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Enhanced Vitamin B12 Production using *Chlorella vulgaris*

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ABSTRACT

Microalgae *Chlorella Vulgaris* is enriched in vitamin B12 and cobalt ion which is positioned in the center of the vitamin molecule. This study aimed to investigate how different concentrations of cobalt chloride salt affected the vitamin B12 production by utilizing CO_2 gas, to assess *C. vulgaris* biomass. Therefore, Bold's basal medium used as the medium and 0.5, 1.5, 2 and 2.5 μM cobalt chloride salt was added to *C. vulgaris* culture. Under four cobalt chloride salt treatments, the best growth rate was obtained at the 2 μM of cobalt chloride salt (0.186 + 0.07 g /L.d). CO_2 gas was supplied by 5% CO_2 gas cylinder and fermented milk as a novel biological CO_2 gas generator (CO_2,10%). Use of fermented milk is a practical approach for elimination of waste gas emission and converting CO_2 into biomass. The results revealed that, in the presence of 5% CO_2 gas, *C. vulgaris* vitamin B12 content at 2 and 2.5 μM cobalt chloride, were 166.23 ± 1.78 and 173.32 ± 4.23 μg /100 g of dry biomass (7 and 12% higher than control), respectively. However, under controlled condition (ambient air and 2 μM cobalt chloride) vitamin B12 content was 154.9 ± 1.14 μg / 100 g of dry biomass.

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1. INTRODUCTION

Vitamin B12 (Cobalamin) is a standout amongst the most indispensable biomolecules in the realm of drug and science. In the early 1920s, since two American physicians, Minot and Murphy, indicated that they could cure severe anemia called "pernicious anemia" with a special diet contained entire liver or liver extracts, vitamin B12 which is the main substance in the liver attracted scientific attention [1]. Cobalamin is a unique vitamin with the complex structure that holds cobalt ion in the center of its molecule and plays an important role in energy producing metabolisms and protein synthesis in the human's body and many organism cells [2]. Vitamin B12 is an essential dietary supplement because it is a necessary cofactor for two enzymes, methionine synthase (*meth*) and methylmalonyl-CoA mutase [3]. Vitamin B12 is vital for sustaining good health in humans. Fungi and Land plants neither synthesize nor require vitamin B12 because they do not have methylmalonyl-CoA mutase, and have a substitute B12-independent methionine synthase (methe) [4]. To avoid cobalamin deficiency disorderliness in humans, consuming foodstuffs with cobalamin source and nourishing supplements is generally practiced. Natural sources of vitamin B12 are, egg, meat, fish and dairy products [5]; these main sources of vitamin B12 is a major challenge for vegetarians, this problem could be solved by using microalgae supplements as a rich and biological source for vitamin B12. Some species of microalgae contains a sustainable amount of vitamin B12, *Chlorella* species is one these microalgae [6]. Annually, thousand tons of *Chlorella* powders and tablets are produced in the United States, Taiwan, Japan, Indonesia and China, and they are sold extensively as nutraceutical crops and supplementary food [7, 8]. *C. vulgaris* biomass is available product which consists of sustainable amounts of nutrients like proteins, carbohydrates, lipids, minerals and vitamins. Among different types of vitamins in *C. vulgaris* biomass, biologically active form of vitamin B12 (methylcobalamin), that is rare among foodstuffs identified in the biomass and attracted attention [9].

Some researcher scientists [9-11] have evaluated vitamin B12 content of *C. vulgaris* tablets but there was no specific research on enhancing vitamin B12

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biosynthesis besides of biomass productivity. Therefore, an especial study with the aim of examining the microalgae growth and impact of the culture medium on the vitamin B12 production is required.

The objective of this investigation is assessing the impact of $CoCl_2.6H_2O$ salt for the biosynthesis of the vitamin B12 in *C. vulgaris* biomass; as mentioned above, cobalt ion is the vitamin B12 central metal ion in its complex molecule, on the other hand, *Chlorella* needs cobalt ion in a trace amount for growing; thus, it could be a cost-effective technique for improving vitamin B12 production. Furthermore, for growing *C. vulgaris* biological CO_2 generator have been designed and utilized here for the first time. In this research, produced CO_2 gas from milk fermentation process, rather than purging into the atmosphere injected into the algae medium to produce the next valuable food product.

