



Original Research article

Investigation of Physicochemical Parameters and Measurement of Ascorbate Peroxidase and Catalase Activity in the Presence of Different Concentrations of Folic Acid in *Propionibacterium freudenreichii*



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Catalase

Ascorbate peroxidase

ABSTRACT

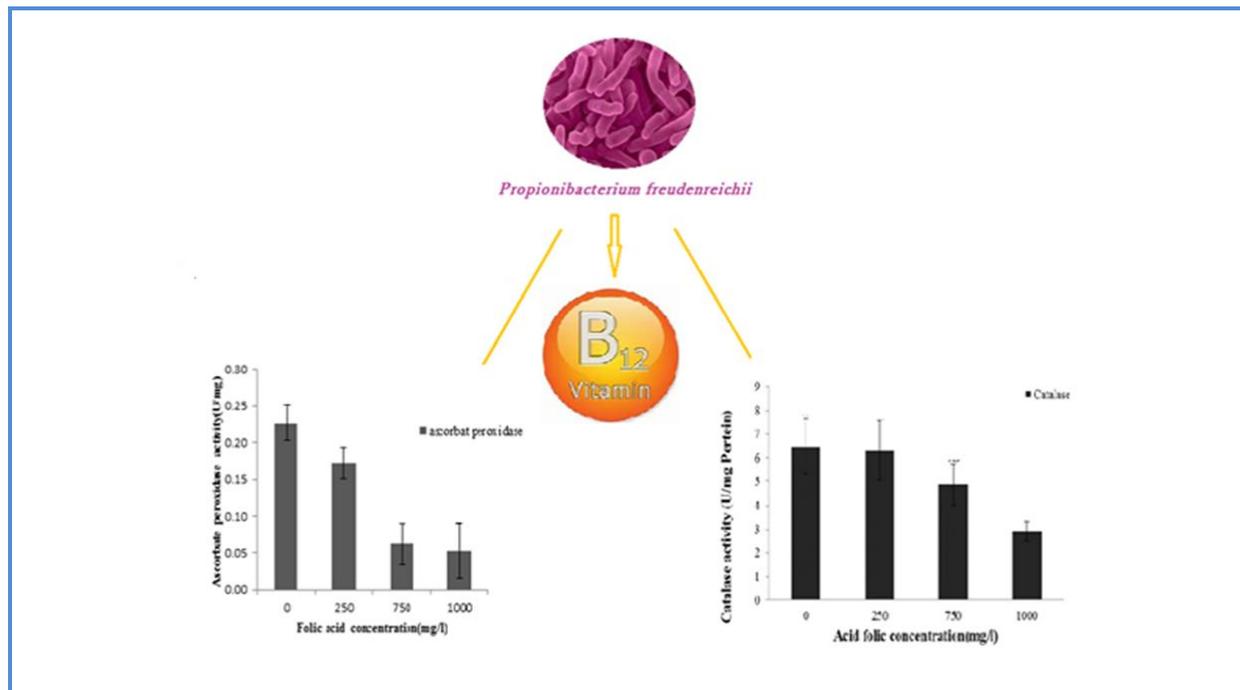
Propionibacterium is a gram-positive genus, anaerobic to microaerophilic, and rod-shaped from propionic bacteria family that is capable of producing propionic acid using transcarboxylase enzymes. The aim of this study was to investigate the effect of physicochemical parameters (temperature and pH) and ascorbate peroxidase and catalase levels at different concentrations of folic acid in *Propionibacterium freudenreichii* to produce better and more vitamin B₁₂. The *Propionibacterium freudenreichii* was inoculated into fermented medium containing soaked corn. Folic acid was added to the fermentation medium at different concentrations, then its absorption at 600 nm, at different temperatures and pH, was investigated. The results revealed that, the optimum pH for bacterial growth was 6.6 and the optimum bacterial growth was at 25-40 °C. Addition of folic acid at lower concentrations to the culture medium of *Propionibacterium freudenreichii* increased the UV absorption at 600 nm. It was found that, high concentration of folic acid reduced the growth rate which, indicating the negative effect of the folic acid on bacterial growth. Catalase and ascorbate peroxidase activity was determined based on H₂O₂ consumption at 240 nm and 290 nm. In this bacterium, CAT and ascorbate peroxidase activity decreased significantly with increasing folic acid concentration. It was also found that folic acid functioned as a toxin for this bacterium at high concentrations and demonstrated antioxidant properties.

