International Food Research Journal 31(2): 387 - 397 (April 2024)
Journal homepage: *http://www.ifrj.upm.edu.my*

Optimisation of biotransformation conditions of theaflavin-3'-gallate

[1]Wu, P., [1]He, R. Y., [1]Chen, H., [1]Gan, Q., [1]Chen, Y., [1]Zhang, X. M., [1]*Hu, T. and [1,2]*Li, S. M.

[1]Hubei Provincial Collaborative Innovation Centre of Dabie Mountain, Characteristic Resources Development, Hubei Provincial Key Laboratory of Economic Forest Tree Germplasm Improvement and Comprehensive Utilisation of Resources, Hubei Zhongke Industrial Technology Research Institute, Huanggang Normal University, College of Biology and Agricultural Resources, Huanggang, Hubei 438000, People's Republic of China
[2]Department of Food Science, Rutgers University, New Brunswick, New Jersey 07102, United States of America

Article history

Received:
29 November 2022
Received in revised form:
8 August 2023
Accepted:
4 January 2024

Keywords

theaflavin-3'-gallate, enzymatic reaction, conditions optimisation

DOI
https://doi.org/10.47836/ifrj.31.2.10

Abstract

Theaflavin-3'-gallate was synthesised using polyphenol oxidase (PPO) from gallocatechin (EGC) and epicatechin gallonic acid (ECG). Using PPO, Box-Behnken design, and single factor test, the optimal reaction conditions were determined: ECG/EGC ratio of 3:7, magnetic stirring speed of 200 rpm, reaction temperature of 37°C, and enzyme concentration of 20 mg/100 mL. Under these conditions, the yield of TF-3'-G was 18.1%. These parameters represent the optimal conversion conditions for theaflavin-3'-monogallate.

Introduction

Tea is a beverage cherished by people globally. It offers not only a pleasing taste but also multiple health benefits (Ding *et al*., 2017). Tea leaves are abundant in polyphenols which exhibit strong antioxidant properties (Moldoveanu and Oden, 2021). Theaflavin (TF1) is a principal functional component in black tea (Ilacqua *et al*., 2017; Qi, 2019) (Figure 1).

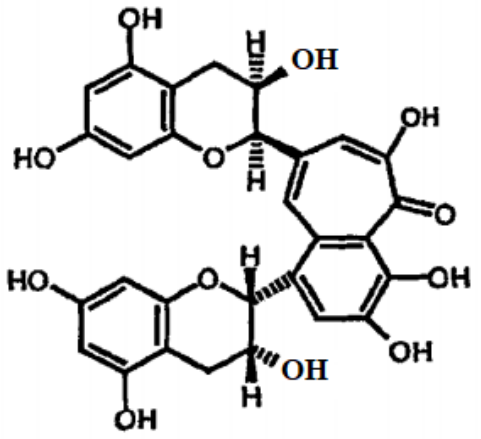

Figure 1. Chemical structure of theaflavin.

It is an orange substance produced from the oxidation and condensation of tea polyphenols (Xue *et al*., 2019). While it constitutes only 3 - 6% of the dry weight, it critically influences the quality of black tea (Gosslau *et al*., 2018; Peng *et al*., 2020). Theaflavin possesses distinct properties such as antioxidant capacity, reduction of cardiovascular and cerebrovascular diseases, induction of tumour cell apoptosis, regulation of immune cell function, and combatting the influenza and HIV viruses (Shan *et al*., 2021). Its robust antioxidant capabilities have demonstrated substantial effects in treating and preventing cancers and cardiovascular diseases (Ohba *et al*., 2017; Jang *et al*., 2020). Theaflavin is a prominent compound in tea research, and holds vast potential in the fields of chemistry and medicine (Wang *et al*., 2017). However, challenges arise due to its low concentration in black tea, the complexities of its separation and purification processes, its low yield, and the high costs of industrial production, all of which hinder the comprehensive research and application of theaflavin (Chen *et al*., 2020). To address the market demand and further the research and application of theaflavin, the present work used

*Corresponding author.
Email: 13419602772@163.com ; shiming@rutgers.edu

TF-3'-G as a model to investigate the factors influencing the biotransformation of the theaflavin monomer, offering insights for its industrial preparation (Wang *et al*., 2020).

Currently, the prevalent synthesis pathways include enzymatic and oxidation syntheses (Mhatre *et al*., 2021). The enzymatic synthesis method offers benefits such as high efficiency and superior safety. Polyphenol oxidase (PPO) is a primary enzyme source for the preparation of the large-scale monomer TF-3'-G. In the present work, PPO was employed to catalyse the formation of ECG and EGC monomers. Additionally, the Box-Behnken design was utilised to optimise the conditions for the TF-3'-G substrate, including the ECG to EGC mass ratio, reaction temperature, magnetic stirring speed, and enzyme concentration (Zheng *et al*., 2020).

