View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by AJOL - African Journals Online

African Journal of Biotechnology Vol. 10(51), pp. 10551-10561, 7 September, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1869
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Optimization of nutritional requirements and ammonium feeding strategies for improving vitamin B_{12} production by *Pseudomonas denitrificans*

Ze-Jian Wang[1]*, Ping Wang[2], Ju Chu[1] and Si-Liang Zhang[1]

[1]State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, P. O. Box 329, 130 Meilong Road, Shanghai 200237, People's Republic of China.
[2]Hebei Medical University, Shijiazhuang 050017, China.

Accepted 6 May, 2011

Statistical experiment design and data analysis were used to establish the major factors in a chemically defined medium and to develop an ammonium control strategy to optimize the specific vitamin B_{12} production rate (*Yp*) of *Pseudomonas denitrificans*. Through Plackett-Burman design, the major factors of glucose, ammonium sulfate and KCl were selected as the significant factors affecting vitamin B_{12} biosynthesis and these were further optimized by central composite design with response surface methodology. The maximum *Yp* of 34.2 µg/gDCW/h was obtained in batch cultivation under the estimated optimal initial composition of glucose (93.6 g/l), $(NH_4)_2SO_4$ (7.93 g/l) and KCl (1.24 g/l). Ammonium control strategies in fed-batch fermentation showed that when ammonium concentration was maintained at 40 mmol/l, the maximum *Yp* reached 36.0 ± 1.31 µg/gDCW/h, which was 57.2% higher than that of the control (22.9 ± 0.83 µg/gDCW/h). This ammonium control strategy successfully enhanced the industrial production, resulting in a stable high vitamin B_{12} production of 212.02 ± 3.03 mg/l and *Yp* of 37.1 µg/gDCW/h.

Key words: Statistical designs, *Pseudomonas denitrificans*, chemically defined medium, ammonium controlling strategy, vitamin B_{12}.

INTRODUCTION

Two aspects are of critical importance in the successful industrial optimization of industrial vitamin B_{12} fermentation processes. Firstly, a full understanding is needed regarding the influence of nutritional composition on microbial fermentation. Secondly, the metabolism and physiological characteristics of the industrial microbial strains must be known in detail. A study on changes in intracellular metabolic flux distributions under different environments, combined with the real-time process parameters, would provide important information for fermentation optimization. Analysis of the correlation between primary metabolism and biosynthesis of the product of interest would also direct rational strain improvement through genetics and modification by molecular biology.

Vitamin B_{12} has a highly complicated molecular structure (Martens et al., 2002; Wang et al., 2010) that is biosynthesized from eight molecules of δ-aminolevulinic acid (δ-ALA). This δ- ALA precursor can be generated by either of two pathways (Figure 1): in the C4 pathway, δ-ALA is made from glycine and succinyl CoA by the action of ALA synthase. In the C5 pathway, the production of δ-ALA begins with glutamate (Lotfy, 2007). The results for a genetically engineered multi-enzyme synthesis of corrin showed that large amounts of the precursors, SAM, ATP and cofactor NADPH, generated via central carbon metabolism, are required for vitamin B_{12} production (Sano, 2009). Therefore, investigation of the relationship between central carbon metabolic pathways and secondary vitamin B_{12} biosynthesis is important. To better understand the metabolic flux distributions in vitamin B_{12} fermentation in *P. denitrificans*, ^{13}C-labeled metabolic flux analysis provides a powerful tool for effective quantification of all intracellular fluxes. This method has been widely utilized in a number of microorganisms including *Escherichia coli* (Dejaegher et al., 2007), *Bacillus clausii*

*Corresponding author. E-mail: wzjvictory@163.com. Fax: +86-21-64253702.

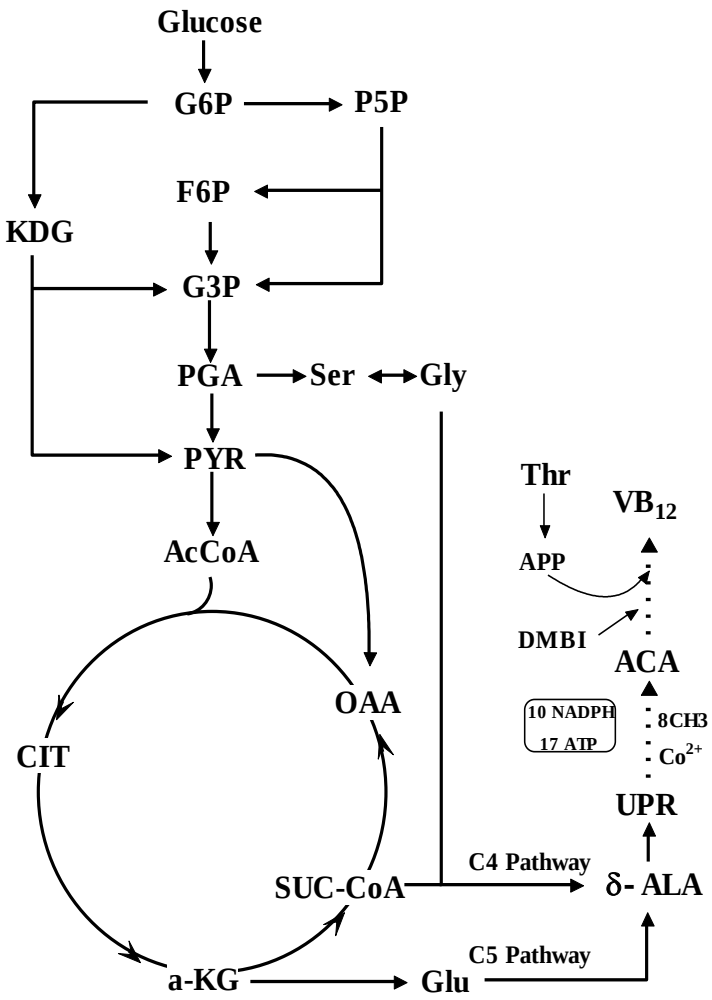

Figure 1. Proposed overview of the pathway of vitamin B_{12} biosynthesis by *P. denitrificans* connected with the central metabolism. ACA, adenosyl cobyric acid; AcCoA, acetyl-CoA; APP, aminopropanol-o-2-phosphate; CIT, citrate; DMBI, dimethyl benzimidazole; F6P, fructose-6-phosphate; Gly, glycine; Glu, glutamate; G3P, glyceraldehyde-3-phosphate; G6P, glucose-6-phosphate; KDG, 2-Keto-3-deoxy-6-phosphogluconate; OAA, oxaloacetate; P5P, pentose-5-phosphate; PGA, 3-phosphate-glycerate; PYR, pyruvate; Ser, serine; SUC-CoA, succinyl-CoA; Thr, threonine; UPR, uroporphyrinogen Ⅲ; α-KG, α-ketoglutarate; δ-ALA, δ-aminolevulinic acid.

