FERMENTATION, CELL CULTURE AND BIOENGINEERING

# Interactive performances of betaine on the metabolic processes of *Pseudomonas denitrificans*

**Wei Xia · Wei-fu Peng · Wei Chen · Kun-tai Li**

**Abstract** The performances of betaine on the metabolic processes of vitamin $B_{12}$-producing *Pseudomonas denitrificans* were investigated in this paper. The results showed that betaine was an indispensable methyl-group donor for vitamin $B_{12}$ biosynthesis, but large amounts of the extracellular glycine accompanied by betaine metabolism would impose a severe restriction on the cell growth of *P. denitrificans*. By further using a comparative metabolomics approach coupled with intracellular free amino acids analysis for the fermentation processes with betaine addition (10 g/l) or not, it was found that betaine could highly strengthen the formation of some key precursors and intermediates facilitating vitamin $B_{12}$ biosynthesis, such as δ-aminolevulinic acid (ALA, the first precursor of vitamin $B_{12}$), glutamate (an intermediate of ALA via $C_5$ pathway), glycine (an intermediate of ALA via $C_4$ pathway), and methionine (directly participating in the methylation reaction involved in vitamin $B_{12}$ biosynthetic pathway). Therefore, the performances of betaine on *P. denitrificans* metabolic processes were not only serving as a decisive methyl-group donor for vitamin $B_{12}$ biosynthesis, but also playing a powerfully promoting role in the generation of vitamin $B_{12}$ precursors and intermediates.

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

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## 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 [15]. Adenosylcobalamin and methylcobalamin are the biological forms of vitamin $B_{12}$, and they are the essential cofactors for methionine synthase and (R)-methylmalonyl-CoA mutase in animals and humans [17].

Due to the chemical synthesis processes being highly complicated and costly, some industrial microorganisms have been successfully exploited for the commercial production of vitamin $B_{12}$, such as *Pseudomonas denitrificans*, *Propionibacterium freudenreichii*, *Propionibacterium shermanii* and so on [7]. In recent years, more and more attentions have been paid to the strain of *P. denitrificans*, due to its higher vitamin $B_{12}$ productivity.

During vitamin $B_{12}$ biosynthetic pathway in *P. denitrificans*, there are eight methylation reactions catalyzed by six different methyltransferases, and the order of the methyl group attachment occurs in the sequence C-2, C-7, C-20, C-17, C-11, C-1, C-5 and C-15, respectively [18]. In these methylation steps, the methyl groups are derived by eight *S*-adenosyl-methionine (SAM) molecules. Although SAM directly participates in the methylation reaction, betaine (*N,N,N*-trimethylglycine) is usually used as the typical methyl-group donor for vitamin $B_{12}$ fermentation by *P. denitrificans*, due to three methyl group involved in its molecule. It was proved that betaine-homocysteine transmethylase (BHTase) could use betaine as a methyl donor and homocysteine as an acceptor to produce *N,N*-dimethylglycine and methionine, then the generated methionine was further turned into SAM by methionine adenosyltransferase [14, 19].

In our previous researches, the large-scale (120,000 l fermenter) vitamin $B_{12}$ production of *P. denitrificans* was

largely improved by optimizing many key fermentation parameters, like fermentation medium components [10], dissolved oxygen (DO) [12] and pH [8]. In addition, the requirement characteristics of betaine were also investigated during the industrial *P. denitrificans* fermentation, and an effective and economical feeding strategy was accordingly established [9].

To date, the role of betaine is mainly considered as the methyl-group donor for vitamin $B_{12}$ biosynthesis, but other biological functions of betaine on *P. denitrificans* fermentation processes are still ambiguous. Based on this fact, a metabolomic approach coupled with intracellular free amino acids analysis was applied to elucidate the metabolic characteristics of *P. denitrificans* under betaine addition.