Materials and methods

Materials and reagents

EGC, ECG, and theaflavin (98%) were used. Polyphenol oxidase (≥ 500 U/mg) was sourced from Sangon Biotech. Reagents, including methanol, acetonitrile, and acetic acid were obtained from TEDIA Tiandi Reagent. Other chemicals used were phosphate buffer, $FeSO_4$ solution, anhydrous ethanol, potassium ferricyanide, and ferric trichloride. The equipment utilised included Thermo Fisher UltiMate 3000 HPLC from Thermo Fisher Technologies, AL204 electronic balance from Shanghai Mettler-Toledo Instrument Co., Ltd., and ZNCL-GS intelligent magnetic stirrer from Gongyi Yuhua Instrument Co., Ltd. Additionally, HH-6 digital display constant temperature water bath, microporous filter membrane (nylon, 0.45 μm), and needle microporous filter (nylon, 0.45 μm) were sourced from Tianjin Jinteng Experimental Equipment Co., Ltd. The GM-1.0A diaphragm vacuum pump was also acquired from Tianjin Jinteng Experimental Equipment Co., Ltd.

Methods

For the enzyme solution, 20 mg of PPO (≥ 500 U/mg) was dissolved in 100 mL of pH 5.6 citrate-phosphate buffer solution to obtain a 0.2 g/mL solution, as described by Huang *et al*. (2017). ECG and EGC ratios were determined, and 10 mL of PPO solution underwent an enzymatic reaction at 200 rpm for 150 min. This was followed by heating in a water bath set at 100°C for 5 min. Following heating, the reaction mixture was filtered using a needle membrane. The filtrate was then analysed using HPLC to determine the content of each component in the reaction liquid, as per the methodology outlined by Li *et al*. (2019). The yield of TF-3'-G was calculated using Eq. 1:

$$\text{TF-3'-G yield (\%)} = a/b * 100\% \quad \text{(Eq. 1)}$$

where, a = amount of substance synthesised by TF-3'-G; and b = amount of substance added to EGC (Tu, 2019).

Univariate test

The parameters such as the quantity ratio of ECG/EGC, reaction temperature, magnetic stirring speed, and enzyme concentration were studied. For ECG/EGC, the volume ratio was set at 3:7. The conditions were as follows: temperature of 37°C, magnetic stirring speed of 200 rpm, and enzyme concentration of 10 mg/mL. The selected control enzyme was PPO.

The effect of the quantity ratios of ECG/EGC (with variations of 1:9, 2:8, 3:7, 4:6, 5:5, and 6:4) on TF-3'-G yield was investigated, maintaining other parameters constant as follows: PPO as the control enzyme, magnetic stirring speed of 200 rpm, and enzyme concentration of 10 mg/mL.

Similarly, the influence of temperature on TF-3'-G yield was explored by varying the temperature (27, 32, 37, 42, and 47°C) while keeping other conditions constant as follows: PPO as the control enzyme, quantity ratio of ECG/EGC at 3:7, magnetic stirring speed of 200 rpm, and enzyme concentration of 10 mg/mL.

Further, the effect of varying the magnetic stirring speed (100, 150, 200, 250, and 300 rpm) on TF-3'-G yield was studied. The conditions were kept constant as follows: PPO as the control enzyme, quantity ratio of ECG/EGC at 3:7, temperature of 37°C, and enzyme concentration of 10 mg/mL.

Lastly, the influence of enzyme concentration variations (10, 15, 20, 25, and 30 mg/mL) on TF-3'-G yield was analysed under the following consistent conditions: PPO as the control enzyme, quantity ratio of ECG/EGC at 3:7, temperature of 37°C, and magnetic stirring speed of 200 rpm (Gong *et al*., 2020).

Analysis and testing

HPLC analyses were carried out using an Agilent liquid chromatograph. Specific parameters set for the analysis were: column type as Inertsil ODS-SP (4.6 × 250 mm, 5 μm), flow rate at 1.0 mL/min, column temperature maintained at 40°C, detection wavelength set to 288 nm, a sample volume of 10 μL, and an analysis duration of 60 min. The mobile phase comprised a C phase (with a mixture of acetic acid, acetonitrile, and water in a 2:9:89 volume ratio) and a D phase (comprising acetonitrile and ultrapure water in a 4:1 volume ratio). A gradient elution was applied. For sample preparation, 0.125 g of ascorbic acid was dissolved in 500 mL of 10% acetonitrile solution. TF-3'-G (10 mg) was precisely weighed and dissolved in this protective solution to achieve 1 mg/mL TF-3'-G control. The resultant peak area measured by HPLC was 54.6018 mAU*min, and subsequently aligned for analysis.

