International Journal of Scientific Engineering and Applied Science (IJSEAS) – Volume-8, Issue-12,December 2022
ISSN: 2395-3470
www.ijseas.com

Optimizing Conditions For The Cultivation Of *Bacillus subtilis* RGT2 Capable Of Producing Flavonoid

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ABSTRACT

Background: Flavonoids are considered as a group of natural compounds with diverse biological activities beneficial to human health. **Objective:** This study was carried out to determine the conditions in which the culture of Bacillus subtilis RGT2 produced many flavonoid compounds. **Research subjects and methods:** Flavonoids produced by Bacillus subtilis RGT2 isolated from Houttuynia cordata in Kien Giang province. The surface response method with the Box-Behnken design and analysis of variance (ANOVA) were used to determine the optimal culture conditions for the flavonoid producing Bacillus subtilis RGT2. Factors such as: D-glucose content (5-35 g/L), pH level of culture medium (5-8), culture temperature (30-42°C) and culture time (12-84 hours). **Results:** The coefficient of determination (R^2) and the model that have significant interactions among all variables are 99.65% and $p<0.0001$, respectively. In addition, the test of lack of suitability for the model is also not significan with $p=0.8354$. The optimal conditions for D-glucose content, pH level, time and culture temperature were found to be 15.57 g/L; 7.6; 47 hours and 35°C respectively with the predictive value of Bacillus subtilis RGT2 flavonoid production of 52.82 mg QE/mL extracellular fluid. Using statistically optimized conditions showed that the actual flavonoid content obtained was 54.95±1.04 mg QE/mL of extracellular fluid. **Conclusion:** In this study, Bacillus subtilis RGT2 produced the most flavonoids when cultured in potato D-glucose broth with pH 7.6 at 35^{o}C, 47 hours of extraction time and 15.57 g/L of D-glucose concentration.

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

I. INTRODUCTION

The outbreak of COVID-19 infection caused by SARS-CoV-2 coronavirus that emerged in December 2019 poses significant threats to medical security and global economy [12]. Although there are currently a number of vaccines used to prevent COVID-19 but not really effective. Therefore, worldwide researchers are exploring alternative methods to manage this new coronavirus through prevention and control of the spread of COVID-19. Antiviral treatments, effective natural inhibitors of COVID-19 invading proteins such as ACE2, approaches to enhancing the host immune against infection and therapeutics passive immunoassay therapeutics among many others. Research by Ngwa et al. (2020) has shown the potential of flavonoid-inspired treatments or prophylaxis against COVID-19 [5]. Research indicates that flavonoids such as caflanone, hesperetin, myricetin and flavonoid derivatives such as equivir can bind with high affinity to the spiking protein, helicase and protease sites on the ACE2 receptor. Interestingly, caflanone can inhibit viral entry factors including tyrosine-protein kinase ABL-2, cathepsin L, cytokines (IL-1β, IL-6, IL-8, macrophage inflammatory protein 1α (Mip-1α), TNF-α), and lipid kinase PI4Kiiiβ, as well as receptor tyrosine-protein kinase AXL-2. From the above analysis, it can be seen that the potential application in the prevention and treatment of diseases of compounds belonging to the flavonoid group can be seen. The addition of flavonoids for healthy people as well as sick people with microbial infections to help increase resistance against infectious diseases and improve human health is a necessity during this epidemic time.

Bacterial strains of the genus Bacillus have been widely used to serve humans for a long time. Many studies have demonstrated that bacterial strains belonging to the genus Bacillus have the ability to produce secondary metabolites that are diverse in chemical structure and biological activity [8]. In our study, the endogenous strain of *Bacillus subtilis* RGT2 inside the *Houttuynia cordata* was isolated and identified by Huynh Van Truong in 2019 [9]. The *Bacillus subtilis* RGT2 strain initially showed the ability to produce flavonoids, flavonoids, antioxidant, antibacterial and anti-inflammatory [10]. The research team recognized the potential for flavonoid production as well as the importance and applicability of flavonoids to human health. The study conducted to develop and determine the culture conditions of *Bacillus subtilis* RGT2 that produce effective flavonoids to initially serve as a source of raw materials for the production of health foods. The study used the Box-Behnken model to optimize the culture conditions for *Bacillus subtilis* RGT2. The Central Composite Design model was applied in the process of optimizing experimental parameters and showing the high efficiency in many fields [3], [4]. The combination of mathematical model into the culture design of *Bacillus subtilis* RGT2 is a scientific basis for the efficient exploitation of secondary metabolites of flavonoid group effectively produced by bacteria.