2. MATERIAL AND METHODS

2. 1. Chemicals and Solvents Methyl cobalamin (Me-Cbl) and Cyanocobalamin (CN-Cbl) were from Sigma-Aldrich. Amberlite XAD-2 was supplied by Supelco Sigma-Aldrich. Methanol, acetonitrile and water were HPLC grade and provided from Merck for vitamin B12 purification and analysis. Ethanol and chloroform were analytical grade and all chemicals were pure and reagent grade.

2. 2. Strains and Cultivation Conditions

2. 2. 1. Microalgae Strain and Culture The microalgae used in this experiment was *C. vulgaris* obtained from Iranian Research Institute of Science, Iranian Academic Center for Education, Culture & Research (ACECR). *C. vulgaris* was maintained in Bold's basal medium (BBM). The medium contained 0.25 g/L of $NaNO_3$, 0.075 g/L of K_2HPO_4, 0.175 g/L of $KH_{(2)}PO_4$, 0.075 g/L of $MgSO_4$, 0.025 g/L of $CaCl_2.2H_2O$, 0.025 g/L of NaCl, 0.005 g/L of $FeSO_4.7H_2O$, 0.05 g/L of Na_2 EDTA, 0.031 g/L KOH, 0.0015 g/L of $ZnSO_4.7H_2O$, 0.0003 g/L of $CuSO_4.5H_2O$, 0.0003 g/L of $MnCl_2.4H_2O$, 0.0001 g/L of $CoCl_2.6H_2O$, 0.00025 g/L of MnO_3, 0.008 g/L of H_3BO_3. The pH was adjusted to 6.4-6.8 with 1M NaOH solution prior to autoclaving at 120°C for 15 min.

The cultures were maintained in an Erlenmeyer flask (250 mL) containing 120 mL medium with the initial cell density of 0.15 g/L. After 6 days (exponential phase), the seed culture was transferred to 1 L of Erlenmeyer flasks containing 500 mL BBM medium with 0.15 g/L inoculum and cultured for 8 days under the same condition. Cultures were aerated with sterile air at a gas flow rate of 0.3 vvm (volume gas per volume medium per min). After 8 days cells were collected for further experiments.

Cultures were placed in a white cabin equipped with two rows of 5630 SMD cool white aluminum rigid LED strip with 2000 lux light intensity measured by digital light meter (Mastech, MS6610, USA) and 16:8 light: dark photoperiod. Cabin temperature was adjusted to 26 ± 2 ºC.

Cobalt chloride concentration, in the BBM medium, is 2 μM. To observe effects of different Cobalt chloride concentration on *C. vulgaris* growth pattern and vitamin B12 production, separated BBM media were prepared with 0.5, 1.5 and 2.5 μM Cobalt chloride concentration. Cultures incubation conditions were the same for all samples. Tests repeated in triplicate.

2. 2. 2. Gas Injection To evaluate *C. vulgaris* growth pattern in the presence of CO_2 gas, *C. vulgaris* cells were cultured in 110 mL serum bottles with 40 mL medium and initial cell density of 0.15 g/L. 5% CO_2 gas was injected to the bottles. Gas flow rate was adjusted to 1 L/min in the room temperature. The methods of injection were the same for all bottles. Bottles were placed in the cabin and they were shaken 3 times a day. For drawing growth pattern, for each day of growth, individual bottle was prepared to confirm that all parameters were constant and medium levels were the same. The collected biomass from each day was analyzed for different purposes. This procedure repeated more than 4 times.

2. 2. 3. Bacteria *Lactobacillus delbrueckii* PTCC 1057 was purchased for vitamin B12 microbiological assay. Two mediums used for this organism. De Man, Rogosa and Sharpe (MRS) broth medium as maintenance medium was obtained from the Iranian Research Organization for Science and Technology (IROST) and vitamin B12 assay medium, was obtained from Biomark (India). All media sterilized by HIRAYAMA model HV-25 autoclaving at 121°C for 15 minutes but glass wares at 125°C for 20minutes. Cultures prepared with 3% (v/v) inoculum and incubated in the orbital incubator (IKA, KS4000I CONTROL) for 24h in 37 °C and 130 rpm.

2. 2. 4. Kefir Grains For milk fermentation process kefir grain was employed. The samples of Kefir grains were obtained from a dairy company (Iran Dairy Industries Corporation). Grains cultured in Damdaran semi fat pasteurized milk with 2, 5 and 10% (w/v) inoculation portion. Cultures placed in the incubator for 24-48 hours with rotating speed of 125 rpm at 26±2ºC [12]. For collecting produced gas and further analyses, culture was distributed in the serum bottles sealed with a rubber crimp seal and aluminum cap.