Determination of hydroxyl radical (OH•) scavenging capacity

To determine the hydroxyl radical (OH) scavenging capacity, solutions of theaflavin-3'-gallate were diluted to various concentrations: 0.05, 0.1, 0.2, 0.3, and 0.5 mg/mL. To each tube, 1.0 mL of 5 mmol/L phenanthroline was added, followed by the addition of 2.0 mL of 0.2 mol/L phosphate buffer (pH 7.4) and 1.0 mL of the sample solution. After thorough mixing, 1.0 mL of 7.5 mmol/L $FeSO_4$ solution was added and mixed immediately. Subsequently, 1.0 mL of H_2O_2 was added, mixed, and incubated at 37°C for 1 h. The absorbance value (A_i) was measured at 536 nm. Aqueous ethanol replaced the sample solution to serve as the blank control (A_0). The hydroxyl radical (OH•) clearance percentage was calculated using Eq. 2:

$$\text{Hydroxyl radical (OH) clearance (\%)} = \frac{A_0 - A_i}{A_i} \times 100(-) \quad \text{(Eq. 2)}$$

where, A_0 = blank absorbance, and A_i = sample absorbance.

Determination of total reducing capacity

Different volumes of samples were diluted with distilled water to yield 1 mL. To this, 1 mL of pH 6.6, 0.2 mol/L phosphate buffer solution, and 1 mL of 1% potassium ferricyanide solution were added. The resulting mixture was incubated in a water bath at 50°C for 20 min. Then, 1 mL of 10% trichloroacetic acid was added and mixed thoroughly. From this mixture, 1 mL of supernatant was taken, and to it, 1.0 mL of distilled water and 0.2 mL of 0.1% ferric trichloride solution were added. After 10 min incubation at room temperature, the absorbance was measured at 700 nm. Distilled water replaced the sample solution in the blank control test. All measurements were conducted in triplicate. An equivalent added amount of 1 g/L of ascorbic acid served as a control. A higher absorbance value indicates a stronger antioxidant capacity (Liu *et al.*, 2021).

Statistical analysis

Statistical analyses were performed using SPSS software. One-way analysis of variance (ANOVA) was used to evaluate differences in TF-3'-G yields across varying experimental conditions. By subjecting the experimental data to variance analysis, we determined the presence of significant differences among treatment groups, and gained deeper insights into the individual contributions of each factor to TF-3'-G yield. This robust statistical approach not only facilitated the interpretation of our experimental findings, but also strengthened the validity of our research conclusions.

Data processing

The range established by the univariate test was utilised, and the Design-Expert 8.0.6 software was employed to design a response surface test with four factors at three levels. This was to determine the optimal test conditions for TF-3'-G (Lei *et al*., 2017). The test factors and levels of the response surface are provided in Table 1.

Results and discussion

The PPO enzyme catalysed the synthesis of TF-3'-G by ECG and EGC, yielding a detectable amount of TF-3'-G after 150 min. This indicated PPO efficient catalytic capability. The present work used the level of TF-3'-G yield as a benchmark to explore the ideal conditions for PPO-catalysed synthesis of TF by ECG and EGC. Factors under consideration included substrate ratio, reaction temperature, rotational speed, and enzyme concentration (Lin *et al*., 2017; Liang *et al*., 2022).

Table 1. Levels and codes of factors used in the Box-Behnken design.

Factor	Horizontal		
	-1	0	1
(A) ECG/EGC mass ratio	2:8	3:7	4:6
(B) Reaction temperature (°C)	-1	37	42
(C) Magnetic stirring speed (rpm)	150	200	250
(D) Enzyme concentration (mg/100 mL)	15	20	25

Effect of ECG/EGC quantity ratio on TF-3'-G yield

Catechins undergo enzymatic oxidation to form quinones. These quinones then pair and react to synthesise theaflavin. The present work found that the ECG/EGC ratio significantly impacted the yield of TF-3'-G (Figure 2). The lowest yield occurred at ECG/EGC ratio of 2:8, whereas a ratio of 3:7 resulted in the highest yield of 3.6%. As the ECG/EGC ratio increased, the TF-3'-G yield initially increased, and then decreased. The optimal substrate ratio was identified as an ECG/EGC ratio of 3:7.