II. RESEARCH SUBJECTS AND METHODS

2.1. Research subjects

Flavonoid compounds are produced by *Bacillus subtilis* RGT2. *Bacillus subtilis* RGT2 is an endogenous bacterial strain isolated from Houttuynia cordata in Kien Giang province [Truong et al., 2019].

2.2. Research Methods

2.2.1. Determining the effect of single factors on the flavonoid production capacity 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 to 8 respectively. Potato D-glucose broth was supplemented with D-glucose with concentrations from 5; 10; 15; 20; 25; 30 to 35 g/L respectively. The culture temperature of *Bacillus subtilis* RGT2 was changed from 30; 32; 34; 36; 38; 40 to 42^{o}C respectively. Culture time was surveyed from 12 ; 24; 36; 48; 60; 72 to 84 hours respectively. During the survey of the influence of single factors, the pH of 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 in 10 minutes to collect the supernatant fluid called extracellular fluid. Extracellular fluid of *Bacillus subtilis* RGT2 was used for the quantification of flavonoids as described in section 2.2.3.

2.2.2. Optimization of culture conditions for flavonoid-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 flavonoid content in the extracellular fluid of *Bacillus subtilis* RGT2 to build the culture process. Optimal. The standard surface response method according to the Box-Behnken experimental design with four factors (pH, D-glucose content, temperature and culture time), three levels in Design expert 11.0 software was used. used to design experiments and evaluate models. *Bacillus subtilis* RGT2 after being grown proliferating according to the culture conditions proposed by Box-Behnken, will also centrifuge the proliferation culture at 3000 rpm at room temperature for 10 minutes to collect the foreign fluid. cell. Extracellular fluid of *Bacillus subtilis* RGT2 was used for the quantification of flavonoids as described in section 2.2.3.

2.2.3. Determination method of flavonoids in the extracellular fluid of *Bacillus subtilis* RGT2

Total flavonoid content in the extracellular fluid of *Bacillus subtilis* RGT2 was performed as described by Akanni et al. (2014) [1]. Extracellular fluids of *Bacillus subtilis* RGT2 with a volume of 500 µL were reacted with 100 µL of 5% NaNO2, incubated for 5 min at room temperature, and then continued to add 100 µL of 10% AlCl3 and mixed. After 5 min of

incubation at room temperature, the reaction mixture was added with 1000 µL of NaOH 1M and 800 µL of deionized water. Finally, the reaction mixture was measured for absorbance spectroscopy at 510 nm at room temperature. Flavonoid content was expressed as mg quercetin (QE) per 1 g extract, based on a standard: y=0.0073x+0.049 (R^2=0.9985).

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 using Minitab 16.0 software with the verification of ANOVA-Tukey's. The chart is drawn by Microsoft excel 2016 software. The data in the optimal model are processed by Design expert 11.0 software.

III. RESEARCH RESULTS AND DISCUSSION

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

Bacillus subtilis RGT2 has the ability to produce flavonoids increased from 22.19±0.25 mg QE/mL, extracellular fluid at pH=5 to 37.81±0.87 mg of QE/mL of extracellular fluid at pH=7,5. At pH=8, the flavonoid production capacity of *Bacillus subtilis* RGT2 was reduced to 35.37±0.39 mg QE/mL of extracellular fluid. Thus, *Bacillus subtilis* RGT2 has the ability to produce flavonoids well in the environment with pH from 7 to 8.

The flavonoid content produced by *Bacillus subtilis* RGT2 increased from 25.55±0.68 mg QE/mL of extracellular fluid when the medium was supplemented with 5 g/L D-glucose to 36.69±0.79 mg QE /mL of extracellular fluid when the medium was supplemented with 15 g/L of D-glucose. When more than 15 g/L D-glucose was added, the flavonoid content began to decrease. Therefore, the D-glucose content from 10 to 20 g/L was selected in the Box-Behnken design.