(Choi et al., 1999), *Penicillium chrysogenum* (Capp et al., 1996) *Pseudomonas putida* (Berthouex and Brown 1994), hepatic cells (Demain et al., 1968) and *Streptomyces tenebrarius* (Blank et al., 2008).

To carry out ^{13}C-based metabolic flux analysis, a chemically defined medium is usually necessary in order to eliminate interference from unknown carbon sources on the isotopmer balance and metabolic flux ratio calculations. However, the productivity obtained under ^{13}C-based chemically defined media is always dramatically lower than that obtained using the complex media commonly used in industrial production. As well, the physiological characteristics of the microorganisms can also dramatically differ when grown under these two diverse conditions. Therefore, an appropriate medium is necessary for the effective ^{13}C metabolic network analysis in industrial microbial strains.

A clearly defined compositional medium could also provide an effective platform for investigating the effects of various factors on metabolism and productivity. In industrial vitamin B_{12} fermentation, the by-products like beet molasses and corn steep liquor are extensively used as carbon and nitrogen sources. However, the uncertain component and different quality of materials

frequently results in large fluctuations of industrial fermentation. These have been also reported in fermentations of cephalosporin C (Li et al., 2008b), erythromycin (Warren et al., 2002) and avermectins. Therefore, determining the significant factors seriously affect the industrial productivities and developing an optimal controlling strategy would prove useful for improving these fermentation techniques.

This study presented experiments designed to screen and optimize a chemically defined medium for vitamin B_{12} production by *P. denitrificans,* using Plackett-Burman design and central composite design. The effects of ammonium concentrations on vitamin B_{12} fermentation, based on this medium, were also investigated in fed-batch fermentation. An optimal ammonium control strategy was also successfully applied to enhance industrial vitamin B_{12} production.

MATERIALS AND METHODS

Media for *P. denitrificans*

Pre-culture of *P. denitrificans* (donated by Huarong Pharmacy Corporation, Shijiazhuang, China) (Kanda, 1995) was performed in shake flasks (2000 ml) containing 250 ml medium composed of 0.5 g/l $(NH_4)_2SO_4$; 1.5 g/l $(NH_4)_2HPO_4$; 0.5 g/l $MgSO_4{\cdot}7H_2O$; and 0.1 g/l $ZnSO_4{\cdot}7H_2O$ adjusted to pH 7.2 to 7.4 with 5 N NaOH. Separately sterilized glucose as the carbon source was added into the medium to a final concentration of 18 g/l just before inoculation. The composition of the initial medium for shake flask fermentation was as follows (g/l): glucose, 40; $(NH_4)_2HPO_4$, 7; KCl, 0.2; $MgSO_4{\cdot}7H_2O$, 1.4; $(NH_4)_2SO_4$, 5; betaine, 12.0; 5,6-dimethylbenzimidazole (DMBI), 0.0065; trace element solution (TES) (100 ml). TES was composed of (g/l): $MnSO_4{\cdot}H_2O$, 0.2; $ZnSO_4{\cdot}7H_2O$, 0.2; $CoCl_2{\cdot}6H_2O$, 0.025; $FeSO_4{\cdot}7H_2O$, 0.03; and Na_2MO_4, 0.02. The pH of the medium was adjusted to 7.2 to 7.4. The medium used in the researches of Li et al. (2000) was adapted here in the industrial bioreactor fermentation (10,000 L), with corn steep liquor as the main nitrogen source.

Shake flask culture

Pre-culture was carried out in a 500 ml Erlenmeyer flask containing 100 ml of seed medium inoculated with cells from a fresh slant and cultured at 28°C on a rotary shaker at 260 rpm for 18 h. The seed culture was then transferred to a 500 ml Erlenmeyer flask containing 50 ml fermentation medium with 10% inoculum and incubated at 32°C on a rotary shaker at 260 rpm for 120 h.

15 L bioreactor fed-batch culture

Fed-batch fermentations were performed in a 15 L turbine-agitated bioreactor (Shanghai Guoqiang Inc., China) with a 10 L working volume. The inoculum was 10% (v/v) of the whole volume of the culture broth. The fermentation temperature and agitation speed were adjusted to 32°C and 400 rpm, respectively. The aeration rate was 0.8 vvm and the pH was controlled automatically at 7.0 to 7.2 with 3 M NaOH. The fed-batch processed combined pulse feeding of ammonium sulfate solution (400 g/l) to control the ammonium concentration. Each condition was tested twice and the data shown represent the mean values. Previous research found that dissolved oxygen concentration was sustained at a zero level constantly during fermentation because of the high oxygen consumption rate of *P. denitrificans* and an uptake rate controlling strategy was also applied in this research (Scott, 2003). When the total sugar in the broth dropped to 20 g/l, feeding of glucose solution (500 g/l) began and the residual glucose concentration was maintained at 1.5 to 2.0% throughout the entire fermentation process.

10,000 L bioreactor fed-batch culture

In a 10,000 L fermenter, the first seed culture was prepared in a 150 L seed fermenter containing 100 L sterile medium with inoculums from eight 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 (70 to 80 L) was transferred into a 1,000 L secondary seed fermenter with 700 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 10,000 L fermenter equipped with three 3-bladed propeller impellers, a temperature probe, pH probe (Mettler Toledo) and dissolved oxygen probe (Mettler Toledo). The pH of the fermentation medium was adjusted to 7.0 and 7.2 before inoculation. The second seed (about 5% of the final working volume) was transferred into 10,000 L fermenter with 7,500 L working volume. The fermentation process was carried out at 32°C for 166 h. The glucose feeding strategies were similar to that mentioned earlier in the 15 L bioreactor.

Determination of dry cell weight (DCW) and vitamin B_{12}

Samples were centrifuged at 8000 *g* for 10 min and washed twice with distilled water; the cell precipitate was dried to a constant weight at 105°C. The vitamin B_{12} concentration was determined by HPLC. Broth samples (25 ml), to which 2.5 ml of 8% $NaNO_2$ and 2.5 ml of glacial acetic acid were added, were boiled for 30 min, then the upper aqueous phase was measured using a HPLC system equipped with a C_{18} column, (4.6 mm, 25 cm and 5 µm) with an UV detector (254 nm). 85% of 0.25 M sodium acetate anhydrous (pH was adjusted to 3.6 with acetic acid) and 15% of methanol in water was used as the mobile phase with a flow of 1.0 ml/min at 40°C (Kamarthapu et al., 2008).