2. 3. Growth and Biomass Productivity

2. 3. 1. Biomass Harvest *C. vulgaris* biomass was harvested by centrifuge at 8500 rpm for 10 minutes. Supernatant was discarded and Pellets washed with

deionized water and centrifuged for a second time, this procedure repeated three times. The harvested biomass was lyophilized and used for analysis.

2. 3. 2. Dry weight quantification and biomass productivity A correlation between *C. vulgaris* cell dry weight and optical density was pre-determined. Optical density (OD) was daily measured by UNICO model 2100 spectrophotometer (UNICO Corporation, New Jersey, U.S.A) at 690 nm [13]. For determining cell dry weight, the harvested microalgae biomass was dried in an oven at 80ºC for 24 h until constant weight. To ensure the accuracy of the data samplings were done in triplicate. Based on cell dry weight with respect to optical density, the calibration curve was developed. Correlation is shown in Equation (1), growth rate was determined according to Equation (2) [14] where X is biomass concentration (g/L) and t is cultivation time (day):

$$\text{Dry Weight (g/L)} = 0.8204\, OD_{690} + 0.039 \quad R^2=0.9932 \tag{1}$$

$$P_X\,(g/L.\,d) = \frac{X_1 - X_0}{t_1 - t_0} \tag{2}$$

2. 4. Analytical Methods

2. 4. 1. Vitamin B12 Extraction and Purification *C. vulgaris* freeze dried biomass was suspended in the specific amount of deionized water. For vitamin B12 extraction, a green method has been selected, extraction by autoclaving at 121°C for 10 min [15]. Autoclaved suspension cooled and centrifuged at 6000 rpm for 10 min. The supernatant contains vitamin B12. The supernatant collected and its pH adjusted to 6.0 to be ready for purification. Purification process consists of two stages. Passing samples through Amberlite XAD-2 column and then Sep-Pak cartridge. Prepared Amberlite XAD-2 column was used for purification. Column chromatography was filled with the slurry of pure methanol and Amberlite XAD-2 resins to a bed height of 15–16 cm. Most of the methanol was drained and replaced with deionized water for equilibrium. After 15 min, the column was drained and vitamin B12 extracts were loaded on the column. For increasing purification efficiency, Vitamin B12 extracts drained very slowly (3 hours) and eluted with 80% methanol. Further purification was performed by passing the samples through a Sep-Pak cartridge. Before using the cartridge is washed with 75% ethanol and then equilibrated with deionized water. Purified samples loaded on Sep-Pak cartridge and eluted with 25% ethanol. Purified vitamin B12 was concentrated under reduced pressure for further analysis [16]. To ensure the accuracy of the purification process, samples were analyzed by HPLC moreover their concentrations determined by the biological method.

2. 4. 2. HPLC Analysis of Vitamin B12 HPLC (Knauer Smartline, Germany) with UV detector was performed on a C18 column reversed-phase (Eurospher 100mm×5mm; particle size 5µm, Germany). The mobile phase of the column was composed of HPLC grade water and acetonitrile, the flow rate was 1.0 ml/min at room temperature. The elution was conducted as follows: 0-7 min, linear gradient from 0-5% (v/v) aqueous acetonitrile, 7-9 min, 5-15% (v/v), 9-16 min, 15-20% (v/v), 16-19 min, 20 to 100% and from 19-22 min, linear gradient from 100% to 0% (v/v) aqueous acetonitrile. Finally, 22-27 min 100% HPLC grade water was injected. Methyl cobalamin (Me-Cbl) besides Cyanocobalamin (CN-Cbl) were used as vitamin B12 standard forms. Me-Cbl and CN-Cbl retention times (RTs) were 19.767 min and 24.517 min, respectively. Purified samples were injected into HPLC to confirm the presence of vitamin B12 in the sample based on standards RTs.

2. 4. 3. Vitamin B12 Quantification Vitamin B12 concentration determined by the most frequent method (microbiological assay of vitamin B12). In this method, vitamin B12 concentration is proportional to turbidity, caused by Lactobacillus delbrueckii cell growth [17]. Based on, Lactobacillus delbrueckii growth in the presence of different concentrations of Me-Cbl and CN-Cbl standards, a linear correlation has been determined to measure vitamin B12 concentration of unknown samples. The standard vitamin B12 (Me-Cbl and CN-Cbl) was prepared within the range of 0-0.25 µg/mL. The turbidity of Lactobacillus delbrueckii culture was measured at 574nm (the best wavelength from different experiments) using UNICO spectrophotometer model 2100 (UNICO Corporation, New Jersey, U.S.A). *C. vulgaris* vitamin B12 contents in different growth condition have been quantified based on this method.

