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Two-Step Optimization for Improving Prodigiosin Production Using a Fermentation Medium for *Serratia marcescens* and an Extraction Process

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Abstract: Prodigiosin (PG) is a secondary metabolite produced by *Serratia marcescens* which has a promising future in food, textile, and other industries due to its bright color and diverse biological activities. Currently, the production of PG is mainly restricted by the components of the fermentation medium and large losses during its extraction process, making large-scale industrial production impossible. In this study, a Box–Behnken design (BBD) was used to optimize the response surface of the fermentation medium of *S. marcescens.* The optimum medium composition was found to be sucrose, 16.29 g/L; peptone, 11.76 g/L; and tween 80, 2.64 g/L. This composition produced a PG amount of 1653.95 ± 32.12 mg/L, which is a 64-fold increase compared to the initial medium. A Box–Behnken design (BBD) was then used to optimize the response surface of the extraction process of PG, aiming to reduce loss during extraction. The optimal extraction conditions were determined to be a solvent fermentation liquid ratio of 9.12:1, an extraction temperature of 25.35 °C, and an extraction time of 30.33 min. These conditions resulted in a final PG production amount of 2142.75 ± 12.55 mg/L, which was nearly 84 times higher than the initial production amount of PG. These results provide essential theoretical and experimental support for the industrial production of PG.

Keywords: prodigiosin; *Serratia marcescens*; fermentation; extraction process; response surface methodology (RSM)

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1. Introduction

There are numerous microorganisms that produce pigments in nature which are found in various ecological environments, such as soil, air, and water [1]. Prodigiosin is a red microbial pigment with a tripyrrole ring structure, which is naturally produced by *Serratia marcescens*, *Serratia puccinata*, *Pseudomonas aeruginosa*, and some *actinomycetes* [2]. Although the significance of PG as a secondary metabolite for producers is not yet clear, it is of interest due to its anticancer, antimicrobial, antimalarial, and immunosuppressive properties [3]. PG has demonstrated its high apoptotic activity against lung, colon, breast, and other cancer cell lines [4–6]. Several in vitro experiments reported that PG not only has a significant inhibitory effect on pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Escherichia coli*, and *Staphylococcus aureus* [7–9], but can also inhibit the fungi of the genera *Epidermomyces*, *Trichoderma*, and *Microsporum* [10]. The antimalarial activity of PG is mainly reflected in its terminal disinfection on *Trypanosoma cruzi*, *Trypanosoma brucei*, *Aedes aegypti*, and *Anopheles stephensi* [11,12]. In terms of immunosuppressive activity, Huh et al. [13] found that PG alters the function of macrophages and NK cells, as well as the proliferative capacity of splenocytes in mice.

Due to the diverse biological activities and high commercial value of PG, there has been an increasing focus on improving its production. Nguyen et al. [14] used marine debris, specifically shrimp heads, as a source of carbon and nitrogen for fermentation. This resulted in a 6.11–10.89-fold increase in PG production compared to previous reports. Gohil et al. [15] used agro-industrial waste soya bean meal as the sole source of nutrients for *S. marcescens*, followed by the addition of sucrose and glycine. The amount of PG achieved was 5.19 times higher than the amount attained using a commercial culture medium. Tao et al. [16] added glucose to the initial medium to promote bacterial growth and added glycerol during the fermentation process to induce the production of PG, resulting in a 7.8-fold increase in production (583 mg/L). Aruldass et al. [17] utilized brown sugar as a cost-effective carbon source. They added lactose and L-tryptophan during fermentation, resulting in a 32-fold increase in the final production of PG in a 5 L bioreactor. These previous reports indicate that the composition of the medium is crucial for PG production. Therefore, determining a suitable fermentation medium was one of the aims of this study.

PG can accumulate in the intracellular medium of *S. marcescens* in large quantities during the fermentation process, making it difficult to acquire [18]. A reliable method for extracting PG is crucial for its industrial production. As an indirect way of increasing PG production, it is also of great value to reduce PG degradation. However, there are limited reports on the optimization of the extraction technique for PG [19]. Ultrasound-assisted cell fragmentation is a popular method [20–22]. Paul et al. [23] utilized Taguchi's method to optimize the parameters of ultrasound-assisted extraction, including the solid/liquid ratio, duty cycle, medium pH, and acoustic intensity, in order to improve the extraction rate of PG. However, parameters such as time and the temperature during the ultrasound still have a high influence on the extraction rate of PG. Further optimizing the conditions for PG extraction using ultrasound-assisted cellular fragmentation to reduce its irreversible degradation during the extraction process is another aim of this study.

