Fed Batch Production of a Fermented Beverage Containing Vitamin B_{12}

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ABSTRACT: *Production of fermented functional foods containing micronutrients is required for their health beneficial properties. Impact of 11 process variables on vitamin B12 production in a dairy beverage containing propionic acid was investigated. Propionibacterium freudenreichii ssp. shermanii was applied in a 3-l fermentor in fed-batch fermentation system. The most suitable conditions for vitamin B12 production were achieved by 5% v/v inoculum size containing Propionibacterium freudenreichii (without Lactobacillus acidophilus) and continuous feeding of lactose with the rate of 0.04 l/h at 36°C in a medium containing 25 g/l molasses, 10 g/l corn steep liquor, at pH=6.5, after 96 h fermentation. Maximum vitamin concentration (30 mg/l) and productivity (7.5 mg/l.day) were obtained in trial 9. Organoleptic properties of the fermented beverage were also acceptable for panelists and no significant difference was observed between samples and control during 6 days refrigerated storage.*

KEYWORDS: *Co-culture, Fed batch fermentation, Plackett-Burman, Process variables, Propionibacterium freudenreichii, Propionic acid, Vitamin B12.*

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INTRODUCTION

Vitamin B_{12} (Cobalamin) is a water soluble vitamin, which plays key role in the nervous system's function. Typically, this micronutrient contributes in fatty acid synthesis and energy production [1-3]. Vitamin B_{12} was firstly extracted from liver and kidney [2,4]. Chemical production of vitamin B_{12} includes 70 steps (due to the complexity of vitamin B_{12} molecule)[5]. So, scale up production of vitamin B_{12} has been limited to fermentation process by *Propionibacterium* (*P.*) *freudenreichii* ssp. shermanii and *Pseudomonas denitrificans* [6,7]. *P. freuden-reichii* sp. shermanii is preferred, because is generally recognized as safe by United States, Food and Drug Administration [6].

Several investigations have been conducted so far to produce vitamin B_{12} using different carbon sources such assucrose [8,9], whey [10], glucose [11], molasses [8], crude glycerol [12], and dairy waste [13], in batch [14], continuous [15] and fed-batch fermentation [11]. In previous reports, 11 process var-iables have not been considered in a screening design for simultaneous production of vitamin B_{12} and propionic acid in fermented dairy beverage by the co-culture of *P. freudenreichii* together with *L. acido-philus.* Such inoculation may produce fermented milk containing short-chain organic acids such as propion-ic acid, which leads to satiety in consumer due to delay in gastric emptying and secretion of special signals [16]. Moreover, the produced fermented milk contains vitamin $B_{12}$with high nutritional value resulted from growth of probiotic microorganisms.

The aim of this study was to evaluate the impact of 11 process variables on vitamin B_{12} production in a fermented dairy beverage by Plackett-Burman design by using the adjunct starter cultures. There is no comprehensive study till now to show influencing process variable on vitamin B_{12} production in such fermented drink. Also organoleptic properties of product was investigated.

EXPERIMENTAL SECTION

Microorganisms and media

P. freudenreichii ssp. shermanii DSM 20270 and *L. acidophilus* LA5 were obtained from the DSMZ collection (DSM Gist Brocade Australia Pty. Ltd., Sydney, Australia) and Chr-Hansen (Chr-Hansen Pty. Ltd., Horsholm, Denmark). *P. freuden-reichii* ssp. shermanii was grown in conservation medium, which contained per liter of deionized water: 1 g KH_2PO_4, 2 g $(NH_4)_2HPO_4$, 5 mg $FeSO_4{\cdot}7H_2O$, 10 mg $MgSO_4{\cdot}7H_2O$, 2.5 mg $MnSO_4{\cdot}H_2O$, 10 mg $CaCl_2{\cdot}6H_2O$, 10 mg $CoCl_2{\cdot}6H_2O$, 5.0 g yeast extract, 5.0 g sodium lactate, and 7.0 g Agar. The pH value was adjusted to 6.8 before autoclaving. The pre-culture and the inoculum media had the same composition as the conservation medium, except that sodium lactate and yeast extract concentration was increased to 20 and 10 g/l, respectively in a broth [17]. The fermentation medium was 1 l of basal medium with the same composition as pre-culture and 200ml of molasses. The basal medium, nitrogen source and fed were autoclaved separately. Feeding solution contained 300ml of skim milk or 0.3ml of sugar solution (both contains 23g of lactose).

