بهینه‌سازی تولید سیانوکوبالامین توسط *Propionibacterium freudenreichii* در غلظت‌های مختلف فولیک‌اسید و متیونین

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چکیده

مقدمه: ویتامین B_{12}، عاملی کلیدی در بسیاری از فرایندهای مرتبط با رشد در بدن انسان است و تولید صنعتی آن در مقیاس وسیع با استفاده از ریزموجودات به‌ویژه *Propionibacterium freudenreichii* انجام می‌شود. مطالعهٔ حاضر با هدف بررسی تأثیر فولیک‌اسید، متیونین و اکسیژن بر تولید بهینهٔ ویتامین B_{12} و وزن خشک سلولی (DCW) در این باکتری انجام شد.

مواد و روش‌ها: باکتری در چهار شرایط مختلف شامل شرایط بی‌هوازی و غلظت‌های مختلف فولیک‌اسید، شرایط هوازی و غلظت‌های مختلف فولیک‌اسید، شرایط بی‌هوازی و غلظت‌های مختلف فولیک‌اسید و متیونین ۰/۰۵ درصد (w/v)، شرایط هوازی و غلظت‌های مختلف فولیک‌اسید و متیونین ۰/۰۵ درصد (w/v) کشت داده شد.

نتایج: بیشترین تولید ویتامین B_{12} و DCW در غلظت ۷۵۰ میلی‌گرم‌درلیتر فولیک‌اسید و در حضور متیونین و در شرایط هوازی به دست آمد؛ با افزایش غلظت فولیک‌اسید تا ۱۰۰۰ میلی‌گرم‌برلیتر، میزان تولید ویتامین B_{12} و وزن خشک باکتری کاهش یافت.

بحث و نتیجه‌گیری: باتوجه‌به تأثیر فولیک‌اسید در فرایندهای تخمیری *Propionibacterium freudenreichii*، مطالعهٔ حاضر می‌تواند بهینه‌سازی مؤثر برای افزایش تولید نهایی ویتامین B_{12} باشد. باتوجه‌به یافته‌های پژوهش حاضر، شرایط هوازی به همراه حضور متیونین بر بهبود تولید ویتامین B_{12} مؤثر است و این بهینه‌سازی می تواند به‌عنوان راهبردی مؤثر بر بهبود تولید ویتامین B_{12} در سطح صنعتی عمل کند.

واژه‌های کلیدی: ویتامین B_{12}، *Propionibacterium freudenreichii*، فولیک‌اسید، متیونین، کروماتوگرافی مایع با کارایی بالا (HPLC)

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Optimization of Cyanocobalamin Production by *Propionibacterium Freudenreichii* in Different Concentrations of Folic Acid and Methionine

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Abstract

Introduction: Vitamin B_{12} is a key factor in many growth-related processes in the human body and its large-scale industrial production is carried out using microorganisms, especially *Propionibacterium freudenreichii*. The aim of this study was to evaluate the effect of folic acid, methionine, and oxygen on the optimal production of vitamin B_{12} and Dry Cell Weight (DCW) of this bacterium.

Materials and methods: Bacteria were maintained in four different conditions including anaerobic conditions and different concentrations of folic acid, aerobic conditions and different concentrations of folic acid, anaerobic conditions and different concentrations of folic acid and methionine 0.05% (w/v), aerobic conditions and different concentrations of folic acid and methionine 0.05% (w/v).

Results: The maximum production of vitamin B_{12} and DCW was obtained in 750 mg / L of folic acid in the presence of methionine and aerobic conditions. By increasing the concentration of folic acid up to 1000 mg / L, the amount of vitamin B_{12} production and dry weight of the bacterium decreased.

Discussion and conclusion: Due to the effect of folic acid on the fermentative processes of *Propionibacterium freudenreichii*, the present study could be an effective optimization to increase the final production of vitamin B_{12}. Based on the above findings, aerobic conditions along with the presence of methionine can be effective in improving vitamin B_{12} production and this optimization may help as an effective strategy to improve vitamin B12 production at the industrial level.