## Materials and methods

### Microorganism and media

An industrial vitamin $B_{12}$-producing strain, *P. denitrificans*, was used in this study, which was maintained on agar slant containing (g/l): sucrose, 30; peptone, 10; $(NH_4)_2SO_4$, 0.25; $(NH_4)_2HPO_4$, 1.5; $MnSO_4·H_2O$, 0.1; $ZnSO_4·7H_2O$, 0.1; agar, 20. The initial pH was adjusted to 7.2 with 1 mol/l NaOH prior to sterilization.

Seed medium was composed of (g/l): maltose, 35; peptone, 20; $KH_2PO_4$, 5.0; $(NH_4)_2SO_4$, 2.0; $(NH_4)_2HPO_4$, 0.8; $MnSO_4·H_2O$, 0.2; $MgSO_4$, 1.5; $ZnSO_4·7H_2O$, 0.02; $CoCl_2·6H_2O$, 0.02; 5,6-dimethylbenzimidazole (DMBI), 0.0045. The pH was adjusted to 7.2 prior to sterilization.

Fermentation medium contained the following ingredients (g/l): maltose, 80; peptone, 25; betaine, 10; $(NH_4)_2SO_4$, 1.0; $MgSO_4$, 2.0; $KH_2PO_4$, 1.0; $ZnSO_4·7H_2O$, 0.08; $CoCl_2·6H_2O$, 0.15; DMBI, 0.08. The pH was adjusted to 7.4 with 1 mol/l NaOH before autoclaving.

### Fermentation in shake flasks

*Pseudomonas. denitrificans* was grown on agar slant (18 × 180 mm) at 28 °C for 48 h, and the fresh cell was washed with 10 ml 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 fermentation medium with 10 % inoculum, and incubated at 30 °C on a rotary shaker at 180 rpm for 120 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 of the fermentation broth was determined by high-performance liquid chromatography (HPLC) [9].

Betaine concentration in fermentation broth was determined by HPLC [9].

Glycine concentration in fermentation broth was assayed according to the method reported by Ebert [5].

The δ-aminolevulinic acid (ALA) concentration was determined according to the literature [3].

#### *Procedures of comparative metabolomics approach*

The extraction and derivation of the samples for gas chromatography–mass spectrometry (GC–MS) analysis were performed according to the methods reported by Robinson et al. [16]. GC–MS was performed with a Thermo Scientific ITQ 1100™ GC/MSn, which was equipped with a capillary column (30 m × 0.25 mm × 0.25 μm, TRACE TR-5MS, ThermoFisher). The temperature programming of the column is as follows: Initially setting at 80 °C for 2 min, then increasing to 300 °C with the speed of 10 °C per min, and holding for 6 min. The spectrometers were operated in electron-impact (EI) mode, and the mass range was 50–600 *m/z*. The temperatures of the inlet, ionization source and quadrupole rods were set at 280, 230 and 150 °C, respectively. 1 μl of injection volume was conducted with an unsplit injection, with high-purity Helium as carrier gas (flow rate 1 ml/min). Each sample was analyzed in six replicates. For GC–MS data processing, the total ion chromatogram (TIC) of each sample was transformed into NetCDF format, and then used XCMS software (an on-site integrated metabolomics analysis platform) to determine the metabolic profile differences and identify the metabolite. Principal component analysis (PCA) was employed to analyze the metabolomic dataset using the Simca-P software (version 12.0, Umetrics Sweden), and the variations of the identified metabolites were ranked by the values of variable importance in the projection (VIP).