Inoculum preparation

An isolated colony from deep agar plate was transferred into 2ml of the pre-culture medium, and incubated at 30°C for 48 h. Then small amount of this culture (400µl) was transferred into 40ml screw-cap flask containing 40ml of the inoculum medium broth. Propionibacter was cultured without agitation for 24-36h at 30°C (until A660≈0.8). According to the results of Farhadi *et al*., the best ratio for *P. freudenreichii* sp. shermanii and *L. acidophilus* was adjusted by 4:1 in 30°C [18]. Inoculum volume was another factor evaluated in the present study.

Fed-batch fermentation

Molasses and nitrogen source were added to the fermentation medium. The fermenter was fed by using different concentrations of lactose or milk (depending on trial combination). Feeding was initiated 35 h after inoculation. Fed-batch ferment-ations were performed under anaerobic conditions using a 3-L bioreactors (2 L working volume). Temperature and pH were controlled to maintain at 30°C and 6.8, respectively.

Sensorial evaluation

Evaluation of organoleptic properties were carried out by scoring of the fermented beverage and Doogh (Iranian dairy beverage) control sample by thirty trained assessors with 48 h interval for 6 days after the production. The samples were served in plastic cups with no color or odor at 20º C. The assessors were asked to evaluate each sample for flavor, odor and color. An evaluation sheet with a 1-5 scales was utilized to indicate the score of the samples as extre-mely dislike equaled = 1, and extremely like equaled = 5 [19,20].

Table 1: The experimental Plackett-Burman design and corresponding vitamin B_{12} production in a dairy beverage.

Trial	pH	Temperature (°C)	Molasses con (g/l)	Feeding strategy	Feeding rate (l/h)	Feeding substrate	Inoculum % v/v	Co-culture	Time (h)	Type of nitrogen source	Nitrogen source conc. (g/l)	Vitamin B_{12}						Propionic acid (g/L)
												Concentration (mg/l)		Yield (mg/g)		Productivity (mg/lday)		
												exp§	pred§§	exp	pred	exp	pred	
1	7.5	30	45	Step	0.03	Milk	5	P.f †+L.a‡	96	CSL§	10	3.00	3.01	1.01	0.16	0.75	0.75	6.66
2	7.5	36	25	Continuous	0.03	Milk	1	P.f +L.a	96	Y.E¶	5	4 .00	4.01	0.56	0.00	1.00	1.00	0.29
3	6.5	36	45	Step	0.04	Milk	1	P.f	96	Y.E	10	11.00	10.99	1.56	5.71	2.75	2.75	0.35
4	7.5	30	45	Continuous	0.03	Lactose	1	P.f	144	Y.E	10	0.00	-0.01	0.00	0.00	0.00	0.00	5.57
5	7.5	36	25	Continuous	0.04	Milk	5	P.f	144	CSL	10	23.00	22.99	14.37	10.63	3.83	3.83	1.28
6	7.5	36	45	Step	0.04	Lactose	1	P.f +L.a	144	CSL	5	3.00	3.03	1.28	0.00	0.50	0.50	0.49
7	6.5	36	45	Continuous	0.03	Lactose	5	P.f	96	CSL	5	29.00	28.97	8.38	10.43	7.25	7.25	2.85
8	6.5	30	45	Continuous	0.04	Milk	5	P.f +L.a	144	Y.E	5	2.00	2.01	0.44	2.49	0.33	0.33	5.83
9	6.5	30	25	Continuous	0.04	Lactose	1	P.f +L.a	96	CSL	10	30.00	30.01	10.75	14.48	7.50	7.50	3.95
10	7.5	30	25	Step	0.04	Lactose	5	P.f	96	Y.E	5	0.05	0.07	0.07	0.00	0.01	0.01	1.67
11	6.5	36	25	Step	0.03	Lactose	5	P.f +L.a	144	Y.E	10	0.05	0.03	0.01	2.04	0.01	0.01	2.73
12	6.5	30	25	Step	0.03	Milk	1	P.f	144	CSL	5	0.05	0.03	0.01	3.75	0.01	0.01	5.37