Effect of reaction temperature on TF-3'-G yield

Temperature influences the enzymatic reaction's activity. Observations indicated that as the temperature increased, the enzymatic reaction's rate increased, thus enhancing the TF-3'-G production rate (Figure 3). However, as the temperature continued to increase, enzyme activity decreased, leading to a decrease in the TF-3'-G production rate. The yield was optimal in the temperature range of 32 - 42°C, reaching a peak of 2.71% around 37°C, and then decreased. The most favourable temperature for theaflavin synthesis was determined to be 37°C.

Effect of rotational speed on TF-3'-G yield

During the reaction under a magnetic stirrer, the rotational speed affected the reaction. Experimental data revealed that a rotational speed of 200 rpm was optimal, with yields increasing up to 10.75% as the rotational speed increased. Based on these findings, 200 rpm was selected as the response value's midpoint (Figure 4).

Effect of enzyme concentration on TF-3'-G yield

The concentration of PPO played a significant role in this reaction. The yield of theaflavin progressively increased as the enzyme concentration ranged between 5 - 20 mg/100 mL (Figure 5). The peak yield, 9.53%, was attained at 20 mg/100 mL, after which the yield began to decrease. Consequently, an enzyme concentration of 20 mg/100 mL was chosen as the response value's midpoint.

Analysis of response surface test results

The ANOVA results, derived from data in Table 2 using the Design-Expert 8.0.6 software, are presented in Table 3. Table 3 reveals that the substrate ratio and reaction temperature of ECG/EGC exerted significant effects on theaflavin production. The *p*-value of the regression model was 0.0153, suggesting that the model was highly significant. Among the variables, A, C, AD, and BD emerged as significant factors. Further analysis indicated that the ranking of the independent variables based on their effect on TF-3'-G yield is as follows: ECG/EGC mass ratio > reaction temperature > enzyme concentration > magnetic stirrer speed. Graphical analysis reveals that steeper curves correspond to greater factor influences. The 2D diagram's shape can highlight insignificant interactions; notably, an elliptical shape indicates significant interactions. The mass ratio and reaction temperature of ECG/EGC exerted a profound effect on TF-3'-G yield, and exhibited significant interactions, corroborating the findings in Table 3. The 2D contour plot of the ECG/EGC mass ratio and magnetic stirrer speed also appears elliptical, signifying a significant interaction and a pronounced effect on TF-3'-G production (Figure 6), consistent with the data in Table 3.

The optimal conditions for TF-3'-G synthesis using PPO, as deduced from response surface analysis, are as follows: ECG/EGC ratio of 3:7, reaction temperature of 37°C, magnetic stirring speed of 200 rpm, and enzyme concentration of 20 mg/100 mL. Under these conditions, the theoretically computed TF-3'-G generation rate aligned closely with the actual experimental outcomes, making them apt for establishing the optimum conditions. The ANOVA results, based on data from Table 2 analysed

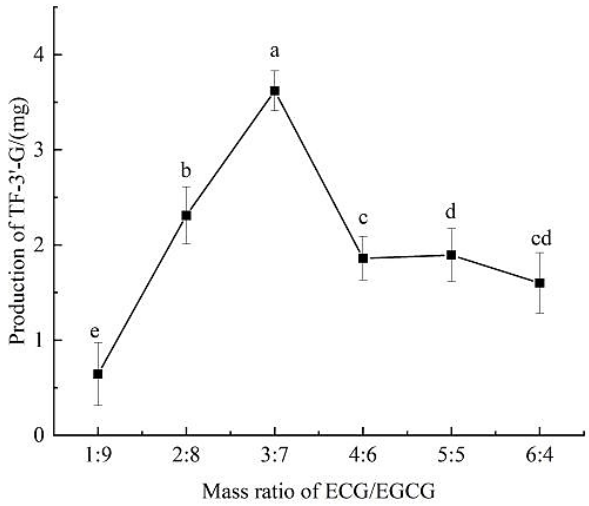

Figure 2. Effect of ECG/EGCG mass ratio on synthesis of TF-3'-G.

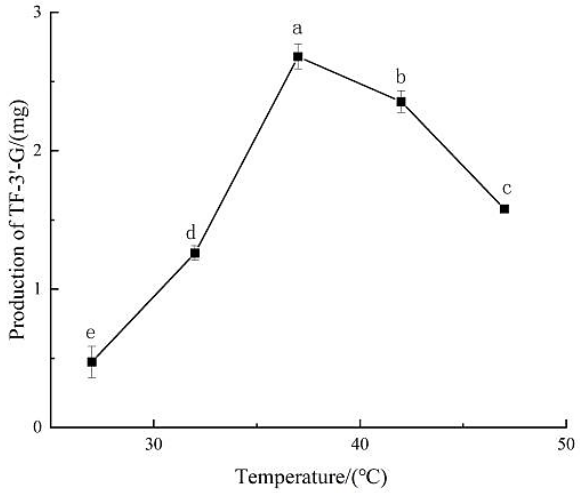

Figure 3. Effect of reaction temperature on synthesis of TF-3'-G.