When surveying temperature factors at 30, 32, 34, 36, 38, 40 and 42°C, respectively, the team found that the highest flavonoid content was 42.24±0.79 mg QE/mL of extracellular fluid at 36°C. Thus, the culture temperature was determined to have a significant effect on the flavonoid production ability of *Bacillus subtilis* RGT2. The research team selected a culture temperature range from 34 to 38°C for the follow-up investigations.

The study showed that the flavonoid content produced by *Bacillus subtilis* RGT2 was best obtained after 48 hours of culture (40.11±1.43 mg QE/mL of extracellular fluid) and then decreased with prolongation of culture time. Therefore, a culture time between 36 and 60 hours was chosen for further optimization experiments.

Table 1. Effect of pH, D-glucose content, temperature and culture time on flavonoid production ability of *Bacillus subtilis* RGT2

Factors	**Total Flavonoid content (TFC, mg QE/mL of extracellular fluid) under the influence of single factors**						
A	**5,00**	**5,50**	**6,00**	**6,50**	**7,00**	**7,50**	**8,00**
TFC	$22,19^{f}$±0,25	$25,89^{e}$±0,31	$28,68^{d}$±0,46	$29,52^{cd}$±0,12	$30,25^{c}$±0,04	$37,81^{a}$±0,87	$35,37^{b}$±0,39
B	**5**	**10**	**15**	**20**	**25**	**30**	**35**
TFC	$25,55^{ef}$±0,68	$30,48^{bc}$±0,30	$36,69^{a}$±0,79	$31,58^{b}$±2,13	$28,93^{cd}$±0,40	$27,24^{de}$±0,55	$24,16^{f}$±0,11
C	**30**	**32**	**34**	**36**	**38**	**40**	**42**
TFC	$30,25^{e}$±0,11	$31,85^{cde}$±0,91	$33,08^{cd}$±1,61	$42,24^{a}$±0,79	$37,76^{b}$±0,41	$33,65^{c}$±0,41	$30,94^{de}$±0,17
D	**12**	**24**	**36**	**48**	**60**	**72**	**84**
TFC	$19,64^{d}$±1,09	$30,23^{c}$±0,11	$32,28^{c}$±0,40	$40,11^{a}$±1,43	$37,15^{b}$±0,40	$35,21^{b}$±0,27	$30,18^{c}$±0,72

Note: A is the effect of pH on the flavonoid production capacity of Bacillus subtilis RGT2; B is the effect of D-glucose content on the flavonoid production capacity of Bacillus subtilis RGT2; C is the

effect of temperature on the flavonoid production capacity of Bacillus subtilis RGT2; D is the effect of time on the flavonoid production capacity of Bacillus subtilis RGT2.

3.2. Optimization of culture conditions affecting flavonoid production capacity of *Bacillus subtilis* RGT2

The response surface experiments were designed according to the Box-Benken model to obtain a design matrix of variables consisting of 29 treatments of which the central 5 treatments are shown in Table 2 along with the experimental values. Experimental results show that *Bacillus subtilis* RGT2 has the ability to produce flavonoids, but the flavonoid content depends on a combination of factors. Specifically, when changing culture conditions, the flavonoid content also changed and ranged from 35.23±2.40 to 51.99±2.67 mg QE/mL of extracellular fluid. The flavonoid content produced by *Bacillus subtilis* RGT2 in 5 central treatments (treatments 25 to 29) was similar and the difference was not statistically significant (p>0.05).

Table 2. Experimental results and prediction of flavonoid content in extracellular fluid of *Bacillus subtilis* RGT2

