Vitamin B₁₂ biosynthesis over waste frying sunflower oil as a cost effective and renewable substrate

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Abstract Statistical experimental designs were used to develop a medium based on waste frying sunflower oil (WFO) and other nutrient sources for production of vitamin B₁₂ (VB12) by *Propionibacterium freudenreichii* subsp. *freudenreichii* PTCC 1674. The production of acetic acid and propionic acid were also evaluated using the same micro-organism. The amount of WFO in the media was initially optimized. The amount of 4 % w/v of oil found to be an appropriate amount for production of VB12. A Plackett Burman design was then employed to identify nutrients that have significant effect on the production of VB12 in the WFO media. Dimethylbenzimidazolyl (DMB), cobalt chloride, ferrous sulfate, and calcium chloride were the most important compounds. The level optimization of nutrients as the significant factors was finally performed using response surface methodology based on a central composite design. The model predicted that a medium containing 35.56 mg/L DMB, 14.69 mg/L CoCl₂·6H₂O, 5.82 mg/L FeSO₄·7H₂O, and 11.41 mg/L CaCl₂·2H₂O gives the maximum VB12 production of 2.60 mg/L. The optimized medium provides a final concentration of vitamin 170 % higher than that by the original medium. This study offers valuable insights on a cost-effective carbon source for industrial production of food-grade VB12.

Keywords *Propionibacterium freudenreichii* · Vitamin B₁₂ · Plackett Burman · Response surface

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Introduction

The production of vitamin B₁₂ (VB12) has received great attention because of the growing global needs. This vitamin has several physiological functions and is widely used in pharmaceutical and food industries (Wang et al. 2012). The introducing of VB12 into the scientific world is referred to the early 1920s when two American physicians, Minot and Murphy, described their method for curing pernicious anemia (Martens et al. 2002). Many efforts have been made in the subsequent decades to improve the yield of VB12 production through optimization routes.

VB12 can only be produced by microorganisms. The industrial production of VB12 is carried out by fermentation using *Propionibacterium* and *Pseudomonas* species (Martens et al. 2002; Kosmider et al. 2012). During the fermentation of *Propionibacterium freudenreichii* (*P. freudenreichii*) for production of food-grade VB12, the extracellular products such as propionic acid and acetic acid are also produced (Hajfarajollah et al. 2014). Propionic acid is an important chemical with applications such as mold inhibitor, preservatives in animal feed and human foods, the substrate for synthesis of cellulose fibers, herbicides, perfumes, and pharmaceuticals (Suwannakham et al. 2006; Zhang et al. 2009). However, the production of these acids has an inhibiting effect on the cell growth and thus on the production of VB12 (Wang et al. 2012; Miyano and Shimizu 2000).

As many other biomaterials, the production of VB12 is an expensive task due to its requirement to synthetic culture media and that its extraction from the culture broth is costly because of very low concentration of vitamin, which is usually less than 10 mg/L (Kosmider et al. 2012). Using low-cost raw materials such as agricultural, industrial or food wastes such as frying oils, molasses, and starch rich wastes is a possible way to reduce the costs. These wastes may be used in future as

substrates for large-scale production of biomaterials like vitamins.

Propionibacteria are able to produce VB12 from a variety of carbon sources, such as tomato pomace (Haddadian et al. 2001), sucrose (Li et al. 2008a, b), household sugar (Quesada-Chanto et al. 1994), molasses (Quesada-Chanto et al. 1994), glycerol (Kosmider et al. 2012), whey (Hugenschmidt et al. 2010; Marwaha and Sethi 1984), and kefir (Wyk et al. 2011). Molasses has been proved to be one of the cheapest substrates for the production of a variety of compounds (Quesada-Chanto et al. 1994). WFO can also be another low-cost substrate. However, biosynthesis of VB12 using waste frying sun flower oil (WFO) has not been studied yet. Large quantities of frying oils are being used in food processing at domestic and industrial levels. Since the used frying oil contains more than 30 % polar compounds (Khuri and Cornell 1987), if these wastes are disposed without proper treatment, it can cause serious environmental hazards to the aqueous community. The greasy components of vegetable oil wastes (VOWs), produces a hydrophobic shield over the aqueous phase of effluent streams and partially blocks the penetration of air, which is required for the living activities (Zulfiqar et al. 2007).