Here, we isolated *S. marcescens* ZPG19 from compost generated by aerobic composting *Flammulina velutipes* residue. The optimal carbon source, nitrogen source, inorganic salt, additives, and amino acids in the fermentation medium were selected using a single-factor experiment. A Plackett–Burman design was used to identify the important factors affecting PG production. The key factors were then identified through the path of steepest ascent method to find the concentration interval for the maximal production of PG. Finally, response surface optimization was carried out to obtain the optimal fermentation medium. The optimal conditions for PG extraction were also determined through a single-factor experiment, the path of steepest ascent method, and response surface optimization. PG production was improved significantly after a series of optimizations. The data obtained from this study provide valuable insights into the industrial production of PG.

2. Materials and Methods

2.1. Microorganism

The strain used for the experiments was *S. marcescens* ZPG19, which had been stored at −80 °C in a refrigerator in a laboratory.

2.2. Fermentation Media and Culture Condition

LB agar and an LB liquid medium were used to activate *S. marcescens*. The composition of the media was as follows (g/L): tryptone, 10; yeast extract, 5; NaCl, 10; and agar, 20. The final pH of 7.5 was adjusted using a 1 M NaOH solution and/or 1 M HCl.

The composition of the initial fermentation medium was as follows (g/L): soluble starch, 5; peptone, 10; and $MgSO_4$ and K_2HPO_4, 1 (inorganic salt ratio of 50:1). The initial pH of 7.5 was adjusted using a 1 M NaOH solution and/or 1 M H_2SO_4.

All media were sterilized at 121 °C for 15 min. Amino acids were filtered through sterile membranes and added to the shake flasks after sterilization to protect their structures at high temperatures.

The bacteria were transferred from the preservation tube to an LB agar plate using an inoculation loop. The plate was then incubated at 20 °C for 24 h in a thermostat. A single colony was picked and cultivated in LB liquid medium with a shaking speed of 180 rpm for 42 h. Then, the bacteria were cultivated with 2% of the inoculation amount at 30 °C with a speed of 200 rpm for 48 h.

2.3. Extraction and Analytical Methods for PG

The fermentation liquid mixed with the acidic organic solvent were fragmentated and collected using centrifugation (4000× *g*, 4 °C, and 15 min). The absorbance was measured at 535 nm [24]. The purity was determined using high-performance liquid chromatography (HPLC) (Aligent, LC-20A, Palo Alto, CA, USA) at 535 nm. Acetonitrile and 10 mM ammonium acetate (85: 15 v/v) were used as a mobile phase at a flow rate of 1.0 mL/min through the column (Agilent, Zorbax SB C18, Palo Alto, CA, USA) at 40 °C [25]. The cell concentration was measured at a wavelength of 600 nm.

2.4. A Single-Factor Experiment of Fermentation Media

A single-factor experiment was used to screen the most suitable types of carbon, nitrogen, inorganic salts, surfactants, and amino acids for the fermentation medium. Various carbon sources, including glucose, sucrose, ethanol, glycerol, peanut seeds, mannitol, brown sugar, maltose, sesame flour, farina tritici, and lactose, were tested. An optimal nitrogen source was selected from CH_3COONH_4, NH_4Cl, tryptone, peptone, yeast powder, yeast extract paste, beef extract, $CO(NH_2)_2$, and $(NH_4)_2SO_4$. Combinations of the most suitable inorganic salt were selected from $MgSO_4/FeSO_4$, $MgSO_4/MnSO_4$, $KCl/FeSO_4$, $KCl/MnSO_4$, $KCl/CaCl_2$, $NaCl/FeSO_4$, $NaCl/MnSO_4$, $NaCl/CaCl_2$, $K_2HPO_4/FeSO_4$, $K_2HPO_4/MnSO_4$, $K_2HPO_4/CaCl_2$, $Na_3C_6H_5O_7/FeSO_4$, $Na_3C_6H_5O_7/MnSO_4$, and $Na_3C_6H_5O_7/CaCl_2$. The surfactant was selected from polysorbate 80, DMSO, sodium dodecyl sulfate, and $NaHCO_3$. A combination of amino acid was selected from proline (Pro)/serine (Ser)/methionine (Met), Pro/Ser, Pro, Ser, Met, Ser/Met, Pro/Met, Pro/Ser/Met/glycine (Gly), Pro/Ser/Met/histidine (His), Pro/Ser/Met/tryptophan (Trp), and Pro/Ser/Met/glutamine (Gln). Fermentation medium was prepared according to the ratio of inorganic salts at 50:1 and 2% inoculation amounts. Each experiment was repeated three times.