Treatments	Conditions				Flavonoid content (mg QE/g extract)	
	pH	D-glucose (g/L)	Temperature (°C)	Time (hour)	Experiment	Prediction
1	7	10	36	48	$40,32^{def}$±3,75	40,36
2	8	10	36	48	$47,06^{a-d}$±0,54	46,84
3	7	20	36	48	$46,05^{a-e}$±2,98	46,47
4	8	20	36	48	$47,26^{a-d}$±0,42	47,43
5	7,5	15	34	36	$48,13^{a-d}$±0,41	48,22
6	7,5	15	38	36	$35,25^{f}$±0,20	35,29
7	7,5	15	34	60	$50,11^{abc}$±0,42	50,28
8	7,5	15	38	60	$42,31^{b-f}$±6,86	42,42
9	7	15	36	36	$40,69^{d-f}$±2,55	40,30
10	8	15	36	36	$43,79^{a-f}$±1,89	43,63
11	7	15	36	60	$44,80^{a-e}$±2,67	44,51
12	8	15	36	60	$48,68^{a-d}$±3,71	48,61
13	7,5	10	34	48	$49,27^{a-d}$±3,49	49,14
14	7,5	20	34	48	$51,99^{a}$±2,67	51,39
15	7,5	10	38	48	$37,49^{ef}$±2,71	37,64
16	7,5	20	38	48	$42,42^{b-f}$±2,34	42,10
17	7	15	34	48	$49,25^{a-d}$±4,20	49,46
18	8	15	34	48	$49,09^{a-d}$±4,24	49,34
19	7	15	38	48	$35,23^{f}$±2,40	35,22
20	8	15	38	48	$42,76^{a-f}$±1,45	42,79
21	7,5	10	36	36	$41,03^{c-f}$±1,01	41,18
22	7,5	20	36	36	$44,22^{a-f}$±3,65	44,48
23	7,5	10	36	60	$45,73^{a-e}$±6,90	45,72
24	7,5	20	36	60	$49,04^{a-d}$±2,45	49,13
25	7,5	15	36	48	$50,16^{abc}$±1,45	51,07
26	7,5	15	36	48	$51,28^{ab}$±0,59	51,07
27	7,5	15	36	48	$51,35^{ab}$±0,42	51,07
28	7,5	15	36	48	$51,23^{ab}$±0,48	51,07
29	7,5	15	36	48	$51,32^{ab}$±0,87	51,07

Note: The following characters in the same column are not significantly statistical (p<0.05).

Based on experimental values, the study formed a quadratic polynomial equation to predict the flavonoid content produced by *Bacillus subtilis* RGT2 with the form $Y_{Flavonoid}$ = –1280,17523 +

140,95510 × A + 5,42002 × B + 43,28463 × C + 0,359335 × D – 0,552511 × A × B + 1,92352 × A × C + 0,032344 × A × D + 0,055365 × B × C + 0,000476 × B × D + 0,052797 × C × D – 13,31659 × A^2 – 0,098577 × B^2 – 0,884370 × C^2 – 0,024150 × D^2. Y is the predicted flavonoid content collected from *Bacillus subtilis* RGT2. A, B, C, D are the factors of pH, D-glucose content, temperature and culture time, respectively.

Basing on the quadratic polynomial equation, the team predicted the flavonoid content that *Bacillus subtilis* RGT2 could produce when cultured under varying conditions (Table 2). The results shows that the predicted flavonoid content is almost equivalent to the experimental value. From that, it can be seen that the quadratic polynomial equation that the study has built has high reliability.

Table 3. Analysis of correlation coefficients of factors affecting the capability of flavonoids production of bacteria

Source	ANOVA flavonoid analysis				
	Sum of squares	**Df**	**Mean square**	**F-value**	**p-value**
Model	668,54	14	47,75	288,79	<0,0001
A- pH	41,46	1	41,46	250,76	<0,0001
B- D-glucose	33,64	1	33,64	203,44	<0,0001
C-Temperature	324,21	1	324,21	1960,75	<0,0001
D-Time	63,28	1	63,28	382,72	<0,0001
AB	7,63	1	7,63	46,15	<0,0001
AC	14,80	1	14,80	89,50	<0,0001
AD	0,1506	1	0,1506	0,9110	0,3560
BC	1,23	1	1,23	7,42	0,0165
BD	0,0033	1	0,0033	0,0197	0,8904
CD	6,42	1	6,42	38,84	<0,0001
A^2	71,89	1	71,89	434,78	<0,0001
B^2	39,39	1	39,39	238,25	<0,0001
C^2	81,17	1	81,17	490,89	<0,0001
D^2	78,44	1	78,44	474,40	<0,0001
Residual	2,31	14	0,1654		
Lack of Fit	1,28	10	0,1275	0,4905	0,8354
Pure Error	1,04	4	0,2600	N=29	CV=0,8885%
Cor Total	670,85	28	R^2=0,9965	R^2_{Adj}=0,9931	R^2_{Pre}=0,9866