#### *Procedures of intracellular free amino acids analysis:*

The fermentation broths (5 ml) were diluted to the identical optical density values with distilled water, then 15 ml of quenching solutions [60 % (v/v) methanol and 0.85 %

(w/v) $Na_2CO_3$] was added. After quenching at $-20$ °C for 30 min, the samples were centrifuged at 8,000 rpm at 4 °C for 5 min. Cell pellets were suspended in 5 ml ice water, and then added 5 ml ice methanol. After being subjected to three cycles of freezing at $-80$ °C and thawing at room temperature, the thawing solutions were centrifuged at 8,000 rpm at 4 °C for 5 min. The supernatants were dried by means of vacuum freeze drying, and then resuspended in 1 ml ddH$_2$O and 1 ml 10 % trichloroacetic acid at 4 °C for 2 h. After centrifuging at 8,000 rpm at 4 °C for 15 min, the supernatants were filtered with a 0.22 μm water-phase filter, and then the amino acids were measured with an automatic amino acid analyzer (Sykam S-433D, Germany) according to the Ninhydrin post-column derivatization method using amino acid standards. The determination parameters are as follows: separation column, filled with Li$^+$ type sulfonic acid exchange resin; reactor temperature, 130 °C; column oven temperature, 58 °C; injection volume 50 μl; detector wavelengths, 440 nm and 570 nm.

## Results and discussion

### Effects of betaine on the cell growth and vitamin $B_{12}$ biosynthesis by *P. denitrificans*

To investigate the effects of betaine on the fermentation processes of *P. denitrificans*, various concentrations of betaine (0, 5, 10, 15, and 20 g/l) were added to the fermentation medium, respectively. Figure 1 summarized the time courses of cell growth and vitamin $B_{12}$ biosynthesis in shake-flask cultivation.

From Fig. 1a, the DCW presented an obvious downtrend with the increases of betaine addition, which revealed that betaine would cause a certain inhibition to the cell growth of *P. denitrificans*. Although lack of betaine was more favorable to cell growth, there was almost no vitamin $B_{12}$ production during the whole fermentation processes, as shown in Fig. 1b. Among the above concentrations of betaine addition, the maximum vitamin $B_{12}$ yield achieved at $58.61 \pm 3.21$ mg/l under 10 g/l of betaine utilization. It is well known that betaine is indispensable to vitamin $B_{12}$ biosynthesis by acting as the methyl-group donor [4]. Similarly, our results also demonstrated that betaine was the essential component for vitamin $B_{12}$ production.

### Negative regulation mechanism of betaine on the cell growth of *P. denitrificans*

As mentioned above, betaine would exert an inhibitory effect on cell growth by *P. denitrificans*, although it is generally known to play an important role in osmoregulation [2]. To illuminate the negative influence of betaine
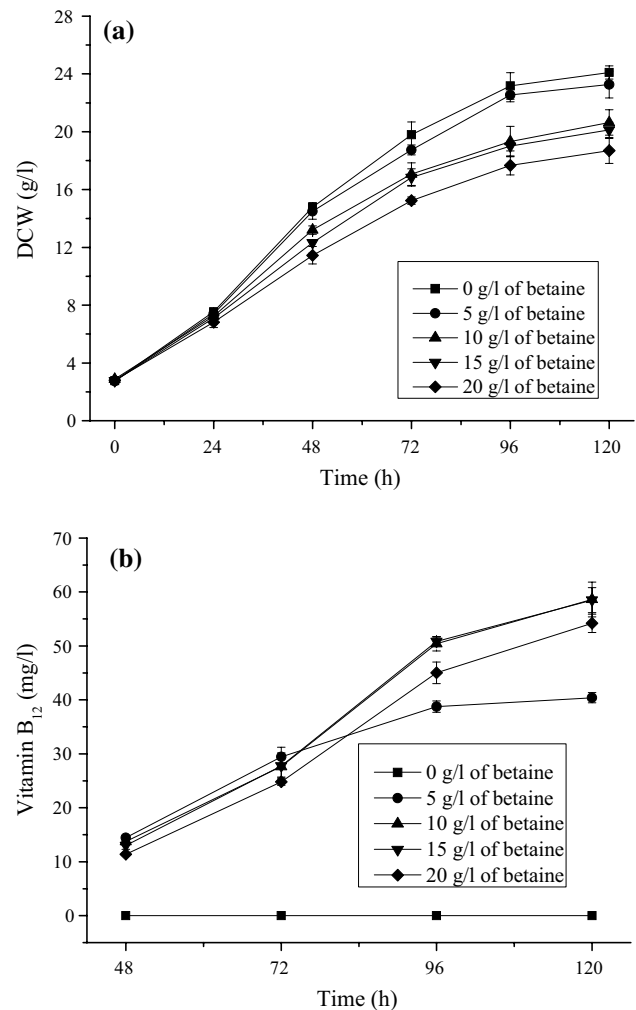