2.5. Plackett–Burman Design of Fermentation Media

PG was used as the response value, and the factors that had a greater influence on it were sought. And three key factors were determined by the response surface design through 12 trials [26]. Duplicate experiments (three times each trial) were carried out. Table 1 shows the factors involved, including sucrose (A), peptone (B), magnesium sulphate (C), tween 80 (D), and proline (E). The experimental design, data analysis, and statistical analysis were conducted using Minitab software (Version 21).

Table 1. Plackett–Burman design of fermentation media.

Test Number	A	B	C	D	E
1	−1	−1	1	1	1
2	−1	−1	−1	−1	−1
3	1	−1	−1	−1	1
4	−1	1	1	−1	1
5	−1	1	1	1	−1
6	−1	1	−1	−1	−1
7	1	1	−1	1	−1
8	1	1	1	−1	1
9	1	−1	1	−1	−1
10	1	1	−1	1	1
11	−1	−1	−1	1	1
12	1	−1	1	1	−1

2.6. Response Surface Design of Fermentation Media

The path of steepest ascent method was conducted for each key factor to maintain maximum production values within the selected concentration interval. Each set of tests was repeated three times. The independent variables and their corresponding levels are displayed in Table 2. The conditions for each factor of the medium were optimized using a Box–Behnken design. To avoid bias, 17 runs were performed in a completely random order. Each set of trials was repeated three times, including five centroid replicates [27]. The key factors in this study were sucrose (A), peptone (B), and tween 80 (C). The production of PG as the response or dependent variable (Y) is shown in Table 3. The Box–Behnken test was analyzed using ANOVA in Design-expert software (Version 13.1.0). A second-order polynomial linear equation was fitted to determine the optimal fermentation medium, and the fermentation process was subsequently verified.

Table 2. Determining the selection level for path of steepest ascent method of fermentation media.

Parameters	Level		
	−1	**0**	**1**
Polysorbate 80 (g/L)	1.6	2.6	3.6
Sucrose (g/L)	5	15	25
Peptone (g/L)	5	10	15

Table 3. Response surface design for media and extraction processes.

Run No	A	B	C
1	1	0	−1
2	−1	−1	0
3	0	0	0
4	−1	0	1
5	0	−1	1
6	0	1	1
7	0	0	0
8	−1	0	−1
9	1	1	0
10	0	−1	−1
11	0	1	−1
12	0	0	0
13	0	0	0
14	1	−1	0
15	−1	1	0
16	1	0	1
17	0	0	0

Note: A is sucrose or solvent/fermentation liquid ratio, B is peptone or extraction time, and C is tween 80 or extraction temperature, respectively.

2.7. A Single-Factor Experiment of Extraction Processes

A single-factor experiment was conducted to optimize the parameters of the PG extraction process. This included determining the solvent-to-fermentation liquid ratio during extraction (19:1, 11:1, 9:1, 7:1, 5:1, 3:1, 1:1, 1:3), the concentration of the extraction solvent (20%, 40%, 60%, 80%, 100%), the extraction time (5 min, 10 min, 20 min, 30 min, 40 min, 60 min), and the extraction temperature (10 °C, 20 °C, 40 °C, 60 °C, 80 °C). The fermentation medium was chosen after optimization with a pH of 2. Each set of experiments was repeated three times.

2.8. Response Surface Design of Extraction Processes

After determining the three key factors of the extraction process, we conducted the path of steepest ascent method on these factors. The independent variables and their

corresponding levels are listed in Table 4. The extraction process was optimized for each factor using a Box–Behnken design. To avoid bias, 17 runs were carried out in a completely random order, including five centroid replicates. Each set of experiments was repeated three times. The key factors studied were the solvent/fermentation liquid ratio (A), extraction temperature (B), and extraction time (C). Table 3 shows the production of PG as the response or dependent variable (Y). The Box–Behnken test was analyzed using an ANOVA and Design-expert software (Version 13.1.0). A second-order polynomial linear equation was fitted to obtain the optimal extraction conditions and validate them.

Table 4. Variables used to determine the selection level for the path of steepest ascent method of extraction processes.

Parameters	Level		
	−1	**0**	**1**
Solvent/Broth	1:11	1:9	1:7
Temperature	10 °C	25 °C	40 °C
Time	20 min	30 min	40 min

2.9. Statistical Analysis

Statistical analyses using GraphPad Prism 6.0 were conducted to differentiate between the experimental and control groups in this study. Each value is the mean of three replications. The data are expressed as the mean ± standard deviation (SD), and a *p*-value < 0.05 is considered significant.

3. Results

3.1. Morphology of *Serratia marcescens*

The *S. marcescens* ZPG19 was characterized by its reddish colonies with smooth edges and a peculiar odor. Short rods were observed using a scanning electron microscope (Figure 1).

