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Optimization of Culture Conditions for *Bacillus Subtilis* Rgt2 Bacteria Capable Of Producing Polyphenols by Response Surface Method

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ABSTRACT

Polyphenols are a group of natural compounds with many important pharmacological activities for human health. This study was conducted to optimize the culture conditions for Bacillus subtilis RGT2 to produce polyphenols. ***Research*** *Bacillus subtilis RGT2 used in the study is an endogenous strain isolated from Houttuynia cordata in Kien Giang province. The response surface method with the Box-Behnken design was used to determine the optimal culture conditions. In the study, four variables were investigated: D-glucose content (5-35 g/L), pH level of culture medium (5-8), culture temperature (30-42°C) and incubation time (12-84 hours). The data were collected and analyzed for variance (ANOVA). The coefficient of determination (R^2) and the model with significant interaction between all variables are 98.05% and p<0.0001, respectively. In addition, the test for the lack of fit for the model is also not significant, with p=0.4223. The optimal conditions for D-glucose content, pH level, time and culture temperature were found to be 13.93 g/L, 7.6, 51 hours and 34°C, respectively with predicted values of the polyphenol production of Bacillus subtilis RGT2 was 74.58 mg GAE/mL of extracellular fluid. Using statistically optimized conditions showed that the actual polyphenol content was 75.75±1.44 mg GAE/mL of extracellular fluid. In this study, Bacillus subtilis RGT2 produced the highest polyphenols when cultured in potato D-glucose broth with pH 7.6 at 34°C with the extraction time of 51 hours and 13.93 g/L of D-glucose content.*

Keywords: *Bacillus subtilis RGT2, Box-Behnken, response surface, polyphenols, optimization.*

I. INTRODUCTION

Polyphenols are a group of natural compounds with antioxidant capabilities that play an important role in maintaining human health [7]. During energy production in mitochondria, some oxygen or nitrogen molecules are converted into dangerous free radicals known as reactive oxygen species (ROS) and reactive nitrogen groups (RNS). Important cellular components, including proteins, lipids, and DNA may get oxidative damage if ROS and RNS production exceeds the cell's capacity to produce antioxidants. Cell damage caused by ROS and RNS is a key factor in the development of various degenerative diseases such as: heart, cancer, Alzheimer's and autoimmune diseases [2]. Cells in the body are protected from the harmful effects of ROS and RNS thanks to the addition of polyphenolic compounds in foods and pharmaceuticals of plant origin [10]. Polyphenols are commonly produced during plant growth and development. Currently, plants with medicinal properties are exploited to extract compounds of the polyphenol group, leading to the risk of depletion. Therefore, it is necessary to research and find a new source of polyphenols to replace plants.

Many studies have demonstrated that endogenous bacteria living inside medicinal plants have the ability to produce natural compounds similar to host plants [13]. The endophytic strain of *Bacillus subtilis* RGT2 inside the Houttuynia cordata has been shown to be able to produce secondary metabolites of the polyphenol group [14]. The present study has determined the appropriate culture conditions to help the *Bacillus subtilis* RGT2 strain produce polyphenols effectively. Response surface Method is often used by scientists to optimize microbial culture conditions to obtain biomass or secondary metabolites from microorganisms [8]. The response surface method has been applied in the optimization of

experimental parameters and has shown high efficiency in many fields [3], [5]. The response surface method has been developed based on mathematical and statistical techniques based on the fit of experimental and predictive models so that the obtained experimental data is relevant to the predictive design of the experiment. The response surface method used contributes to orientation and time saving in building experimental models [1]. In addition, the study has contributed to creating a premise as a scientific basis for the exploitation of secondary metabolites of the polyphenol group from endogenous bacteria in medicinal plants.

II. RESEARCH SUBJECTS AND METHODS

2.1. Research subjects

Bacillus subtilis RGT2 used in the study is an endogenous bacterial strain isolated from Houttuynia cordata in Kien Giang province with the ability to produce polyphenols and was selected and identified in 2019 [15].