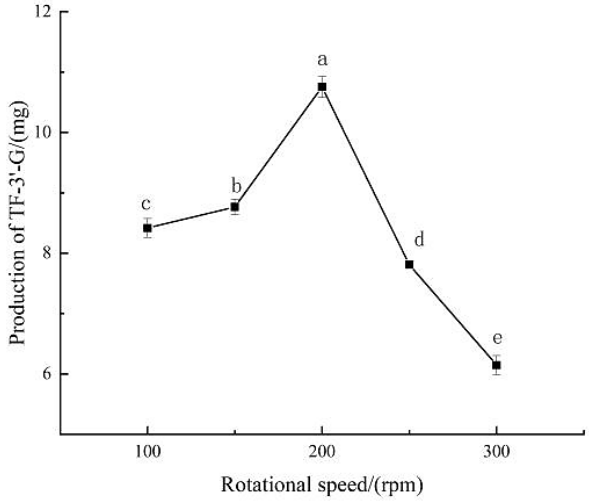

Figure 4. Effect of magnetic stirring speed on synthesis of TF-3'-G.

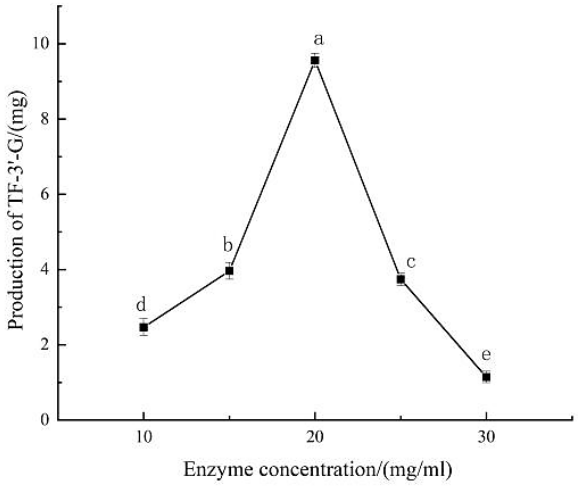

Figure 5. Effect of enzyme concentration on synthesis of TF-3'-G.

Table 2. Experimental design and response surface analysis.

Test number	(A) ECG/EGC mass ratio	(B) Reaction temperature (°C)	(C) Magnetic stirring speed (rpm)	(D) Enzyme concentration (mg/100 mL)	Product quality (mg)
1	3:7	42	200	15	3.94
2	4:6	37	200	25	8.83
3	4:6	32	200	20	12.7
4	3:7	37	150	15	4.10
5	3:7	32	250	20	3.60
6	3:7	37	200	20	18.1
7	3:7	42	150	20	1.94
8	3:7	37	200	20	14.40
9	2:8	42	200	20	9.98
10	2:8	32	200	20	8.39
11	4:6	42	200	20	16.26
12	3:7	32	200	25	11.30
13	3:7	32	150	20	9.52
14	3:7	37	200	20	12.85
15	3:7	42	200	25	12.15
16	3:7	37	200	20	16.20
17	3:7	42	*250*	20	12.83
18	3:7	37	*200*	20	15.16
19	3:7	32	*200*	15	11.42
20	2:8	37	200	15	7.91
21	4:6	37	200	15	8.70
22	3:7	37	250	15	7.50
23	3:7	37	250	25	10.53
24	2:8	37	150	20	10.00
25	3:7	37	150	25	1.31
26	2:8	37	250	20	5.00
27	2:8	37	200	25	17.12
28	4:6	37	250	20	13.13
29	4:6	37	150	20	11.92

Table 3. Analysis of variance for the quadratic polynomial model 1.

Source of variance	**Quadratic sum**	**Free degree**	**Mean square**	**F-value**	***p*-value**	
Model	392.82	14	28.06	3.36	0.0153	**
(A) ECG/EGCG mass ratio	22.41	1	22.41	2.68	0.1238	*
(B) Reaction temperature	0.043	1	0.043	5.177	0.9437	*
(C) Magnetic stirring speed	81.96	1	81.96	9.80	0.0074	
(D) Enzyme concentration	0.31	1	0.31	0.037	0.8497	
AB	9.00	1	9.00	1.08	0.3171	**
AC	27.20	1	27.20	3.25	0.5339	**
AD	3.40	1	3.40	0.41	0.0257	
BC	18.90	1	18.90	2.26	0.1549	
BD	70.65	1	70.65	8.45	0.0115	
CD	17.37	1	17.37	2.08	0.1714	
A^2	2.83	1	2.83	0.34	0.5697	**
B^2	102.98	1	102.98	12.32	0.0035	**
C^2	28.83	1	28.83	3.45	0.0845	**
D^2	52.08	1	52.08	6.23	0.0257	**
Residual	117.04	14	8.36			
Unplanned item	109.45	10	10.95	5.77	0.0529	
Pure error is poor	7.59	4	1.90			
Total difference	509.86	28				

(*) The difference was significant, with a $p < 0.05$; (**): The difference was extremely significant, with a $p < 0.01$.