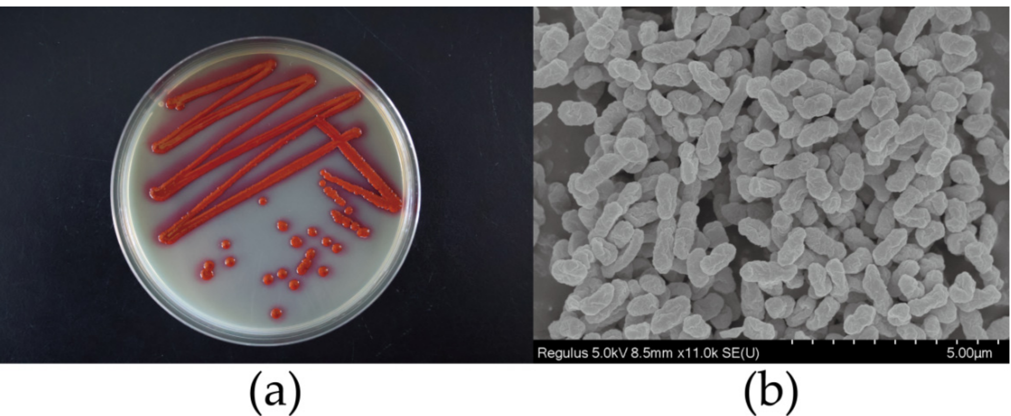

Figure 1. Morphology of *S. marcescens* ZPG19: (**a**) strain colonies; (**b**) scanning electron microscopy images of the strain.

3.2. A Single-Factor Experiment of Fermentation Media

3.2.1. Effect of Carbon Source on PG Production and Bacterial Growth

Carbon, as a fundamental component of the microbial metabolism, plays a crucial role in providing energy for cell growth. The growth of PG was not significantly affected by different carbon sources, as seen in the results in Figure 2a. The growth rate of the cells was marked higher when using sucrose as the carbon source at OD_{535} among the eleven carbon sources tested. The inhibitory effect of glucose on PG was consistent with the findings of Su et al. [28]. Thus, sucrose should be considered as the optimal carbon source for PG production through fermentation.