† Propionibacterium freudenreichii, ‡ Lactobacillus acidophilus, § corn steep liquor, yeast extract, § experimented, §§ predicted yield which was calculated for each experiment by $Y_i = \sum_{i=0}^{N} Ai.Ki$*, where, A_i is each coefficient's value and K_i is the code of each parameter according to the design*

Design of experiments

The Plackett-Burman design (PBD) [21] was used to evaluate the relative importance of various factors for vitamin B_{12} production in the fermented milk product. The design is useful in biotechnology screening research [22,23]. The application of PBD is for evaluating the multi-variables experiments (e.g. with variable of 7, 11 and 15 numbers). The effective factors and their levels were selected based on the literature review and pre-experience [2]. The numerical range of each variable should be wide enough but practically reasonable [22]. The important criteria to choose each factor settings for any two-level screening design have been mentioned elsewhere [22]. Table 1 shows the selected exper-imental variables (i.e. pH, temperature, molasses concentration, type of feeding, feeding strategy, rate of feeding, inoculum volume, co-culture, time, type of nitrogen source, and nitrogen source) and their levels, as well as a PBD for conducting 12 treatment combinations for fermented drink production.

Dry weight

The dry weight of biomass was estimated after centrifugation at 15350 ×g for 10 min at 4°C and drying to constant weight using freeze dryer interval of 12 h.

Vitamin B_{12} concentration

Each run was conducted in triplicate measurement of vitamin B_{12} concentration. Extraction of this intracellular metabolite was performed by boiling in 0.1 M potassium cyanide for 15 min at the pH of 6.0. After filtration of the obtained solution with 0.45 µm syringe filters, vitamin B_{12} concentration was measured by HPLC system equipped with Backman pump (B110, Shimadzu, 25 mm× 4.6 mm; 5 µm). A mixture of phosphate buffer and 10% of

Table 2: Biomass concentration during 6 days of fed-batch production of fermented beverage containing vitamin B_{12} using Plackett-Burman design.

Biomass (g dry weight/L)						
Trials	24 (h)	48 (h)	72 (h)	95 (h)	120 (h)	144 (h)
1	0.38±0.05	0.73±0.03	1.79±0.06	2.98±0.01	-	-
2	0.88±0.03	1.45±0.02	4.60±0.02	5.08±0.06		
3	1.40±0.02	4.46±0.05	5.05±0.03	5.40±0.06		
4	0.30± 0.09	0.67± 0.06	0.95±0.03	1.14±0.05	1.82±0.02	2.10±0.06
5	0.09±0.03	0.36±0.03	0.55±0.04	0.83±0.03	1.34±0.04	1.61±0.04
6	0.67±0.03	1.52±0.02	2.15±0.05	2.24±0.03	2.31±0.04	2.35±0.04
7	0.86±0.03	3.08±0.04	3.36±0.04	3.46±0.03		-
8	1.68±0.07	2.95±0.04	3.23±0.03	3.88±0.02	4.27±0.05	4.56±0.03
9	1.42±0.05	2.55±0.04	2.82±0.04	2.79±0.03		-
10	0.17±0.04	0.36±0.04	0.67±0.03	0.75±0.05		-
11	0.96±0.07	1.39±0.04	2.12±0.04	2.97±0.09	3.34±0.04	3.55±0.04
12	3.65±0.04	4.33±0.06	5.52±0.02	5.55±0.04	6.16±0.03	6.22±0.04

acetonitril as rectifier was the mobile phase. HPLC grade water with the EC of 0.8 microsiemense was used.

Statistical analysis

Statistical analysis of the results was performed using Design Expert software (version 9.0). All data are presented as mean value ± standard deviation (±SD) of two independent experiments at different days. A *P*-value equal or below 0.05 (presented as $P \leq 0.05$) was considered statistically significant.

RESULTS AND DISCUSSION

The experimental levels of 11 variables that are investigated in this design 1 and corresponding vitamin B_{12} production are illustrated in Table 1. The results of produced biomass concentrations at the end of the fermentation and during each trial are shown in Table 2.

Also Fig. 1 shows the concentration of produced vitamin B_{12} and dry biomass profiles during the fermentation in each run. TABLE 3illustrates the coefficient of each parameter on vitamin B_{12} production in a dairy beverage. The positive value of A_i indicates that the high level of variable leads to more production, and the negative value of A_i indicates that the low level caused to more response. Significant parameters were identified by the student's *t*-test. Degree of freedom is 10 (number of variables – 1), and α is 0.05. Statistical calculations are shown in Table 3.