2.2. Research Methods

2.2.1. Determining the effect of single factors on the polyphenol production ability of *Bacillus subtilis* RGT2

Bacillus subtilis RGT2 was grown in potato D-glucose broth with pH from 5; 5.5; 6; 6.5; 7; 7,5 and 8, respectively. Potato D-glucose broth was supplemented with D-glucose with concentrations from 5; 10; 15; 20; 25; 30 and 35 g/L, respectively. The culture temperature of *Bacillus subtilis* RGT2 was changed from 30; 32; 34; 36; 38; 40 and 42^{o}C, respectively. Culture time was surveyed from 12; 24; 36; 48; 60; 72 to 84 hours, respectively. During the investigation of the influence of single factors, the pH=7, the temperature of 30^{o}C, the culture time of 24 hours and the D-glucose content of 10 g/L were fixed. Then, the proliferation culture was centrifuged at 3000 rpm at room temperature for 10 min to collect the supernatant called extracellular fluid. The extracellular fluid of *Bacillus subtilis* RGT2 was used for the quantification of polyohenols as described in section 2.2.3.

2.2.2. Optimization of culture conditions for polyphenol-producing *Bacillus subtilis* RGT2

After conducting a survey of the single factors, the research team selected the factors that have the greatest influence on the polyphenol content in the extracellular fluid of *Bacillus subtilis* RGT2 to build the optimal culture process. The standard response surface method according to the Box-Behnken experimental design with four factors (pH, D-glucose content, temperature and incubation time), three levels in Design expert 11.0 software was used to design experiments and evaluate models. *Bacillus subtilis* RGT2 after being proliferated according to the culture conditions proposed by Box-Behnken also centrifuges the growth culture at 3000 rpm at room temperature for 10 minutes to collect the extracellular fluid. The extracellular fluid of *Bacillus subtilis* RGT2 was used to quantify polyphenols as described in section 2.2.3.

2.2.3. Polyphenols Quantitative methods in the extracellular fluid of *Bacillus subtilis* RGT2

The total polyphenol content was measured by the Folin-Ciocalteu colorimetric method [12]. The reaction consisted of 50 µL of extracellular fluid; 50 µL of deionized water and 50 µL of Folin-Ciocalteu reagent (25%). After 8 min, 50 µL of 10% sodium carbonate solution was added and well shaken. The absorbance of the reaction mixture was measured by spectrophotometer at 765 nm after incubation for 30 min at 40 °C. The polyphenol content was determined equivalent to milligrams of gallic acid per gram of extract (mg GAE/g extract) based on the equation: y=0.0098x + 0.0592 (R2=0.9996).

2.2.4. Data processing and analysis

The data in the single-factor survey are presented in the form of MEAN±STDEV and processed by ANOVA-Tukey's Minitab 16.0 software. The chart is drawn by Microsoft excel 2016 software. The data in the optimal model are processed by Design expert 11.0.

III. RESEARCH RESULTS AND DISCUSSIONS

3.1. Effect of single factors: pH, D-glucose content, temperature and culture time on polyphenol production ability of *Bacillus subtilis* RGT2

The results show that the polyphenol content was determined to increase from 39.17±0.19 mg GAE/mL extracellular fluid at pH=5 to 47.89±0.61 mg GAE/mL extracellular fluid at pH =7.5. The polyphenol content started to decrease when *Bacillus subtilis* RGT2 was cultured in medium with pH=8 (44.50±0.13 mg GAE/mL of extracellular fluid). Research results show that *Bacillus subtilis* RGT2 has a good ability to produce polyphenols in the environment with pH from 7 to 8, so it was selected in the Box-Behnken design in the optimization experiment.

