generations of the anterior precursors for vitamin B_{12} biosynthesis, the time courses of glutamate and ALA were compared under 0 and 5 mg/L rotenone treatment. As shown in Fig. 3, when *P. denitrificans* fermentation process was treated with 5 mg/L rotenone at 12 h, the generations of glutamate and ALA were all obviously higher than those obtained without rotenone addition, which implied that more anterior precursors could be afforded to produce vitamin B_{12}.

During the central carbon metabolism in most microorganisms, the pyruvic acid produced in glycolytic pathway would flow to TCA cycle to generate α-ketoglutarate [19]. Then, the generated α-ketoglutarate could further convert into glutamate via the glutamine synthetase/glutamate synthetase (GS/GOGAT) pathway [20]. As mentioned above, compared to *P. denitrificans* fermentation process without rotenone treatment, 5 mg/L rotenone addition would improve the specific activities of phosphofructokinase and pyruvate kinase involved in glycolytic pathway (Fig. 1), resulting in more generation of pyruvic acid (Fig. 2). Afterwards, the enhanced pyruvic acid was favorable for the generation of α-ketoglutarate, which would

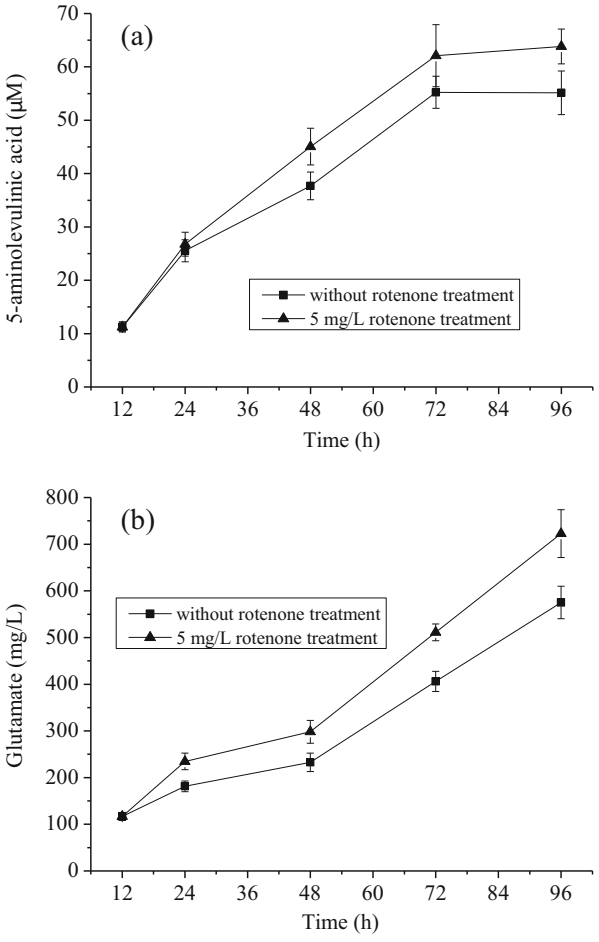

Fig. 3 Time courses of the generations of 5-aminolevulinic acid and glutamate under 0 and 5 mg/L rotenone treatment during *Pseudomonas denitrificans* cultivation in shake flasks

further accelerate the biosynthesis of amino acids and provide more amino acids substrates for ALA biosynthesis (Fig. 3).

Based on the metabolic diagram of *P. denitrificans* under 5 mg/L rotenone treatment (as shown in Fig. 4), it could be concluded that the addition of 5 mg/L rotenone could break the balance of carbon metabolism via strengthening the glycolytic flux, resulting in the promotion of anterior precursors for vitamin B_{12} biosynthesis, such as glutamate and ALA. As a result, the vitamin B_{12} productivity had an improvement compared to the fermentation without rotenone treatment. Admittedly, exogenous addition of rotenone was an impracticable strategy to improve the industrial vitamin B_{12} production. However, the metabolic characteristics of *P. denitrificans* under various rotenone concentrations again proved that the DO control level would be one of the critical parameters to vitamin B_{12} productivity.

It should be noted that *Pseudomonas* species mostly utilized the Entner-Doudoroff pathway for glucose metabolism. This study only examined the kinetics of the key glycolytic enzymes (phosphofructokinase and pyruvate kinase), pyruvic acid, and the anterior precursors (glutamate and ALA) under *P. denitrificans* fermentation processes with 0 and 5 mg/L rotenone treatment. Although these information could preliminarily arrive at the conclusion that the strengthened glycolytic flux metabolism was more favorable for vitamin B_{12} biosynthesis, further studies should be carried out to establish the networks of central carbon metabolism based on ^{13}C labeling analysis, especially the relationship between the Entner-Doudoroff and glycolytic pathway in *P. denitrificans*. Furthermore, it was also required to illustrate the complete dynamic changes of enzymes and intermediates involved in vitamin B_{12} biosynthetic pathway.

Conclusions

The present study investigated the effects of various rotenone concentrations on the metabolic processes of *Pseudomonas denitrificans*. Compared to the fermentation without rotenone

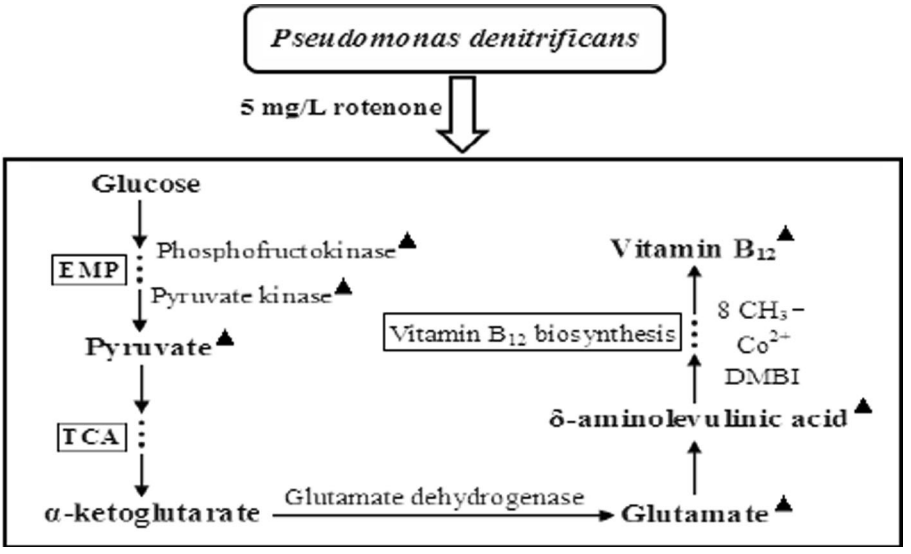

Fig. 4 The metabolic diagram of *P. denitrificans* under 5 mg/L rotenone treatment (meant increase)

addition, it was observed that 5 mg/L rotenone treatment could not only significantly accelerate the glycolytic flux, but also provide more anterior precursors vitamin B_{12} biosynthesis, such as glutamate and 5-aminolevulinic acid. As a result, higher vitamin B_{12} productivity could be obtained, which was parallel to our previous research that a limiting dissolved oxygen concentration was more favorable for vitamin B_{12} biosynthesis.

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