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Optimization of Polyphenol Oxidase and Peroxidase Production Using Native *Bacillus* spp. Isolated From Fully Fermented Tea

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Abstract

Tea (*Camellia sinensis*) is a species of evergreen shrubs or small trees in the flowering plant family Theaceae. One of the major steps in black tea manufacturing is the fermentation process, in which bacteria may be incorporated. This study aimed at using the one-factor-at-a-time (OFAT) and Taguchi methods to optimize the production of polyphenol oxidase (PPO) and peroxidase (POD) by *Bacillus* spp. isolated from fully fermented tea. The results showed that sucrose and glucose exhibited the greatest effects on the production of polyphenol oxidase and peroxidase by the strains *Bacillus* sp. (TB3) and *B. licheniformis* (TB14), respectively. In addition, the results indicated that the best nitrogen sources for the production of PPO by *Bacillus* sp. (TB3) and POD by *B. licheniformis* (TB14) were sodium nitrate and ammonium carbonate, respectively. Based on the obtained results, the medium components for the production of PPO by *Bacillus* sp. (TB3) at a pH of 7 were as follows: 0.5% sucrose, 1.0% peptone, 0.8% yeast extract, 0.2% hydrolyzed casein, 0.02% potassium dihydrogen phosphate, 0.005% magnesium sulfate heptahydrate, and 0.1% sodium nitrate. Moreover, the optimal culture medium for the production of POD by *B. licheniformis* (TB14) at a pH of 7 was as follows: 0.3% of glucose, 1.0% of peptone, 0.8% of yeast extract, 0.2% of hydrolyzed casein, 0.02% of potassium dihydrogen phosphate, and 0.005% of magnesium sulfate heptahydrate. Increased production of PPO and POD enzymes was obtained, about 8- and 6-fold more than in the basal culture media, respectively.

Key words: *Bacillus* spp.; Optimization; Peroxidase; Polyphenol oxidase

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Introduction

Tea (*Camellia sinensis*) is a species of evergreen shrub or small tree in the flowering plant family Theaceae. About three million tons of tea are annually cultivated in more than 32 countries. The world's main tea producers are India, China, Sri Lanka, Indonesia, Kenya, Malawi, Tanzania, Zimbabwe, New Guinea, Bangladesh, Rwanda, and Cameroon. As one of the main tea producers in the world, India accounts for about 30% of the world's tea plantations, with an area of more than 400,000 hectares. Sri Lanka is considered the world's largest exporter of tea; this tropical country exports tea to other countries under the brand Ceylon. China is the world's second-largest producer and exporter of tea. However, most of the tea produced in this country is dedicated to domestic consumption. Based on recent data, Iran is the 9th largest tea producer in the world, with 35,000 hectares under tea cultivation. The only regions of Iran suitable for the cultivation of tea are located in the north. The high, rainy, and fertile plains with a temperate climate throughout the year are the best places for the cultivation and processing of tea with the desired smell and color. Iran began the semi-industrial cultivation and harvest of tea in the 1940s. The area under cultivation for tea in Iran is currently about 35,000 hectares, which is mainly located in Guilan province. Although Iran has always been among the pioneers of rural arts and agricultural products, this country has never played a decisive role in the global and even domestic markets for tea (Faezi & Tayeri, 2007; Hafezi et al., 2006).

After harvesting, there are seven stages in tea processing: harvesting, withering, rolling, fermenting, drying or heating, and sorting. In the withering process, the number of bacteria increases by 5–50 times. For example, in Indian tea, the microbial population reaches 0.5 to 2 million. Molds and yeasts are usually are found in small quantities in this process.

Fermentation produces heat by consuming oxygen and generating water-soluble, colored polyphenolic compounds. During the fermentation process, flavanols are the main substrates for polyphenol oxidase (tyrosinase) (PPO; EC 1.14.18.1), which is the key enzyme and has an important role in the formation of black tea components such as bisflavanol, theaflavins (TFs), thearubigens (TRs) and epitheoflavicacids. In addition, peroxidase (EC 1.11.1.7) may contribute to the oxidation of tea flavanols (Crocker, 2003; Faezi & Tayeri, 2007; Hafezi et al., 2006; Michels, 2000).

Depending on the temperature and type of process, fermentation may last between 40 minutes and 3 hours. Fermentation results in the production of theaflavins and thearubigins, which are abundantly found in black tea. Microbial flora incorporates itself into the fermentation processes of tea through polyphenol oxidase and peroxidase activities. The effects of these enzymes produced by bacterial strains, such as Bacillus spp., result in the production of compounds that improve the quality and taste of tea.