The survey results showed that the polyphenol content increased from 37.95±0.21 mg GAE/mL extracellular fluid when adding 5 g/L D-glucose to 50.72±1.26 mg GAE/mL extracellular fluid and 15 g/L D-glucose to the culture medium. The polyphenol content started to decrease when D-glucose content increased higher than 15 g/L. Therefore, a D-glucose content of 10 to 20 g/L was selected in the Box-Behnken design in the optimization experiment.

When conducting a survey of temperature factors at 30, 32, 34, 36, 38, 40 and 42°C, respectively, the research team found that the most obtained polyphenol content was 53.48±1.07 mg GAE/mL extracellular fluid at 36°C. Thus, the culture temperature was determined to have a significant effect on the polyphenol production ability of *Bacillus subtilis* RGT2. The research team selected a culture temperature range of 34 to 38°C for the follow-up investigations.

The results showed that the polyphenol content produced by *Bacillus subtilis* RGT2 was best obtained after 48 hours of culture (52.93±0.21 mg GAE/mL of extracellular fluid) and then decreased when prolonging the incubation time. Therefore, a culture time between 36 and 60 h was chosen for further optimization experiments.

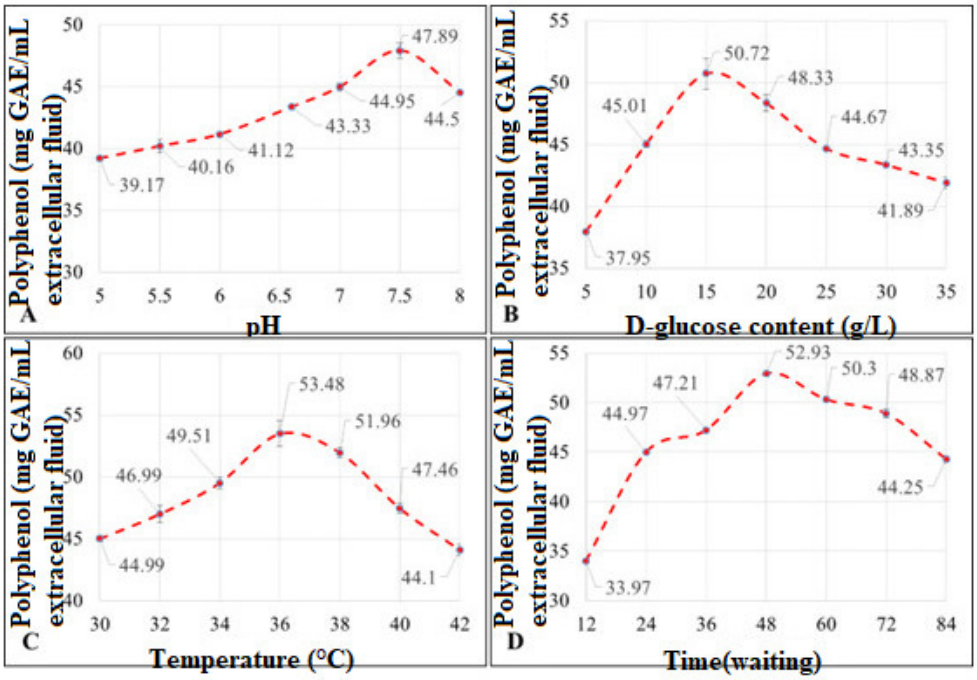


Figure 1. Effect of pH (Figure A), D-glucose content (Figure B), temperature (Figure C) and culture time (Figure D) on the polyphenol production capacity of *Bacillus subtilis* RGT2

Note: A is the effect of pH on the polyphenol production ability of *Bacillus subtilis* RGT2; B is the effect of D-glucose content on the polyphenol production ability of *Bacillus subtilis* RGT2; C is the effect of temperature on the polyphenol production ability of *Bacillus subtilis* RGT2; D is the effect of time on the polyphenol production ability of Bacillus subtilis RGT2