Several nutrients with appropriate contents are needed to be provided in the broth medium for biosynthesis of the vitamin. However, the optimization of medium contents by the classical method, in which one variable is changed while the others are fixed at a certain level, is laborious and time-consuming especially when there are large number of variables (Pereira et al. 2010). An alternative and more efficient approach in microbial systems is to use statistical methods. Response surface methodology (RSM) is a commonly used method to assess the optimal fermentation conditions. It is also an efficient statistical technique for optimization of multiple variables with minimum number of experiments. Plackett–Burman design allows testing large number of parametric effects with the least number of observations, and allows random error variability estimation and testing of the statistical significance of the parameters (Plackett and Burman 1946).

In the present study, WFO was selected among several studied carbon sources for detailed investigations on production of VB12, acetic acid, and propionic acid by *P. freudenreichii*. The experiments were accomplished in three steps; the weight percent of WFO in the media was initially optimized. A Plackett–Burman experimental design was then performed to screen the nutrients in the media, which significantly affect the production of VB12. Screened factors were then applied to find the best concentrations of nutrients for the production of VB12 based on an experimental design with response surface method.

Materials and method

Chemicals and microorganisms

α -(5,6-dimethylbenzimidazolyl) cyanocobamid (Vitamin B₁₂) was purchased from da jung Co. (South Korea). All other chemicals were provided from Merck (Germany). WFO, which was used for frying potato, was supplied from a local restaurant. The data on composition of WFO are brought in the supplementary data (Online Resource 1). Prior to use, the WFO samples were filtered through a cellulose filter to remove any suspended materials. WFO was also tested for any possible contamination by VB12. Since VB12 is a water soluble vitamin, WFO was contacted with distilled water and shaken for an hour. The water was examined using HPLC (described later) to see if there is any VB12. *Propionibacterium freudenreichii* subsp. *freudenreichii* PTCC 1674 was purchased from Persian Type Culture Collection in lyophilized state and stored at -70°C in glycerol stocks. This strain is gram-positive, non-spore forming, rod-shape, and aerotolerant anaerobic bacterium and produces VB12 intracellularly and propionic acid and acetic acid extracellularly (Ye et al. 1996).

Media and cultivation

For inoculum preparation, the lyophilized bacterium from glycerol stock (1.5 ml) was thawed and precultured in medium containing (g/L): glucose 20, peptone 5, yeast extract 10, KH_2PO_4 2, $(\text{NH}_4)_2\text{NO}_3$ 4 for 18 h at 120 rpm and 30°C . The amount of 4 % (v/v) inoculation was performed from this culture into 100 cm³ of the production medium containing (g/L): peptone 5, yeast extract 10, KH_2PO_4 1, K_2HPO_4 1, $(\text{NH}_4)_2\text{NO}_3$ 4, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 1, $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$ 0.002 and betaine 5 and incubated on a temperature controlled Kuhner shaker incubator (Germany) at 130 rpm and 30°C . Other compounds such as 5,6 dimethylbenzimidazole (DMB), $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, EDTA, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, H_3BO_3 and $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ were prepared according to the optimization experiments. pH before autoclaving was adjusted between 6.7 and 7 using 3 N NaOH.

Primary experiments were performed in the medium supplemented with 5 and 15 mg/L of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ and DMB respectively, as recommended by some previous researches (Berry and Bullerman 1996; Marwaha et al. 1983).

