Optimal conditions for vitamin B_{12} production from *Lactobacillus rhamnosus*

Ali Bader Najim and Athraa Harjan Muhsen

Faculty of Agriculture - University of Kufa, Najaf, Republic of Iraq

Corresponding author Email: athraa.alduhaidahawi@uokufa.edu.iq

https://doi.org/10.36077/kjas/2022/v14i2.3687

Received date: 12/6/2022

Accepted date: 21/7/2022

Abstract

This research was conducted to study the effect of some nutrients on the growth and production of vitamin B_{12} by Lactobacillus genus *L.rhamnosus*, when their ability to produce vitamin B_{12} was tested under different conditions. The results indicate that a pH of 7 is optimal for vitamin B_{12} production by the bacteria *L.rhamnosus* and it was found that the optimum incubation temperature for the production of vitamin B_{12} ranged between 30 - 35 °C, with regard to the effect of the incubation period on the biological activity and productivity of vitamin B_{12}. The results showed that 3 days was the best in the production of vitamin B_{12}. The results also showed that adding the lowest concentration of cobalt chloride gave a high productivity of the vitamin compared to the highest added concentrations and that the best carbon source was sucrose among the three carbon sources that included glucose, fructose, and sucrose, and the results of the study showed that the best nitrogen source It is was peptone compared to yeast extract, which gave the highest production of the vitamin B_{12}.

Keywords: Vitamin B_{12} production, optimum conditions, *Lactobacillus rhamnosus*.

Introduction

Probiotic as a term is a relatively new word meaning for life and it is currently used to describe a group of bacteria when administered insufficient quantity, confer beneficial effects for humans and animal (19). Probiotic bacteria are applied to balance disturbed intestinal microflora and important in the treatment of a wide range of human disorders including lactose intolerance, diarrhea, food allergies, intestinal infection, constipation gastroenteritis, hepatic, flatulence, colitis, gastric acidity, osteoporosis, high blood cholesterol and cancer (11 and 25).The most organisms used as probiotic belong to *Bifidobacterium*, Lactobacillus and some Enterococcus ssp. (34). Lactic acid bacteria (LAB) and *Bifidobacterium* are amongst the most important groups of microorganisms used in the food industry, to products, such as yoghurts, cheese and pickled vegetables (31).

Lactobacillus rhamnosus benefits are reducing the activity of fecal enzymes such as β-glucuronidase and azoreductase which contribute to the risk of colon, mammary, and prostate cancer (17). *Lactobacillus rhamnosus* increases the number and the activity of natural killer cells and exerts immune stimulating effects, including on fetuses (23). The *L. rhamnosus* strain, was one of the most well-documented probiotic microorganisms (9). It has been deemed a probiotic because of its resistance to acid and bile as well as its good growth characteristics that allow it to survive and persist within the gastrointestinal tract (12). It has also been reported to be highly resistant to technological processes and has a great adhesion capacity to the intestinal epithelial layer to subsequently inhibit the growth and adherence of several pathogens (9 and 12). *Lactobacillus rhamnosus* can survive and thrive through the gastrointestinal tract while adhering to the intestinal epithelial cells. This strain has been displayed as an excellent mucus-adhering Lactobacillus strain when compared to related strains such as *Lactobacillus johnsonii* and *Lactobacillus casei* (33). *L.rhamnosus* is a safe probiotic with non-transferable antibiotic resistance (27). There is a good information in the literature that shows that *L. rhamnosus* can tolerate stresses during manufacturing, specifically when it is incorporated into food products. This ability of *L. rhamnosus* to survive these technological stresses means that it can be easily incorporated into different food products ,and consequently increases its accessibility to various consumers (29).

The studies also demonstrated the ability of L. rhamnosus to survive under osmotic stress caused by sucrose. Their results showed that the strain could tolerate the sucrose even at extreme concentrations, suggesting that it can safely be used in sugar-based foods. (30).

Materials and Methods

A number of factors affecting the production of vitamin $B_{12}$from *Lactobacillus rhamnosus* was studied. These factors including carbon sources, nitrogen source, and its concentration, pH of the medium, incubation temperature, the incubation period, and the cobalt concentration.

The organisms used in this study namely: *Lactobacillus rhamnosus*.

Media used**:** MRS broth, glucose, peptone, Sucrose, yeast extract, fructose, $CoCl_2$.