3.2. Optimization of the effects of culture conditions on the polyphenol production ability of Bacillus subtilis RGT2

As a result of the Box-Behnken design, the response surface experiments were designed to obtain a design matrix of variables consisting of 29 treatments of which 5 central treatments are shown in Table 1 along with with experimental values. Experimental results show that *Bacillus subtilis* RGT2 is capable of producing polyphenols, however, polyphenol content depends on a combination of factors. Specifically, when changing culture conditions, polyphenol content also changed and ranged from 50.14±2.43 to 74.08±1.80 mg GAE/mL of extracellular fluid. The polyphenol content produced by Bacillus subtilis RGT2 in the 5 central treatments (treatment 25 to 29) was similar and the difference was not statistically significant ($p>0.05$).

Table 1. Experimental results and prediction of polyphenol content in extracellular fluid of Bacillus subtilis RGT2

Treatment	Conditions				Polyphenol content (mg GAE/g extract)	
	pH	D-glucose (g/L)	Temperature (^oC)	Time (hour)	Experiment	Prediction
1	7	10	36	48	$58,38^{hij}$±1,29	58,07
2	8	10	36	48	$67,51^{c-f}$±2,10	67,32
3	7	20	36	48	$67,12^{c-f}$±2,11	67,92
4	8	20	36	48	$67,99^{b-f}$±0,78	68,91
5	7,5	15	34	36	$69,20^{a-e}$±0,85	69,98
6	7,5	15	38	36	$50,14^{k}$±2,43	51,07
7	7,5	15	34	60	$71,92^{abc}$±2,10	71,60
8	7,5	15	38	60	$60,52^{ghi}$±2,30	60,35
9	7	15	36	36	$57,35^{ij}$±0,60	58,10
10	8	15	36	36	$62,19^{f-i}$±3,13	63,13
11	7	15	36	60	$63,78^{e-h}$±1,38	63,46
12	8	15	36	60	$68,80^{a-e}$±0,05	68,67
13	7,5	10	34	48	$71,29^{abc}$±3,03	71,04
14	7,5	20	34	48	$74,08^{a}$±1,80	74,97
15	7,5	10	38	48	$54,44^{jk}$±0,19	54,17
16	7,5	20	38	48	$60,80^{ghi}$±1,76	61,68
17	7	15	34	48	$71,69^{abc}$±2,89	71,29
18	8	15	34	48	$71,29^{abc}$±2,07	70,59
19	7	15	38	48	$50,91^{k}$±0,52	50,38
20	8	15	38	48	$62,16^{f-i}$±0,62	61,32
21	7,5	10	36	36	$59,44^{g-j}$±1,97	58,87
22	7,5	20	36	36	$60,34^{g-j}$±2,42	66,50
23	7,5	10	36	60	$64,64^{d-g}$±2,05	66,23
24	7,5	20	36	60	$70,71^{abc}$±0,47	70,04
25	7,5	15	36	48	$71,65^{abc}$±1,89	71,79
26	7,5	15	36	48	$71,93^{abc}$±1,36	71,79
27	7,5	15	36	48	$73,74^{ab}$±0,45	71,79
28	7,5	15	36	48	$70,54^{a-d}$±3,41	71,79

29	7,5	15	36	48	$71,10^{abc}$±1,39	71,79

The following characters in the same column do not have statistical meanings ($p<0.05$).

Based on experimental values, the study built a quadratic polynomial equation to predict the polyphenol content produced by Bacillus subtilis RGT2 with the form $YPolyphenol = –1377.21263 + 161.53770 \times A + 6.81041 \times B + 45.63353 \times C + 0.403760 \times D – 0.825850 \times A \times B + 2.91156 \times A \times C + 0.007370 \times A \times D + 0.089456 \times B \times C – 0.015923 \times B \times D + 0.079790 \times C \times D – 16.61332 \times A^2 – 0.083353 \times B^2 – 1.06129 \times C^2 – 0.029848 \times D^2$. In which, Y is the predicted polyphenol content obtained from Bacillus subtilis RGT2; A, B, C, D are the factors of pH, D-glucose content, temperature and culture time, respectively.