Extraction and analysis

The concentration of VB12 (cyanocobalamin) was determined by high performance liquid chromatography (HPLC) with the method reported previously (Hugenschmidt et al. 2010; Zhang et al. 2010). Hydroxocobalamin is a natural form of cobalamin, which is formed in the cell during time.

However, this product is unstable when it is out of the bacterial cell. Cyanocobalamin, vitamin B₁₂ by definition, is a stable form of cobalamin compound (Piao et al. 2004). Therefore, hydroxocobalamin should rapidly convert to cyanocobalamin using KCN. This is because of the affinity of the molecule for cyanide ion. If this conversion does not occur, the unstable hydroxocobalamin may degrade and it cannot be detected by HPLC. Hence, in order to detect VB12, the culture broth was centrifuged (7000×g, 15 min) to harvest cells. The pellet was then washed with 0.2 M phosphate buffer (pH 5.5), and was centrifuged again (7000×g, 15 min). The cells were then dissolved in 0.1 % KCN (pH 6.0) and autoclaved at 121 °C for 15 min. After cooling, the sample was centrifuged to remove solids. The supernatant was filtered by 0.45 µm nylon filters and analyzed by HPLC.

A reversed-phase C18 column (Shimadzu 250 mm×4.6 mm; 5 µm particle size) was used for HPLC where the UV detection wavelength, flow rate of mobile phase, oven temperature, and injection volume were set to 361 nm, 1 mL/min, 25 °C, and 20 µL, respectively. VB12 at concentrations ranging from 0.5 to 10 µg/ml in MilliQ water were used for calibration. Each sample was injected twice and the mean values were reported (additional data are given in Online Resources 2 and 3).

The concentrations of propionic acid and acetic acid in the broth were determined using the same HPLC system with the mobile phase containing 1 mmol/L sulfuric acid and 8 mmol/L Na₂SO₄ in deionized water (pH 2.8). The UV detection wavelength, flow rate of mobile phase, oven temperature, and injection volume were set to 215 nm, 1 ml/min, 25 °C, and 20 µL, respectively. The standard solutions of propionic and acetic acids with concentrations ranging from 1 to 30 g/L in MilliQ water were used for calibration. Each sample was injected twice and the mean values were reported.

Carbon source evaluation

Some carbon sources other than WFO such as glucose, starch, kerosene, and glycerol were primarily studied to compare the VB12 production by those resources as well as the amounts of acid production. The production medium for these experiments was (g/L): peptone 5, yeast extract 10, KH₂PO₄ 1, K₂HPO₄ 1, (NH₄)₂NO₃ 4, MgCl₂·6H₂O 1, MnCl₂·2H₂O 0.002, betaine 5, CoCl₂·6H₂O 5 and DMB 15 mg/L. All experiments were performed with three replicates.

Design of experiments

The amount of WFO in the medium was optimized with one factor at a time method. WFO in different weight percents of 2, 4, 6 and 8 %w/v was used as a carbon source in the culture media. The best amount was selected for further experiments.

The concentrations of nutrients have important roles in the production of VB12 (Kosmider et al. 2012). The Plackett-Burman Design (Plackett and Burman 1946) generated by using multiple regression and ANOVA with the Minitab V. 16 software were used for screening the nutrients to find the significant ones. The parameters were the concentrations of DMB, CoCl₂·6H₂O, FeSO₄·7H₂O, EDTA, CaCl₂·2H₂O, H₃BO₃ and Na₂MoO₄·2H₂O. Dummy 1, Dummy 2, Dummy 3 and Dummy 4 were also included in this screening process. Selection of the nutrients parameters was based on our comprehensive study of related literature and some primary experiments. All the parameters were prepared in two levels according to Table 1. The experiments were repeated twice. The significant parameters (nutrients) were chosen based on their effect on the final concentration of VB12. This experimental design is unable to describe the interaction between factors but can be used to screen the factors that significantly affect the measured response (Lofty et al. 2007). The *p*-value was considered as a tool for evaluating the significance of each coefficient. The parameters with confidence levels greater than 95 % were considered as the significant influencing parameters on the response.