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Graphical Abstract



Introduction

Propionibacterium freudenreichii is one of the non-motile gram-positive bacteria. One notable feature is that it produces large amounts of propionic acid and acetic acid. These bacteria can ferment sugars and polyhydroxy alcohols, producing lactate as long as the bacteria present near their fermentation activities [1]. Members of the genus *Propionibacterium* are destructive parasites of the human body and other animals found in sweat glands, sebaceous glands and other parts of the skin. They are almost epidemic and not a problem for most people; however, but *Propionibacteria* may cause acne and other skin problems [2]. *Propionibacteria* are commonly used to produce vitamin B₁₂, tetrapyrrol and propionic acid [3]. Vitamin B₁₂ and folate are involved in some of the biochemical reactions, affecting the production and transport of the methyl group. Vitamin B₁₂ is a methionine synthase (MS) cofactor, acting as a catalyst in the conversion of homocysteine to methionine. Methionine can function as a general methyl donor for DNA and RNA methylation by converting *S*-adenosyl methionine (SAM) [4]. DNA methyl transferase enzymes catalyze the transfer of a methyl group to the 5-carbon cytosine residue by SAM [5]. Folate is a type of vitamin that can receive units of carbon through donor molecules and is found in many metabolic pathways such as formation of an asyl group and synthesis of nucleotides, vitamins, and some amino acids. Folate is also involved in improving the efficiency of DNA replication, repair, and methylation [6]. In addition, increased folate intake in patients with

inflammatory bowel disease may help regulate the turnover of rectal cells [7]. Human colon fluoro microbes produce vitamin K (monokinins) and most water soluble vitamins of group B including biotin, nicotinic acid, folate, riboflavin, thiamine, pyridoxine, pantothenic acid, and cobalamin [8]. Diet vitamins are mainly absorbed in the small intestine; however, microbial vitamins are mainly absorbed in the colon [9]. It seems that, the colonocytes can assist in the uptake of biotin, thiamine, folate, riboflavin, pantothenic acid, and monokinins, suggesting that microbial vitamins play a significant role in systemic vitamin levels, and in particular in the homeostasis of vitamins in local epithelial cells [9, 10]. All cells, both prokaryotic and eukaryotic, require reduced folate cofactors in the acceptor/donor reactions of single carbon units in biosynthetic processes such as thymine, methionine and purine formation and in some degradative reactions. Generally, all cells need folates, but the methods of obtaining them may vary between organisms. For instance, animals cannot synthesize folate and combine these derivatives with their diet using active transport systems. Plants, fungi, specific protozoa, and several archaea and bacteria can synthesize folates, which can probably be synthesized *via* the same general biosynthetic pathway [11] with some variation [12-16]. In fact, the current interest in replacing animal protein with vegetable protein might be due to the reduced B₁₂ intake in the future [17]. Therefore, B₁₂ deficiency can be offset by enrichment of plant products using natural methods such as fermentation strength by B₁₂ production of food microorganisms [18]. *Propionibacterium freudenreichii* is the only bacterium known to safely synthesize B₁₂ [19]. Applying *P. freudenreichii* to cereal-based products (such as bread) helps improve product shelf life [20, 21]. Recent studies indicate the ability of organisms to produce active B₁₂ in cereal-based matrices [22, 23]. The present work was carried out to study the effect of physicochemical parameters (temperature and pH) and ascorbate peroxidase and catalase enzyme levels on different concentrations of folic acid in *Propionibacterium freudenreichii* in order to produce better and more vitamin B₁₂. In a similar study, the effects of different sources of carbon, nitrogen and some elements such as Cu, Mn, Zn, Mg and Fe on vitamin B₁₂ production by *Propionibacterium freudenreichii* and *Bacillus megaterium* were investigated [24].