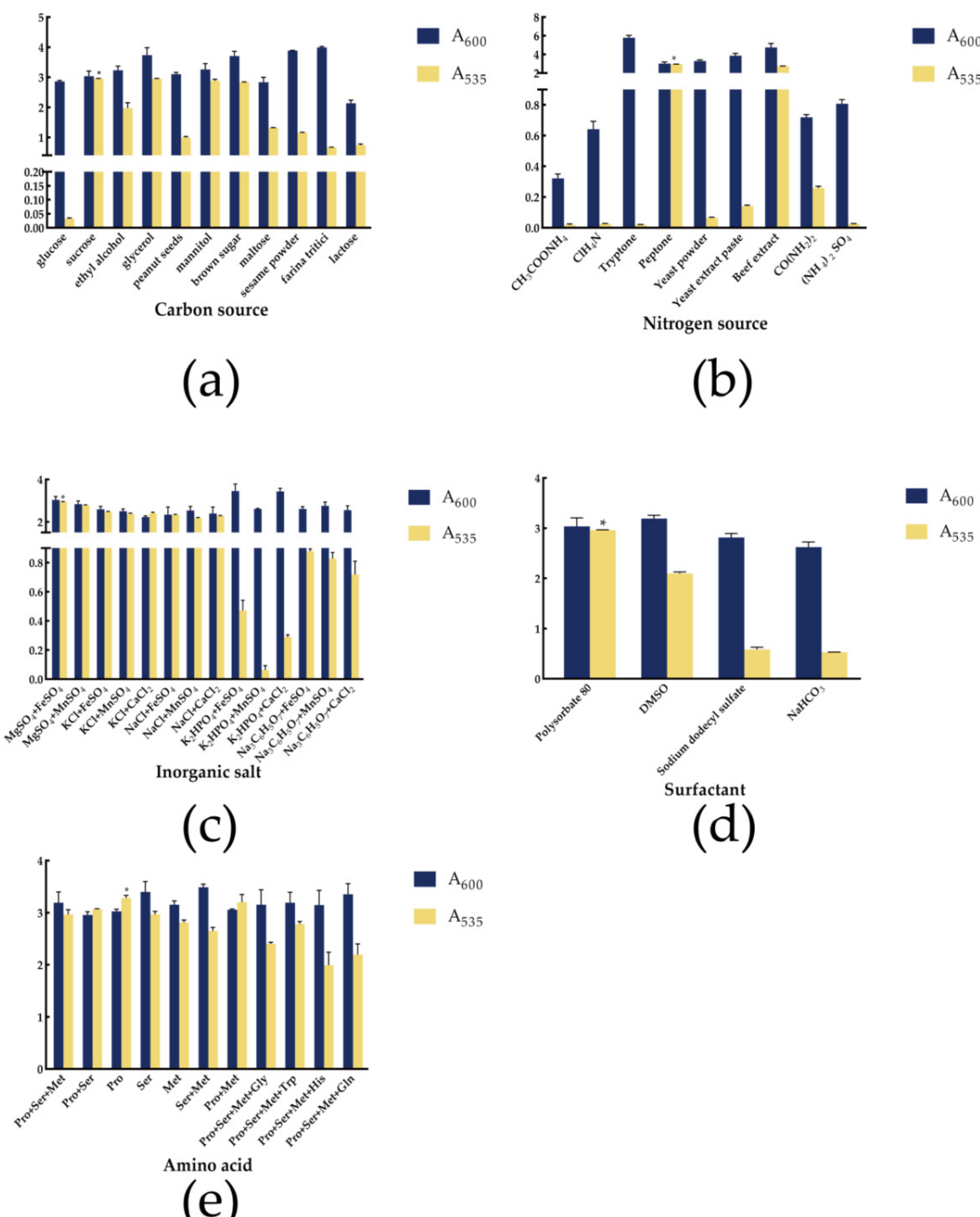

Figure 2. Shake flask fermentation screening for optimal medium composition: (**a**) fermentation results of different carbon sources; (**b**) fermentation results of different nitrogen sources; (**c**) fermentation results of different inorganic salts; (**d**) fermentation results of different surfactants; (**e**) fermentation results of different amino acids. (A_{600} denotes the concentration of *S. marcescens*, A_{535} denotes the concentration of PG, and * represents $p < 0.05$.)

3.2.2. Effect of Nitrogen Source on PG Production and Bacterial Growth

A nitrogen source is essential for the synthesis of pyrrole rings in PG [29]. The results showed that organic nitrogen sources, such as peptone, were not only significantly beneficial for PG synthesis but also more favorable for the growth of *S. marcescens* compared to inorganic nitrogen sources, such as CH_3COONH_4 at OD_{535} (Figure 2b). Therefore, peptone was considered as the optimal nitrogen source.

3.2.3. Effect of Inorganic Salt on PG Production and Bacterial Growth

Inorganic salts can promote the production of target products by activating enzymes involved in product synthesis. Mg^{2+} plays a crucial role in maintaining intracellular acid–

base balance and assisting in the nutrient utilization of bacteria [30]. Na^+ and K^+ can be conducive to maintaining a constant osmotic pressure [31,32]. Fe^{2+} is a constituent of important enzymes for cellular pigment synthesis. However, an excess of Fe^{2+} can inhibit pigment synthesis [33]. Ca^{2+} can protect the permeability of cell membranes [34], and Mn^{2+} can promote cell growth [35]. Additionally, it is reported that low concentrations of phosphate encourage PG synthesis, while the precursors for PG synthesis would be inhibited as the phosphate concentration increases [36]. The results illustrate that the effect of inorganic salts on bacterial growth is limited, and the optimal combination is $MgSO_4/FeSO_4$ (Figure 2c).

3.2.4. Effect of Surfactant on PG Production and Bacterial Growth

An excessive accumulation of PG would lead to feedback inhibition [18]. Surfactants can facilitate the transfer of PG from the intracellular level to the extracellular level, timely releasing feedback inhibition and increasing PG production. The results showed that the surfactants had a limited impact on bacterial growth, while tween 80 significantly increased PG production compared to the other three surfactants (Figure 2d).

3.2.5. Effect of Amino Acids on PG Production and Bacterial Growth

Considering the involvement of some amino acids in the process of PG synthesis [37], a combination of amino acids was added to the fermentation medium as a substrate to increase PG production. Figure 2e demonstrates that the growth of the bacteria was not significantly affected by the addition of a single amino acid or a combination of amino acids, and the addition of proline was found to be the most favorable for pigment production.

3.3. Results of the Plackett–Burman Test for Fermentation Media

The Plackett–Burman test was analyzed using an analysis of variance (ANOVA) (refer to Table 5). The results showed that the main parameters affecting PG production were A (sucrose), B (peptone), and C (tween 80), as indicated by their relatively small *p*-values. Therefore, parameters A, B, and C were selected for the path of steepest ascent method and response surface design.

Table 5. Analysis of variance (ANOVA) of the Plackett–Burman test for fermentation medium optimization.