After identifying the critical medium nutrients by Plackett-Burman design, RSM was employed to optimize the nutrient concentrations, which maximize the production of VB12. Central composite design was used to determine the optimum levels of the significant variables and the effects of their mutual interactions on VB12 production. A central composite design coupled with a full quadratic polynomial model is a powerful combination that efficiently provides an adequate representation of most continuous response surfaces without expending many resources (Sen and Swaminathan 1997). A total of 31 experiments were carried out for four test variables. Each independent variable was studied at three different levels (low, medium and high, coded as −1, 0 and +1, respectively). The axial distance (alpha) was chosen to be 1.45 to make this design rotatable. Therefore, five levels were ultimately employed. The center point of the design was replicated two times for the estimation of error. A center point is a point in which all variables are set at their mid value.

Results and discussion

Primary evaluation of carbon sources

Prior to medium optimization experiments, *P. freudenreichii* was grown in batch cultures on different carbon sources in order to investigate and compare the ability of the microorganism to utilize various carbon sources.

Five carbon sources from different origins (hydrocarbon, carbohydrate, and vegetable oil) were examined for VB12

Table 1 Experimental ranges and levels of the 7 factors tested in the Plackett–Burman design

Factor	Symbol	Level (mg/L)	
DMB	X ₁	5	20
CoCl ₂ ·6H ₂ O	X ₂	2	10
Na ₂ MoO ₄ ·2H ₂ O	X ₃	0.1	10
H ₃ BO ₃	X ₄	1	4
EDTA	X ₅	0.5	5
FeSO ₄ ·7H ₂ O	X ₆	2	20
CaCl ₂ ·2H ₂ O	X ₇	2	10

biosynthesis and acids production. It is noticeable that WFO was free from any VB12 contamination. Table 2 shows the amount of produced VB12. The maximum concentrations of propionic and acetic acids are also reported. As seen, the production of VB12 using WFO is comparable with glucose as a carbon source. The considerable amount of VB12 produced on the fresh and waste sunflower oils suggests the possibility of using these substrates as renewable low cost sources. Hence, WFO was selected for further optimization studies.

The production of VB12 was detected to be low on starch and kerosene, possibly because the microorganism cannot utilize kerosene and starch efficiently. Furthermore, polymeric structure of starch may affect its weak utilization by the microorganism. It is worth mentioning that the production of acids is lower when the vegetable oils are used as the substrate. It is known that the produced acids, particularly propionic acid, has an inhibitory effect on the biosynthesis of VB12 (Miyano and Shimizu 2000). Therefore, lower production of acetic and propionic acids on sun flower oils is a reason for increasing the VB12 production on these substrates compared to that on glucose.

P. freudenreichii produces a considerable amount of acetic and propionic acids as byproducts. The amount of produced

Fig. 1 Production of VB12 vs. time for different amounts of WFO

acids in the present work is comparable with those in previous ones. Similar result was obtained by Gardner and Champagne (2005) who obtained between 10 and 20 g/L propionic acid using different native and spent MRS media by *P. freudenreichii* subsp. *shermanii*. However, more propionic acid was obtained in spent media than in native MRS. *Propionibacterium acidipropionici* is a main producer of propionic acid. Yang and Huang (1995) produced about 70 g/L propionic acid in recycle batch fermentations using immobilized cells of *P. acidipropionici*. 16.2 and 17 g/L propionic acid were obtained by *P. acidipropionici* in a two-stage fermentation system using sugar and whey, respectively (Quesada-Chanto et al. 1994). Amounts of 3 and 4.7 g/L acetic acid were produced by the same way using sugar and whey, respectively (Quesada-Chanto et al. 1994). However, acetic acid bacteria are among the major acetic acid producers. They divided into five to six genera of which *Acetobacter* and *Gluconabacter* species can tolerate high concentration of acetic acid (Yamada et al. 2000).