Also, the changes in the chemical ingredients and bioactivities of tea during microbial fermentation have been studied in recent years and were related to the bioconversion of active components by microorganisms. In recent years, the study on using bacteria to ferment tea has mainly focused kombucha. During the fermentation process of kombucha, changes in sugar and acid content give the tea a new taste, the production of aromatic substances increases the aroma of the tea, and changes in phenolic substances increase the antioxidant capacity of the tea.

Zhao et al. (2016) reported that lactic acid bacteria are involved in the fermentation of tea. Furthermore, kombucha is fermented by acetic acid bacteria and yeast, and during fermentation, beneficial ingredients such as vitamin C and glucuronic acid increase (Neffe-Skocińska et al., 2017). In addition, kombucha has significant changes in antioxidant potential, pH, acetic acid, alcohol, and sugar contents, and beneficial ingredients such as organic acids, minerals, vitamins, amino acids, and polyphenols can be produced in the fermentation process (Hu et al., 2022; Jakubczyk et al., 2020).

Isolation and characterization of polyphenol oxidase and peroxidase-producing *Bacillus* strains from fully fermented tea (*Camellia sinensis*) were studied by Faezi and Tayeri (2007). According to results obtained from this study,

most isolates of bacteria from fermented tea leaves possessed typical cellular and colonial morphologies and physiological, biochemical, and nutritional features that resembled Bacillus spp. senso stricto. The isolated strains were identified and belonged to the species *B. subtilis, B. licheniformis, B. sphaericus, B. pumilus, and B. cereus*, respectively. The isolates showed polyphenol oxidase (tyrosinase) and peroxidase activities. The highest polyphenol oxidase and peroxidase activities were observed for *Bacillus* sp. TB3 and *B. licheniformis* (TB14), respectively, where values of 5.48 and 3.73 units ml–1 were observed (Faezi & Tayeri, 2007).

In all fermentation processes, culture conditions and nutrients should be optimized. In fact, optimization of the culture conditions is one of the most common ways to improve final products. Medium-scale optimization is still one of the most investigated phenomena that is carried out before any large-scale metabolite production.

Before the 1970s, optimization was carried out using classical methods, which were expensive and time-consuming. With the advent of modern mathematical and statistical techniques, media optimization has become more effective and economical. In optimization, the most suitable fermentation conditions (e.g., pH, temperature, agitation speed, etc.) and the appropriate medium components (e.g., carbon, nitrogen, etc.) must be identified and optimized accordingly. An increase in productivity reduces the overall cost of the product as well as the production cost; hence, it is one of the important topics for research (Singh et al., 2017; Wang et al., 2011).

Although there have been many studies regarding the optimization of different enzymes from different sources, to the best of our knowledge, there is insufficient information about the optimization of polyphenol oxidase (tyrosinase) and peroxidase enzymes produced by native *Bacillus* strains isolated from fermented tea. This study aims to investigate the optimization of polyphenol oxidase (tyrosinase) and peroxidase enzymes produced by *Bacillus* spp. isolated from fully fermented tea leaves.

Material and methods

Bacterial strains and culture conditions

Frozen vials containing a suspension of *Bacillus* strains were unpacked under aseptic conditions and cultured on a nutrient agar medium. Incubation was performed at 30°C for 24 hours. A basal medium containing 0.2% of peptone, 0.2% of hydrolyzed casein, 0.1% of glucose, 0.02% of potassium dihydrogen phosphate, and 0.005% of magnesium sulfate heptahydrate was used for assessing polyphenol oxidase (tyrosinase) and peroxidase enzyme activities. The pH of the medium was set at 7. The *Bacillus* strain cultured on the broth-nutrient medium was cultured on the basal medium in 250-ml Erlenmeyer flasks containing 50 ml of the culture medium in such a way that there were 108 bacterial cells per ml. Fermentation was performed in an incubator shaker at 150 rpm at 30°C.

Measurement of polyphenol oxidase (tyrosinase) and peroxidase activity

For determining enzyme activities, culture flasks containing *Bacillus* spp. were centrifuged at 12,000-×g at 4 °C for 20 min. The biomass pellets were discarded, and the supernatants were used for determining polyphenol oxidase and peroxidase activities. The activity of polyphenol oxidase was determined based on the Duckworth and and Coleman method (Duckworth & Coleman, 1970).