Source	df	Adj SS	Adj MS	F-Value	*p*-Value	Significant
model	5	1,949,046	389,809	15.26	0.0001	**
linearity	5	1,949,046	389,809	15.26	0.0001	**
sucrose	1	1,396,667	1,386,667	54.67	0.0001	**
peptone	1	336,114	336,114	13.16	0.0012	
$MgSO_4$	1	4276	4276	0.17	0.6851	
polysorbate 80	1	106,489	106,489	4.17	0.0502	
proline	1	105,499	105,499	4.13	0.0511	
error	30	766,402	25,547			
lack of fit	6	758,040	126,340	362.60	0.0001	**
pure error	24	8362	348			
cor total	35	2,715,448				

Note: ** represents $p < 0.01$.

3.4. Response Surface Design to Optimize Optimal Fermentation Media

Based on the results of the single-factor experiment and the Plackett–Burman test, a carbon source, nitrogen source, and surfactant were selected as parameters to carry out the path of steepest ascent method. A three-factor, three-level Box–Behnken test was then

conducted with PG as the response value, and a second-order polynomial equation was obtained using a multiple regression analysis, which is shown as follows:

$$Y = -2564.48046 + 65.02227A + 311.37782B + 1371.62672C - 0.566067AB + 3.81883AC - 0.637833BC - 2.10068A^2 - 12.77781B^2 - 270.02525C^2 \quad (1)$$

where Y denotes the production of PG (mg/L), A denotes sucrose, B denotes peptone, and C denotes tween 80, respectively.

The analysis of variance and significance tests for the equations are presented in Table 6. The statistical significance was tested using the F-value [38]. The results indicated that the fitted model was highly significant and statistically significant, with an F-value of 42.7 and $p < 0.0001$. Additionally, the out-of-fit term $p > 0.05$ suggested that the out-of-fit term was not significant. After comparing the F-values of different factors, it was discovered that peptone (107.8) had a greater impact on the production of PG than sucrose (8.13) and tween 80 (0.6998). Therefore, it can be concluded that peptone had a more significant effect on the production of PG, while tween 80 had a relatively minor effect. Table 6 showed that the model's most significant factors were B, A^2, B^2, and C^2, with p-values less than 0.01. The next most significant factor was A, with a p-value of less than 0.05. The p-value (>0.05) for the interaction terms AB, BC, and AC suggests that there was no significant interaction between the factors. Second-order polynomial equations were used to plot three-dimensional response surfaces, which visualized the influence of each factor on the response values. As shown in Figure 3a,c,e, combined with the analysis of the slopes of the response surfaces, the effects of sucrose and tween 80 (AC) on PG production were more significant, followed by sucrose and peptone (AB), and lastly, peptone and tween 80 (BC), which was in agreement with the results of the analysis of variance (ANOVA). As shown in Figure 3b,d,f based on contour shape, the interaction between the factors was not significant, which is in line with the data in Table 6.

Table 6. Analysis of variance (ANOVA) of the Box–Behnken design experiments for fermentation medium optimization.

Source	Sum of Square	df	Mean Square	F-Value	p-Value	Significant
Model	1.487×10^6	9	1.652×10^5	42.70	<0.0001	**
A	31,452.83	1	31,452.83	8.13	0.0247	
B	4.172×10^5	1	4.172×10^5	107.80	<0.0001	**
C	2708.36	1	2708.36	0.6998	0.4305	
AB	3204.31	1	3204.31	0.8280	0.3931	
AC	5833.40	1	5833.40	1.51	0.2592	
BC	40.68	1	40.68	0.0105	0.9212	
A^2	1.858×10^5	1	1.858×10^5	48.01	0.0002	**
B^2	4.297×10^5	1	4.297×10^5	111.02	<0.0001	**
C^2	3.070×10^5	1	3.070×10^5	79.33	<0.0001	**
Residual	27,089.85	7	3869.98			
Lack of Fit	22,826.83	3	7608.94	7.14	0.0539	
Pure Error	4263.02	4	1065.75			
Cor Total	1.514×10^6	16				

Note: A is sucrose, B is peptone, and C is polysorbate 80. ** represents $p < 0.01$.