Fermentation process:

The effect of the carbon source

The method described by Cheng *et al*.(6).was followed with some modification as it inoculated 250 ml flasks containing the production medium and replaced the carbon source of the medium with 3 other carbon sources, including (glucose, fructose, sucrose) and at and concentrations (3, 7, 10) $gm.L^{-1}$ with the pH was fixed at 7, three replicates per flask was done, and all of them were incubated at 37°C for 72 hours, the absorbance was read at wavelength 356 nm using a UV Visible Spectrophotometer.

The effect of the nitrogen source

The culture medium for the production of vitamin B_{12} was prepared and the original nitrogen source was replaced with other nitrogen sources to determine the most efficient ones in the production of the vitamin, which included (peptone and yeast extract) at concentrations (3, 7, 10) $gm.L^{-1}$ and three replicates per beaker was done and incubated at 37°C for a period of 72 hours and The vitamin intake was measured by a spectrophotometer at the wavelength of 356 nm(6).

Effect of pH

The effect of pH on the production of vitamin B_{12} was studied. The vials containing in the production medium were inoculated with different numbers of pH (5, 6, 7, and 8) using a solution of 0.1 KM HCl and 0.1 M NaoH, and the amount of vitamin B_{12} produced was measured. By a spectrophotometer at a wavelength of 356 nm (17).

Effect of temperature

The production medium was inoculated in 250 ml flasks with the studied isolates at different incubation temperatures included (28, 30, 35,and 37) °C for 5 days under anaerobic conditions, then the amount of vitamin B12 was measured (25).

Effect of incubation time

The method described by Chowdhury (7) was followed with some modification, as the flasks containing the production medium were incubated in a shaking incubator at different times (3, 5, 7) days, with fixation of the optimal conditions obtained from previous experiments, with three replications. The amount of vitamin B_{12} was measured as described by Pirtino(24).

The effect of cobalt chloride $CoCl_2$

Cobalt is also an important factor for the production of vitamin B_{12} The fermentation medium was made from the basic medium and a series of $CoCl_2$ of (1, 0.1 and 0.01) $gm.10ml^{-1}$ was added with modification at a temperature of 37 °C for 72 hours after which vitamin B_{12} was measured as described by Chowdhurg (7).

Results and Discussion

The effect of the carbon source

The results shown in Figure (1) indicate the use of multiple sources of carbon, including (fructose, glucose, sucrose) for the purpose of determining the most efficient ones in the production of vitamin B_{12}.

The results showed that *L.rhamnosus* bacteria gave the highest production of the vitamin when using the carbon source represented by sucrose with a concentration of 7 g/L and a vitamin production rate of 393.01 µg.L^{-1}, followed by glucose and fructose at a concentration of 3 and 10 g/L with a production rate of vitamin 332.10 and 322.66 µg.L^{-1} respectively, but the carbon source represented by sucrose is the most efficient, and the reason can be attributed to the fact that sources are from the sugars that are consumed quickly and enhance the speed of cell growth, which is directly proportional to the amount of vitamin produced as indicated by Ni.naomichi (25)

Carbohydrates are one of the essential nutrients for the growth of microorganisms, as all kinds of carbon sources can be absorbed and used by bacteria and can be represented, as it is one of the most important sources for the components of the cell membrane and bacteria differ in their synthesis from one type to another and their consumption in the medium by microorganisms moreover, the carbon source is considered a source of energy for the purpose of bacterial metabolism (6).