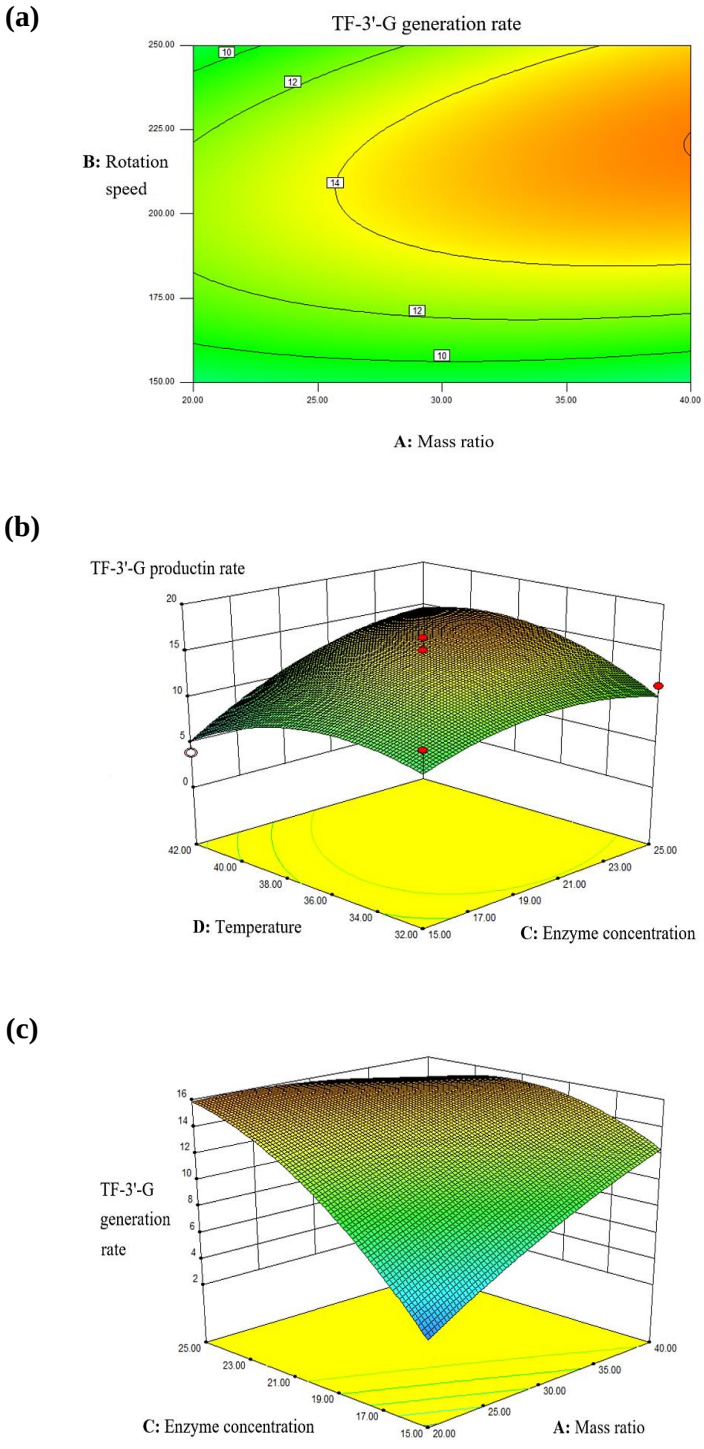

Figure 6. Interaction effects between mass ratio and rotation speed **(a)**, enzyme concentration and reaction temperature **(b)**, and enzyme concentration and mass ratio **(c)** on TF-3'-G yield.

with the Design-Expert 8.0.6 software, can be found in Table 3. As depicted in Table 3, both the mass ratio of ECG/EGC and the reaction temperature exerted significant effects on TF-3'-G yield ($p < 0.01$).