Determination of ammonium and δ-aminolevulinic acid (δ-ALA) concentrations

The concentrations of ammonium-nitrogen (NH_4^+-N) in broths were quantified by the method described by Kanda, which uses O-phenylphenol as a substitute for liquid phenol (Hofmann et al., 2008). δ-ALA concentration in the fermentation broth was determined according to the method reported by Choi et al. (1999).

Experimental design and data analysis

For selection of significant factors for vitamin B_{12} production, eight independent variables, including glucose, KCl, Na_2HPO_4, $MgSO_4$, betaine, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$ and TES, were tested and identified via the Plackett-Burman design (Christensen and Nielsen, 2000). All trials were carried out in triplicate and the average vitamin B_{12} yields were treated as responses. The main effect of each variable was simply calculated as the difference

Table 1. Plackett-Burman design matrix for eight variables with the observed vitamin B_{12} production.

Run	X_1 Glucose (g/l)	X_2 KCl (g/l)	X_3 Na_2HPO_4 (g/l)	X_4 $MgSO_4$ (g/l)	X_5 Betaine (g/l)	X_6 $(NH_4)_2HPO_4$ (g/l)	X_7 $(NH_4)_2SO_4$ (g/l)	X_8 TES (ml)	VB_{12} (mg/l)
1	-1(40)	-1(0.2)	-1(1.5)	1(1.4)	-1(2.5)	1(10)	1(9)	-1(80)	36.0± 0.32
2	-1	-1	-1	-1(0.8)	-1	-1(6)	-1(5)	-1	39.0± 0.37
3	1(70)	-1	1(2.0)	1	-1	1	1	1(120)	52.5± 0.84
4	-1	1(1)	1	-1	1(5.5)	1	1	-1	31.5± 0.43
5	-1	1	-1	1	1	-1	1	1	28.5± 0.72
6	-1	-1	1	-1	1	1	-1	1	40.5± 0.83
7	1	-1	-1	-1	1	-1	1	1	39.1± 0.53
8	1	1	-1	-1	-1	1	-1	1	49.5± 0.64
9	1	1	1	-1	-1	-1	1	-1	42.0± 0.96
10	1	1	-1	1	1	1	-1	-1	46.5± 0.50
11	1	-1	1	1	1	-1	-1	-1	54.2± 1.02
12	-1	1	1	1	-1	-1	-1	1	37.5± 0.74
13	0(55)	0(0.6)	0(1.75)	0(1.1)	0(4)	0(5)	0(7)	0(100)	41.2± 0.49
14	0	0	0	0	0	0	0	0	41.3± 0.78
15	0	0	0	0	0	0	0	0	43.2± 0.80
16	0	0	0	0	0	0	0	0	40.6± 1.14

between the average of measurements made at the high setting (+1) and low setting (-1) for that factor. The experimental design is shown in Table 1. The Plackett-Burman experimental design is based on the first order model (Equation 1):

$$Y = \beta_0 + \sum \beta_i X_i \quad (1)$$

Where, Y is the response (vitamin B_{12} yield), β_0 is the models intercept, β_i is the linear coefficient and X_i is the level of the independent variable.

This model does not describe the interactions among factors; it is used to screen and evaluate the important factors that influence the response. The significance of each variable was determined via Student's *t*-test using SAS software.

The next step in the formulation of the medium was to determine the optimum levels of significant variables for vitamin B_{12} production. For this purpose, the response surface methodology (RSM) was used for the augmentation of total vitamin B_{12} production (Li et al., 2008a). The significant variables utilized were as follows: glucose, KCl and $(NH_4)_2SO_4$, each of which was assessed at five coded levels (-1.68, -1, 0, +1 and +1.68) and a total of 20 experiments were carried out, as shown in Table 3. All variables were taken at a central coded value, which was designated as zero. The minimum and maximum ranges of the variables were used and the full experimental plan with regard to their values in actual and coded form is provided in Table 3. The statistical software package Design-Expert-7.0 was used to generate the model to predict the effect of combined variables on vitamin B_{12} production. For a three-variable system, the responses can be predicted by the following second-order regression equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (2)$$

Where, Y is the dependent variable (vitamin B_{12} yield or specific vitamin B_{12} production rate); X_1, X_2 and X_3 are the independent variables as
mentioned earlier; β_0 is the regression coefficient at center point; β_1, β_2 and β_3 are linear coefficients; β_{12}, β_{13} and β_{23} are second order interaction coefficients; β_{11}, β_{22} and β_{33} are quadratic coefficients. The values of the coefficients, the optimum levels and R^2 were determined as mentioned earlier.

RESULTS AND DISCUSSION

Evaluation of significant variables by Plackett-Burman Design.

The design matrix selected for the screening of significant variables for vitamin B_{12} production and the corresponding responses are shown in Table 1. The adequacy of the model was calculated and the variables showing statistically significant effects were screened via Student's *t*-test for ANOVA with SAS statistical software (Table 2). Factors showing p-values of less than 0.05 were considered to have significant effects on the response and were therefore, selected for further optimization studies. Glucose, with a probability value of 0.0018 was determined to be the most significant factor, followed by $(NH_4)_2SO_4$ (0.0115) and KCl (0.0298).

Glucose had a positive effect on the vitamin B_{12} production. Vitamin B_{12} is a highly complicated molecule as it contains a tetrapyrrole-derived macrocycle (Martens et al., 2002; Wang et al., 2010) and somewhere around 30 genes are necessary for its complete *de novo* biosynthesis (Wang et al., 2010). In these processes, large amounts of energy and cofactors were required for maintenance and vitamin B_{12} production in the oxygen-dependent fermentation by *P. denitrificans* (Sano, 2009). Therefore, a high glucose concentration is necessary for greater vitamin B_{12} production. Nitrogen as $(NH_4)_2SO_4$ also plays an important role in cell

Table 2. Model coefficient estimated by multiplies linear regression.