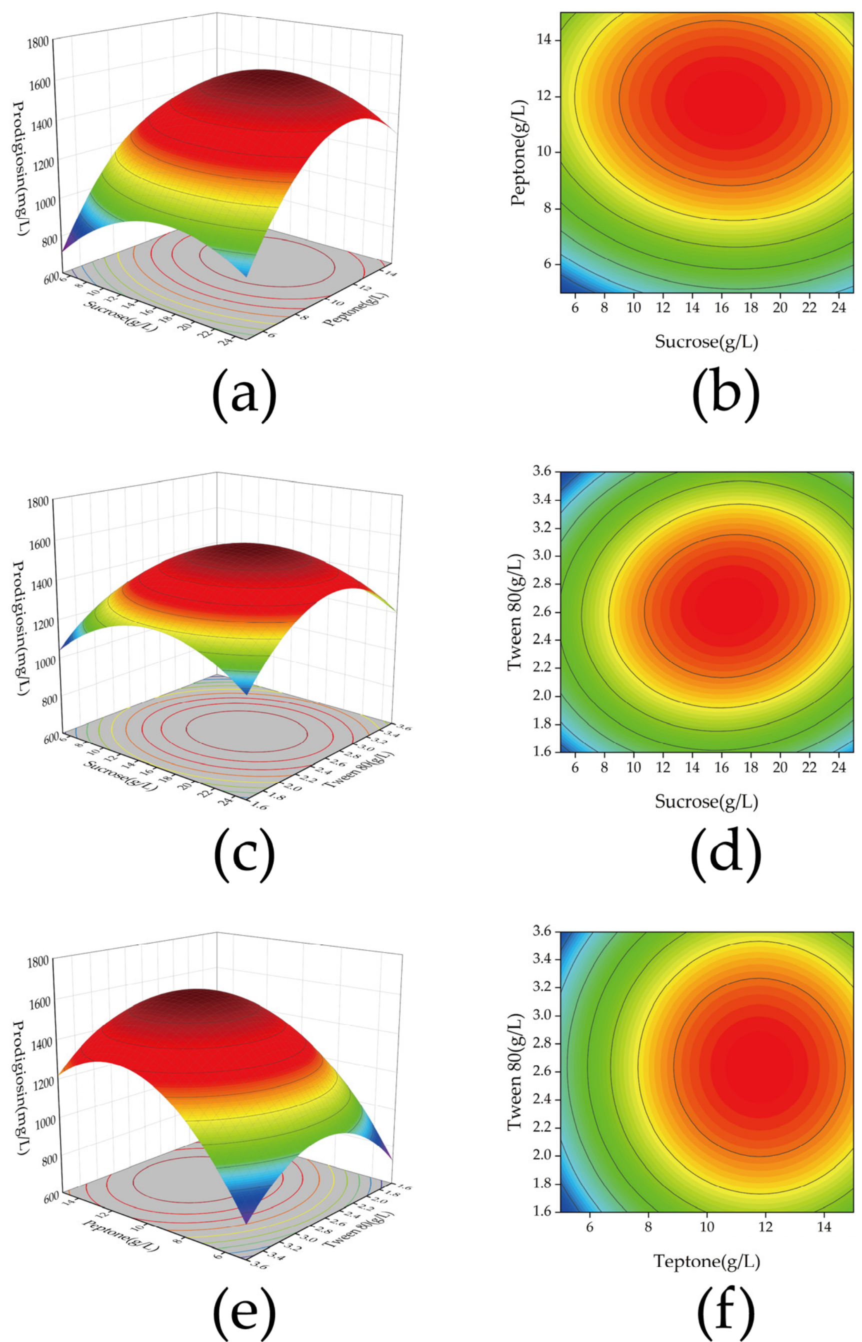

Figure 3. Three-dimensional plots and corresponding contour plots of the three variables for the second-order polynomial function model response (PG (mg/L) production). ((**a**,**b**) Effect of sucrose and peptone; (**c**,**d**) effect of sucrose and tween 80; (**e**,**f**) effect of peptone and tween 80).

3.5. Fermentation Validation of Optimal Fermentation Media

The fermentation medium of *S. marcescens* was optimized based on the second-order polynomial model obtained from the regression analysis. The best-predicted fermentation medium consisted of sucrose (16.29 g/L), peptone (11.76 g/L), tween 80 (2.64 g/L), $MgSO_4$ and $FeSO_4$ (2 g/L), and proline (1 g/L) at a pH of 7.2–7.4. The predicted production amount of PG was 1607.07 mg/L. Fermentation was conducted in shake flasks with a 2% inoculation amount and a speed of 180 rpm for 48 h at 28 °C. The production of PG was measured using high-performance liquid chromatography (HPLC). The amount of obtained PG was 1653.95 ± 32.12 mg/L, which was in agreement with the model's prediction. There was a 65-fold increase compared to the initial medium (25.03 mg) in PG production.