Keywords: Vitamin B_{12}, *Propionibacterium Freudenreichii*, Folic Acid, Methionine, High-performance Liquid Chromatography (HPLC).

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Introduction

Vitamin B_{12} or cobalamin, is a water-soluble vitamin that belongs to the corrinoid compound group (1) which has various exclusive biochemical functions in the brain, nervous system, and cellular metabolisms. It especially plays an important role in fatty acids synthesis and energy production in the human body (2). Vitamin B_{12} is essential for the function of the nervous system (3), and its deficiency may have a variety of symptoms such as neuropathy, nervous system disease, and megaloblastic anemia (4). The chemical production of vitamin B_{12} is not profitable due to its complicated process which involves 70 steps (1). Therefore, vitamin B_{12} production is limited to the industrial fermentation process of bacteria such as *Propionibacterium freudenreichii* and *Pseudomonas denitrificans,* which is a rapid and low-cost procedure (5). Two different pathways are applied for this purpose, including the aerobic pathway in *Pseudomonas denitrificans* and the anaerobic pathways in *Salmonella typhimurium*, *Propionibacterium freudenreichii* subspecies *shermanii,* and *Bacillus megaterium* (6). *Propionibacterium freudenreichii* belongs to the *Propionibacteriaceae* family and uses various sources of carbon to produce vitamin B_{12}, such as glucose (7), molasses (8), and crude glycerol (9). *Propionibacterium* ssp. are capable of producing a wide range of biological compounds that enhance human health such as folic acid, proline, conjugated linoleic acid, and vitamin B (10). Folate is the generic name for a large number of chemical derivatives of pteroylglutamic acid (PGA), is also known as folic acid. It has interference with the basic activities of cellular metabolisms, such as DNA repair, methylation, and the biosynthesis of nucleotides, as well as vitamins and amino acids (11). Folate deficiency is associated with a large number of disorders, including Alzheimer's disease, cardiovascular diseases, osteoporosis, the increased risk of breast and colorectal cancer (12-14). Folic acid is commonly applied to improve the nutritive value of the foods and is used as a nutritional supplement (15). Various compounds are necessary in the synthesis of vitamin B_{12} including 5, 6-dimethylbenzimidazole (DMBI). The presence of oxygen in the growth medium of *Propionibacterium* plays an essential role in the production of DMBI (16, 17). Cobalt and DMBI are often added to the industrial production of vitamin B_{12} because they are required for the formation of the corrin ring and the lower ligand molecule respectively and are considered limiting factors in vitamin B_{12} (18, 19). The role of amino acids on the growth and production of vitamin B_{12} has been studied. For example; the presence of methionine increases the levels of vitamin B_{12} because methionine is needed for various methylation processes in the ovarian environment (20). Although many studies have been proposed on the anaerobic biosynthesis of Vitamin B_{12} in *Propionibacterium* in terms of its genetic and biochemical characterization, the effect of folic acid on this bacterium has not been addressed yet. In this study, the authors have tried to develop new strategies for *Propionibacterium freudenreichii* to enhance vitamin B_{12} production. In particular, this study aimed to determine the effect of methionine and different concentrations of folic acid on the optimal production of vitamin $_{B12}$ and dry cell weight in aerobic and anaerobic conditions.

Materials and Methods

Microorganisms and Mediums: Based on a preliminary investigation regarding the effect of folic acid on the cell growth and vitamin B_{12} biosynthesis in *Propionibacterium freudenreichii,* four concentrations of folic acid (control, 250, 750, and 1000 mg/L) were added to the medium culture. Table 1 shows various methods for optimizing vitamin B_{12} production used in this study, including the effect of anaerobic and aerobic conditions and the presence of methionine on bacterial culture medium at different concentrations of folic acid.

Table 1. Different Methods for Optimizing the Production of Vitamin B12

Determined Vitamin B_{12} production in:
1) Anaerobic conditions and different concentrations of folic acid.
2) Aerobic conditions and different concentrations of folic acid.
3) Anaerobic conditions and diverse concentrations of folic acid and methionine 0.05% (v/w).
4) Aerobic conditions and different concentrations of folic acid and methionine 0.05% (v/w).