Variable	DF	PE	SE	t-value	Pr>|t|
Intercept	1	0.27615	0.00489	56.53	<.0001
X_1	1	0.0375	0.00508	7.37	0.0018
X_2	1	-0.0125	0.00508	-4.46	0.0298
X_3	1	0.00917	0.00508	1.8	0.1458
X_4	1	0.00583	0.00508	1.15	0.3152
X_5	1	-0.0075	0.00508	-1.47	0.2142
X_6	1	0.0075	0.00508	1.47	0.2142
X_7	1	0.0225	0.00508	4.72	0.0115
X_8	1	-0.0025	0.00508	-0.49	0.6487

DF, Degree of freedom; PE, parameter estimate; SE, standard error.

Table 3. Central composite design matrix for the three variables along with the observed and predicted vitamin B_{12} production and specific vitamin B_{12} production rate (µg/gDCW/h).

Serial	X_1	X_2	X_3	VB_{12} (mg/l)	Predicted	*Yp*	Predicted *Yp*
1	-1 (50.0)	-1 (0.7)	-1 (7.0)	49.5± 1.34	50.1	24.0± 1.21	23.2
2	1 (90.0)	-1	-1	53.3± 0.67	53.6	19.4± 0.94	19.8
3	-1	1(1.3)	-1	45.2± 0.35	46.6	28.8± 0.87	28.9
4	1	1	-1	47.3± 0.63	48.0	25.6± 1.03	25.7
5	-1	-1	1 (9.0)	42.4± 0.96	42.8	27.1± 0.69	26.8
6	1	-1	1	48.2± 0.37	47.9	29.0± 1.25	28.7
7	-1	1	1	46.5± 0.86	47.3	31.1± 1.46	30.5
8	1	1	1	50.0± 0.94	50.5	31.8± 0.97	32.5
9	-1.68 (36.4)	(0)1.0	0(8.0)	41.9± 1.67	40.5	29.0± 1.44	29.9
10	1.68 (103.6)	0	0	46.3± 0.65	46.1	29.4± 1.15	28.8
11	0(70.0)	-1.68 (0. 5)	0	52.9± 0.86	52.8	22.8± 0.94	23.3
12	0	1.68 (1.5)	0	53.3± 1.37	51.9	31.4± 1.85	31.2
13	0	0	-1.68 (6.32)	59.4± 1.20	54.1	20.5± 0.47	20.6
14	0	0	1.68 (9.68)	50.3± 0.97	50.0	29.3± 1.07	29.4
15	0	0	0	64.7± 0.89	63.9	29.0± 0.96	30.5
16	0	0	0	63.3±1.67	63.9	31.4±1.27	30.5
17	0	0	0	64.2± 0.85	63.9	32.7±1.95	30.5
18	0	0	0	63.8± 0.53	63.9	29.8± 0.88	30.5
19	0	0	0	63.1± 0.79	63.9	29.2± 1.43	30.5
20	0	0	0	64.6± 0.63	63.9	31.0± 0.96	30.5

R^2 (coefficient of determination) = 0.99; X_1, glucose (g/l); X_2, KCl (g/l); X_3, $(NH_4)_2SO_4$ (g/l).

growth and product fermentation and higher vitamin B_{12} production was achieved as N source concentrations were increased. Statistical analysis also showed that KCl had a significant effect on vitamin B_{12} production. As is well known, K^+ has a significant function in the regulation of intracellular osmosis. The K^+ concentrations in the cytoplasm of growing cells increases greatly with increases in osmolarity of the defined growth medium to allow expulsion of redundant putrescine (Borodina et al., 2005).

Vitamin B_{12} biosynthesis requires eight methylation reactions, with S-adenosylmethionine (SAM) serving as the methylating agent. Kamarthapu et al. (2008) proposed that, SAM synthetase was stimulated by the monovalent cation K^+ at 100 mM and the further research demonstrated the presence of conserved amino acids at positions D286 for the K^+ binding site, confirming the need for K^+ for SAM synthetase activity. Betaine is an important methyl-group donor for vitamin B_{12} biosynthesis in *P. denitrificans* (Lofty et al., 2007). In this study, betaine concentrations from 2.5 to 5.5 g/l had little effect on vitamin B_{12} production, so betaine concentration was selected at 2.5 g/l. All other non significant variables were neglected and the optimum

Table 4. Model coefficients estimated by multiple linear regression (ANOVA).

Parameter	Vitamin B_{12} production			Specific VB_{12} production rate		
	SS	F Value	P Level	SS	F Value	P Level
Model	1181.6	112.11	< 0.0001*	255.9	21.19	< 0.0001*
X_1	37.4	31.94	0.0002*	1.5	1.08	0.3226
X_2	1.0	0.87	0.3733	76.3	56.86	< 0.0001*
X_3	20.6	17.60	0.0018*	94.3	70.25	< 0.0001*
X_1^2	769.5	657.15	< 0.0001*	2.4	1.77	0.2124
X_2^2	245.3	209.45	< 0.0001*	19.3	14.35	0.0036*
X_3^2	255.9	218.52	< 0.0001*	54.2	40.38	< 0.0001*
$X_1 X_2$	2.0	1.71	0.2205	0.0	0.01	0.9324
$X_1 X_3$	1.4	1.23	0.2926	13.8	10.28	0.0094*
$X_2 X_3$	32.8	28.01	0.0004*	2.1	1.57	0.2384

X_1, glucose; X_2, KCl; X_3, $(NH_4)_2SO_4$; SS, sum of squares; *P level less than 0.05 indicate the model terms were significant.

levels of the three variables, glucose, $(NH_4)_2SO_4$ and KCl were further determined by RSM design.

Optimization of fermentation conditions by central composite design

The experimental design, the levels of vitamin B_{12} production and the specific productivity obtained from different concentrations of additives are summarized in Table 3.

The experimental levels of vitamin B_{12} production and the specific productivity were used to generate the second-order regression models. A second order polynomial function was fitted to the experimental results of vitamin B_{12} production and the specific productivity with statistical software. The response of vitamin B_{12} production (Y) and the specific vitamin B_{12} production rate (Y_p) can be expressed in terms of the following regression equations (3, 4), respectively:

$$Y = 63.99+1.65X_1 - 0.27X_2 - 1.23X_3 - 7.31X_1^2 \\ -4.13X_2^2 - 4.21X_3^2 - 0.5X_1X_2 + 0.43X_1X_3 + 2.03X_2X_3 \quad (3)$$

$$Yp = 30.5-0.33X_1 + 2.36X_2 + 2.62X_3 - 0.41X_1^2 \\ -1.16X_2^2 - 1.94X_3^2 + 0.04X_1X_2 + 1.31X_1X_3 - 0.51X_2X_3 \quad (4)$$

The quality of fit of the second-order regression equations was checked using the coefficient of determination R^2 (multiple correlation coefficient) and was calculated to be 0.9902 and 0.995 with vitamin B_{12} production and the Y_p as the response, respectively, indicating that 99.02 and 99.5% of the variability in the response could be explained by the model. The ANOVA table is presented in Table 4. The model F-values of 112.11 and 21.19 showed the models were significant and the adequacy of second-order regression models. According to the linear coefficients of second-order equations, statistical analysis demonstrated that glucose and $(NH_4)_2SO_4$ had significant effects on vitamin B_{12} production, while KCl and $(NH_4)_2SO_4$ both had considerable effects on the Y_p within the tested ranges, at the probability level of 95%.