Experimental

Materials and methods

Propionibacterium freudenreichii PTCC (1647) bacteria were purchased from the fungi and bacteria center of Iran scientific research organization. Bacteria in anaerobic conditions on sodium lactate medium (smachme chemical) containing 10 g/L casein peptone-tryptic digest (Merck, Germany), 5 g/L yeast extract (Merck, Germany), 10 g/L lactate (Sigma-Aldrich) and 1000 mL of deionized

water were incubated at 30 °C for 3 days. Then, the bacterium was transferred to pre-culture medium containing 200 g/L filtered tomato juice, 10 g/L yeast extract, 10 g/L tryptone (biolife), and kept for 2 days at 30 °C [25, 26]. The bacterium was then transferred to the pre-culture medium 2, containing 20 g/L filtered soaked corn, 90 g/L glucose to increase the bacterial growth rate. After bacterial inoculation, the containers were incubated at 30 °C for 24 h without aeration. Subsequently, folic acid was added to fermentation medium with various concentrations (0, 30, 250, 500, 750, and 1000 mg/L), then the medium was stored for 4 days at 30 °C under the anaerobic conditions.

Determination of optimum pH at different concentrations of folic acid

Bacteria were cultured in sodium lactate medium with different pH (5, 6.6, and 9). Then, the optical absorption of the medium containing bacteria was read at 600 nm (UV-spectrophotometer, Shimadzu Japan model: UV- 2550) [27, 28]. The concentration of 0 mg/L of folic acid was considered as the control.

Determination of optimum temperature at different concentrations of folic acid

To study the thermal stress, the tubes containing culture medium were incubated for 72 h at 25, 30, 40, and 50 °C and then their optical absorption was read at 600 nm. The concentration of 0 mg/L of folic acid was considered as the control.

Measurement of ascorbate peroxidase activity

The reaction solution of the ascorbate peroxidase enzyme (APX-EC 1.11.1.1) consisted of 50 mM phosphate buffer (pH=7) containing 0.2 mM EDTA and 50 µL of 200 mM hydrogen peroxide. 900 µL of the reaction solution was mixed with 50 µL of the enzyme extract and the absorbance at 290 nm was investigated for 60 s. One unit of ascorbate peroxidase activity was expressed at the rate of 1 µmol/min ascorbic acid at 25 °C. The enzyme activity was calculated using the Equation 1 [29, 30].

$$(\Delta OD/\Delta t) \times V / (\epsilon \times v \times p) \quad (1)$$

Where ΔOD is absorption difference, Δt is temperature difference, V is enzymatic extract volume, v is buffer volume, p is protein concentration, and ϵ is extinction coefficient (2.8 Mm/cm).

Measurement of catalase (CAT-Ec 1.11.1.6) activity

The reaction mixture was 950 µL containing 900 µL phosphate buffer 50 mM (pH 0.7) and 50 µL of H₂O₂ (200 mM) was mixed with 50 µL of the bacterial enzyme extract. Determination of CAT activity was

performed based on the H_2O_2 consumption (the extinction coefficient, 39.4 mm/mm^{-1}) at 240 nm, over 60 s intervals (at 25°C) and based on the standard curve of absorption of 0 to 11 mm H_2O_2 [31-33].

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 are represented as the mean \pm 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 and discussion

Initial experiments were carried out at pH: 5, 6.6 and 9. There was no UV absorption at pH=5 and pH=9 and the results showed that the optimum pH for bacterial growth was 6.6 at 250 mg/L of folic acid. As folic acid concentration increased, the rate of absorption decreased and the lowest level of absorption was at 1000 mg/L of folic acid. Figure 1, is shown the bacterial growth at different concentrations of folic acid at pH=6.6.

As seen in Table 1, at 25°C , the best bacterial growth at 250 mg/L of folic acid was statistically higher than the control group but the rest of the concentrations were not significantly different from the control and 250 mg/L folic acid. At 30°C the bacterial growth rate at all folic acid concentrations was similar and higher than the control group. At 30, 250, and 1000 mg/L of folic acid at 40°C , the bacteria had the highest growth rate and were statistically similar.