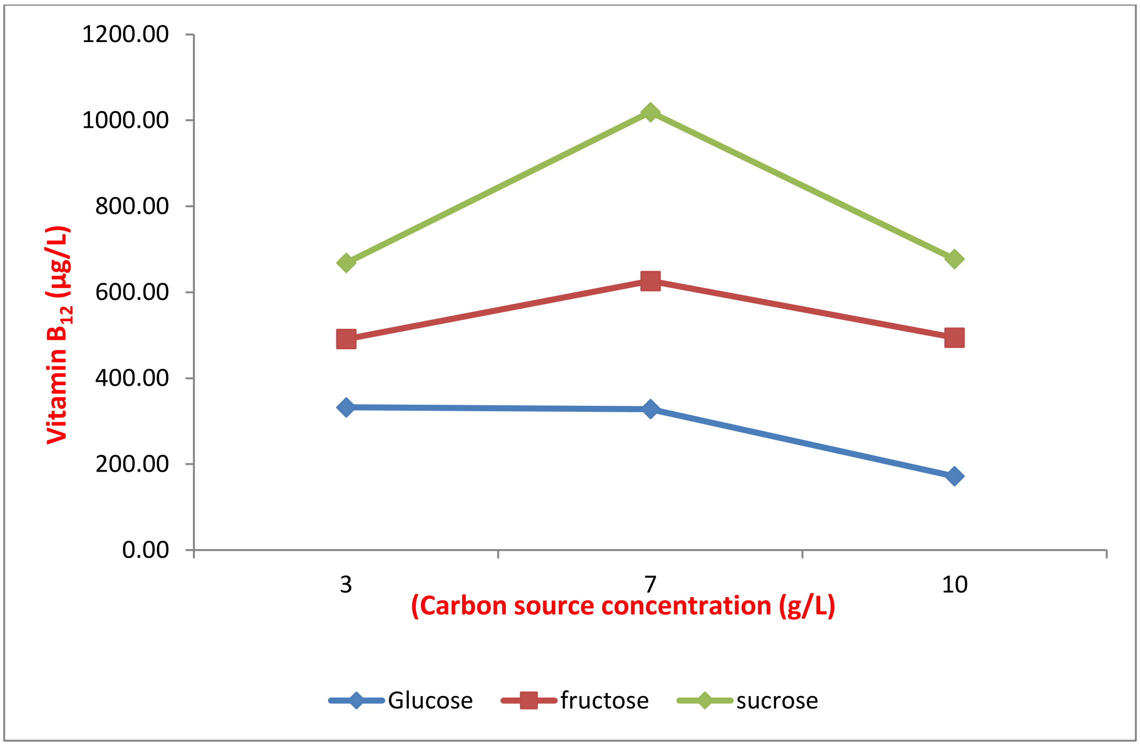

Figure 1. the effect of different carbon sources on the production of vitamin B_{12} by *Lactobacillus rhamnosus*

The effect of the nitrogen source

The effect of the nitrogen source on the production of vitamin B_{12} from *L. rhamnosus* bacteria was studied at concentrations of 3, 7 and 10 g.L^{-1} that included yeast extract and peptone. The results shown in Figure (4-6) showed that the best nitrogen source for vitamin production is peptone at a concentration of 3 g.L^{-1}. The amount of vitamin production was 453.52 µg.L^{-1}, followed by yeast extract at a concentration of 10 g.L^{-1}, as the amount of vitamin production was 301.07

µg.L^{-1}, while the lowest production of vitamin was recorded for the same isolate using yeast extract at a concentration of 3 g.L^{-1} as a nitrogen source, as the amount of vitamin produced was 203.80 µg.L^{-1}.

Sources such as peptone and others in the fermentation medium act as sources of nitrogen, most of them, as a buffer system such as ammonia, as its presence in the medium reduces or equalizes the pH of the medium, which affects the synthesis of metabolites during the fermentation process, in addition to that, the nitrogen source affects a number of enzymes glucose-6-phosphate dehydrogenase, hexokinase, gluconate dehydrogenase, which participate in the production of acids and vitamins, and free amino acids are somewhat required to improve the work of enzymes inside cells, so the nitrogen sources help in raising the requirements for growth and fermentation (22).

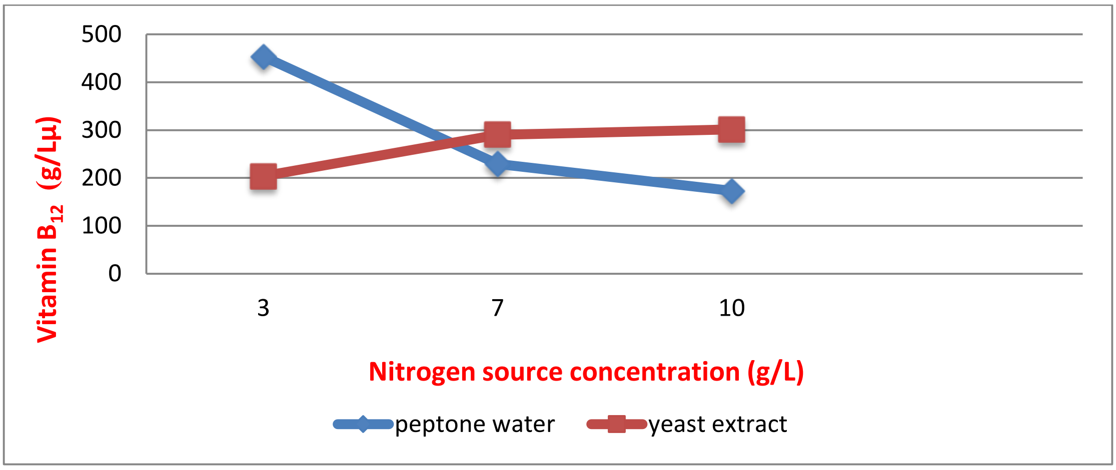

Figure 2. the effect of different nitrogen sources on the production of vitamin B_{12}

According to the previous results, peptone was chosen as the best nitrogen source for vitamin B_{12} production and was used in all subsequent stages of the study. The scientist worked on different nitrogen sources, including peptone, casein, and yeast extract (4), also indicated that peptone is an essential substance for *Lactobacillus* bacteria, which increases the efficiency of vitamin production.