Based on the quadratic polynomial equation, the team predicted the polyphenol content that *Bacillus subtilis* RGT2 could produce when cultured under varying conditions (Table 1). The results show that the predicted polyphenol content is almost equivalent to the experimental value. It can be seen that the quadratic polynomial equation that the study has built has high reliability.

Table 2. Analysis of correlation coefficients of factors affecting the ability of bacteria to produce polyphenols

Source	ANOVA polyphenol Analysis				
	Sum of squares	**Df**	**Mean square**	**F-value**	**p-value**
Model	1274,90	14	91,06	50,29	<0,0001
A	78,65	1	78,65	43,44	<0,0001
B	98,18	1	98,18	54,23	<0,0001
C	682,57	1	682,57	376,98	<0,0001
D	89,23	1	89,23	49,28	<0,0001
AB	17,05	1	17,05	9,42	0,0083
AC	33,91	1	33,91	18,73	0,0007
AD	0,0078	1	0,0078	0,0043	0,9485
BC	3,20	1	3,20	1,77	0,2049
BD	3,65	1	3,65	2,02	0,1775
CD	14,67	1	14,67	8,10	0,0129
A^2	111,89	1	111,89	61,80	<0,0001
B^2	28,17	1	28,17	15,56	0,0015
C^2	116,90	1	116,90	64,56	<0,0001
D^2	119,83	1	119,83	66,18	<0,0001
Residual	25,35	14	1,81	Adeq precision=25,4106	
Lack of Fit	19,47	10	1,95	1,33	0,4223
Pure Error	5,87	4	1,47	N=29	CV=2,05%
Cor Total	1300,24	28	R^2=0,9805	R^2_{Adj}=0,9610	R^2_{Pre}=0,9067

Note: A is the pH, B is the D-glucose content, C is the culture temperature and D is the culture time.

The optimal levels of each variable for the polyphenol content produced by Bacillus subtilis RGT2 were determined by generating three-dimensional (3D) response surface histograms as shown in Figure 2. Interpretation of the 3D response surface and contour plots are graphical representations of the regression equation. Response surface plots provide visual interpretations of the relationship between factors, the degree of experimentation of each variable, and the type of interaction between the two experimental variables [6]. As shown in Figure 2, the structures of the reaction surface between pH (A) and D-glucose

content (B) (Figure 2A, 2A')), pH (A) and temperature (C)) (Figure 2B, 2B'), pH (A) and time (D) (Figure 2C, 2C'), D-glucose content (B) and time (D) (Figure 2D, 2D'), temperature (C) and time (D) (Figure 2E, 2E'), D-glucose content (B) and temperature (C) (Figure 2F, 2F') all have a sagging shape with downward opening, therefore, it is shown that the total polyphenol content produced by Bacillus subtilis RGT2 according to the design model is predicted to be high.