Table 2 Production of biomaterials using various carbon sources, All experiments were performed in triplicate

carbon source	VB12 (μg/mL)	Propionic (g/L)	Acetic (g/L)
Glucose ^a	1.3±0.07	19.1±0.44	15.3±0.21
Glycerol ^b	1.1±0.05	15±0.54	12.45±0.31
Kerosene ^b	1±0.09	N.D.	N.D.
Starch ^b	<0.5	11.5±0.12	6.6±0.12
Sunflower oil ^b	1.56±0.09	15.33±0.23	8.1±0.11
Waste frying sun flower oil ^b	1.6±0.12	14.91±0.31	8.6±0.36

N.D. not determined

^a 36 g/L was used

^b 4% w/v was used

Fig. 2 Production of acetic acid vs. time for different amounts of WFO

Fig. 3 Production of propionic acid vs. time for different amounts of WFO

Optimization of carbon source

To find the optimum amount of WFO in the medium for acetic acid, propionic acid, and VB12 biosynthesis, the concentration of WFO in a mineral salt medium was varied between 2 and 8 % (w/v). The highest amount of VB12 was produced when 4 % WFO was used in the cultivation of *P. freudenreichii* (Fig. 1). With the WFO concentration of 4 % (w/v), the production of about 1.6 mg/L VB12 after 120 h cultivation time was obtained. Therefore, a mineral salt medium with 4 % WFO was chosen as the appropriate culture medium for the further experiments. The biosynthesis of VB12 was inhibited after 120 h in the conventional process, in which the concentration of propionic acid had reached to approximately 14 g/L (Fig. 3).

In addition, the production of two important acids i.e. acetic and propionic acids were evaluated. Figures 2 and 3 show the variation of acid concentrations over the cultivation time on different amounts of WFO. Maximum acetic and propionic acids productions were about 9 and 15 g/L, respectively, and were the same for both 4 and 2 % (w/v) WFO.

The concentration of acetic acid rises just in the early hours of fermentation, reaches to about 2 g/L, and keeps rising till it reaches to a maximum value in the 4th day (9 g/L). This value is kept constant for about 1 day, and then is decreased because of possible oxidation of acetic acid that means the maximum concentration for acetic acid is reached in the 4th day. Similar manner can be seen for propionic acid (Fig. 3) in which the concentration is reached to about 6 g/L in the early hours of process and is attained to its maximum level after about 96 h.

Plackett-Burman design

A total 11 parameters including seven media components and four dummy variables were screened in 12 runs to investigate VB12 production in 4 % (w/v) WFO medium. According to Fig. 1, the production of VB12 after 120 h was considered as a response. Analysis of variance (ANOVA) result is represented in Table 3. In addition, the values of VB12 concentration in Plackett-Burman experiments along with the complete table of the design are inserted in the supplementary data as online resource 4. The value of R^2 for this experiment is 0.83. The Adj R^2 is 0.75. It is obviously seen from Table 3 that among the seven tested media components, only DMB, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ are significant parameters since the parameters show the confidence level of above 95 % and p -value of less than 0.05. All the other variables

Table 3 Results of statistical analysis according to ANOVA for Plackett–Burman design

Source	DF	Seq SS	Adj SS	Adj MS	F-value	p-value
Main Effects	7	2.09746	2.09746	0.299638	10.88	<0.0001
DMB (X1)	1	0.97445	0.97445	0.974454	35.39	<0.0001
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (X2)	1	0.51509	0.51509	0.515094	18.71	0.001
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (X3)	1	0.01411	0.01411	0.014114	0.51	0.484
H_3BO_3 (X4)	1	0.00004	0.00004	0.000038	0	0.971
EDTA (X5)	1	0.09028	0.09028	0.090283	3.28	0.089
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (X6)	1	0.19838	0.19838	0.19838	7.21	0.016
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (X7)	1	0.3051	0.3051	0.305102	11.08	0.004
Residual Error	16	0.44052	0.44052	0.027532		
Lack of Fit	4	0.09156	0.09156	0.02289	0.79	0.555
Pure Error	12	0.34895	0.34895	0.02908		
Total	23	2.53798				