**Fig. 1** Time courses of cell growth (**a**) and vitamin $B_{12}$ biosynthesis (**b**) during *Pseudomonas denitrificans* fermentation processes with various concentrations of betaine addition. Values represent the averages of three measurements and *error bars* indicate standard deviation

on cell growth, the fermentation processes of *P. denitrificans* were further carried out under betaine addition (10 g/l) or not, in which the kinetics of betaine consumption and glycine accumulation were investigated and compared.

Accompanied by the increase of cell growth, the betaine concentration decreased sharply from the initial $9.87 \pm 0.66$ to $1.42 \pm 0.02$ g/l at 72 h (Fig. 2a), which indicated that betaine was rapidly consumed by *P. denitrificans*. As shown in Fig. 2b, the initial concentrations of glycine were approximately up to 150 mg/l in both the two fermentation schemes. Without betaine addition to the fermentation medium, a gradual downtrend of the glycine concentration occurred along with the fermentation processes. However, the extracellular glycine concentrations under 10 g/l betaine addition dramatically rose to
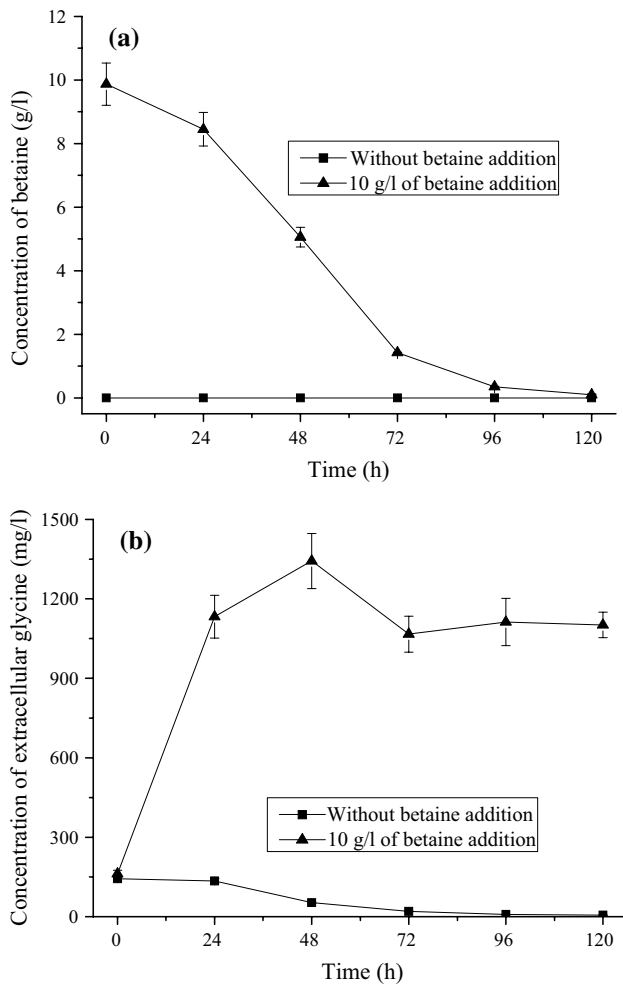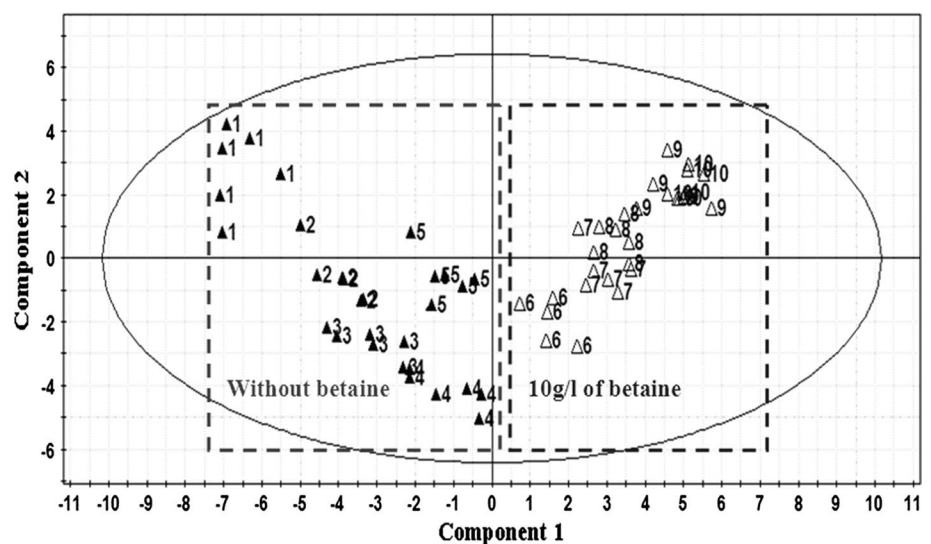
**Fig. 2** Time courses of the concentrations of betaine (**a**) and extracellular glycine (**b**) during *Pseudomonas denitrificans* fermentation processes with 10 g/l betaine addition or not. Values represent the averages of three measurements and *error bars* indicate standard deviation