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 flavonoid content produced by *Bacillus subtilis* RGT2 were determined by generating three-dimensional (3D) response surface charts as shown in Figure 1. Interpretation of the 3D response surface charts and contour plots are graphical representations of the regression equation. Response surface charts 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 1, the structures of the reaction surface between pH (A) and D-glucose content (B) (Figure 1A), pH (A) and temperature (C) (Figure 1B)), pH (A) and time (D) (Figure 1C), D-glucose content (B) and time (D) (Figure 1D), temperature (C) and time (D) (Figure 1E), the D-glucose content (B) and the temperature (C) (Figure 1F) are all sag-shaped with downward opening, thus it shows the total flavonoid content produced by *Bacillus subtilis* RGT2 according to predictive design model is high.

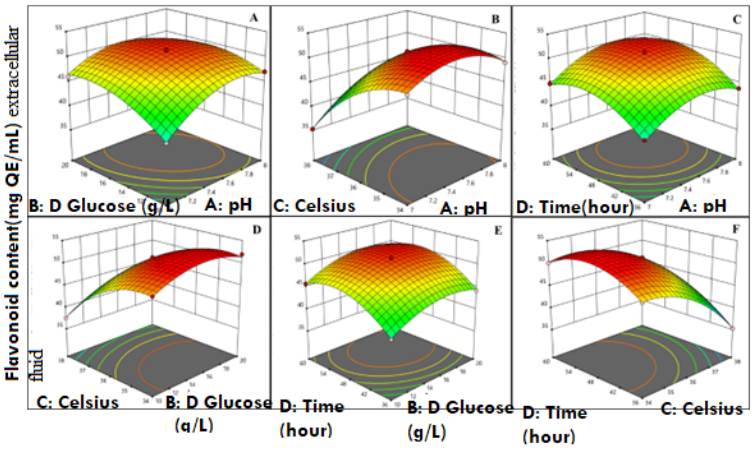

Figure 1. 3D graph showing the interaction between the factors

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

Based on the results of the analysis of the best prediction for culturing the flavonoid-producing *Bacillus subtilis* RGT2 shown in Figure 2.

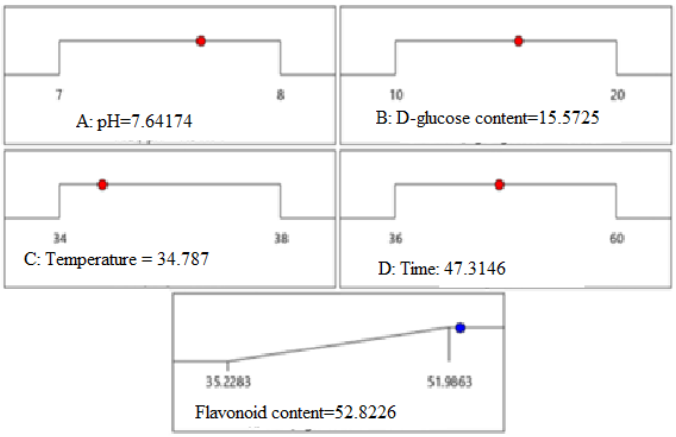

Figure 2. Optimal extraction procedure and predicted optimal flavonoid content

Thus, *Bacillus subtilis* RGT2 was cultured in potato D-glucose broth supplemented with 15.57 g/L D-glucose at pH=7.6, incubation temperature at 34.79^{o}C and culture time of 47.31 hours, the flavonoid content was 52.82 mg QE/mL of 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 15.57 g/L D -glucose at pH=7.6, incubation temperature at 35^{o}C and culture time of 47 hours, the flavonoid content obtained was 54.95±1.04 mg QE/mL of extracellular fluid. The test results showed that the flavonoid content produced by *Bacillus subtilis* RGT2 between the prediction and the experiment was completely consistent.

IV. DISCUSSION

4.1. On the influence of single factors: pH, D-glucose content, temperature and culture time on flavonoid production ability of *Bacillus subtilis* RGT2.