Accordingly, PPO oxidizes tyrosine into dihydroxyphenylalanine and then o-quinone. This oxidation increases the absorption at a wavelength of 280 nm. The absorption increases with the enzyme concentration and then begins a linear trend after 5–10 minutes. The spectrophotometer was set at 280 nm and 25°C, and the following compounds were added to each spectrophotometer cuvette: 1 ml of 0.5-M phosphate buffer with a pH of 6.5, 1 ml of 0.0001-M tyrosine, and 0.9 ml of reagent water. This mixture was oxygenated by injecting oxygen into cuvettes for 4-5 minutes. The cuvettes were then transferred to a spectrophotometer, and the absorption was recorded at 280 nm for 4-5 minutes until reaching a temperature equilibrium. In the next step, 0.1 ml of the culture supernatant was added to the cuvettes in the culture medium of each of the iso-

lated bacteria, and the adsorption was measured again at 280 nm. The linear part of the curve presented the difference in adsorption. Accordingly, one unit of enzyme activity was equal to a change in the absorption of 0.001 at 280 nm and a pH of 6.5.

Peroxidase activity was measured using Trinder's method. In this method, 4-aminoantipyrine acts as an electron donor. The intensity of the reaction, which finally results in the decomposition of hydrogen peroxide, is determined by measuring the increased absorption at 510 nm. The following materials were used to measure the activity of POD: 0.2-M potassium phosphate buffer at a pH of 7, 0.0017-M hydrogen peroxide solution, 0.0025-M aminoantipyrine solution, and 0.17-M phenol. For this purpose, the spectrophotometer was set to 510 nm and 25°C. The following solutions were added to each cuvette: 1.4 ml of phenol aminoantipyrine solution and 1.5 ml of 0.0017-M hydrogen peroxide solution. The mixture of these two solutions was incubated in the spectrophotometer for 4-5 minutes. Then 0.1 ml of the culture supernatant was added to the cuvettes in the culture medium of each of the isolated bacteria, and the adsorption was measured again at 510 nm. The linear part of the curve presented the difference in adsorption. Accordingly, one unit of enzyme activity was equal to the decomposition of 1 mmol of hydrogen peroxide per minute at 25°C and a pH of 7 (Trinder, 1969).

Optimization of carbon and nitrogen sources by the one-factor-at-a-time (OFAT) method

Different carbon sources, including sucrose, fructose, lactose, maltose, galactose, starch, inositol, mannitol, and glucose at 1% w/v were used for the production of polyphenol oxidase (tyrosinase) and peroxidase enzymes. The effects of yeast extract and peptone at concentrations of 0.2% to 2.0% on the production of polyphenol oxidase and peroxidase enzymes by *Bacillus* sp. (TB11) and *B. licheniformis* (TB14) were studied by the OFAT method. In other words, once the concentration of yeast extract was kept constant and the peptone concentration was changed, and vice versa. After obtaining the best concentrations of yeast extract and peptone for the production of PPO and POD by *Bacillus* sp. (TB11) and *B. licheniformis* (TB14), the effects of sources of mineral nitrogen on the production of PPO and POD by these two strains were investigated. Mineral nitrogen sources, including sodium nitrate, ammonium sulfate, ammonium carbonate, ammonium dihydrogen phosphate, ammonium acetate, and ammonium chloride, were selected at a concentration of 0.1% w/v. In addition, organic nitrogen sources include soybean flour, corn steep liquor, whey, wheat extract, and barley flour extract. All organic sources at a concentration of 0.1% w/v were considered in the basal medium for optimization procedures.

Taguchi's arrays for medium optimization

To investigate the interaction among nutritional components of the production medium and optimize the concentrations for polyphenol oxidase and peroxidase enzyme production by *Bacillus* spp. (TB11) and *B. licheniformis* (TB14) strains, Taguchi's arrays were used. An L18 (2^1&3^5) array was selected to determine the effects of five 3-level and one 2-level factor on polyphenol oxidase and peroxidase production. In the second step, another L18 (2^1&3^5) array was used to adjust the effects of five 3-level factors and one 1-level factor on polyphenol oxidase and peroxidase production.

Results

The maximum activities of polyphenol oxidase and peroxidase enzymes by *Bacillus* sp. (TB3) and *B. licheniformis* (TB14) were about 5.48 and 3.73 units per mL-1 in our previous study. Table 1 shows polyphenol oxidase and peroxidase activities in the presence of different carbon sources by *Bacillus* spp. (TB3) and *B. licheniformis* (TB14). As shown, the maximum polyphenol oxidase and peroxidase activities by the strains were about 26.52 and 15.78 units per mL-1 in the presence of sucrose and glucose, respectively.