The superiority of peptone in vitamin production from the isolate used in this study can be attributed to its containing many components that meet the complex needs of Lactobacillus bacteria, as it contains short-chain amino acids and nitrogen (5)

After determining the best nitrogen source for vitamin production, the optimal concentration of the nitrogen source was using peptone at a concentration of 3 g.L^{-1}.

Effect of pH

The effect of pH on the production of lactic acid from the producing isolate *L.rhamnosus* was studied. As it is noted in Figure (3) that the best pH for the production of vitamin B_{12} is 7, where the amount of vitamin produced was 160.51 µg.L^{-1} for the produced isolate, as indicated by some studies the pH 7 was used in the production of vitamin B_{12} from Propionibacterium freudenreichii and it achieved the best growth and the best production of the vitamin (3)

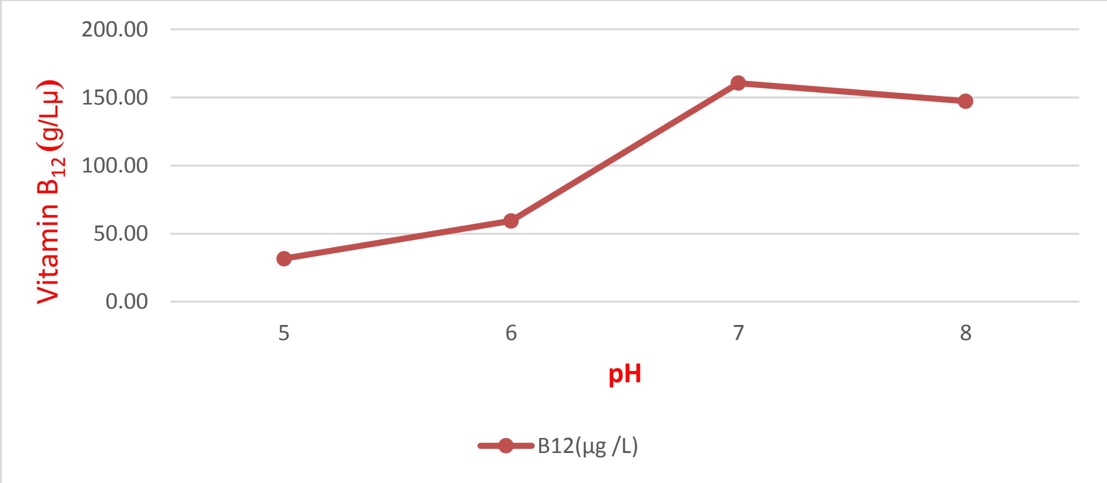

Figure 3. the effect of different degrees of pH on the production of vitamin B_{12}

The pH of the medium is very important for the growth of microorganisms and to direct their metabolism. Changes in the pH are very important for the effectiveness of the enzymes of the microorganisms and the intermediate products and the degree of their dissolution and solubility. In addition, these changes have an effect on the outcome of the final products of the metabolism of microorganisms (11) and the pH affects In general, in the function of metabolic enzymes and the transport of nutrients in the cell (13).

The pH affects the production of any specific metabolic product, so a narrow range of pH must be maintained because determining the pH of the culture medium is one of the very important factors in increasing productivity, so compounds are added to the culture media that act as acidity regulators for the purpose of maintaining the pH as well as being sources of nutrients for microorganisms, so calcium carbonate is used for the purpose of maintaining the neutral pH in the culture medium. When the pH decreases, the carbonates decompose and when the pH increases, the acids released into the culture medium through microorganisms reduce the pH (1).

It was also reported by Abdel-Hafez *et al.*(2) and Marwaha *et al*. (22), who found that the optimum pH for vitamin B_{12} production by strains of Propionibacterium was 7.0 ± 2, in addition, as mentioned by Snydman (27) also showed that vitamin B_{12} production by *B.megaterium* reached its highest level at an initial pH of 6.0.

Effect of temperature

The results indicated in Figure (4) showed that the highest production of vitamin B_{12} for isolate L. rhamnosus using different temperatures was at a temperature of 30 °C, where the amount of vitamin production was 248.46 µg/L compared with the rest of the temperatures, the amount of vitamin production was 16.51,88.01 $\mu g.L^{-1}$ at (35 and 37) °C, respectively.