$1{,}132 \pm 80.78$ mg/l at 24 h, and then maintained among 1,100–1,300 mg/l until the end of fermentation.

According to the above results, it could be concluded that betaine metabolism would accompanied by large generation of glycine. Using the synthetic medium, our previous study had been clearly illustrated that glycine in fermentation broth would impose a severe restriction on the cell growth of *P. denitrificans* [11]. Thus, the highly vast amounts of generated extracellular glycine attributed to the actual reason why the cell growth rate under 10 g/l betaine was dramatically lower than that in the case of betaine absence (as shown in Fig. 1a), especially after 24 h.

**Positive regulation mechanism of betaine on vitamin $B_{12}$ biosynthesis of *P. denitrificans***

Betaine is generally considered as the essential methyl-group donor for vitamin $B_{12}$ biosynthesis by *P. denitrificans*. Apart from this role, there is very little information on other biological functions of betaine during *P. denitrificans* metabolic processes. Based on this fact, a comparative metabolomics approach coupled with intracellular free amino acids analysis was carried out to investigate the metabolic differences of *P. denitrificans* under 10 g/l of betaine addition or not.

Figure 3 shows the score plots performed by PCA analysis of the comparative metabolomics study. It was observed that the two fermentation processes were clearly separated, indicating that betaine had significant effects on the metabolism of *P. denitrificans*. By further identifying the intracellular metabolites, it was found that there were 14 different metabolites (VIP value >1) mainly contributed to the discriminating metabolic profiles between the two fermentation processes with betaine addition or not, in which VIP values sorted in descending order are

**Fig. 3** PCA scores' scatter plots. 1, 2, 3, 4, and 5 mean the fermentation samples of 24, 48, 72, 96 and 120 h without betaine addition, respectively. 6, 7, 8, 9, and 10 mean the fermentation samples of 24, 48, 72, 96 and 120 h under 10 g/l betaine addition, respectively

as follows: 9,12,15-Calendic acid (3.10797), Palmitic acid (2.9145), Gluconic acid (2.41038), Citric acid (1.77461), Tripalmitin (1.7484), Linoleic acid (1.73114), Octadecanoic acid (1.70652), α-ketoglutarate (1.60257), Pyruvic acid (1.57038), Decanedioic acid (1.51068), Glutamate (1.46877), Succinic acid (1.27418), Glycine (1.19136), and Proline (1.05742).