Propionibacterium freudenreichii (PTCC: 1674) was purchased from the Iranian Research Organization for Science and Technology (IROST, Karaj, Iran) (ptcc.irost.org). Each culture medium was composed of the following components (per liter of deionized water): lactate sodium 1%, extract yeast 1% (as nitrogen source) (Merck, Germany), trypticase soy broth 1% (Sigma-Aldrich), 10 g of tryptone (Biolife), 90 g of glucose (as carbon source), and 20 g of Corn Steep Liquor (CSL). The fermentation medium supplements were composed of the following (per liter of deionized water): control, 250, 750 and 1000 mg/L concentrations of folic acids (single carbon donor or a recipient) (Merck, Germany), 100 mg of DMBI (Sigma-Aldrich), 100 mg of cobalt chloride (2), 0.05% (v/w) of methionine as methyl donor (20Marwaha et al. 1983), KCN: 1% (w/v) (21), 2.5 mL of glacial acetic acid, and $NaNO_2$ 8 %(w/v) (22).

Production of Vitamin B_{12} in the Anaerobic Conditions and Different Concentrations of Folic Acid: The culture medium was incubated for 80 h at 30 ºC in the anaerobic jar systems and the fermentation medium (50 mL) was approximately normalized at the pH of 7. Folic acid was added to the different fermentation media at the concentration of control, 250, 750, and 1000 mg/L and subsequently, the media were kept for 4 days at 30 ºC.

Production of Vitamin B_{12} in the Aerobic Conditions and Different Concentrations of Folic Acid: *Propionibacterium freudenreichii* were cultured under the same conditions as done in the anaerobic ones, but the aerobic conditions were created for them. The culture medium was extracted from jars anaerobic system; subsequently, the cap was removed and placed on a shaker (Lap companion) for a period of 72 h at 134 rpm (23, 24).

The Effect of Folic Acid in the Anaerobic Conditions and the Presence of Methionine: The bacteria were cultured similar to the anaerobic conditions, and methionine 0.05 % (w/v) was simultaneously added to the different media with folic acid (at the concentration of control, 250, 750, and 1000 mg/L) and subsequently, the media were kept for 4 days at 30 ºC.

The Effect of Folic Acid in the Aerobic Conditions and the Presence of Methionine: The bacteria were cultured in anaerobic conditions the same as previous conditions, and folic acid (at concentrations of control, 250, 750 and 1000 mg/L) along with methionine 0.05% (w/v) were added to the different media. Subsequently, the aerobic conditions were created for them, as done before.

Quantification of the Dry Cell Weight: The cultures medium of *Propionibacterium freudenreichii* were sterilized by autoclaving at 121 ºC for a period of 15 min followed by centrifugation at 14000 rpm for 10 minutes at 4 ºC. The cell precipitation was oven-dried at 70 ºC for 48 h and then weighted.

Determination of the Produced Vitamin B_{12}: The concentrations of the fermentation products were determined through HPLC (model: SY-8100). Biosynthetic vitamin B_{12} was used in this study as the standard for quantification (Merck Company). The broth sample (25mL) was added with 2.5 mL of $NaNO_2$ 8 % (w/v) and 2.5 mL of glacial acetic acid. The mixture was boiled for 30 min, followed by adding potassium cyanide 1% (w /v) and filtering. The mixture was injected by a C18 column (5µm, 4.6µm× 25cm) with a flow rate of 1.0 mL/min and a wavelength of 254 nm in 25 ºC. The mobile phases consisted of KH_2PO_4 (.02 M) methanol (70/30 v/v).

Statistical Analysis: The mean values were obtained based on the measurements of three replicates and all quantifications were done based on Duncan's multiple range tests for the discrimination of significance (defined as $P<0.05$). Data were represented as the mean ± standard errors of the mean (SEM). Analyses were carried out using the statistical analysis system software (SPSS 19) and graphs were drawn by Excel 2007.