Effect of theaflavin concentration on hydroxyl radical (OH•) scavenging

Figure 7 reveals that within a specific concentration range, an increase in theaflavin concentration corresponds to an increase in the hydroxyl radical (OH•) scavenging rate. The strong antioxidant activity of the theaflavin molecule arises from the stability of the resulting benzoquinone product. Consequently, theaflavin's antioxidant effect *in vitro* may be attributable to its capacity to scavenge free radicals. Theaflavins can undergo oxidation to produce phylloquinone compounds when exposed to air, light, and elevated temperature. During the free radical scavenging experiments involving theaflavin-3'-monogallate, light-protected experimental conditions were employed to minimise the synthesis of leaf quinone products, and eliminate their potential influence on the results.

Effect of theaflavin concentration on total reducing capacity

Figure 8 indicates a linear relationship between theaflavin concentration and its total reducing capacity, observed within the mass concentration range of 0 - 50 μg/mL.

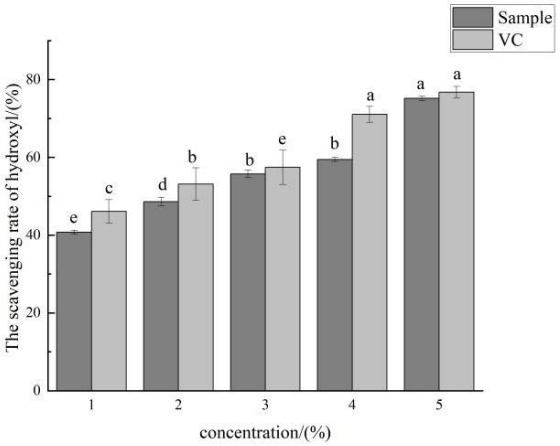

Figure 7. Effect of theaflavin concentration on hydroxyl radical (OH•) scavenging.

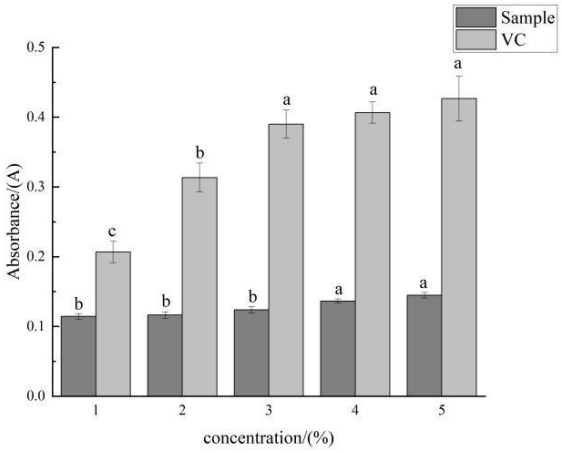

Figure 8. Effect of theaflavin concentration on total reducing capacity.

Conclusion

The present work explored the effect of the ECG/EGC ratio on TF-3'-G yield. The optimal conditions determined were ECG/EGC ratio of 3:7, reaction temperature of 37°C, magnetic stirring speed of 200 rpm, and enzyme concentration of 20 mg/100 mL. The potential application and technical benefits of optimising theaflavin biotransformation in the present work include the techniques employed were environmental friendly and non-polluting reagents, thus aligning with market preferences for green and potent theaflavin products. For every single factor set in the present work, four replicate groups were established, thus accounting for potential experimental randomness and ensuring precise experimental point selection. The enzyme source used in the present work was PPO. Numerous studies confirm its superior catalytic potential, thus enhancing the conversion rate of theaflavin. The high-performance liquid chromatography (HPLC) utilised boasts high detection sensitivity, automation, and optimised mobile phase, thus ensuring efficient analysis. Response surface optimisation aids in examining every test level. Primary or quadratic polynomial models can simplify complex function relationships in the experimental data. This method offers a straightforward and efficient approach to problem-solving.

Acknowledgement

The authors are grateful to High-level Cultivation Project of Huanggang Normal University, Hubei Provincial Key Laboratory of Industrial Microbiology of Hubei University of Technology, and Hubei Provincial Key Laboratory of Economic Forest Germplasm Improvement and Comprehensive Utilisation of Resources for financial support.

References

Chen, Z., Xu, L., Zhang, Y., Xie, W. and Tan, L. 2020. Effect of ultrasound-assisted leaching on leaching rates of theaflavin and tea in in black tea. Newsletter of Sericulture and Tea 208(4): 27-31.

Ding, Q.-H., Zi, C.-T., Zhou, Z.-Z. and Lu, C. Y. 2017. Research progress in the physico-chemical properties, extraction separation and biological activities of theaflavins. Journal of Anhui Agricultural Sciences 45(11): 85-87.