Temperature is important during the microbial fermentation processes and the production of important metabolic materials, as the high and low temperature during the incubation period is a reason for the production of a small or large quantity and according to the conditions present during the fermentation and the presence of the carbon source, and the temperature 28-30 °C was the optimal temperature for the production of vitamin B_{12} by Lactobacillus bacteria (28).

Several studies have indicated that the temperature of 30°C is optimal for vitamin production from *Lactobacillus* bacteria, Different temperatures were used to produce the vitamin from *Lactobacillus* bacteria(18), and the highest production of the vitamin was achieved at a temperature of 30°C, as mentioned Liu and Moon (20) which showed that the optimum temperature for the growth of Propionibacterium spp. is approximately 30°C.

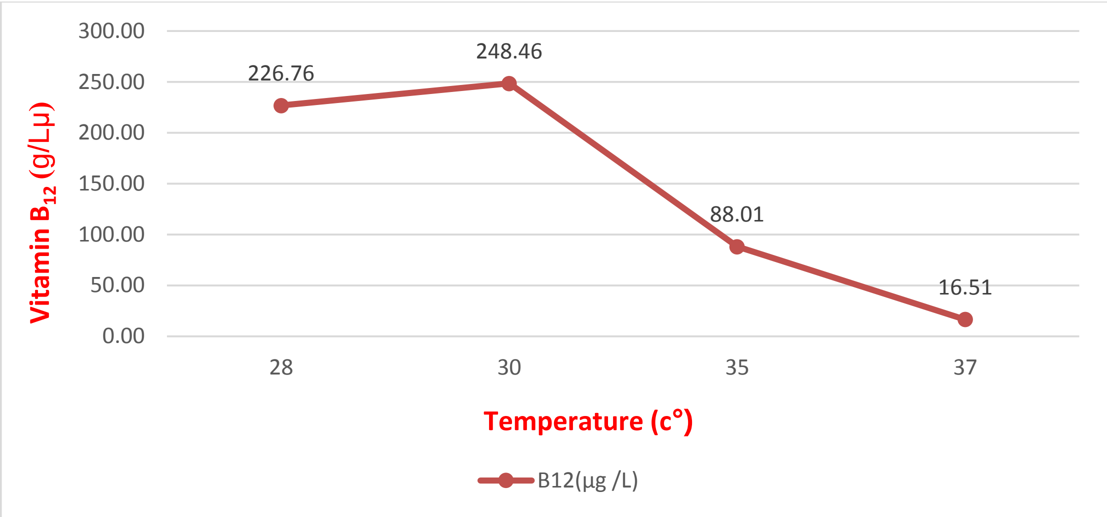

Figure 4. the effect of different temperatures on the production of vitamin B_{12}

Heyssel *et al.* (14) and Schwartz (26) found that oxygen inhibits the activity of two key enzymes in the production of vitamin B_{12} (δ-amino-levulinic acid dehydratase and δ-amino-levulinic acid synthetase). of the most efficient isolation under study by incubation at a temperature of 30 °C, it can be attributed to the fact that this temperature is the optimal for the work of the materials necessary to convert the basic substance into a vitamin, and that any temperature higher or lower than this temperature affects the metabolism and thus affects the process of vitamin production.

Effect of incubation time

The results shown in Figure (5) showed the application of different incubation periods that included (3, 5, and 7) days, as the results show that the incubation period had an effect on the production of vitamin B_{12}, as the best incubation period was 3 days, where the amount of vitamin produced was 259.03 $\mu g.L^{-1}$ and followed by a period of 7 where the amount of vitamin production reached 181.19 $\mu g.L^{-1}$ days compared to the rest of the days. The results showed that the best incubation period was 3 days, as it gave the highest production of vitamin B_{12}.