Results

Vitamin B_{12} Production and Cell Growth under Aerobic and Anaerobic Conditions: Production levels of vitamin B_{12} with different concentrations of folic acid increased in the aerobic conditions, compared to the non-aerobic ones. In the anaerobic conditions, there was no significant difference in vitamin B_{12} production between the various concentrations of folic acid. At 750 mg/L concentration of folic acid, the amount of vitamin B_{12} production in the aerobic conditions was about 5-times ($P<0.05$) more than that obtained in similar conditions in the anaerobic medium. However, at the 1000 mg/L and 250 mg/L concentrations of folic acid vitamin B_{12} level increased 3 and 2.1 times in comparison to the anaerobic conditions, respectively, but the differences were not significant ($P>0.05$). Under the anaerobic conditions, no significant difference was observed between different concentrations of folic acid (Fig. 1).

In the aerobic conditions, the highest cell dry weight was recorded in the concentration of 750 mg/L of folic acid which was significantly different from other concentrations ($P<0.05$), while there was no significant difference between other concentrations ($P>0.05$). In the anaerobic conditions, there was no significant difference between each concentration ($P>0.05$). The dry cell weight increased in the aerobic conditions in comparison to the anaerobic conditions with a 1.8-time increase at the concentration of 750 mg/L (Fig. 2).

The Effect of Folic Acid and Methionine in the Anaerobic Conditions on the Production of Vitamin B_{12} and the Cell Growth: The results of HPLC indicated a significant difference in the production of vitamin B_{12} in the presence of methionine and at the 750 and 1000 mg/L concentrations of folic acid, in which the highest level was observed at 750 mg/L of folic acid. In the presence of methionine, the level of vitamin B_{12} increased significantly, as compared to its absence. Also, at 750 mg/L concentration of folic acid together with methionine, the production of vitamin B_{12} was approximately 6-folds more and in 1000 mg/L concentration of folic acid, it was 4 times more than the recorded level in the absence of methionine. However, no significant difference was observed in the control group (Fig. 3).

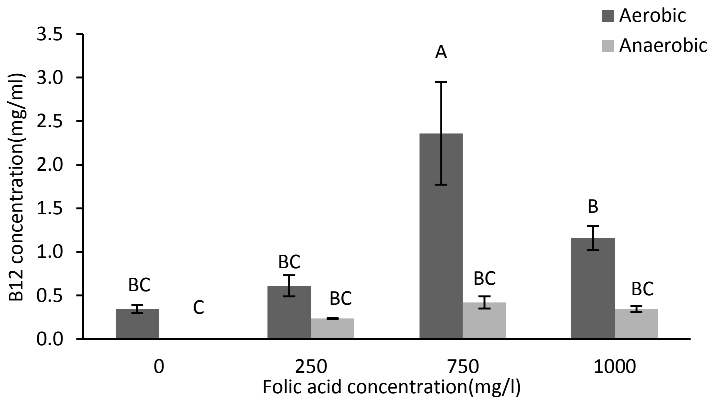

Fig. 1- The effect of different concentrations of folic acid (control, 250, 750, and 1000 mg/L) on the amount of vitamin B12 production by *P.freudenreichii* in the aerobic and anaerobic. The data are expressed as the mean of three replicates ± standard error of the mean. Non-similar letters express the meaning of the data according to the Duncan's ($P<0.05$) assay: A indicates the highest value, B is the highest value after A, C is the lowest value, and BC is the intermediate value between B and C.