To determine biomass protein and carbohydrate content, 10 mg of *C. vulgaris* freeze dried biomass were immersed in 5 mL deionized water. The Cell suspension was homogenized by IKA T18 basic homogenizer for 15 min with maximum speed (6) to break down the cell wall. For each experiment, 1 mL of extraction was used for protein and carbohydrate quantification.

2. 4. 4. Protein Extraction and Quantification Microalgae proteins extracted by biomass solubilization in an alkaline solution. For this purpose, 1 mL of microalgae suspension was added to 1 mL of NaOH 2 M and heated in a water bath at 95ºC for 10 min. The suspension was centrifuged. The supernatant was collected for protein assay and the pellets were re-extracted with 2mL of NaOH 1 M. The experimental procedure was the same as the first extraction. Two supernatants were combined together and the pellets were discarded. Three mL of deionized water was added,

and thus, the final volume of the extract was 6 mL [18]. Protein content was determined by Bradford method using the Coomassie Brilliant Blue dye [19]. Bovine Serum Albumin used as the standard of protein.

2. 4. 5. Carbohydrate determination Microalgae carbohydrate content was determined according to Phenol–Sulfuric Acid method developed by DuBois et al. [20]. Glucose was used as carbohydrate standard. Solutions preparation was carried out based on the procedure published by Albalasmeh et al. [21]. All experiments repeated more than 3 times.

2. 4. 6. Total Lipid Content The total lipids extraction were performed according to a modified Folch's method [22]. 0.25 mg freeze dried microalgae was transferred into pre-weighted glass vial. 5 mL chloroform/ methanol (2:1, v/v) mixture was added to vial to dissolve the biomass. The mixture was vortexed for 30 s at room temperature. This was followed by stirring the mixture for 15–20 min. The lower chloroform phase contains lipids and cell debris were in methanol phase. Upper phase discharged gently. 0.9 % NaCl was added to mixture for washing residual particles and vortex for few minutes. The upper methanol layer removed carefully and the stated procedure repeated until upper phase became clear. The glass vial with chloroform phase containing lipids was placed inside oven at 60° C to remove the chloroform until constant weight of lipid was achieved. The lipid content was gravimetrically calculated [23, 24].

2. 4. 7. Chlorophyll Extraction and Quantification 5 mg of *C. vulgaris* freeze dried biomass were immersed in 3 mL pure methanol (99.9%). The suspension was lysed by sonication (Elmasonic, S10H, Germany) (15min, 30 watts, 30°C). Each sample heated at 70°C for 5 min in a water bath and then centrifuged at 500×g for 15 min at 10°C. The supernatant was collected and pellets re-extract with methanol under the same condition. Two supernatants combined together and the absorbance was measured at 653 and 666 nm. Chla, Chlb and Chla + b concentrations were determined using the following Equations (3, 4, 5) [25]:

$$\text{Chl } a \text{ (mg /L)} = 17.12 \times A_{666} - 8.86 \times A_{653} \quad (3)$$

$$\text{Chl} b \text{ (mg/L)} = 32.23 \times A_{653} - 14.55 \times A_{666} \quad (4)$$

$$\text{Chl } a+b \text{ (mg/L)} = 2.57 \times A_{666} + 23.6 \times A_{653} \quad (5)$$

2. 4. 8. CO_2 Gas Analysis CO_2 generation during milk fermentation process analyzed by gas chromatography (GC, Agilent 7890A) equipped with Supelco Propak Q column and a thermal conductivity detector (TCD).The injector temperature was kept at 150°C. Initial oven temperature was 40°C which increased 10°C/min and the final temperature was 180°C. The carrier gas was helium and TCD temperature was set at 220°C.

2. 5. Experimental Setup Kefir is a naturally fermented dairy beverage with a slightly acidic taste, yeasty flavor and creamy consistency. Kefir's unique flavor is a blend of ethanol, lactic acid, carbon dioxide and flavoring products, such as acetoin and acetaldehyde [26]. The suitable period of fermentation for kefir making is 24 h. But the aim of using kefir grains in this study is to produce CO_2 gas at a suitable concentration, not to produce kefir beverage. Therefore, according to experimental data, 48 h fermentation period has been selected. During the milk fermentation process, CO_2 gas produces. On the other hand, microalgae need CO_2 for starting their photosynthesis process. For this reason, a continuous system was designed to connected milk fermentation flask to the microalgae culture. Setup was placed in shaking incubator (Noorsanat ferdous) and operation condition set in the optimal state for the best growth for both kefir grains and *C. vulgaris*. Temperature sets on 28°C, light intensity was 2000 lux, and photoperiod sets to 16:8 of light/dark. To avoid exposure of kefir container, the flask was covered with aluminum foil. After a period of fermentation lasting 48 h, the grains are removed by filtration and the fermented milk replaced with fresh milk to control CO_2 production. Inoculation of grains as a starter was constant in all experiment. *C. vulgaris* growth pattern and biological characteristics have been evaluated like other samples. Experimental setup is shown in Figure 1.