For predicting these optimal points mathematically, second-order regression models were used to develop response surface curves relating vitamin B_{12} productivity to various levels of additives. Figures 2 and 3 showed the response surface plot for vitamin B_{12} production and Y_p as a function of KCl and $(NH_4)_2SO_4$ and glucose, respectively. By moving along the X and Y axes, it can be demonstrated that increasing the levels of KCl and $(NH_4)_2SO_4$ had a conspicuous effect on vitamin B_{12} production (Figure 2). As a result, the stationary ridge shape was observed on the surface plot of the two fermentation parameters and the maximum response inside the design boundary. Based on the medium optimization evaluated from the model of Equation 3, the predicted largest vitamin B_{12} yield was 67.2 mg/l when the glucose, $(NH_4)_2SO_4$ and KCl concentration were 72.2, 7.85 and 0.98 g/l, respectively. Figure 3 showed the interaction of glucose and $(NH_4)_2SO_4$ had a significant effect on Y_p, the plot exhibited that higher concentrations of glucose and $(NH_4)_2SO_4$ were favorable for the Y_p. The maximum predicted Y_p of 33.3 µg/gDCW/h was attained when the glucose, $(NH_4)_2SO_4$ and KCl concentration were 93.6, 7.93 and 1.24 g/l, respectively. The results showed that higher initial concentrations of glucose and KCl were important for accelerating the vitamin B_{12} biosynthesis rate of *P. denitrificans*, while lower glucose concentration was favorable for higher vitamin B_{12} production.

The verification experiments carried out at two optimal conditions showed the observed experimental vitamin B_{12} production and the Y_p was 65.8 ± 1.5 mg/l

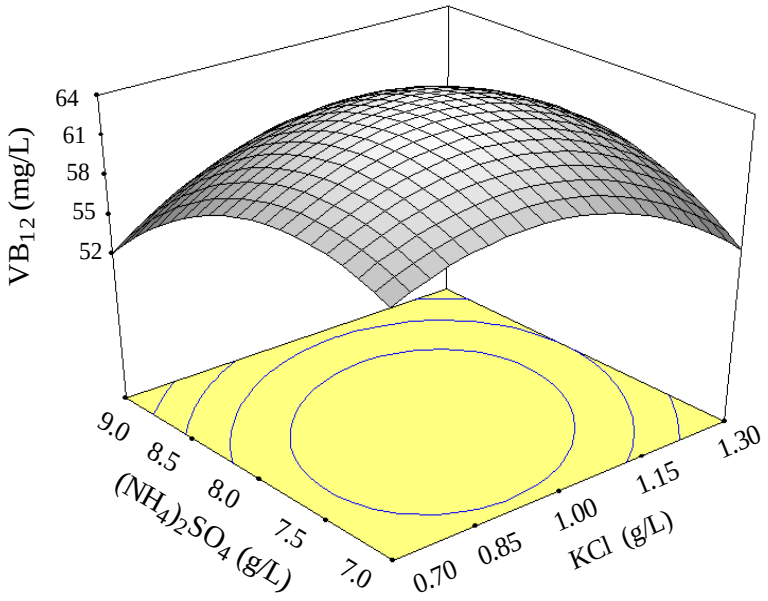

Figure 2. The response of vitamin B_{12} yield as a function of $(NH_4)_2SO_4$ and KCl concentration based on the central composite experimental results.

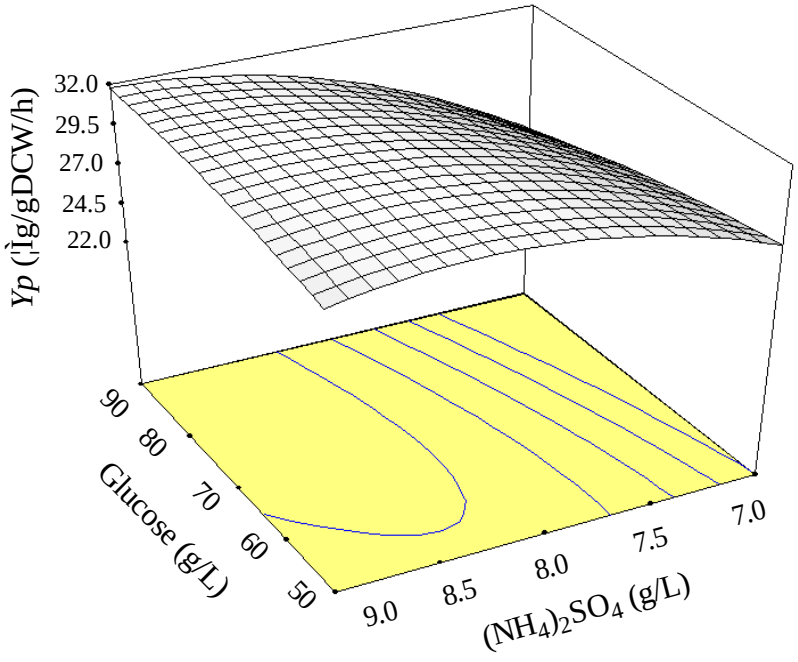

Figure 3. The response of specific vitamin B_{12} production rate as a function of $(NH_4)_2SO_4$ and glucose concentration based on the central composite experimental results.

and 34.2 ± 1.0 µg/gDCW/h, respectively, which were approximately 103.3 and 87.5% higher than that of the control (32.4 ± 1.3 mg/l and 18.2 ± 0.7 µg/gDCW/h with basal medium before applying P-B optimization). The great similarity between the predicted and observed results demonstrated the accuracy and applicability of