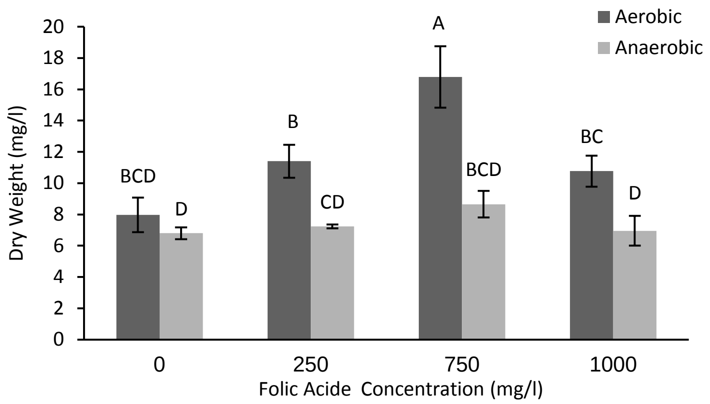

Fig. 2- The effect of different concentrations of folic acid (control, 250, 750, and 1000 mg/L) on the bacterial growth in aerobic and anaerobic conditions. The data are expressed as the mean of three replicates ± standard error of the mean. Non-similar letters express the meaning of the data according to the Duncan's ($P<0.05$) assay: A indicates the highest value, B is the highest value after A, C is the value after B, and BC is the intermediate value between B and C, D indicates the lowest value, BCD is the average value between B, C and D, and CD is the intermediate value between C and D.

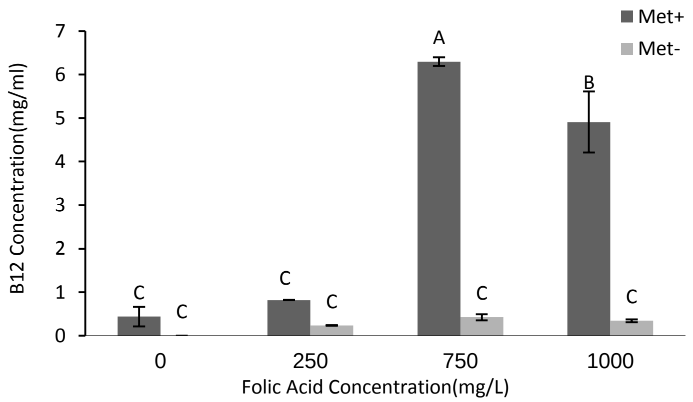

Fig. 3- The effect of different concentrations of folic acid (0, 250, 750, 1000mg/L) on the production of B12 in the presence (open bar) and absence (close bar) of methionine (Met) under the anaerobic conditions by *P.freudenreichii*. The data are expressed as the mean of three replicates ± standard error of the mean. Non-similar letters express the meaning of the data according to the Duncan's ($P<0.05$) assay: A indicates the highest value, B is the highest value after A, and C is the lowest value (Met=Methionine).

In anaerobic conditions, there was no significant difference in the dry cell weight between the groups whether methionine was available in the medium or not (*P*>0.05). At 750 mg/L concentration of folic acid in combination with methionine, the dry weight decreased significantly in relation to the control group. Besides, adding methionine to the fermentation medium increased the dry weight of the control group approximately 2.4 times more than the recorded data in the absence of it. The dry weight of the bacteria decreased as folic acid concentration increased the concomitant with methionine in the anaerobic conditions, which showed the negative effect of the high concentration of folic acid on the bacterial growth (Fig. 4).

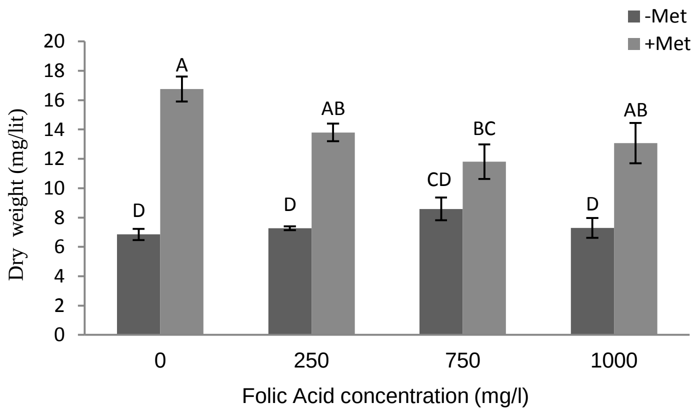

Fig. 4- The effect of folic acid (control, 250, 750, and 1000 mg/L) on the bacterial growth and dry cell weight in the presence and absence of methionine under the anaerobic conditions. The data are expressed as the mean of three replicates ± standard error of the mean. Non-similar letters express the meaning of the data according to the Duncan's ($P<0.05$) assay (Met=Methionine). A indicates the highest value, B is the highest value after A, AB is the intermediate value between A and B, C is the value after B, and BC is the intermediate value between B and C, D indicates the lowest value, CD is the intermediate value between C and D.