Table 4 shows organoleptic properties of produced fermented beverage in conditions of treatment combination number 9 of Plackett-Burman design. As the results shows the beverage has total actability from view of flavor, color and odor of the new introduced beverage. This is similar to the find-ings of some researchers who showed that probiotics did not affect the overall acceptance of functional foods [25-27]. The results showed wide concentration of produced vitamin B_{12} in the twelve trials (0 to 30 mg/l). This wide spectrum of results reveals the great impact of process variables on vitamin B_{12} production and correct range finding which has led to changes in response by changing the levels of independent variable. Like other acid producing microorganisms, growth of *P. freudenreichii* ssp. shermanii strongly depends on pH [9].It has been reported that growth of *P. freudenreichii* will be suppressed in a culture with pH less than 4.5 [18]. Previous reports showed that pH range of 6.5-8.5 is suitable for vitamin B_{12} production by this microorganism [9]. In the present study, the pH of 6.5 was found to be the most suitable for biomass and vitamin B_{12} production in pH range of 6.5 and 7.5. Moreover, maximum productivity was obtained in trial 9 when the pH of fermentation was 6.5 (Table 1).

Table 3: Statistical data for analysis of the effect of process variables on vitamin B_{12} production in fed-batch fermentation based on Plackett-Burman design†.

Variables	Vitamin B_{12}						Biomass Concentration (g/l)	
	Concentration (mg/l)		Yield (mg/g)		Productivity (mg/lday)			
	Coeff§.	t-value	Coeff.	t-value	Coeff.	t- value	Coeff.	t-value
pH	-3.25	184.65	-0.322	0.128	-0.980	9.544	-0.896	56.709
Temperature (°C)	2.91	165.34	1.157	0.459	0.561	5.462	0.473	29.936
Molasses concentration (g/l)	-0.76	43.18	-1.092	0.433	-0.065	0.633	0.042	2.633
Feeding strategy	5.90	335.28	2.547	1.015	1.323	12.882	-0.108	6.854
Feeding rate (L/h)	2.74	155.68	1.542	0.611	0.491	4.781	-0.528	33.418
Feeding substrate	1.51	90.34	0.212	0.084	0.550	5.355	-1.206	76.329
Inoculum volume (%)	0.75	42.61	0.002	0.001	0.035	0.341	-0.89	56.329
Co-culture	-1.7 5	99.43	-0.862	0.342	-.313	2.950	0.178	11.266
Time (h)	4.08	231.82	0.518	0.205	1.215	11.830	0.31	19.620
Type of nitrogen source	-5.91	335.80	-2.763	1.096	-1.311	12.765	0.473	29.936
Nitrogen concentration (g/l)	2.41	136.93	1.413	0.560	0.478	4.654	-0.363	22.975

§The coefficient of each parameter was computed by $A_i = \frac{1}{N}\sum_1^N xiki$, where, A is the effect of the evaluated parameter, X_i is the response (vitamin B_{12} production) from the experimental trials in which the variables being tested are at the maximum and minimum levels (shown by K_i), and N is the number of trials totally. A_0 is sum of yields divided by N.

†Standard error is given by $Se^2=\sum_{i=1}^{12}(Yi-yi)$, where, ($Y_i$ –y_i) is the difference between the experimental and predicted yields. The estimated error is computed by $S_b=\sqrt{se^2/N}$. Amount of t for each variable is the ratio of coefficient to the estimated error.

Quesada *et al*. had reported that the optimal temperature for vitamin B_{12} production is 40° C [9]. The results of this study showed 30° C as optimal temperature in the fed-batch fermentation of vitamin B_{12} by *P. freudenreichii*.

Growth of many microorganisms depends on the substrate concentration; indeed, high substrate con-centration cause inhibition of the growth. The data of present work shows that increased concentration of substrate stimulate biomass production of *P. freuden-reichii* ssp. shermanii. As shown in Table 3, high substrate concentration has no inhibitory effect on the growth of *P. freudenreichii* ssp. *Shermanii*. So, the biomass production continues at high substrate con-centration that ultimately leads to a prolonged logari-thmic growth. It can be concluded that the micro-organism does not enter the stationary phase and produce of secondary metabolite of vitamin B_{12}.