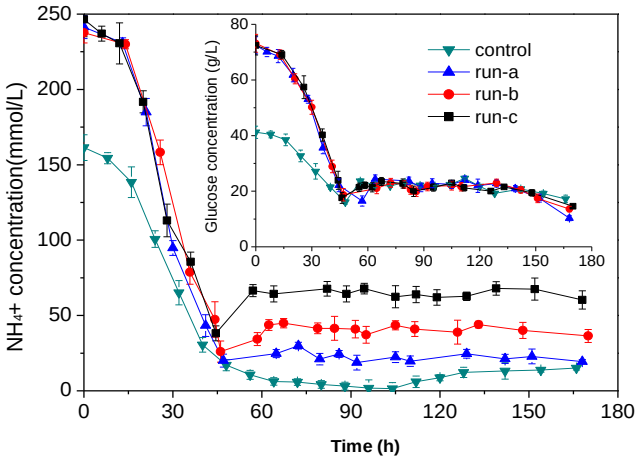

Figure 4. Profiles of ammonium and glucose concentration with different ammonium controlling strategies in the fed-batch vitamin B_{12} fermentation in 15 L bioreactors with chemically defined medium. Ammonium concentration were controlled by feeding ammonium sulfate solution at Run-a, 22 ± 1.8 mmol/l (▲); Run-b, 40 ± 2.0 mmol/l (●); Run-c, 65 ± 2.5 mmol/l (■); the control (with the basal fermentation medium and not ammonium controlled, ▼).

Table 5. The physiological parameters of the fed-batch vitamin B_{12} fermentation by *P. denitrificans* under various ammonium controlling strategies after 168 h cultivation.

Culture	Control	Run-a	Run-b	Run-c
X_{max}	23.2 ± 1.14	26.0 ± 2.17	27.3 ± 0.91	29.2 ± 1.19
Growth rate	0.48 ± 0.03	0.68 ± 0.07	0.74 ± 0.11	0.79 ± 0.09
Time of batch phase	48 ± 1.5	38 ± 1.1	37 ± 0.9	37 ± 1.6
Vitamin B_{12} (mg/l)	89.5 ± 4.3	133.5 ± 5.5	165.6 ± 4.9	93.8 ± 6.3
Yp (μg/gDCW/h)	22.9 ± 0.83	30.6 ± 1.77	36.0 ± 1.31	19.1 ± 2.07
Glycine (μg/gDCW/h)	139.6 ± 13.8	226.4 ± 14.2	408.0 ± 13.4	176.2 ± 9.3
Glutamate (μg/gDCW/h)	193.7 ± 12.3	188.9 ± 19.8	297.6 ± 20.4	137.3 ± 18.7
Succinate (μg/gDCW/h)	81.1 ± 7.1	124.7 ± 12.8	94.2 ±7.5	73.1 ± 11.4
Pyruvate (μg/gDCW/h)	11.3 ± 2.9	15.4 ± 3.7	13.2 ± 4.2	121.1 ± 13.8

X_{max}, Maximum cell concentration (g/l); growth rate: maximum cell density divided by the bath culture time (g/L/h); *Yp*, specific vitamin B_{12} production rate (μg/gDCW/h). Ammonium controlling strategies: control, with the basal fermentation medium and not ammonium controlled; Run-a, Run-b and Run-c: the ammonium concentration were controlled at 22 ± 1.8, 40 ± 2.0 and 65 ± 2.5 mmol/l by feeding ammonium sulfate in vitamin B_{12} fermentation with optimal medium, respectively.

the central composite design as an extremely powerful method for optimizing the vitamin B_{12} medium (Li et al., 2000).

Ammonium controlling strategies in fed-batch fermentation (15 L) with chemically defined media

Vitamin B_{12}, a secondary metabolite of *P. denitrificans,* was mainly synthesized during the stationary growth period, and the fed-batch cultivation was applied in vitamin B_{12} fermentation of *P. denitrificans*. Except for the initial nutrients, the components of the medium in the stationary state of cell growth also exerted significant effects on vitamin B_{12} biosynthesis. The earlier mentioned results had proved that $(NH_4)_2SO_4$ was the significant factor for both vitamin B_{12} production and the specific B_{12} production production rate. Therefore, the effects of ammonium concentration on vitamin B_{12} biosynthesis were further investigated in fed-batch culture with a chemically defined medium. With the rapid cell growth, the residual ammonium concentration drastically dropped from 248 ± 2.9 to 23.5 ± 3.2 mmol/l at 47 h (Figure 4) and then the ammonium concentrations were controlled at 22 ± 1.8, 40 ± 2.0 and 65 ± 2.5 mmol/l by feeding ammonium sulfate solution (Figure 4). The fed-batch fermentation with the basal fermentation medium and no ammonium control was

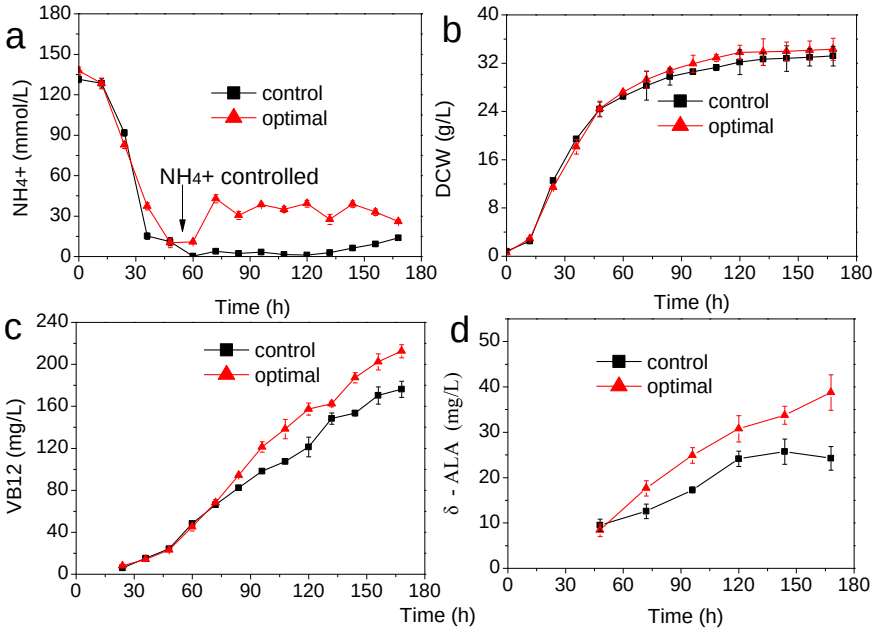

Figure 5. Time course of vitamin B_{12} fermentation with (▲) and without (■) ammonium controlling strategies in 10 m^3 industrial production under complex medium. (a) ammonium concentrations (mmol/l); (b) cells density (DCW g/l); (c) vitamin B_{12} production (mg/l); (d) δ-aminolevulinic acid (δ-ALA mg/l).

used as the control.