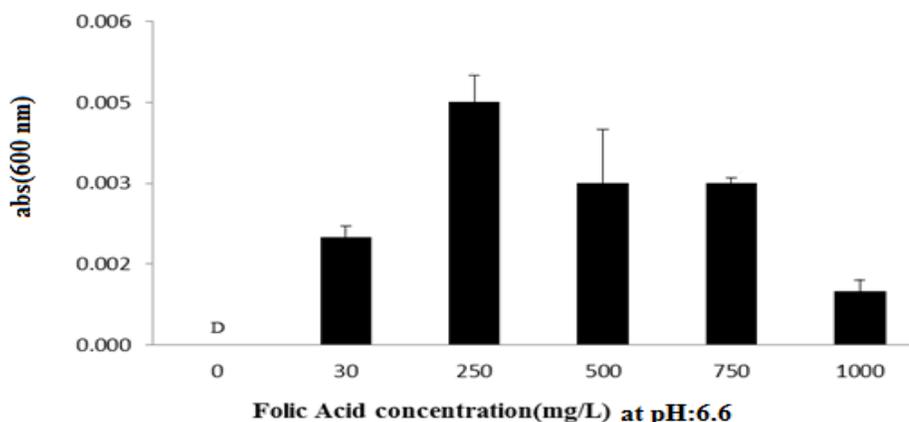


Figure 1. Diagram of bacterial growth at different concentrations of folic acid at pH=6.6

At 500 and 750 mg/L folic acid, the bacterial growth rate was statistically similar and there was no significant difference with the control group. At 50°C , bacterial growth at all concentrations except

1000 mg/L was higher than the control group. Therefore the best temperature for bacterial growth was 40 °C and the best concentration was 250 mg/L of folic acid.

Table 1. The effect of temperature on bacterial growth rate at different concentrations of folic acid

		Folic acid concentrations(mg/L)					
		0	30	250	500	750	1000
Temperature (°C)	25	0 ^b	0.007±0.001 ^{ab}	.0125±.0035 ^a	.009±.001 ^{ab}	.008±.001 ^{ab}	.0065±.0005 ^{ab}
	30	0 ^b	.0085±.0005 ^a	.0135±.0015 ^a	0.0135±.0035 ^a	.011±.002 ^a	0.013±.001 ^a
	40	0 ^c	0.0116±.014 ^a	0.1225±.0025 ^a	0.0495±.0035 ^b	.028±.025 ^{bc}	0.0126±.004 ^a
	50	0 ^b	0.0225±.012 ^a	0.021±.0014 ^a	0.016±.007 ^a	0.0235±.003 ^a	0.0145±.003 ^{ab}

Value are mean ± standard error (n=3). Meanes in each rows having the same letter are significantly different using Duncan's test at (p<0/05)

Ascorbate peroxidase activity increased with decreasing the folic acid concentration and decreased with increasing folic acid concentration. As shown in Figure 2, the enzyme activity increased at concentrations of 0 and 250 mg/L of folic acid while the concentration of ascorbate peroxidase activity decreased significantly with increasing folic acid concentration at 750 and 1000 mg/L of folic acid.

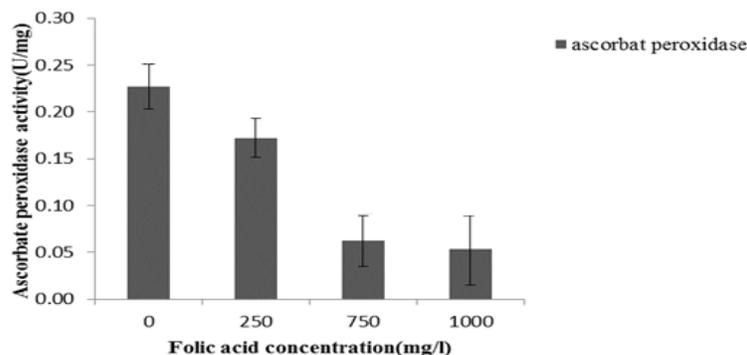


Figure 2. Diagram of ascorbate peroxidase enzyme activity at different concentrations of folic acid

In the study of catalase (CAT) activity at different concentrations of folic acid (0, 250, 750 and 1000 mg/L) in *Propionibacterium freudenreichii*, under aerobic conditions, CAT activity decreased significantly with increasing the folic acid concentration. This decrease in activity was not significant at 750 mg/L folic acid, but it was significant at 1000 mg/L folic acid. The enzyme activity in the control group increased almost twice as compared to the concentration of 1000 mg/L folic acid. At concentrations of 0 and 250 mg/L, the activity increased 2.6 times higher than the concentration of 1000 mg/L. Therefore, it was found that high concentrations of folic acid inhibited the activity of CAT Remy, reducing the bacterial growth and vitamin B₁₂ production. The effect of different concentrations of folic acid on CAT activity is shown in Figure 3.