Gong, L., Bo, J. and Du, Z. 2020. Progress in improving theaflavin. Tea Newsletter 47(3): 375-381.

Gosslau, A., Zachariah, E., Li, S. M. and Ho, C. T. 2018. Anti-diabetic effects of a theaflavin-enriched black tea extract in the obese ZDF rat model. Journal of Food Bioactives 3: 151-160.

Huang, Y. J., Wu, M. Y. and Yao, Y. N. 2017. Effect of different reaction conditions on the synthesis of theaflavin by polyphenol oxidase of Mengku. Food Science 38(22): 54-59.

Ilacqua, A. N., Shettler, J. A., Wernke, K. M., Skalla, J. K. and McQuade, K. J. 2017. Theaflavins from black tea affect growth, development, and motility in *Dictyostelium discoideum*. Biochemical and Biophysical Research Communications 491(2): 449-454.

Jang, M., Park, Y. I., Cha, Y. E., Park, R., Namkoong, S., Lee, J. I. and Park, J. 2020. Tea polyphenols EGCG and theaflavin inhibit the activity of SARS-CoV-2 3CL-protease *in vitro*. Evidence-Based Complementary and Alternative Medicine 2020: 5630838.

Lei, S., Xie, M., Hu, B., Zhou, L., Sun, Y., Saeeduddin, M., … and Zeng, X. 2017. Effective synthesis of theaflavin-3,3'-digallate with epigallocatechin-3-*O*-gallate and epicatechin gallate as substrates by using immobilized pear polyphenol oxidase. International Journal of Biological Macromolecules 94(Pt A): 709-718.

Li, W., Zhang, C. Y., Li, F., Ren, Z., Liang, S. and Liu, D. 2019. Simultaneous determination of catechins and theaflavins in tea by ultra-high performance liquid chromatography-tandem mass spectrometry. Modern Preventive Medicine 46(22): 4179-4184.

Liang, S., Wang, F., Chen, J. X., Granato, D., Li, L. J., Yin, J. F. and Xu, Y. Q. 2022. Optimization of a tannase-assisted process for obtaining teas rich in theaflavins from *Camelia sinensis* leaves. Food Chemistry X 13: 100203.

Lin, C. X., Yang, J. R. and Wang, G. Y. 2017. Catalytic synthesis of theaflavin by polyphenol oxidase. Journal of Plant Physiology 53(8): 1359-1364.

Liu, C. W., Zhang, Z. Y., Wang, J. Y., Zhou, F., Zeng, H., Zhang, S., … and Liu, Z. 2021. Progress in research on the bioactivity of theaflavins. Food Science 43(19): 318-329.

Mhatre, S., Naik, S. and Patravale, V. 2021. A molecular docking study of EGCG and theaflavin digallate with the druggable targets of SARS-CoV-2. Computers in Biology and Medicine 129: 104137.

Moldoveanu, S. C. and Oden, R. 2021. Antioxidant character and levels of polyphenols in several tea samples. ACS Omega 6(15): 9982-9988.

Ohba, M., Oka, T., Ando, T., Arahata, S., Ikegaya, A., Takagi, H., … and Asai, A. 2017. Antiviral effect of theaflavins against caliciviruses. The Journal of Antibiotics 70(4): 443-447.

Peng, Y., Li, G. and Liu, X. Y. 2020. Progress on health efficacy and mechanism of theaflavin in black tea. Tea Newsletter 47(2): 198-203.

Qi, M. 2019. Optimization of theaflavin production process in microwave-assisted extraction of black tea. Fujian Tea Leaf 41(2): 5-6.

Shan, Z., Nisar, M. F., Li, M., Zhang, C. and Wan, C. C. 2021. Theaflavin chemistry and its health benefits. Oxidative Medicine and Cellular Longevity 2021: 6256618.

Tu, Y. F. 2019. Progress in the pharmacological efficacy and isolation and purification of theaflavin. China Tea Processing 4: 77-84.

Wang, F. S. and Wu, Y. F. 2017. Study on preparation conditions of ester teaflavinase. Journal of Longdong College 28(1): 43-47.

Wang, Y. X., Liu, X. S. and Liu, J. J. 2020. Process optimization of high theaflavin black tea by adding exogenous catechins. Guizhou Agricultural Science 48(12): 114-118.

Xue, J. J., Yin, P. and Zhang, J. Y. 2019. Formation of theaflavins and polyester types by plant-derived polyphenols oxidase oxidized catechins. Food Industry Technology 40(20): 76-81.

Zheng, Y. C., Yu, T. and Zheng, Z. G. 2020. Research progress on the biological activity and development and application of theaflavin. Chinese Medicinal Herbs 51(23): 6095-6101.