As shown in Table 5, the period of batch phase in optimal chemically defined medium was only 37.5 ± 0.8 h, less than that of the control (48 ± 1.5 h) and the cell growth rate was 41.7 to 61.2% higher than that of the control. Cell growth rate was enhanced by the increased ammonium concentration. A pronounced enhancement of vitamin B_{12} synthesis was achieved under an ammonium concentration of 40 ± 2.0 mmol/l and the final vitamin B_{12} production was 165 ± 2.2 mg/l, which was 84.4% greater than that of the control. The specific vitamin B_{12} production rate (*Yp*) also increased when the ammonium concentration increased to 40 ± 2.0 mmol/l. The maximum *Yp* reached 36.0 ± 1.31 µg/gDCW/h; 57.2% higher than that of the control (22.9 ± 0.83 µg/gDCW/h) in fed-batch fermentation (Table 5). However, when ammonium concentration increased to 65 ± 2.5 mmol/l, vitamin B_{12} biosynthesis was dramatically inhibited, the vitamin B_{12} production decreased to 93.5 ± 2.2 mg/l and the *Yp* dropped to 19.1 ± 2.07 µg/gDCW/h.

Determination of the intermediates showed that higher specific secretion rates of glycine, glutamate and succinate were obtained when the ammonium concentration was increased to 40 ± 2.0 mmol/l. These intermediates were the critical precursors of δ-ALA biosynthesis and thus, promoted vitamin B_{12} production. Glutamate joint the transaminase for many amino acids biosynthesis and ammonium was necessary for the biosynthesis of glutamate from α-ketoglutarate by reductive ammonia fixation via glutamate dehydrogenase (Muller et al., 1989). Therefore, maintaining the appropriate ammonium concentration at 40 ± 2.0 mmol/l was favorable for higher glutamate production. The other amino acids such as aspartate, serine, alanine and organic acids α-ketoglutarate and acetate showed little difference under all the four conditions. However, under the highest ammonium concentration of 65 ± 2.5 mmol/l, the specific secretion rate of glycine, glutamate and succinate were significantly decreased. This might be caused by the significant inhibition of high ammonium concentration on the activity of pyruvate dehydrogenase and then led to the highest specific puruvate accumulation rate of 121.1 ± 13.8 µg/gDCW/h, which were 9 folds higher than that of the other conditions. Overall, the control of the ammonium concentration at 40 mmol/l led to higher accumulation of relevant intermediates and therefore, greater vitamin B_{12} biosynthesis by *P. denitrificans*.

Scale-up of the optimal ammonium control strategy in a 10 m^3 fermenter

The results from the fed-batch fermentation with a chemically defined medium demonstrated that the nitrate source levels had significant effects on vitamin B_{12} fermentation. In vitamin B_{12} fermentation, the by-products like beet molasses and corn steep liquor are extensively used as nitrogen sources due to their low

cost. The vitamin B_{12} productivities in large-scale industrial fermentation were always tremendously influenced by the uncertainty of the components and changing quality of raw materials, especially the large differences in the nitrogen content of the raw materials. Therefore, in order to eliminate negative effects of nitrogen content fluctuation in the medium on vitamin B_{12} production, the optimal ammonium controlling strategy was implemented on the industrial fermentation in a 10 m^3 fermenter with complex medium. Figure 5 shows the kinetics of ammonium concentration, biomass growth, vitamin B_{12} production and δ-ALA concentration in large-scale fermentations with and without ammonium controlling strategy. As cell growth increased, the residual ammonium concentrations decreased sharply to 15.0 ± 3.0 mmol/l at 37 ± 2.8 h at the two kinds of cultivation and then ammonium concentrations were maintained at 35 to 43 mmol/l by pulse feeding of ammonium sulfate solution, compared with that of the control)(Figure 5a). The cell growth, δ-ALA concentration and vitamin B_{12} production had no different before the ammonium concentration was controlled. Although, the cell growth with the ammonium controlled cultivation had little higher than that of the control (Figure 5b), a remarkable enhancement of vitamin B_{12} biosynthesis was achieved under ammonium controlled cultivation (Figure 5c). A stably vitamin B_{12} production reached 212.0 ± 3.0 mg/l, 20.5% higher than that of the control (176.2 ± 2.9 mg/l). The maximum specific vitamin B_{12} production rate reached 37.1 μg/gDCW/h under ammonium controlling strategy, which was 14.6% higher than that of without ammonium control (31.6 μg/gDCW/h). Analysis of the δ-ALA concentrations showed that maintaining ammonium concentration at 35 to 43 mmol/l would greatly accelerate δ-ALA biosynthesis and the high flux of δ-ALA generation thus improved the vitamin B_{12} production (Figure 5d). This ammonium control strategy was successfully applied to industrial production in a complex medium and a stable and higher vitamin B_{12} production was achieved.

Previous research shown that glutamate and methionine are necessary for growth of *P. denitrificans* in a chemically defined medium (Christiansen et al., 2002), while our results demonstrated that the industrial strain of *P. denitrificans* can grow well with glucose as the sole carbon source. The specific vitamin B_{12} production rate reached 36.0 ± 1.31 μg/gDCW/h under the optimal chemically defined medium in fed-batch fermentation, which was similar to that with a complex medium (37.1 μg/gDCW/h) in large-scale industrial production (Li et al., 2000).. The physiological characteristics of *P. denitrificans* in industrial vitamin B_{12} production can be well implemented in this chemically defined medium. Therefore, the obtained optimal chemically defined medium and ammonium controlling strategy could be used for further ^{13}C-metabolic flux analysis of *P. denitrificans*, so as to reveal the metabolic network distributions and implement a rational optimization on industrial vitamin B_{12} fermentation. Furthermore, a stable high productivity and more econo-mical industrial vitamin B_{12} production were successfully achieved.