2. 6. Statistical Analysis Statistical analysis was evaluated by Microsoft Excel package and a P-value <0.05 was considered significant.

3. RESULTS AND DISCUSSION

3. 1. Effect of CO_2 Concentration on *C. vulgaris* Biomass Productivity and Growth From Figure 2 among three different conditions, *C. vulgaris* was shown the best growth pattern in the presence of 5% CO_2 and microalgae biomass concentration on 8th day was 1.43 g/L. whereas the *chlorella* cultures aerated with 10% CO_2 gas and sterile air have 0.85 and 0.75 g/L biomass concentration, respectively. The biomass concentration obtained from this experiments was similar to those records conducted by Guo et al. [27]. Observations indicated that, higher CO_2 concentration can improve *C. vulgaris* photosynthetic activity and promote them to shorten their cultivation time. However, there is no linear correlation between increasing in CO_2 concentration and biomass productivity. According to data summarized in Table 1, *C. vulgaris* biomass productivity increased from

0.051± 0.02 g/L.d to 0.186 ± 0.07 g/L.d when the aeration condition was shifted from sterile air (0.03% CO_2) to 5% CO_2. In contrast, when input CO_2 concentration increased to 10 % biomass productivitydecreased to 0.088 g/L.d; which was due to culture acidification and biomass growth inhibition. Figure 2 shows the effect of CO_2 of concentration on pH changes in different growth conditions. The initial pH level, for all cultures, was adjusted to 6.65, after inoculation pH level in presence of ambient air increased because of photosynthetic process and consumption of H^+ in the culture medium. In contrast, when the *C. vulgaris* culture was aerated with high concentration of, CO_2, most probably three forms of dissolved organic carbon existed in the system, CO_2, HCO_3^- and CO_3^{2-}. During the course of growth phase, the pH changes were observed. According to Mayo reports [28], the optimum pH range for the growth of *Chlorella* is 6 to 8, although cells can grow in the range of 4 to 10. As illustrated in Figure 2 the pH range for *Chlorella* growth in various CO_2 concentration was from 6 to 10 which is reliable to literature.

3. 2. Effect of Kefir Grains Produced CO_2 on *C. vulgaris* Growth This system is the first multi-target and completely biological design for cultivation of microalgae. Not only, CO_2 gas as a greenhouse gas did not purge to the atmosphere within the milk fermentation process but also utilized to produce another rich food product (*C. vulgaris* biomass). This setup is exceptionally straightforward and cost-effective in comparison with different techniques for developing microalgae. Supply and infusion of gas, other than of microalgae culture air circulation, requires complex and expensive types of equipment. By designing this system, some microalgae production challenges have been comprehended; one of them is preparing gas with a specific concentration which must be suitable for microalgae growth. In such manner and to set up 10% CO_2, kefir grains were utilized. For examination on various CO_2 fixations, different percentages of kefir grains as a starter of milk fermentation were considered. Analysis of generated gas by means of gas chromatography is shown in Figure 3 The best concentration of CO_2 (10% CO_2) was produced after 48 h using inoculation of 2% W/V kefir grain.

Figure 1. Kefir *C. vulgaris* experimental setup

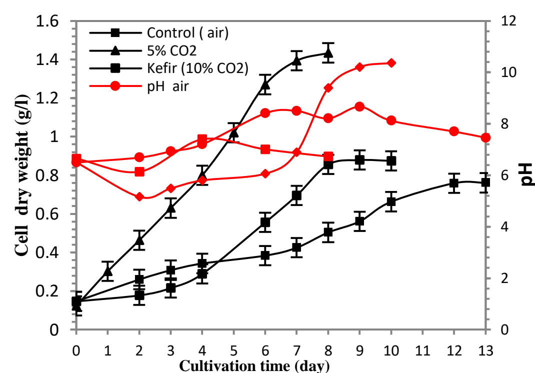

Figure 2. Effect of CO_2 concentration toward biomass yield and pH level

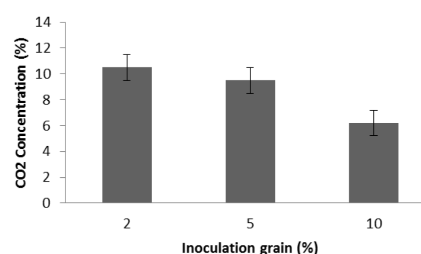

Figure 3. CO_2 Gas analysis during the process of production of kefir drinks, in percentages of different inoculants of kefir grains to milk