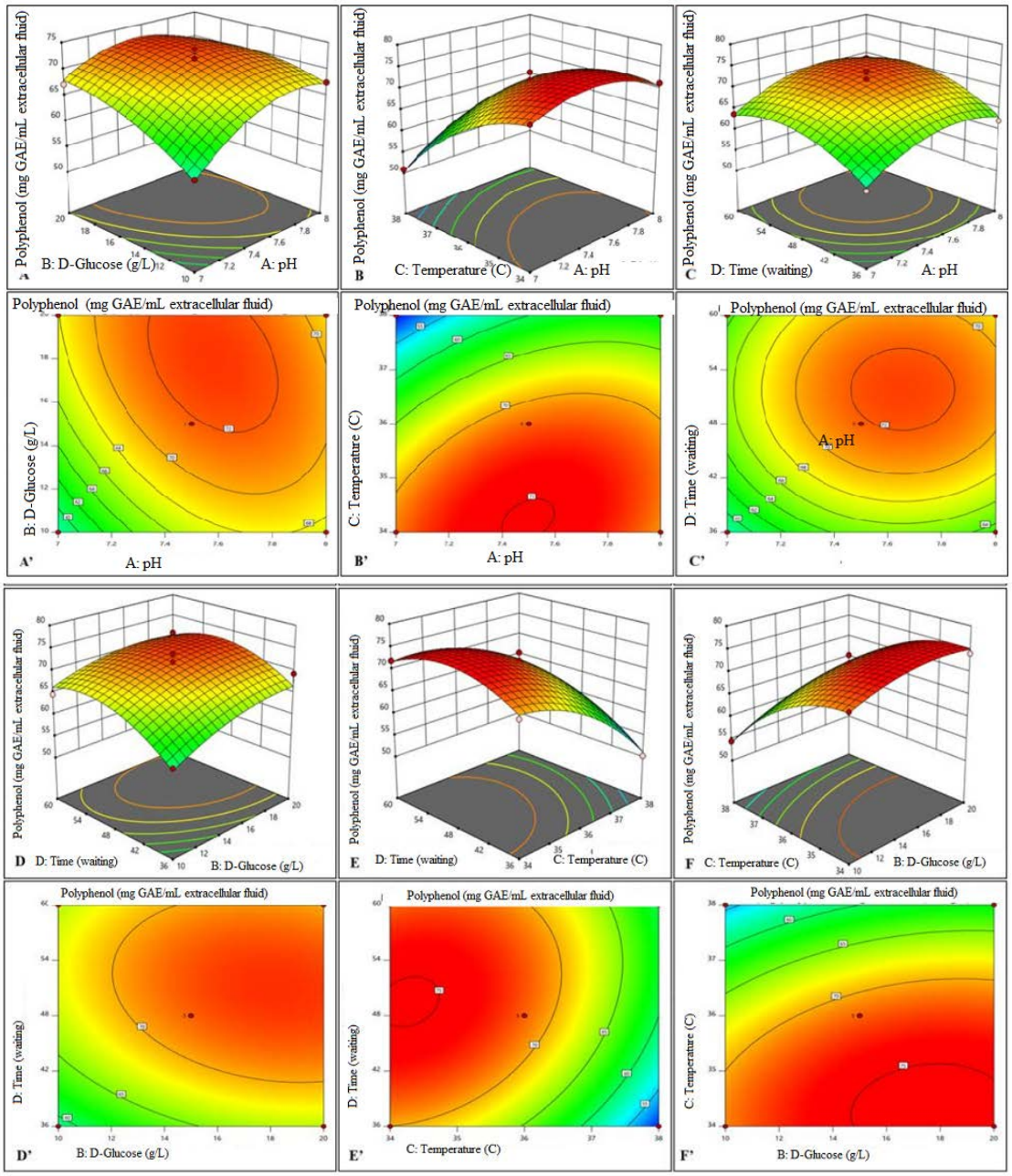
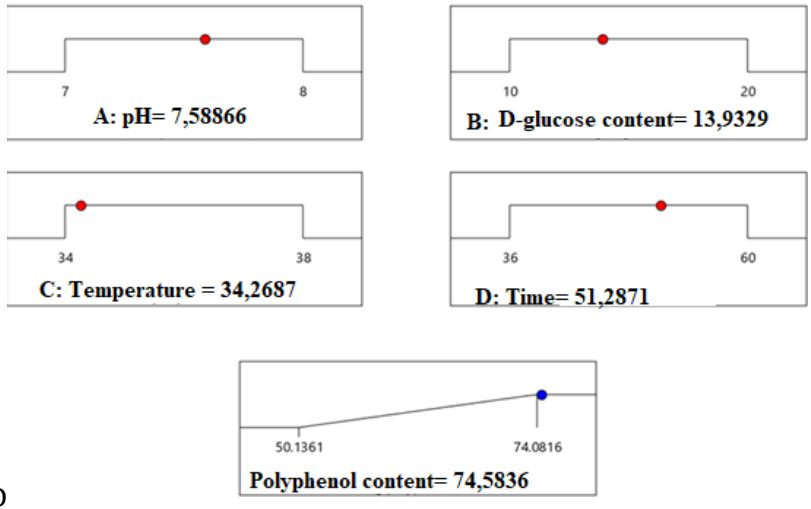