The above 14 different metabolites could be classified into the following three categories: fatty acids, organic acids and amino acids. According to the relative abundance of these metabolites (data not shown), it was found that 10 g/l of betaine addition would cause a significant decrease to the fatty acids and intracellular organic acids during the whole fermentation processes, but had an obviously positive effects on the accumulation of the above amino acids, especially glutamate. It is generally known that glutamate plays a central role in a wide range of metabolic process in bacterial cells, due to its serving to link nitrogen and carbon metabolism [6]. During vitamin $B_{12}$ biosynthetic pathway in *P. denitrificans*, δ-aminolevulinic acid (ALA) is the first precursor, which could synthesized via $C_5$ pathway (glutamate as the substrate) and $C_4$ pathway (glycine as the substrate) [1, 13]. According to the comparative metabolomics study, it seemed that betaine also had a promoting function on vitamin $B_{12}$ biosynthesis by strengthening the precursor formation.

To further validate this hypothesis, the concentrations of intracellular free amino acids were assayed using an automatic amino acid analyzer. Figure 4 represents the heat map of the intracellular free amino acids in *P. denitrificans* under the fermentation processes with 10 g/l of betaine addition or not. Compared to the fermentation processes without betaine addition, it was observed that the concentrations of intracellular glutamate, glycine, methionine, ornithine and proline had an obvious improvement in the case of 10 g/l betaine utilization. Furthermore, the kinetics of ALA was also investigated during the two fermentation processes, as shown in Fig. 5. It was observed that the maximum concentration of ALA only reached approximately 5 μmol/l without betaine addition, which was highly lower than that obtained under 10 g/l of betaine utilization (56.08 ± 3.74 μmol/l).

According to the comparative metabolomics study coupled with intracellular free amino acids analysis, 10 g/l of betaine addition had an obvious enhancement in the biosynthesis of the glutamate group amino acids, such as glutamate, proline, and ornithine. In addition, betaine would also result in a higher accumulation of intracellular glycine (an intermediate for ALA biosynthesis via $C_4$ pathway), methionine (a direct substrate of SAM) and ALA (the first precursor for vitamin $B_{12}$ biosynthesis), compared to the fermentation without betaine addition. In brief, besides its
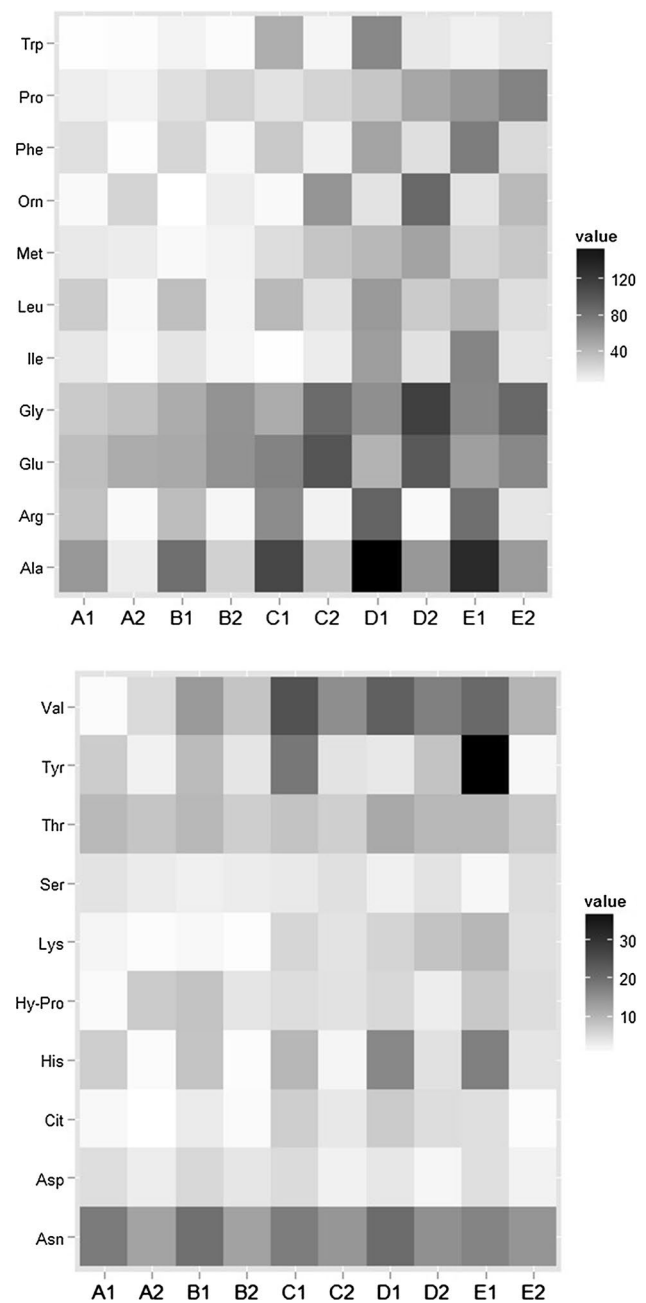