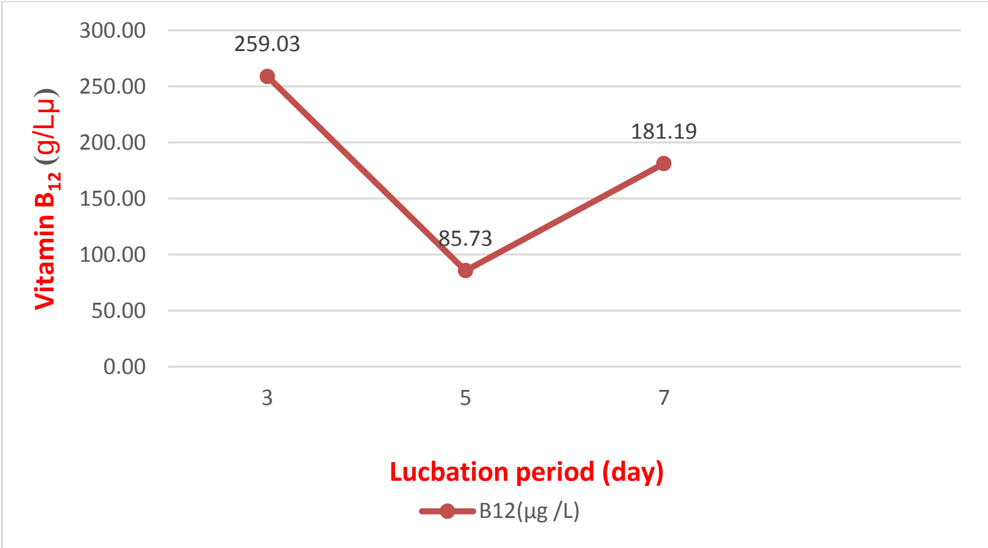

Figure 5. Shows the effect of incubation period on the production of vitamin B_{12}

The effect of cobalt chloride $CoCl_2$

Figure (6) shows that high concentrations of cobalt chloride have a negative effect on the production of vitamin B_{12} and lower the pH, as it was found that the use of the lowest concentration of cobalt chloride (0.01) $g.10ml^{-1}$ gave the highest production of vitamin B_{12} among several concentrations that included (0.01, 0.1 , 1) $g.10ml^{-1}$ with a production rate of 158.57 $\mu g.L^{-1}$, where (32) stated that different cobalt concentrations affected the production of vitamin B_{12} directly, and it was also found that the pH decreased while increasing the amount of cobalt chloride ($Cocl_2$) as well as increasing the concentration of cobalt. result in increased red color and enlargement of the heart or pulmonary fibrosis, so appropriate concentrations of cobalt must be studied and tested in industrial production applications, as well as a natural substance derived from food must replace pure chemicals in order to prevent the harmful effects of cobalt.

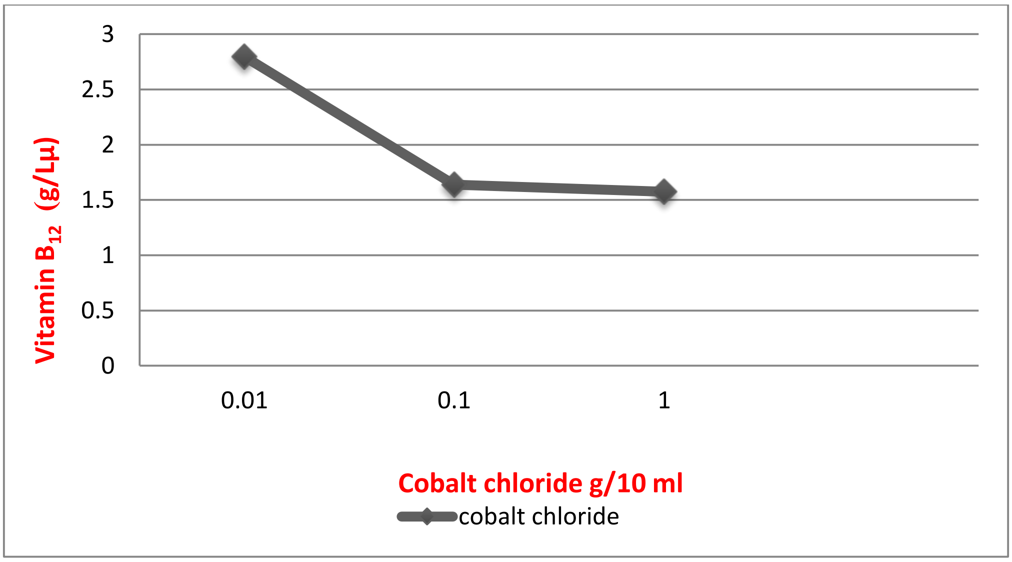

Figure 6. The effect of adding different concentrations of cobalt $Cocl_2$ on B_{12} production.

Conflict of interest

The authors have no conflict of interest.

References

1. **Al-Haidari, N. K. and Al-Musleh, R. M.**1989**.** Industrial Microbiology. First Edition. University of Baghdad. Iraq.

2. **Abdel-Hafez, A. M.; W. A. Mashhoor; R. F. Gamal; A. A. Rafaat and EL-Sayed, A. A.**1981**.** Production of vitamin B12 by propioni acid bacteria. II- Effect of nutritional and environmental factors on vitamin B12 production by Propionibacterium shermanii P-16. Bull.1686, Agric. Microbiol. Dept., Fac. of Agric. Ain Shams Univ. Cairo. Egypt.