Figure 2. 3D graph showing the interaction between factors

A, A': Model of interaction between temperature and D-glucose content; B, B': Model of interaction between pH and culture temperature; C, C': Model of interaction between pH and culture time; D, D': Model of interaction between D-glucose content and culture time; E, E': Model of interaction between temperature and culture time; F, F': Model of interaction between D-glucose content and culture temperature.

Based on the results of the analysis, the predicted best option for culturing polyphenol-producing Bacillus subtilis RGT2 is shown in Figure 3. Thus, Bacillus subtilis RGT2 cultured in potato D-glucose broth has been added with 13.93 g/L D-glucose at pH=7.58, incubation temperature

34.27°C and culture time is 51.29 hours, obtained polyphenol content is 74.58 mg GAE/mL extracellular fluid. To test the predictive model, the research team conducted an experimental setup under the following conditions: Bacillus subtilis RGT2 was cultured in potato D-glucose broth supplemented with 13.93 g/L D -glucose at pH=7.6, incubation temperature 34oC and culture time is 51 hours, obtained polyphenol content is 75.75±1.44 mg GAE/mL extracellular fluid. The test results showed that the polyphenol content produced by Bacillus subtilis RGT2 between the prediction and the experiment was completely consistent.



D

Figure 3. Optimal extraction procedure and predicted optimal polyphenol content

IV. DISCUSS

4.1. The influence of single factors: pH, D-glucose content, temperature and culture time on polyphenol production ability of Bacillus subtilis RGT2.

pH is one of the important factors affecting the growth and development of bacteria. Previous studies have shown that the pH in the culture medium directly affects the ability of Bacillus subtilis to convert and produce secondary products. In this study, the pH from 5 to 8 was selected to investigate the polyphenol production ability of Bacillus subtilis RGT2. The pH affects the concentration of hydrogen ions in the composition of the medium, changing the charge state of the bacterial cell membrane, increasing the permeability, and making metabolism easier. From there, bacteria are able to adapt and grow quickly to produce more secondary metabolic products. However, if the pH is raised too high or too low, it is not suitable for bacteria, it will lead to the breakdown of the plasma membrane and damage to bacterial cells [11, 16]. Carbohydrates are the major source of carbon and energy for most microorganisms. Carbohydrates can be classified into simple sugars, oligo sugars and polymolecular sugars. Not all microorganisms are capable of using polymolecular sugars. Only microorganisms that are able to secrete extracellular enzymes to hydrolyze these sugars into simple sugars can absorb them. Therefore, the research team chose D-glucose sugar to add to the culture medium of *Bacillus subtilis* RGT2. The polyphenol content started to decrease while increasing D-glucose content higher than 15 g/L. Research by Vijayaraghavan et al. (2014) also showed that when the carbon source exceeded the tolerance threshold of bacteria, the density and content of bacterial secondary metabolites also began to decrease [16]. Thus, Bacillus subtilis RGT2 grew and produced good polyphenols in the range of D-glucose content added from 10 to 20 g/L.

Temperature also has many effects on the growth, development and metabolism of bacteria. Many studies have demonstrated the rate of molecular movement, the rate of

diffusion and the metabolism of bacteria with the external environment when the incubation temperature is increased. A suitable increase in bacterial culture temperature can increase the efficiency of metabolism and the production of secondary metabolites by reducing the viscosity of the culture medium, increasing the permeability of the nutrients into cells, increasing the solubility [4]. However, bacteria are also inhibited or killed at inappropriate culture temperatures (too high or too low). Therefore, the temperature factor has both the effect of increasing and decreasing the reproduction and production of bacterial secondary metabolites.