**Fig. 4** Heat map representation of the concentrations of intracellular free amino acids during *Pseudomonas denitrificans* fermentation processes with 10 g/l betaine addition or not. A1, B1, C1, D1, and E1 mean the fermentation samples of 24, 48, 72, 96 and 120 h without betaine addition, respectively. A2, B2, C2, D2, and E2 mean the fermentation samples of 24, 48, 72, 96 and 120 h with 10 g/l betaine addition, respectively

well known function on methyl-group donor for vitamin $B_{12}$ biosynthesis, betaine could also give rise to a significantly higher level of intracellular precursors and intermediates, thus accelerating vitamin $B_{12}$ biosynthesis by *P. denitrificans*.
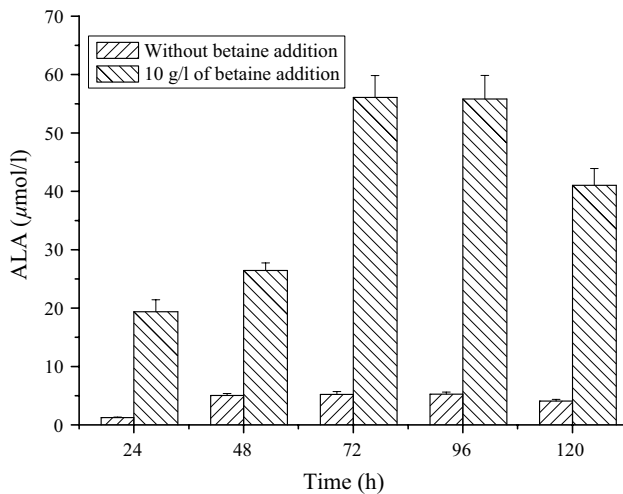
**Fig. 5** Time courses of ALA formation during *Pseudomonas denitrificans* fermentation processes with 10 g/l betaine addition or not. Values represent the averages of three measurements and *error bars* indicate standard deviation

## Conclusion

The present work investigated the performances of betaine on the metabolic processes of *P. denitrificans*. It was found that betaine was the indispensable methyl-group donor for vitamin B$_{12}$ biosynthesis, but large amounts of the extracellular glycine accompanied by betaine metabolism would impose a severe restriction on the cell growth. Apart from its major role in serving as the methyl-group donor, the comparative metabolomics study coupled with intracellular free amino acids analysis revealed that betaine could also significantly accelerate the formation of vitamin B$_{12}$ precursors and intermediates, facilitating vitamin B$_{12}$ biosynthesis.

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