The obtained results were mean values of 3 different runs of experiments for high accuracy and validity of generated data.

Consequences of kefir grain biomass production and pH changes with 2% inoculation of kefir grains to milk are presented in Figure 4 It could be finished up from the results that an initial 24 h, kefir grains demonstrated the most extreme increment in weight and development. After this time, because of the fermentation happened in culture, the development of kefir grains has been hindered [29]. For the insurance of results, all tests were repeated in excess of 6 times.

3. 3. Effect of $CoCl_2$ Salt on *C. vulgaris* Growth It is realized that Cobalt metal is fundamental for micro-algae growth in a trace amount and it could be found in the center of vitamin B12 molecule. To investigate the impact of $CoCl_2.6H_2O$ salt treatments on vitamin B12 synthesis, diverse concentrations of $CoCl_2.6H_2O$ (0.5, 1.5, 2 and 2.5 micro Molar) were added to BBM culture medium. The development of C. vulgaris within the sight of 5% CO_2 is illustrated in Figure 5 From the results, in presence of 5% of CO_2 gas and 2 µM of CoCl2.6H2O salt, the best development of C. vulgaris and the maximum biomass productivity (1.674 g/L) were acquired. As indicated in Table 1, $CoCl_2.6H_2O$ concentration affected on C. vulgaris growth.

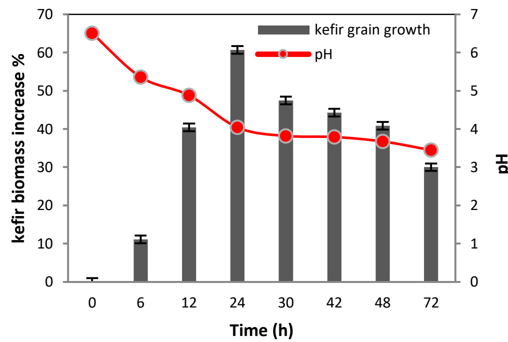

Figure 4. Kefir grains weight diagram over different times and pH changes in fermented kefir drink

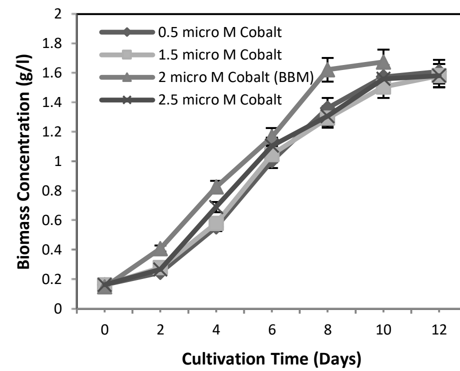

Figure 5. Effect of cobalt chloride salt on *C. vulgaris* growth

TABLE 1. Maximum biomass concentration and productivity in various concentrations of cobalt chloride salt and aeration

	CO_2 Concentration (%)					
	Air	5 %				10 %
Cobalt chloride concentration (µM)	2	0.5	1.5	*2	2.5	2
Biomass concentration (g/L)	0.761± 0.05	1.608	1.582	1.514 ± 0.13	1.581	0.879
Biomass productivity (g/L.d)	0.051± 0.02	0.149	0.147	0.186 ± 0.07	0.157	0.088

An increase in $CoCl_2.6H_2O$ concentration from 0.5 µM to 2 µM resulted in enhancement of growth by 25% higher than control in contrast increase in $CoCl_2.6H_2O$ concentration to 2.5 µM caused growth reduction by 16% lower than corresponding control (2 µM). These results are in agreement with those data reported by Battah et al. [30]. Growth raise at low Co^{2+} concentrations might be caused by Co^{2+} substitution for Zn^{2+} in some metalloenzymes [31]. Interestingly, retardation of plasma membrane function by interacting with sulfhydryl groups on proteinaceous membranes to produce S–metal–S bridges that can change membrane permeability caused by the toxic effect of high Co^{2+} concentrations [32].