The Effect of Folic Acid and Methionine in the Aerobic Conditions on the Production of Vitamin B_{12} and the Cell Growth: In the aerobic and anaerobic conditions and presence of methionine, the highest level of vitamin B_{12} production was at the 750 mg/L of folic acid and subsequently at 1000 mg/L. The production of vitamin B_{12} with control and 250 mg/L of folic acid was approximately the same. However, there was a slight difference between the production of vitamin B_{12} in the presence of methionine under aerobic and anaerobic conditions. Therefore, it is likely to believe that methionine is more effective in vitamin B_{12} production than the aerobic conditions. The highest level of vitamin B_{12} was observed in the 750 mg/L concentration of folic acid; as compared to the control group, in which there was a 7-time increase. On the other hand, in 1000 mg/L concentration of folic acid, there was a 5-time increase in comparison to the control group (Fig. 5).

When methionine was available in anaerobic conditions along with an increasing concentration of the folic acid, the cell dry weight decreased, and there was no significant difference between different groups (250, 750, and 1000 mg/L). In the aerobic conditions, however, there was a significant difference between the concentrations of 750 and 1000 mg/L of folic acid compared to the similar concentrations in anaerobic conditions in term of dry weight with 1.5 and 1.3 times increase in 750 and 1000 mg/L concentration of folic acid respectively as compared to the same treatments in anaerobic conditions (Fig. 6).

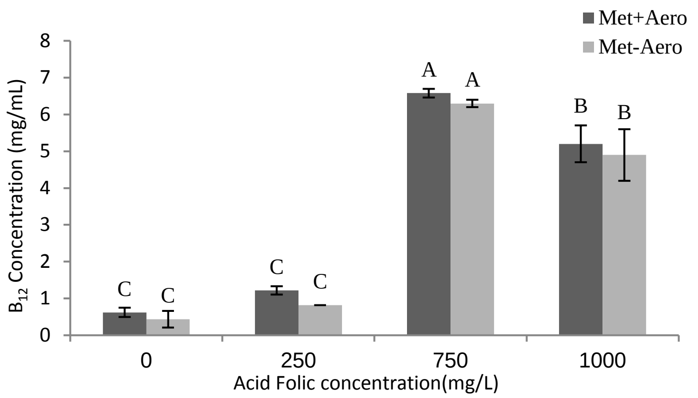

Fig. 5- The effect of different concentrations of folic acid (0, 250, 750, 1000mg/L) on the production of B12 in the presence of methionine under the aerobic and anaerobic conditions by *P.freudenreichii.* The data are expressed as the mean of three replicates ± standard error of the mean. Non-similar letters express the meaning of the data according to the Duncan's ($P<0.05$) assay: A indicates the highest value, B is the highest value after A, and C is the lowest value (Met=Methionine).

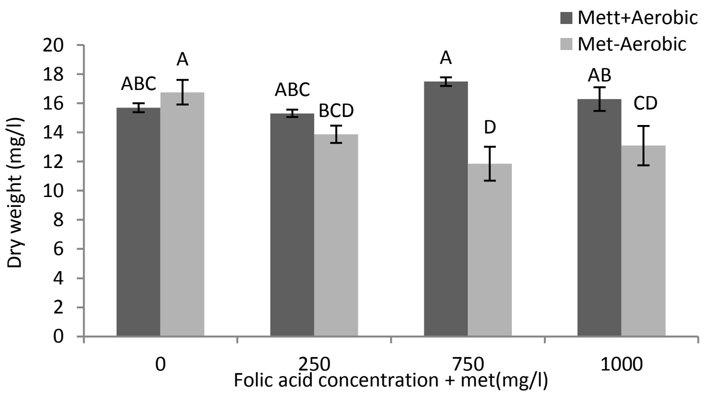

Fig. 6- The effect of different concentrations of folic acid (0, 250, 750, 1000mg/L) on dry cell weight (mg/l) in the presence of methionine under the aerobic and anaerobic conditions by *P.freudenreichii*. The data are expressed as the mean of three replicates ± standard error of the mean. Non-similar letters express the meaning of the data according to the Duncan's ($P<0.05$) assay: A indicates the highest value, B is the highest value after A, and C is the value after B, and BC is the intermediate value between B and C, ABC is the average value between A, B and C, D indicates the lowest value, BCD is the average value between B, C, D, and CD is the intermediate value between C and D (Met=Methionine).