3. **Abou-Taleb Khadija, A. A.; W. A. Mashhoor; A. N. Sohair and Sharaf, M. S.**2010**.** Effect of environmental factors on vitamin B12 production by Propionibacterium freudenreichii and Bacillus Megaterium, J. Biol. Chem. Agric. Microbial. Dept., Fac. of Agric., Ain Shams Univ., Shoubra El-kheima, Environ. Sci., 2010, Cairo, Egypt, 5(4):509-521.

4. **Aeschlimann, A. and U. Von Stockar.**1990. The effect of yeast extract supplementation on the production of lactic acid from whey permeate by *Lactobacillus helueticus.* Applied Microbiology and Biotechnology, 32:398–402.

5. **Al-Khafaji, M. Z.**1990.Biotechnology. Dar Al-Hekma Press for Printing and Publishing. University of Baghdad. Ministry of Higher Education and Scientific Research. Iraq.

6. **Cheng, F.; H. Chen; N. Lei; M. Zhang and Wan, H.**2019**.** Effects of carbon and nitrogen sources on activity of cell envelope 18 proteinase produced by Lactobacillus plantarum LP69. Acta Universitatis Cibiniensis Series E. Food Technology, 23(1):11-18.

7. **Chowdhury, A.; M. N. Hossain; N. J. Mostazir; M. Fakruddin; M. Billah and Ahmed, M. M.**2012**.**Screening of Lactobacillus spp. From Buffalo Yoghurt for Probiotic and Antibacterial Activity. Journal of Bacteriology and Parasitology, 3(8): 1–5. DOI: 10.4172/2155-9597.1000156ز

8. **De Champs, C.; N. Maroncle; D. Balestrino; C. Rich and Forestier, C.**2003**.** Persistence of colonization of intestinal mucosa by a probiotic. Strain, Lactobacillus casei subsp. rhamnosus LCR35, after oral consumption. J. Clin. Microbiol, 41(3):1270–1273. doi:10.1128/JCM.41.3.1270-1273.

9. **Doron, S.; D. R. Snydman and Gorbach, S. L.**2005**.**Lactobacillus GG: Bacteriology and clinical applications. Gastroenterol. Clin. N. Am., 34(3):483–498. DOI: 10.1016/j.gtc.2005.05.011.

10. **Egorov, N. S.**1985**.**Antibiotics Ascientific Approach .Mir Publishers Moscow. Russian.

11. **Fooks, L.; R. Fuller and Gibson, G. R.**1999**.**Prebiotics-probiotics and human gut microbiology. International

Dairy Journal, 9(1):53-61. http://dx.doi.org/10.1016/S0958-6946(99)00044-8

12. **Forestier, C.; C. De Champs; C. Vatoux and Joly, B.**2001.Probiotic activities of Lactobacillus casei rhamnosus: In vitro adherence to intestinal cells and antimicrobial properties. Res. Microbial., 152(2):167–173. DOI:10.1016/s0923-2508(01)01188-3

13. **Harley, J. and L. Prescott.**2002.Laboratory exercises microbiology. 5th ed. WCB. The Mcgraw- Hill, Companies. New York. USA.

14. **Heyssel, R. M.; R. C. Bozian; W. J. Darby and Bell, M. C.**1966.Vitamin B12 turnover in man. The assimilation of vitamin B12 from natural foodstuff by man and estimates of minimal daily dietary requirements. Am. J. Clin. Nutr. 18(3):176-184. DOI: 10.1093/ajcn/18.3.176

15. **Holt, G. and R. Krieg.**1986**.** Bergey's Manual of Systematic Bacteriology.,2, Williams and Wilkins Company. USA.

16. **Hugenschmidt, S; S. Miescher; N. Gnehm and Lacroix, C.**2010**.Screening** of a natural biodiversity of lactic and propionic acid bacteria for folate and vitamin B12 production in supplemented whey permeate / International Dairy Journal., 20(12):852-857. DOI:10.1016/j.idairyj.2010.05.005

17. **Ibrahim. F.; S. Ruvio; L. Granlund; S. Salminen; M. Viitanen and Ouwehand, A. C.**2010**.**Probiotics and immune senescence: Cheese as a carrier. *FEMS Immunology and Medical Microbiology*, 59(1):53-59. https://doi.org/10.1111/j.1574-695X.2010.00658.x

18. **Kious, J. J.**2000**.** Lactobacillus and Lactic acid Production. Prepared in partial fulfillment of the requirements of the Office of Science, DOE ERULF under the direction of Min Zhang in Strain Development at the National Renewable Energy Laboratory. Taiwan.