3. 4. *C. vulgaris* Vitamin B12 Content in Different Growth Condition

Synthesis of vitamin B12 in C. vulgaris cell is profoundly relies upon the substrate. Consequently, no vitamin B12 was added to the culture medium [33]. With the aim of *C.vulgaris* vitamin B12 generation in different media conditions, the *C. vulgaris* biomass was collected and extracted, then purified. Results of HPLC analysis was confirmed the presence of vitamin B12 in *C. vulgaris* biomass via comparison with standard forms of vitamin. The retention time of methylcobalamin and cyanocobalamin were 19.53 min and 24.45 min, respectively. At this point, the presence of two unique types of vitamin B12 (methyl cobalamin and cyanocobalamin) was proved in *C. vulgaris* biomass, vitamin B12 concentration was determined by biological assay and data are reported in Table 2. All data stated in Table 2 are in agreement with literature based on tests conducted on commercial *chlorella* tablets. Experiments revealed that vitamin B12 content varied from trace amount to 415 µg/100 g of dry weight [33]. Nakano et al. [34] demonstrated that supplementation with *Chlorella* decreases the risk of anemia in pregnant women, suggesting that *Chlorella* supplements are natural source of vitamin B12, folic acid and iron. According to the results, the most astounding efficiency of vitamin B12 production was obtained in 2.5 µM $CoCl_2.6H_2O$ salt and 5% CO_2.

TABLE 2. *C. vulgaris* vitamin B12 content at different growth condition

Culture condition	Amount of vitamin B12 (µg/100 g of dry weight)		
	CN-Cbl	Me-Cbl	Total
Air	127.96 ± 3.18	26.94 ± 2.35	154.9 ± 1.14
5% CO_2	134.38 ± 3.90	31.85 ± 2.14	166.23 ± 1.78
5% CO_2 +2.5 µM $CoCl_2$	137.87 ± 2.14	35.45 ± 2.11	173.32 ± 4.23
Algomed tablet (commercial)	121.29 ± 3.27	21.13 ± 3.08	142.42 ± 1.83

The presence of 5% CO_2 gas and employment of BBM medium (2 µM $CoCl_2.6H_2O$) brought about enhancement of vitamin B12 production to 7% higher than the control (ambient air). Moreover, 2.5 µM $CoCl_2.6H_2O$ improved vitamin B12 production approximately 12% higher than corresponding control. These consequences showed that vitamin B12 content altogether relies on the growth condition and substrate content. Algomed tablet was concentrated to affirm the correct extraction and purification procedure. As Table 2 presents, the amount of extracted vitamin B12 from Algomed tablets is less than the vitamin produced in vitro, since the objective of Algomed tablet production is to create more biomass, not a particularly rich in vitamin B12. However, vitamin B12 is a familiar product of a certain bacteria [35], *Chlorella* as a food supplement annually produced in thousand tons in the United States, China, japan, Indonesia and Taiwan [36, 37]. Prime *Chlorella*™, Lucky Vitamin™, Sun *Chlorella*™, Puritan's Pride™ and HerbMark™ more are merely a few examples of commercial *Chlorella* products [7]. Therefore, enhancing vitamin B12 content in this green supplement could be useful.

3. 6. Protein, Carbohydrate, Lipid and Chlorophyll Content in *C. vulgaris* Biomass The biomass at the late growth phase (7th-8th day) was collected and analyzed for determination of biochemical content of the cells. In Table 3 the amount of each substance at different growth conditions, along with comparison with other research data in this area, has been reported. According to the results, in presence of CO_2 gas concentration less than 5%, *C. vulgaris* has created the most noteworthy amount of carbohydrates. Based on literature, it is a reasonable and acceptable result [38] that in the presence of CO_2 gases in the culture medium, mineral carbon, stored in the form of carbohydrates or fat in the cells. Based on other research [39], when stress enters into microalgae environment, microalgae biomass tends to save carbohydrates. As shown in Table 3, the amount of protein produced in the presence of 5% CO_2 gas was higher than air, but no significant change was observed, and the results were similar to those reported in literature [40]. In the presence of 10% CO_2 gas of fermented milk, the intracellular microalgae metabolites have progressed towards fat and protein generation. The amount of carbohydrate produced has not relatively changed compared to the control conditions (aerated with air). Due to increased concentration of carbon in the medium, the production of fatty acids and fat production, has increased. In this situation, the highest amount of protein was produced compared to other conditions, but this change is not exceptionally significant. Proteins play a key role in cell storage and growth as cellular motors, chemical messengers, and regulators of cellular activity [41]. It appears that when the amount of unused nitrogen in the culture medium is high, more protein is produced. When concentration of CO_2 gas increases in the medium, the cell is restricted in growth and respiration in addition to cell division are impaired. According to decrease in cell growth, the amount of nitrogen is less consumed from the medium, then the accumulation of organic nitrogen can be observed. This slight increase in nitrogen concentration promotes protein production [25, 42].