Discussion and Conclusion

Vitamin B_{12} is the most complex and the largest vitamin which has a vital role in nerve tissue health, brain function, and the production of red blood cells with a wide application in medical and nutritional industries (25). Megaloblastic anemia, neurologic impairment, and depressive symptoms are some of the main consequences of vitamin B_{12} deficiency in the human body (26). The biosynthesis of vitamin B_{12} is limited to the industrial fermentation using microorganisms, which is an alternative method to the originally established complicated and expensive chemical synthesis (27). In the present study, the results of HPLC suggests that the production of vitamin B_{12} increased in the aerobic conditions, as compared to the anaerobic ones, in which the highest level of vitamin B_{12} production was at 750 mg/L of folic acid. The data showed that the growth of the microorganism was inhibited at the highest concentration of folic acid (1000 mg/L) followed by the limitation of further production of vitamin B_{12}. It could be argued that folic acid had a toxic effect at 1000 mg/L concentration on the fermentation process.

Turning to the aerobic and anaerobic conditions, it seems that the fermentation process was dependent on oxygen, and the highest rates of vitamin B_{12} could be obtained when oxygen was available. These results supported the hypothesis that the presence of oxygen could be necessary to produce the DMBI rings by *Propionibacterium* (28). In this study, similar to some studies (29-31), it was found that oxygen could lead to the production of DMBI for the synthesis of vitamin B_{12} in the bacteria which is in contrast with the results of other studies (32). The presence of environmental oxygen could lead to the inhibition of aminolevulinic acid synthase and aminolevulinic acid dehydrates that could be regarded as critical enzymes for the synthesis of vitamin B_{12} and its production in the anaerobic conditions can exceed the aerobic conditions (20).

Our results suggested that adding methionine to the fermentation medium could increase the amount of vitamin B_{12} production. It has been reported that the decline of methionine could improve the synthesis of vitamin B_{12} during the fermentation of bacteria (20). On the other hand, methionine appears to be a methyl group donor (27, 33) and the concomitant presence of methionine and oxygen increased the production of vitamin B_{12} and the dry cell weight in comparison to the same treatments without methionine and the anaerobic conditions. Analysis of the control group showed that in the presence of methionine, dry weight was 2.5 times higher than the methionine-free conditions. However, in the methionine-containing medium, the dry weight decreased following the increase of the folic acid concentration, indicating the negative effect of folic acid on cell mass growth. Regarding the optimal conditions, the amount of vitamin B_{12} production in the aerobic conditions increased in comparison to the anaerobic conditions, in the presence of methionine, as compared to the same treatments without methionine. The authors, therefore, designed some new experiments using both aerobic conditions and methionine, and no significant difference was found in the amount of vitamin B_{12} production in the aerobic conditions+ methionine and anaerobic conditions+ methionine. Therefore, an important factor that leads to increased production of vitamin B_{12} in *Propionibacterium* is the presence of methionine. In the anaerobic conditions+ methionine, the dry weight decreased following the increase of folic acid concentration. However, in the aerobic conditions+ methionine, the growth of the bacterial mass increased significantly, which was merely at the concentration of 750 mg/L of folic acid ($P<0.05$).

The maximum production of vitamin B_{12} and DCW was obtained at 750 mg / L folic acid in the presence of methionine and aerobic conditions. By increasing the concentration of folic acid up to 1000 mg / L, the amount of vitamin B_{12} production and dry weight of the bacterium decreased. The present study could be an effective optimization to increase the final production of vitamin B_{12}. Based on the above findings, aerobic conditions combined with the presence of methionine can be effective in improving vitamin B_{12} production, and this optimization may help as an effective strategy to improve vitamin B_{12} production at the industrial level.

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Conflict of Interest

The authors reported no conflict of interest.

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