$R^2 = 0.83$

SS sum of squares

DF degree of freedom

Table 4 A) Experimental range and levels of independent test variables used in central composite design; B) Central composite design matrix with experimental values of VB12 production

A					
Independent variables (mg/L)		Levels			
		− α	−1	0	1
DMB		1	10	30	50
CoCl ₂ .6H ₂ O		0.4	4	12	20
FeSO ₄ .7H ₂ O		0.2	2	6	10
CaCl ₂ .2H ₂ O		0.4	4	12	20
					A
					59
					23.6
					11.8
					23.6
B					
Std Run	DMB	CoCl ₂ .6H ₂ O	FeSO ₄ .7H ₂ O	CaCl ₂ .2H ₂ O	VB12 (mg/L)
19	30	0.4	6	12	2.059
10	50	4	2	20	1.708
12	50	20	2	20	1.747
5	10	4	10	4	0.764
15	10	20	10	20	1.009
8	50	20	10	4	1.774
14	50	4	10	20	1.602
24	30	12	6	23.6	2.331
13	10	4	10	20	0.984
28	30	12	6	12	2.763
9	10	4	2	20	0.889
3	10	20	2	4	0.946
29	30	12	6	12	2.592
20	30	23.6	6	12	2.425
7	10	20	10	4	0.972
16	50	20	10	20	1.649
31	30	12	6	12	2.465
23	30	12	6	0.4	2.285
2	50	4	2	4	1.736
22	30	12	11.8	12	1.712
21	30	12	0.2	12	1.848
17	1	12	6	12	0.438
25	30	12	6	12	2.663
30	30	12	6	12	2.587
27	30	12	6	12	2.469
6	50	4	10	4	1.549
4	50	20	2	4	1.892
1	10	4	2	4	0.658
26	30	12	6	12	2.383
11	10	20	2	20	0.941
18	59	12	6	12	1.526

have comparatively larger *p*-value indicating their comparatively insignificant influence on the VB12 production. DMB is the most significant nutrient due to its low *p*-value. CoCl₂.6H₂O is also a significant nutrient affecting the final concentration of VB12 with the *p*-value of 0.001. The role of DMB and CoCl₂ in the production of VB12 is clear, as they are the part of the metabolic pathway of the VB12 production (Burgess et al. 2009). Kosmider et al. (2012) screened NaH₂PO₄.2H₂O, FeSO₄.7H₂O, casein hydrolysate, and

calcium pantothenate as the significant factors affecting VB12 production by Plackett-Burman design using glycerol as a carbon source.

By using Minitab software, the equation obtained for Plackett-Burman design is as follows:

$$Y = 1.444 + 0.201 X_1 + 0.146 X_2 - 0.048 X_3 - 0.002 X_4 - 0.123 X_5 - 0.181 X_6 + 0.225 X_7 \quad (1)$$