The two strategies of feeding fermentor was applied (continuous and stepwise) and compared in the present study for vitamin B_{12} production. Continuous feeding shows more effective than step feeding which is probably due to less substrate limitation. So length of logarithmic growth increased, and production of secondary metabolite related to the stationary phase would be postponed.

To our knowledge, there is no research report on the effect of feeding rate on vitamin B_{12}production in the fed-batch fermentation of vitamin B_{12} by *P. freudenreichii*. In one report fed-batch fermentation of *P. acidipropionici* was used [28]. In the present study, the effect of two feeding rates (0.03 and 0.04 l/h) was also investigated. The results showed that the higher feeding rate could provide required carbon source, and lead to increased growth and production. The maximum productivity (7.5 mg/l.day), yield (14.37 mg vitamin B_{12}/g biomass) and concentration (30 mg/l) of vitamin B_{12} were obtained in 0.04 l/h rate of feeding.

In this experiment, lactose or milk was used as feed. Because of complex composition of milk, the microorganism showed better growth and vitamin production in the presence of lactose solution.

Coral *et al*. reported no significant impact for inoculum size (1, 2 and 5% v/v) on propionic acid

Table 4: Organoleptic properties of produced fermented beverage in conditions of treatment combination 9 of Plackett-Burman design† .

Sensory characteristics§§	Flavor				Color				Odor			
Day Score	1st	3rd	6th	Control	1st	3rd	6th	Control	1st	3rd	6th	Control
Average score§	4.7	4.8	4.6	4.7	4.6	4.8	4.6	4.8	4.6	4.7	4.7	4.7
Total score‡	145	148	145	146	147	148	147	146	147	146	148	148

§ The range of scores is from 1 to 5

‡ Scored by thirty trained panelists

† Data does not show any significant difference between samples and control during 6 days after production

§§ Doogh as an traditional Iranian dairy beverage was selected as control

production [17]. They used 1% (v/v) inoculum volume. However, from the results of present study, it can be concluded that inoculum size is an important factor for vitamin B_{12} production, such that the increase in inoculum volume led to more vitamin B_{12} production. The maximum yield of vitamin B_{12} (14.37 mg vitamin B_{12} per g biomass) was obtained when the inoculum volume was 1% (v/v).Taking into account that daily recommended dose for vitamin B_{12} is 2.4 micrograms for adults, by consumption of 0.1 ml of this beverage into each drink vitamin is sufficient.

Table 1 shows that the maximum yield of vitamin B_{12} were obtained in trial combinations 5, 7 and 9, respectively. Table 2 shows data about biomass production which are high in trial combination 12. By comparison of results of Tables 1 and 2, it can be concluded that there is no direct relation between biomass and yield production. In run number 9 the fermentation duration was 96 h, so the cells had not enough time (in compared to 144 h of trials 4, 5, 6, 8, 11 and 12) for more proliferation and biomass production. In fact, production of higher amounts of cells may not necessarily cause increased yield of vitamin production. By the other words, some environmental condition acts as stimulator of vitamin production while some are growth promotor. This observation is not far from general definition about second metabolite like vitamin B_{12} which mainly produced in stationary phase. Anyway, in trial number 9 both productivity and yield of productions are in their high amount in compare to other runs.

Results also showed that using the co-culture of *L. acidophilus* and *P. freudenreichii* decreased vitamin B_{12} production. In the presence of *L. acidophilus*, at first, all of lactose was consumed, and a large amount of lactic acid was produced (unpublished data).

Previous reports suggest that 144 his more suitable for vitamin B_{12} and biomass production [17]; however, in this study, the maximum productivity of 7.5 mg/l.day was obtained in 96 hours. Since length of a process is a significant key factor in successful scaled up production, the results of this study justify the requirements of industrial production of vitamin B_{12} in terms of time.

CSL (as a by-product of the corn wet-milling) is a renewable and cheap source of nitrogen, and may have different compositions depending on the methods of preparation. CSL could contain different carbohydrates such dextrose, maltobiose and maltotriose [29] which can be used as extra carbon source for vitamin B_{12} production [29]. Our results showed that vitamin B_{12} is associated with high concentration of the nitrogen source, and high concentration of nitrogen source produces high concentration of vitamin B_{12}.

