Optimal conditions for vitamin B\textsubscript{12} production from \textit{Lactobacillus rhamnosus}

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Abstract

This research was conducted to study the effect of some nutrients on the growth and production of vitamin B\textsubscript{12} by Lactobacillus genus \textit{L.rhamnosus}, when their ability to produce vitamin B\textsubscript{12} was tested under different conditions. The results indicate that a pH of 7 is optimal for vitamin B\textsubscript{12} production by the bacteria \textit{L.rhamnosus} and it was found that the optimum incubation temperature for the production of vitamin B\textsubscript{12} ranged between 30 - 35 °C, with regard to the effect of the incubation period on the biological activity and productivity of vitamin B\textsubscript{12}. The results showed that 3 days was the best in the production of vitamin B\textsubscript{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\textsubscript{12}.

Keywords: Vitamin B\textsubscript{12} production, optimum conditions, \textit{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 B₁₂ 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, CoCl₂.

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 B₁₂ 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 (17).

Effect of pH

The effect of pH on the production of vitamin B₁₂ 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 B₁₂ 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 B₁₂ was measured as described by Pirtino (24).

The effect of cobalt chloride CoCl₂

Cobalt is also an important factor for the production of vitamin B₁₂. The fermentation medium was made from the basic medium and a series of CoCl₂ 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 B₁₂ 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 B₁₂.
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 B12 by *Lactobacillus rhamnosus*

The effect of the nitrogen source

The effect of the nitrogen source on the production of vitamin B12 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 B₁₂

According to the previous results, peptone was chosen as the best nitrogen source for vitamin B₁₂ 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 B₁₂ 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 B₁₂ from Propionibacterium freudenreichii and it achieved the best growth and the best production of the vitamin (3)
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<sub>12</sub> production by strains of Propionibacterium was 7.0 ± 2, in addition, as mentioned by Snydman (27) also showed that vitamin B<sub>12</sub> 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<sub>12</sub> 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 µg.L<sup>-1</sup> 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 B₁₂ by Lactobacillus bacteria (28).

Several studies have indicated that the temperature of 30°C is optimal for vitamin production 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 B₁₂

Heyssel *et al.* (14) and Schwartz (26) found that oxygen inhibits the activity of two key enzymes in the production of vitamin B₁₂ (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 B₁₂, as the best incubation period was 3 days, where the amount of vitamin produced was 259.03 µg.L⁻¹ and followed by a period of 7 where the amount of vitamin production reached 181.19 µg.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 B₁₂.
The effect of cobalt chloride CoCl₂

Figure (6) shows that high concentrations of cobalt chloride have a negative effect on the production of vitamin B₁₂ 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 B₁₂ among several concentrations that included (0.01, 0.1, 1) g.10ml⁻¹ with a production rate of 158.57 μg.L⁻¹, where (32) stated that different cobalt concentrations affected the production of vitamin B₁₂ directly, and it was also found that the pH decreased while increasing the amount of cobalt chloride (CoCl₂) 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 5. Shows the effect of incubation period on the production of vitamin B₁₂

Figure 6. The effect of adding different concentrations of cobalt CoCl₂ on B₁₂ production.
Conflict of interest

The authors have no conflict of interest.

References


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