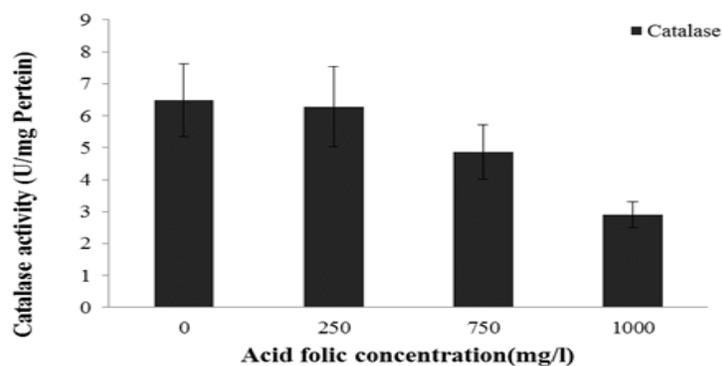


Figure 3. Diagram of catalase activity at different concentrations of folic acid

Studies have shown that microbes have obtained adequate protective measures to prevent detectable toxicity by endogenous reactive oxygen species (ROS) [34]. Increased intracellular levels of these oxidants, especially superoxide (O_2^-) and hydrogen peroxide (H_2O_2), create enzymes that prevent damage to growth and DNA. Glutathione, ascorbate and vitamin E are often added to bacterial cultures to eliminate ROS. These compounds are natural antioxidants in mammalian systems. Glutathione is also required for glutathione peroxidase reductant, H_2O_2 detergent and ascorbate reductant ascorbate peroxidase [35]. Superoxide dismutase is part of the defense systems of aerobic organisms that protect them against oxidative damage [36, 37] and act as a catalyst for the conversion of superoxide anion (O_2^-) to O_2 and H_2O_2 then by the catalase, H_2O_2 is reduced to H_2O [38]. Thus, it appears that superoxide dismutase and catalase limit the accumulation of reactive oxygen species. Environmental bacteria are involved in oxidative stress from various sources. Reactive oxygen species (ROS) are produced in cells during microbial aerobic growth as well as in stress conditions as a byproduct of normal cellular metabolism [39]. Ascorbate peroxidase has a higher affinity for H_2O_2 than catalase and reduces H_2O_2 to H_2O by using ascorbate as a specific electron donor [40].

In general, studying the environmental conditions for bacterial growth and the rate of production of substances such as vitamin B_{12} seems important. B_{12} , or cobalamin, is a water-soluble vitamin that affects the normal functioning of the brain and nervous system and also has a role in the formation of blood. It is usually involved in the metabolism of cells in the human body, especially in DNA synthesis. B_{12} is by far the largest and most complex vitamin in the structure, and the only way for industrial production can be through the synthesis of bacterial fermentation. [41]. Stress tolerance is an important functional property of probiotic bacteria. One study showed that *P. freudenreichii* preferred carbon source and neutral pH in environments containing all growth factors [42]. Previous studies showed that pretreatment can increase tolerance in *P. freudenreichii* [43-46]. In a similar study, different

Pseudomonas aeruginosa strains were exposed to different pHs and temperatures. The results showed that the highest growth rate was at 30 °C and pH=7.5 [47]. In our study for *P. freudenreichii* it was found that the best pH and temperature is 6.6 and 25-40 °C.

Conclusion

This study was carried out to investigate the effect of temperature and pH parameters and assay of ascorbate peroxidase and catalase in the presence of 0, 250, 750 and 1000 mg/L of folic acid on *Propionibacterium freudenreichii* to produce more and better vitamin B₁₂. Adding corn to the bacterial culture medium can increase the production of vitamin B₁₂ and folate in the bacterium and improve the fermentation process. Examination of temperature and pH parameters along with addition of different concentrations of folate to the bacterial culture medium revealed that the optimum pH for growth of the bacterium and production of vitamin B₁₂ is 6.6 and the optimum temperature is 25-40 °C and bacterial growth was reduced at high concentrations of folate. The study also found that, the ascorbate peroxidase activity decreased with increasing the folic acid concentration. The results revealed that, enzyme activity increased at 0 and 250 mg/L of folic acid, whereas ascorbate peroxidase activity decreased with increasing the folic acid concentration at 750 and 1000 mg/L. CAT activity in propionic bacteria, under aerobic conditions, was significantly decreased with increasing folic acid concentration. Therefore, high concentrations of folic acid inhibit catalase activity.

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Conflict of Interest

We have no conflicts of interest to disclose.

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