The present study the most influencing process variables which has impact on vitamin B_{12} production in a dairy beverage containing propionic acid were determined. Further research using central composite design is suggested up to obtain the optimized conditions of fermentation. Also investigation of details of the metbabolic engineering of process of propionic acid is recommended. Although beverage containing vitamin B_{12}, propionic acid, and *P. freudenreichii* has potential for health beneficial properties, their use as probiotic drink still needs further research and more endeavors, including raw material type/ratio, safety, etc. The effect of *P. freudenreichii* and its metabolite on the function of probiotic drink needs more investigation.

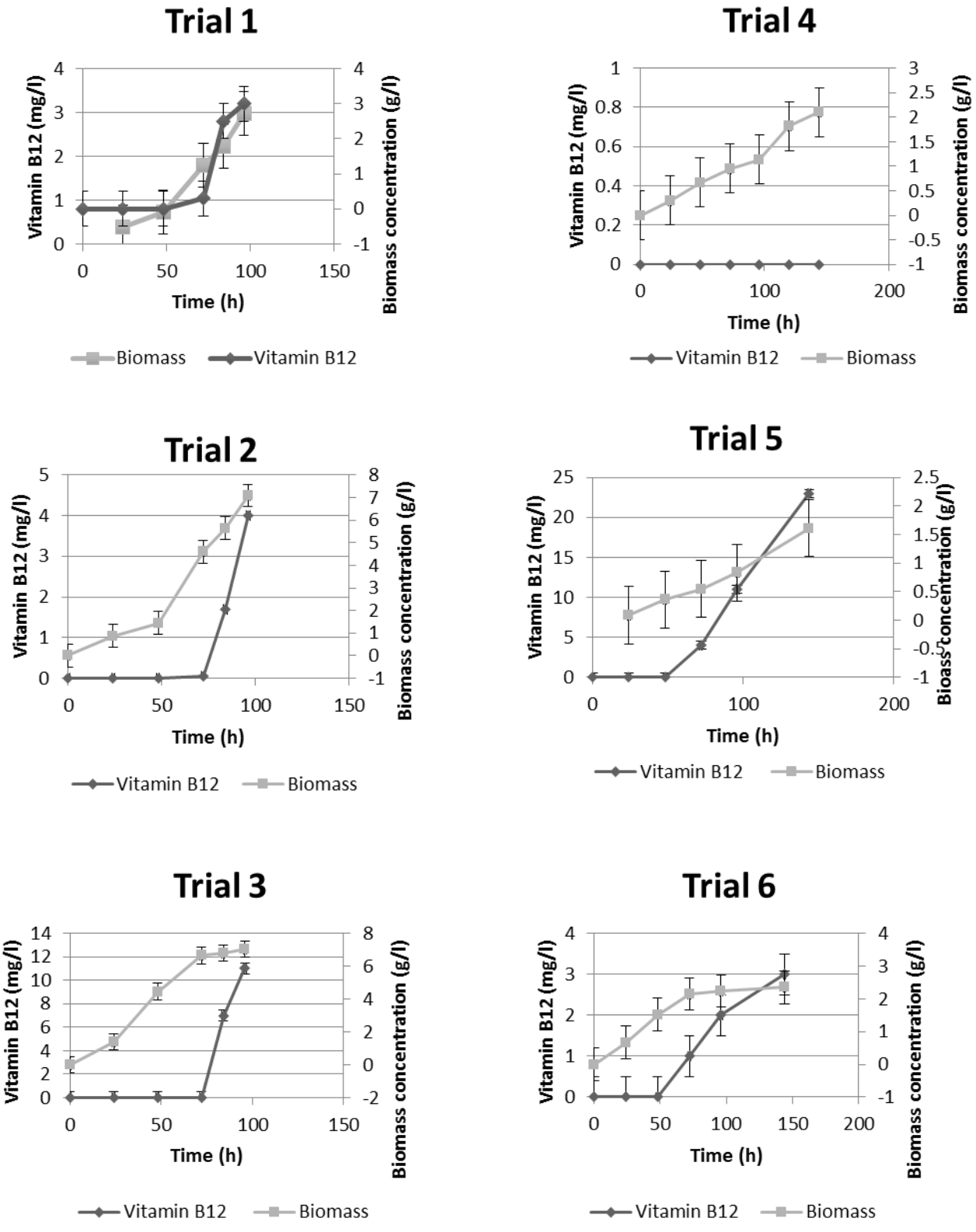
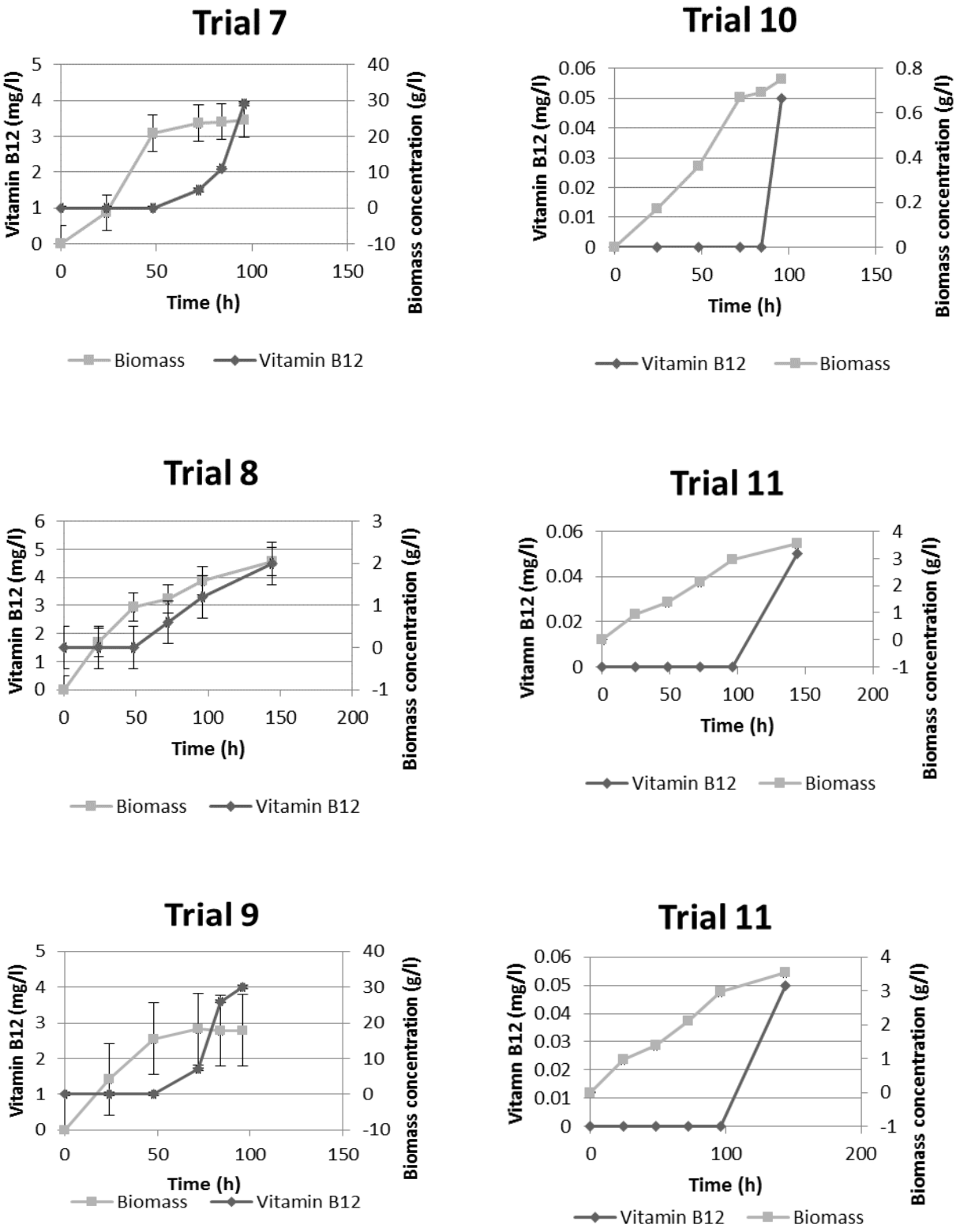


Fig. 2: Vitamin B_{12} and dry biomass production profiles during the fed-batch fermentation in 12 trials of Plackett- Burman design.



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