Table 5 Analysis of RSM model variance (ANOVA) for VB12 production

Source	DF	Seq SS	Adj SS	Adj MS	F	p-value
Regression	14	13.5193	13.5193	0.96567	100.16	<0.001
Linear	4	3.3596	3.3596	0.83989	87.11	<0.001
DMB	1	3.2245	3.2245	3.22449	334.44	<0.001
CoCl ₂ .6H ₂ O	1	0.1221	0.1221	0.12210	12.66	0.003
FeSO ₄ .7H ₂ O	1	0.0084	0.0084	0.00837	0.87	0.0356
CaCl ₂ .2H ₂ O	1	0.0046	0.0046	0.00460	0.48	0.0500
Square	4	10.0526	10.0526	2.51314	260.66	<0.001
DMB ²	1	8.2843	5.2375	5.23745	543.22	<0.001
CoCl ₂ .6H ₂ O ²	1	0.4208	0.1344	0.13442	13.94	0.002
FeSO ₄ .7H ₂ O ²	1	1.2768	1.1478	1.14776	119.04	<0.001
CaCl ₂ .2H ₂ O ²	1	0.0707	0.0707	0.07073	7.34	0.015
Interaction	1	0.1072	0.1072	0.01787	1.85	0.152
DMB*CoCl ₂ .6H ₂ O	1	0.0007	0.0007	0.00070	0.07	0.791
DMB*FeSO ₄ .7H ₂ O	1	0.0404	0.0404	0.04040	4.19	0.057
DMB*CaCl ₂ .2H ₂ O	1	0.0331	0.0331	0.03312	3.44	0.082
CoCl ₂ .6H ₂ O*FeSO ₄ .7H ₂ O	1	0.0001	0.0001	0.00006	0.01	0.940
CoCl ₂ .6H ₂ O*CaCl ₂ .2H ₂ O	1	0.0319	0.0319	0.03186	3.30	0.088
FeSO ₄ .7H ₂ O*CaCl ₂ .2H ₂ O	1	0.0011	0.0011	0.00109	0.11	0.741
Residual Error	16	0.1543	0.1543	0.00964		
Lack-of-Fit	10	0.0521	0.0521	0.00521	0.31	0.952
Pure Error	6	0.1022	0.1022	0.01703		
Total	30	13.6736				

$R^2 = 98.8\%$, predicted $R^2 = 97.3\%$, adjusted $R^2 = 97.8\%$

where Y is the response (VB12 concentration), and X_i is as indicated in Table 1. A large constant coefficient (either positive or negative) indicates that the factor has a large impact on fermentation, while a coefficient close to zero means that the factor has little or no effect. It is another indicator to confirm the selection of the significant nutrients selected according to the *p*-value.

Fig. 4 Time course of VB12 production and CDW with optimized medium in fermenter

Response surface methodology

Response surface methodology (RSM) was applied for data analysis and finding the optimized amount of four nutrients which are screened by Placket Burman design. It should be noted that the insignificant factors identified by Placket Burman design are not used in the further experiments. The mineral salt medium with 4 % w/v WFO as the carbon source was used and the production of VB12 after 120 h was considered as a response. In order to describe the nature of the response surface in the optimum region, a central composite design with five coded levels was performed (Table 4A). The plan applied in this study involved 31 experiments conducted according to Table 4B. The response surface graphs were obtained to understand the effect of variables individually and in combination, and to determine their optimum levels for maximum VB12 production.

Analysis of variance (ANOVA) of the model along with the corresponding *p*-values and the parameter estimated for the VB12 production are shown in Table 5. The value of the correlation coefficient, R^2 (98.8 %), shows that the regression model provides accurate description of the experimental data. A reasonable agreement between predicted (97.3 %) and

◀ **Fig 5** Response surfaces and contour plots showing the mutual effects of interactions between factors

adjusted (97.8 %) values is observed. The lack-of-fit value was not significant (0.0521) indicating that the equation is adequate for predicting VB12 concentration under all conditions. All these evaluations confirm that the model can be used for the prediction of VB12 production within the given range of variables. Eq. (2) shows the relative VB12 concentration (Y) as a function of the test variables (X_i) in uncoded units:

$$\begin{aligned}
 Y = & -1.29252 + 0.134694X_1 + 0.0625489X_2 + 0.26191X_6 \\
 & + 0.0483356X_7 - 0.00178402X_1^2 - 0.00178628X_2^2 - 0.0208788X_6^2 \\
 & - 0.00129579X_7^2 - 4.14063E-5X_1X_2 - 6.28125E-4X_1X_6 \\
 & - 2.84375E-4X_1X_7 - 5.85937E-5X_2X_6 - 6.97266E-4X_2X_7 \\
 & + 0.000257813X_6X_7
 \end{aligned}
 \quad (2)$$