Table 1. Polyphenol oxidase and peroxidase activities of *Bacillus* sp. (TB3) and peroxidase activities of *Bacillus licheniformis* (TB14) in the presence of different carbon sources.

Carbon sources 1%(W/V)	Polyphenol oxidase activity by *Bacillus* sp.(TB3) Unit/ml^{-1}	Peroxidase activity by *Bacillus licheniformis* (TB14) Unit/ml^{-1}
Sucrose	26.52±0.03	15.36±0.09
Fructose	25.31±0.08	15.53±0.11
Lactose	24.28±0.12	14.32±0.15
Maltose	23.37±0.06	13.82±0.08
Galactose	25.36±0.14	15.95±0.16
Starch	23.36±0.17	13.12±0.07
Inositol	25.86±0.09	12.98±0.06
Mannitol	25.37±0.11	13.28±0.02
Glucose	25.98±0.06	15.78±0.03

Note: Values are the mean ± SD of triple determinations.

Table 2 shows the effects of different nitrogen sources on polyphenol oxidase and peroxidase production by *Bacillus* sp. (TB3) and *Bacillus licheniformis* (TB14), respectively. As shown, the maximum polyphenol oxidase and peroxidase activities of the strains were about 25.89 and 15.71 units per mL-1 in the presence of sodium nitrate and ammonium carbonate, respectively.

Table 2. Polyphenol oxidase and peroxidase activities of *Bacillus* sp. (TB3) and peroxidase activities of *Bacillus licheniformis* (TB14) in the presence of different nitrogen sources.

Nitrogen Sources 1%(W/V)	Polyphenol oxidase activity by *Bacillus* sp. (TB3) Units/ml^{-1}	Peroxidase activity by *Bacillus licheniformis* (TB14) Units/ml^{-1}
$NaNO_3$	25.89±0.07	13.35±0.16
NH_4SO_4	24.23±0.13	12.83±0.03
NH_4CO_3	23.38±0.03	15.71±0.19
$NH_4H_2PO_4$	24.29±0.09	13.11±0.21
NH_4-acetate	22.89±0.17	12.22±0.12
NH_4Cl	24.66±0.05	14.08±0.08
Soybean-flour	25.21±0.21	14.39±0.13
Corn-steep liquor	25.33±0.10	13.68±0.11
Whey	25.56±0.06	14.77±0.18
Barely-flour	22.11±0.03	13.73±0.09
Peptone	24.39±0.08	14.17±0.15
Hydrolyzed casein	21.12±0.17	14.03±0.06

Note: Values are the mean ± SD of triple determinations.

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Table 3 shows the experimental conditions for polyphenol oxidase production by *Bacillus* sp. (TB3) using Taguchi's array method. As shown, the maximum polyphenol oxidase activity was about 42.12 units per mL-1 in the 14 experimental runs.

Table 3. Experimental factors and their results of the first L18 (2^1&3^5) Taguchi's array for polyphenol oxidase activity by *Bacillus* sp. (TB3).

Level	Factors					
	A, Sucrose%(W/V)	B, Peptone % (W/V)	C, Yeast extract % (W/V)	D, Hydrolyzed casein%(W/V)	E,KH_2PO_4 %(W/V)	Polyphenol oxidase activity by *Bacillus* sp. (TB3)Units/ml^{-1}
1	0.25	0.5	0.4	0.05	0.005	
2	0.5	1.0	0.8	0.1	0.01	
3	1.0	2.0	1.6	0.2	0.02	
Run	A	B	C	D	E	
1	1	1	1	1	1	20.12±0.12
2	1	1	2	2	2	25.11±0.09
3	1	1	3	3	3	27.06±0.28
4	1	2	1	1	2	20.12±0.09
5	1	2	2	2	3	21.13±0.17
6	1	2	3	3	1	28.41±0.06
7	1	3	1	2	1	32.25±0.13
8	1	3	2	3	2	33.67±0.10
9	1	3	3	1	3	28.94±0.19
10	2	1	1	3	3	37.12±0.22
11	2	1	2	1	1	32.32±0.11
12	2	1	3	2	2	36.85±0.03
13	2	2	1	2	3	37.15±0.17
14	2	2	2	3	1	42.12±0.18
15	2	2	3	1	2	31.11±0.05
16	2	3	1	3	2	35.61±0.16
17	2	3	2	1	3	29.13±0.22
18	2	3	3	2	1	36.18±0.14
Control	2	2	2	2	2	28.13±0.04

Note: Values are the mean ± SD of triple determinations.