The growth and metabolism of bacterial strains are often subject to pH coordination. The pH in the culture medium directly affects the ability to convert to produce secondary products of *Bacillus subtilis*. In this survey, a pH of 5 to 8 was chosen to evaluate the flavonoid production capacity of *Bacillus subtilis* RGT2. Studies show that pH regulates the concentration of hydrogen ions located in the composition of the environment, thereby

directly affecting the state of charge of the bacterial cell membrane, increasing permeability, and making metabolism easier. If the pH is raised too high or too low, it will lead to the breakdown of the plasma membrane and the damage of bacterial cells [7], [11].
D-glucose is a simple sugar that bacteria can use. The D-glucose used in this study is a source of carbon and energy for the flavonoid-producing *Bacillus subtilis* RGT2. Some studies show that only some bacteria have the ability to use polysaccharides, so the use of D-glucose in the study is necessary. However, providing D-glucose too low or too high can lead to a deficiency or excess of carbon sources, which adversely affects the growth and metabolism of *Bacillus subtilis* RGT2. Because if the D-glucose content is too low or too high, it will unbalance the osmotic pressure of substances across the bacterial cell membrane [11].
Bacterial growth, development, and metabolism are significantly influenced by temperature. Temperature affects the movement speed of molecular, the speed of diffusion, and the metabolism of bacteria with the environment. The appropriate culture temperature accelerates the metabolism and the production of secondary metabolite compounds. As the right temperature reduces the viscosity of the culture medium, increases the permeability of nutrients into the cells, and increases the solubility [2]. Bacteria are also inhibited or killed at too high or too low culture temperatures.
According to Huang et al. (2014), suitable culture time helps to obtain effective biomass as well as content of secondary metabolites. The long incubation period may be due to the depletion of the nutrient source and some of the secondary metabolites are degraded by prolonged culture time and light exposure. However, the culture period is too short to allow the bacteria to grow and convert nutrients into bioactive secondary metabolites [2].

4.2. On optimization of culture conditions affecting flavonoid production of *Bacillus subtilis* RGT2

The pH, D-glucose, temperature and culture time all affected the flavonoid production of *Bacillus subtilis* RGT2. Therefore, the study took the above four factors into account to build the optimal culture procedure. The experimental values are used in the ANOVA statistical analysis and are presented in Table 1. The F value of the model is 49.05, which indicates that the factors in the model have a significant influence on flavonoid content and ANOVA analysis also shows that the $p<0.0001$ value of the regression model has a linear relationship between the dependent variable (flavonoid content) and all the independent variables. It means that testing model is reliable. The lack of fit of the model (Lack of Fit) is not significant with $p=0.8354>0.05$, indicating that the obtained experimental data is in good agreement with the predictive model. The value of R2adj of 0.9931 indicates that the total predicted model variation of 99.31% for the flavonoid content produced by *Bacillus subtilis* RGT2 is due to the independent variables. The coefficient of determination ($R2=0.9965$) are commonly used to assess how well a predictive model is, exhibits a good correlation between experimental and predictive response values. The volatility ($CV=0.8885\%$) 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. An appropriate precision measuring the signal-to-noise ratio (Adeq precision) with a ratio greater than 4 is desired by the design model. 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 and BD ($p>0.05$).).
The Box-Benken design is a more efficient model 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 effect of factor interactions under linear and quadratic conditions [6]. In this study, the optimization of flavonoid production conditions by *Bacillus subtilis* RGT2 was divided into two stages: (1) screening for the main effects of selected variables and (2) optimization of response. The response surface method not only helps to

determine 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 flavonoid content produced by *Bacillus subtilis* RGT2 had 54.95±1.04 mg QE/mL of extracellular fluid under optimal conditions of potato D-glucose broth supplemented with 15.57 g/L D-glucose, pH at 7.6, temperature at 35°C and culture time of 47 hours.

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 flavonoid production ability of *Bacillus subtilis* RGT2. After optimizing the reaction surface, the optimal conditions for the production of flavonoids by *Bacillus subtilis* RGT2 were determined as potato D-glucose broth supplemented with 15.57 g/L D-glucose, pH at 7.6, temperature at 35°C and culture time of 47 hours. Under these conditions, the flavonoid content which can be obtained is 54.95±1.04 mg QE/mL of extracellular fluid. Thus, *Bacillus subtilis* RGT2 can be used as a source of flavonoid-producing microorganisms and an alternative to the extraction of flavonoid compounds from plants.

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