TABLE 3. Final content of biochemical components under various aeration condition

Aeration condition	Highest biomass concentration (g/L)*	Final content % (g/ g dry weight)				Cited
		Carbohydrate*	Lipid*	Protein*	Chlorophyll*	References
Air	0.759±0.04	15.34±0.68	9.44±0.43	31.47±0.98	0.91±0.14	Present study
5% CO_2	1.43±0.08	40.11±3.09	20.38±1.27	35.23±2.72	0.87±0.17	Present study
10% CO_2 of kefir	0.879±0.03	22.59±4.63	28.44±0.84	40.68±1.71	0.307±0.09	Present study
Air	1.34±0.08	20.30±0.73	21.69±0.44	33.07±1.42	5.65±0.28	[43]
5% CO_2	1.80±0.13	36.77±0.98	28.07±0.73	28.67±3.76	4.0±0.11	[43]
2% CO_2	1.22±0.12	5.1	42	-	0.51±0.09	[44]

*Values are means ± SD ($n<3$)
*$p<0.05$, differences were significant

4. CONCLUSION

The present study confirmed that by changing the culture medium and CO_2 source, the biochemical composition of *C. vulgaris* biomass, including protein, lipid, carbohydrate, Chlorophyll and vitamin B12 will change. Considering the parameters contemplated in this investigation, vitamin B12 biosynthesis in *C.vulgaris* microalgae biomass depends on cell growth and $CoCl_2.6H_2O$ salt concentration. The optimum

concentration of $CoCl_2.6H_2O$ salt for vitamin B12 enhancement was 2.5 µM which developed vitamin B12 production almost 12% higher than consistent control. Further study is needed to improve other vitamin production in this edible microalgae to determine the suitability of *C. vulgaris* as a feedstock for the production of a supplement rich in vitamins.

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Enhanced Vitamin B12 Production using *Chlorella vulgaris*

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چکیده

ریزجلبک *کلرلا ولگاریس* غنی از ویتامین B12 می‌باشد و یون کبالت در مرکز این ویتامین قرار دارد. این مطالعه با هدف بررسی تاثیرغلظت‌های مختلف نمک کلریدکبالت بر تولید ویتامین B12 و تولید زیست‌توده *کلرلا ولگاریس* در حضور گاز CO_2 انجام پذیرفت. بنابراین محیط‌کشت BBM جهت کشت این ریزجلبک به کار برده شد و غلظت‌های ۰/۵، ۱/۵، ۲ و ۲/۵ میکرومولار از نمک کبالت‌کلرید به محیط کشت اضافه شد. تحت تیمار چهار نمک کلریدکبالت، بهترین میزان رشد در ۲ میکرومولار نمک کبالت‌کلرید حاصل شد (۰/۱۸٦+۰/۰۷ گرم بر لیتر در روز). در این پژوهش گاز CO_2 توسط سیلند گازCO_2 با غلظت ۵٪ و شیر تخمیر شده به عنوان یک مولد جدید گاز CO_2 (CO_2، ٪۱۰) تامین گشت. استفاده از شیر تخمیر شده یک رویکرد عملی برای از بین بردن انتشار گازهای گلخانه ای و تبدیل گاز CO_2 به زیست توده است. نتایج نشان داد، ویتامین B12 تولید شده در حضور گاز CO_2با غلظت ۵٪ و غلظت‌های ۲ و ۲/۵ میکرومولار نمک کبالت کلرید به ترتیب۱/۷۸± ۱٦٦/۲۳ و ۲۳/٤ ± ۱۷۳/۳۲ میکروگرم بر ۱۰۰ گرم زیست‌توده خشک(به ترتیب ۷ و ۱۲٪ بیشتر از شرایط کنترل) می‌باشند. این در حالیست که در شرایط کنترل (هوا و ۲ میکرومولار کبالت‌کلرید) ۱/۱٤ ± ۱٥٤/۹ میکروگرم B12 به ازای ۱۰۰ گرم زیست‌توده خشک تولید گشت.

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