Table 4 shows the experimental conditions for peroxidase production by *B. licheniformis* (TB14) using Taguchi's array method. As shown, the maximum peroxidase production was about 23.86 units per mL-1 in the 17 experimental runs.

Table 4. Experimental factors and their results of the first L18 (2^1&3^5) Taguchi's array for peroxidase activity by *B. licheniformis* (TB14).

Level	Factors					
	A, Glucose % (W/V)	B, peptone % (W/V)	C, yeast extract % (W/V)	D, hydrolyzed casein% (W/V)	E,KH_2PO_4 % (W/V)	Polyphenol oxidase activity by *B. licheniformis* (TB14) Units/ml^{-1}
1	0.15	0.1	0.4	0.2	0.005	
2	0.3	0.2	0.8	0.4	0.01	
3	0.6	1.0	1.6	0.8	0.02	
Run	A	B	C	D	E	
1	1	1	1	1	1	14.36±0.08
2	1	1	2	2	2	15.12±0.12
3	1	1	3	3	3	13.12±0.17
4	1	2	1	1	2	12.31±0.19
5	1	2	2	2	3	16.04±0.03
6	1	2	3	3	1	17.01±0.06
7	1	3	1	2	1	11.09±0.08
8	1	3	2	3	2	18.32±0.04
9	1	3	3	1	3	10.13±0.02
10	2	1	1	3	3	13.18±0.11
11	2	1	2	1	1	16.31±0.07
12	2	1	3	2	2	14.98±0.16
13	2	2	1	2	3	18.19±0.04
14	2	2	2	3	1	16.12±0.23
15	2	2	3	1	2	12.89±0.03
16	2	3	1	3	2	21.44±0.15
17	2	3	2	1	3	23.86±0.05
18	2	3	3	2	1	17.86±0.29
Control	2	2	2	2	2	16.68±0.14

Note: Values are the mean ± SD of triple determinations.

The results also indicated that sucrose at a concentration of 0.5% had a significant effect on polyphenol oxidase activity by *Bacillus* sp. (TB3). In addition, the best carbon source for the production of peroxidase by *B. licheniformis* (TB14) was glucose. As shown in Table 2, among the mineral and complex sources of nitrogen used for optimizing the production of the polyphenol oxidase enzyme by *Bacillus* spp. (TB3), sodium nitrate exhibited the greatest effect. In addition, ammonium carbonate showed a significant effect on peroxidase enzyme activity by *B. licheniformis* (TB14). Based on the obtained results, the medium components for the production of polyphenol oxidase by *Bacillus* sp. (TB3) at a pH of 7 were as follows: 0.5% sucrose, 1.0% peptone, 0.8% yeast extract, 0.2% hydrolyzed casein, 0.02% potassium dihydrogen phosphate, 0.005% magnesium sulfate heptahydrate, and 0.1% sodium nitrate. Moreover, the optimal culture medium for the production of peroxidase by *B. licheniformis* (TB14) at a pH of 7 was as follows: 0.3% glucose, 1.0% peptone, 0.8% yeast extract, 0.2% hydrolyzed casein, 0.02% potassium dihydrogen phosphate, and 0.005% magnesium sulfate heptahydrate. Increased production of polyphenol oxidase and peroxidase enzymes was obtained, about 8- and 6-fold more than in the basal culture medium, respectively.

Discussion

This study optimized the culture medium for producing polyphenol oxidase and peroxidase enzymes by *Bacillus* strains isolated from fully fermented tea, i.e., *Bacillus* sp. (TB3) and POD by *B. licheniformis* (TB14), for the first time using the one-factor-at-a-time (OFAT) and Taguchi methods. Sucrose and glucose were the best carbon sources for polyphenol oxidase and peroxidase enzymes. Among the mineral and complex sources of nitrogen, sodium nitrate and ammonium carbonate were the best sources for polyphenol oxidase and peroxidase activities, respectively. The results also demonstrated that the best concentrations of peptone and yeast extract for producing polyphenol oxidase and peroxidase by *Bacillus* sp. (TB3) and *B. licheniformis* (TB14) were 1.0% and 0.8%, respectively. Since the optimized culture medium is cost-effective, the crude extract of these two enzymes can be used for tea fermentation. In addition, the other methods that can be employed to optimize the production of enzymes by *Bacillus* sp. (TB3) and *B. licheniformis* (TB14) are physical or chemical mutation selection, protoplast fusion, and genetic engineering. Based on these findings, future studies are recommended to determine the optimal conditions for producing these enzymes on semi-industrial and industrial scales and the parameters required for designing bioreactors for the production of these enzymes.

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Conflict of Interest

No conflict of interest was declared.

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