In order to determine the optimum conditions, a solution was given by the optimizer option of the Minitab software. The concentrations of DMB, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were adjusted to be in range while the VB12 production was assumed to be in its maximum amount. The RSM model predicts that a medium containing DMB 35.56 mg/L, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 14.69 mg/L, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5.82 mg/L, and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 11.41 mg/L gives the maximum VB12 production of 2.60 mg/L. A validation experiment (with three replicates) was carried out in a 5 L fermenter (Infors, Switzerland) with 3 L working volume under optimized conditions and the results are shown in Fig. 4 (Online Resource 5 shows the fermenter setup). As seen in the figure, the maximum VB12 concentration of about 2.74 mg/L after 120 h is obtained, which is very close to the predicted value that confirms the validation of the model.

Up to now, many studies have been carried out to improve the productivity of vitamin B₁₂, by optimizing the culture medium composition and cultivation process. Kosmider et al. (2012) optimized a medium using glycerol as a carbon source by Plackett-Burman and RSM designs using *Propionibacterium freudenreichii* sp. *shermanii* as the producer microbe. The optimized medium provided a 93 % increase in final vitamin concentration (4 mg/L) compared to that by the original medium. Chiliveri et al. (2010) in another research reported 43 % increase in vitamin B₁₂ production by medium optimization with the use of statistical methods. However, the carbon source was glucose in that case. VB12 production have also been improved by optimizing cultivation process, such as controlling of dissolved oxygen (Miyano and Shimizu 2000), pH (Li et al. 2008a, b), and reducing the inhibitory effect of acids (Wang et al. 2012).

To further increase in the yield of VB12 production, another strategy that is random mutagenesis can be used to generate strains with higher yields of VB12 production. Zhang et al. (2010) improved the production of vitamin B₁₂ by *Propionibacterium shermanii* using genome shuffling. The genome shuffled strain produced about 61 % higher VB12 (2.85 mg/L) compared to that by the parent strain after 96 h. Production of VB12 using genetically engineered *Propionibacterium freudenreichii* by Piao et al. (2004), resulted in 1.7 mg/L VB12 in a lactate rich medium.

Major interactions between the test variables can be analyzed by the response surfaces from the circular or elliptical nature of the contours (Fig. 5). These contours can be used to predict the VB12 concentrations for different values of the test variables (Khuri and Cornell 1987). Each contour curve represents an infinite number of combinations of two test variables with the other two maintained at their respective zero level. The maximum predicted yield is indicated by the surface confined in the smallest ellipse in the contour diagram. As indicated in Table 5, interactions between DMB and FeSO₄, DMB and CaCl₂ as well as CoCl₂ and CaCl₂ are relatively high compared to those between other nutrients. These results were confirmed by the contour plots of Fig. 5. The slope of each contour curves show that the concentration of one nutrient entirely depends on the concentration of another and vice versa. In other words the effect of one factor solely depends on the concentration of the other.

Conclusions

The capability of *P. freudenreichii* to produce VB12, propionic acid, and acetic acid over the WFO was evaluated. The production of these materials over time with various amounts of WFO concentrations was studied. The medium cultivated on 4 % w/v of WFO showed the most appropriate VB12 production.

Afterward, using two optimization steps, namely Placket-Burman and RSM, the amount of some elemental nutrients were optimized. Placket Burman results showed that the concentrations of DMB, cobalt chloride, ferrous sulfate, and calcium chloride had the most pronounced effects on the biosynthesis of VB12 by *P. freudenreichii*. Using RSM, the optimized concentrations of these nutrients were obtained. 2.74 mg/L VB12 was obtained from the optimized medium after 120 h which is 1.7 times more than that obtained in the primary experiments.

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