Appropriate culture time helps to obtain effective biomass as well as content of secondary metabolites. Therefore, the research team conducted a survey of polyphenol content at different time points of culture from 12 to 84 hours. The reason for this may be due to the depletion of the nutrient source and some of the secondary metabolites degraded by prolonging culture time due to light exposure. If the culture time is prolonged, it will lead to competition of bacteria for survival and lead to a decrease in the number of bacteria [4].

4.2. The optimization of culture conditions affecting the ability to produce polyphenols of Bacillus subtilis RGT2

Single factors such as pH, D-glucose content, temperature and time were determined to have an impact on the polyphenol production ability of Bacillus subtilis RGT2, so four factors were selected to be included in the study of optimal culture conditions. The experimental values used in the ANOVA statistical analysis are presented in Table 2. The F value of the model is 50.29, which indicates that the factors in the model have a significant influence on polyphenol content and ANOVA analysis also showed that the $p < 0.0001$ of the regression models had a linear relationship between the dependent variable (polyphenol content) and all the independent variables, which means that the test model is reliable. The lack of fit of model (Lack of Fit) is not significant with $p=0.4223>0.05$, indicating that the obtained experimental data is in good agreement with the predictive model. The value of R2adj of 0.9610 shows that the total variation of the prediction model of 96.10% for the polyphenol content produced by Bacillus subtilis RGT2 is due to the independent variables. The coefficient of determination ($R2=0.9805$), commonly used to assess how well a predictive model is, exhibits a good correlation between experimental and predictive response values. The volatility (CV=2.05%) shows that the deviation between the experimental value and the predicted value is low and shows not only a high degree of accuracy but also high confidence in the experiments performed. The appropriate precision measuring the signal-to-noise ratio (Adeq precision) with a ratio greater than 4 is desired by the design model. In this study, Adeq precision=25,4106 showed an appropriate signal. Therefore, the quadratic model was chosen in this optimization study. Besides, the research results show that the coefficients in the regression equation all have statistically significant differences ($p<0.05$) except for the coefficients AD, BC and BD ($p>0$). ,05).

The response surface method is a more efficient method that reduces development costs, optimizes experimental conditions, improves production efficiency, and solves real-world production problems. Compared with one-factor-at-a-time experiments, statistically designed experiments can describe the effects of factor interactions under linear and quadratic conditions [9]. In this study, the optimization of the polyphenol production conditions of *Bacillus subtilis* RGT2 was divided into two stages: (1) screening for the main effects of the selected variables and (2) optimization of the response. The response surface method not only helps in determining the optimal level of the most important factors, but has also proved useful and satisfactory in this process optimization practice. Through these optimization experiments, the polyphenol content produced by *Bacillus subtilis* RGT2 was 75.75±1.44 mg GAE/mL extracellular fluid under optimal conditions of potato D-glucose broth medium supplemented with 13.93 g/L D-glucose, pH 7.6, temperature 34°C and 51 h of culture time.

V. CONCLUSION

Factors such as pH, D-glucose content, temperature and culture time interact with each other and all have a significant influence on the ability to produce polyphenols of *Bacillus subtilis* RGT2. After optimizing the response surface, the optimal conditions for the polyphenol production of *Bacillus subtilis* RGT2 were determined as potato D-glucose broth supplemented with 13.93 g/L D-glucose, pH 7.6, temperature 34°C and 51 hours of culture time. Under this condition, the polyphenol content can be obtained as 75.75±1.44 mg GAE/mL extracellular fluid. Thus, *Bacillus subtilis* RGT2 can be used as an alternative source of polyphenol producing microorganisms for the extraction of polyphenol compounds from plants.

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