Conclusion

In this research, glucose, ammonium sulfate and KCl were selected as the major factors affecting vitamin B_{12} productivity of *P. denitrificans* from eight varieties through P-B design and the optimal concentration and the interaction between these factors were estimated with RSM design and a second-order polynomial model. Further experiments showed that maintaining the ammonium concentration at 35 to 43 mmol/l was necessary for enhanced vitamin B_{12} biosynthesis. One advantage of optimizing a chemically defined medium and establishing a control strategy was that a higher specific vitamin B_{12} production rate was achieved, which was similar to that of an industrial complex-medium condition. In addition, the ability to use glucose and inorganic ammonium as the sole carbon and nitrogen sources should allow further metabolic flux analysis on vitamin B_{12} fermentation of *P. denitrificans* by ^{13}C labeling experiments, as the influence of unlabeled carbon sources on labeling abundance is eliminated. The ammonium controlling strategy was able to prevent the fluctuation in total-N concentrations of the raw materials in industrial production, allowing a stable 20.5% augmentation of B_{12} production.

ACKNOWLEDGEMENTS

This work was financially supported by a grant from the major State Basic Research Development Program of China (973 Program), No.2007CB714303 and National Key Technology R&D Program, No.2008BAI63B01. We also thanks Huarong pharmacy corporation (Shijiazhuang, China) for donating the industrial strain *P. denitrificans*.

REFERENCES

Berthouex PM, Brown LC (1994). Factorial experimental designs.Statistics for Environmental Engineers. CRC press, Boca Raton.

Blank LM, Ionidis G, Ebert BE, Buhler BS, Chmid A (2008). Metabolic response of *Pseudomonas putida* during redox biocatalysis in the presence of a second octanol phase. FEBS. J. 275: 5173-5190.

Borodina I, Scholler C, Eliasson A, Nielsen J (2005). Metabolic network analysis of *Streptomyces tenebrarius*, a *Streptomyces* species with an active entner-doudoroff pathway. Appl. Environ. Microbiol. 71: 2294-2302.

Capp MW, Cayley DS, Zhang W, Guttman HJ, Melcher SE, Saecker RM, Anderson CF, Record MT (1996). Compensating effects of opposing changes in putrescine (2^+) and K^+ concentrations on lac repressor-lac operator binding: in vitro thermodynamic analysis and *in vivo* relevance. J. Mol. Biol. 258: 25-36.

Choi C, Hong BS, Sung HC, Lee HS, Kim JH (1999). Optimization of

extracellular 5-aminolevulinic acid production from *Escherichia coli* transformed with ALA synthase gene of Bradyrhizobium japonicum. Biotechnol. Prog. 21: 551-554.

Christensen B, Nielsen J (2000). Metabolic network analysis of *Penicillium chrysogenum* using ^{13}C-labeled glucose. Biotechnol. Bioeng. 68: 652-659.

Christiansen T, Christensen B, Nielsen J (2002). Metabolic network analysis of *Bacillus clausii* on minimal and semirich medium using ^{13}C-labeled glucose. Metab. Eng. 4: 159-169.

Dejaegher B, Capron X, Heyden YV (2007). Fixing Effects and Adding Rows (FEAR) method to estimate factor effects in supersaturated designs constructed from Plackett-Burman designs. Chemometrics and Intelligent Laboratory Systems. 85: 220-231.

Demain AL, Daniels HJ, Schnable L, White, RF (1968). Specificity of the stimulatory effect of betaine on the vitamin B_{12} fermentation. Nature. 220: 1324-1325.

Hofmann U, Maier K, Niebel A, Vacun G, Reuss M, Mauch K (2008). Identification of metabolic fluxes in hepatic cells from transient ^{13}C-labeling experiments: Part I. Experimental observations. Biotechnol. Bioeng. 100: 344-354.

Kamarthapu V, Rao KV, Srinicas, PN (2008). structural and linetic properities of *Bacillus subtilis* S-adenosylmethionine synthetase expressed in *Escherichia coli*. Biochemica. Et. Biophysica. Acta. 1784: 1949-1958.

Kanda J (1995). Determination of ammonium in seawater based on the indophenol reaction with o-phenylphenol (OPP). Water Res. 29: 2746-2750.

Li, H-B, Chen, F, Jiang, Y (2000). Determination of vitamin B_{12} in multivitamin tablets and fermentation medium by high-performance liquid chromatography with fluorescence detection. J. Chromatography 891: 243-247.

Li KT, Liu DH, Li YL, Chu J, Wang YH, Zhuang YP, Zhang SL (2008 a). An effective and simplified pH-stat control strategy for the industrial fermentation of vitamin B_{12} by *Pseudomonas denitrificans*. Bioproc. Biosyst. Eng. 31: 605-610.

Li KT, Liu DH, Li YL, Chu J, Wang YH, Zhuang YP, Zhang SL (2008 b). Influence of Zn^{2+}, Co^{2+} and dimethyl-benzimidazole on vitamin B_{12} biosynthesis by *Pseudomonas denitrificans*. World J. Microbiol. Biotechnol. 24: 2525-2530.

Lofty WA, Ghanem KM, El-Helow ER (2007). Citric acid production by a novel *Aspergillus niger* isolate: II. Optimization of process parameters through statistical experimental designs. Bioresour. Technol. 98: 3470-3477.

Lotfy WA (2007). The utilization of beet molasses as a novel carbon source for cephalosporin C production by *Acremonium chrysogenum*: Optimization of process parameters through statistical experimental designs. Bioresour. Technol. 98: 3491-3498.

Martens JH, Barg H, Warren MJ, Jahn D (2002). Microbial production of vitamin B_{12}. Appl. Microbiol. Biotechnol. 58: 275-285.

Muller B, Collins AN, Ellis MK, Whittingham WG, Leeper FJ, Battersby AR (1989). Syntheses Relevant to Vitamin B_{12} Biosynthesis - the Glutamate Route to (-)-Ring-B Imide and Synthesis of the 2,7,12,20-Tetramethyl-isobacteriochlorin. J. Chem. Soc. Chem. Commun. 16: 1119-1122.

Sano C (2009). History of glutamate production. Am. J. Clin. Nutr. 90: 728S-732S.

Scott AI (2003). Discovering nature's diverse pathways to vitamin B_{12}: a 35-year odyssey. J. Org. Chem. 68: 2529-2539.

Wang ZJ, Wang HY, Li YL, Chu J, Huang MZ, Zhuang YP, Zhang SL (2010). Improved vitamin B_{12} production by step-wise reduction of oxygen uptake rate under dissolved oxygen limiting level during fermentation process. Bioresour. Technol. 101: 2845-2852.

Warren MJ, Raux E, Schubert HL, Escalante-Semerena JC (2002). The biosynthesis of adenosylcobalamin (vitamin B_{12}). Nat. Prod. Rep. 19: 390-412.