19. **Kopp-Hoolihan, L.**2001**.**Prophylactic and therapeutic Uses of probiotics: A Review. Journal of American Dietary Association, 101(2): 229-241. doi:10.1016/S0002-8223(01)00060-8.

20. **Liu, J. A. P. and N. J. Moon.**1982**.**Commensalism interaction between lactobacillus acidophilus and Propionibacterium shermanii. Appl. Environ Microb., 44:715-722.

21. **Marwaha, S. S.; R. P. Sethi and Kennedy, J. F.**1983.Influence of 5, 6 Dimethylbenzimidazole (DMB) on vitamin B12 biosynthesis by strains of Propionibacterium. Enzyme and Microbial Technology, 5:361-364.

22. **Mohammed, Y.; L. Byong; K. Zhen and Guocheng, Du.**2014. Development of a two-step cultivation strategy for the production of vitamin B12 by Bacillus megaterium. Microbial Cell Factories, 13:102.

23. **Moslem, P.; N. Hossein; R. Mahdi; N. H. Seyed; A. S. Seyed.**2017. Lactobacillus rhamnosus Gorbach-Goldin GG: A top well-researched

probiotic strain. J. Med. Microbiol., 5(4-6):46–59.

24. **Nishio, N; Y. T. Ano and Kamikubo, T.1975.** Department of Fermentation Technology.1974, Faculty of Engineering. Hiroshima University. Hiroshima Received April 22, Agr. BioI. Chem., 39: (1):21 – 27.

25. **Pitino, I.; C. L. Randazzo; K. L. Cross; M. L. Parker; C. Bisignano; M. S. J. Wickham; G. Mandalari and Caggia, C.**2012**.**Survival of Lactobacillus rhamnosus strains inoculated in cheese matrix during simulated human digestion. Food Microbial, 31:57–63. DOI: 10.1016/j.fm.2012.02.013

26. **Schwartz, A. C.1973.** Anaerobiosis and oxygen consumption of some strains of Propionibacterium and a modified method of comparing the oxygen sensitivity of various anaerobes. Zeitschrift fur Allgemeine Mikrobiol., 13: 681 - 691.

27. **Snydman, D. R.**2008**.**The safety of probiotics. Clin. Infect. Dis., 46: 104–111. DOI: 10.1086/523331

28. **Song, J.; H. Liu; L. Wang; J. Dai and Zheng, Z.**2014.Enhanced production of vitamin K2 from Bacillus subtilis (natto) by mutation and optimization of the fermentation medium. Braz. Arch. Biol. Technol., 57: 606–612. doi:10.1590/ S1516-8913201402126.

29. **Sunny-Roberts, E.O and D. Knorr.**2008**.** Evaluation of the response of Lactobacillus rhamnosus VTT E-97800 to sucrose-induced osmotic. Stress. Food Microbiol, 25(1):183–189. DOI: 10.1016/j.fm.2007.05.003.

30. **Randazzol, C. L.; I. Pitinoi; F. Licciaedell; G. Mmuratore and Caggia, C.**2013**.**Survival of Lactobacillus rhamnosus probiotic strains in peach jam during storage at different temperatures. Food Sci. Technol., 33(4):652–659.

31. **Tanook, G. W.; K. Munro; H. J. Harmsen; G. W. Welling; J. Smart and Gopal, P. K.** 2000**.** Analysis of feacal micoflora of human subjects consuming aprobiotic products containing Lactobacillus rhamnosus DR20. Appl. Environ Microbil., 66(6):2578-2588. DOI: 10.1128/AEM.66.6.2578-2588.2000.

32. **Tiffany M. E.; V. Fellner and Spears, J. W.**2006**.**Influence of cobalt concentration on vitamin B12 production and fermentation of mixed ruminal microorganisms grown in continuous culture flow-through fermentors. J Anim Sci., 84(3):635-40 DOI: 10.2527/2006.843635x.

33. **Tuomola, E. M.; A.C. Ouwehand and Salminenm, S. L.**1999**.**The effect of probiotic bacteria on the adhesion of pathogens to human intestinal mucus. FEMS Immunol. Med. Microbiol., 26(2):137–142. DOI: 10.1111/j.1574-695X.1999.tb01381.x.

34. **Venema, K.2015.**Health effect of pro and prepiotics: Utilization of sophisticated in-vitro Tools. (In. Beneficial Microorganisms in Medical Health Applications; series:

Microbiology Monographs., 28: 1-18.).