3.6. A Single-Factor Experiment of Extraction Processes

3.6.1. Effect of Solvent-to-Fermentation Liquid Ratio on PG Extraction Rate during Extraction

During the fermentation process, tween 80 transfers intracellular PG from *S. marcescens* to the extracellular medium. Therefore, the PG content in the medium should not be neglected during the extraction process. The ratio of fermentation liquid to solvent used had a direct impact on the efficiency of cell fragmentation, which further affected the rate of PG extraction. We obtained the largest amount of PG when the ratio of solvent to fermentation liquid was 9:1 (Figure 4a).

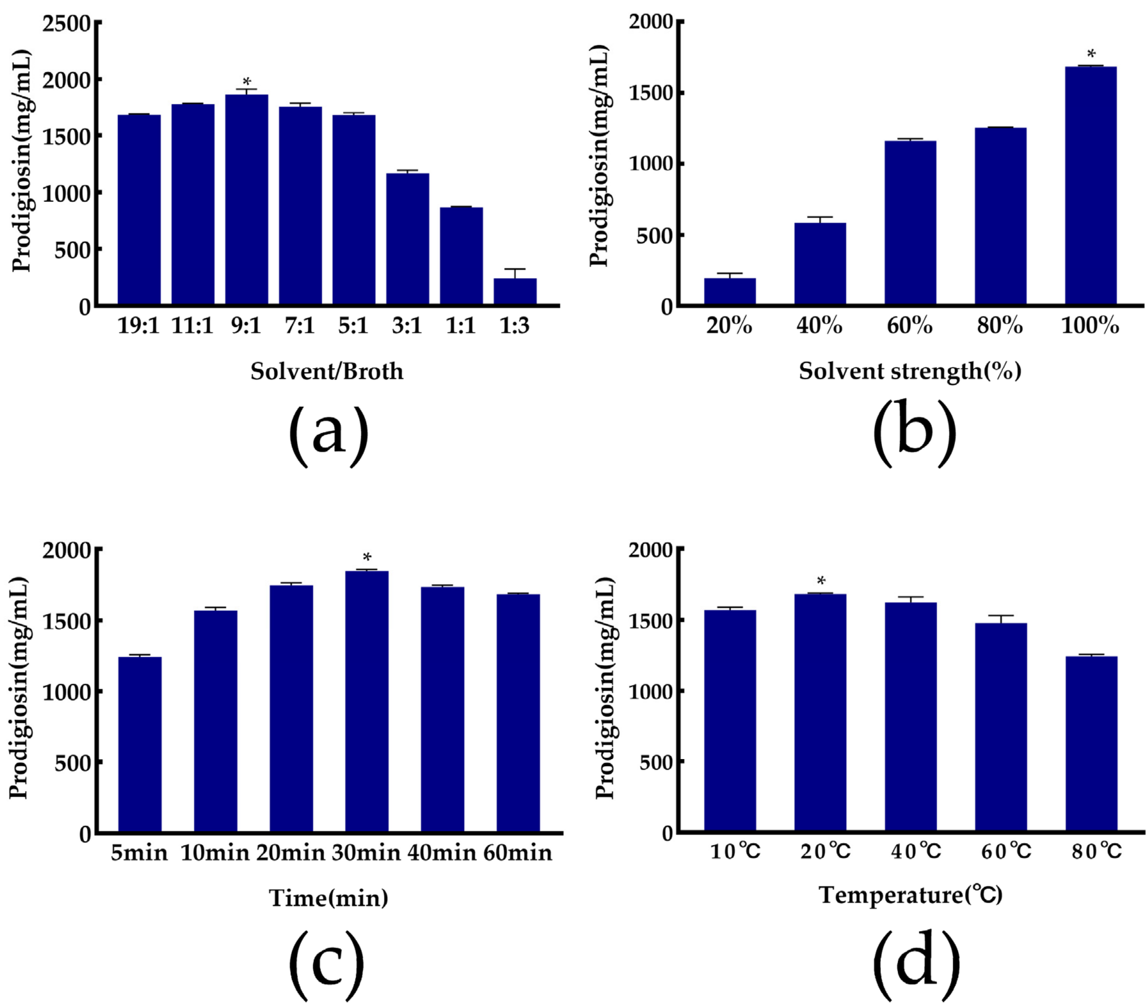

Figure 4. Optimization of the extraction process: (**a**) solvent fermentation liquid ratio; (**b**) extraction solvent concentration; (**c**) extraction time; (**d**) extraction temperature (* represents $p < 0.05$).

3.6.2. Effect of Solvent Concentration on PG Production during Extraction

As a fat-soluble pigment, PG is insoluble in water [22]. Therefore, ultrasound-assisted extraction with organic solvents was used to extract PG. In this study, we chose methanol as the extractant for PG due to its lower toxicity. According to the results presented in Figure 4b, the extraction of PG was the most effective when using a 100% methanol solution.

3.6.3. Effect of Extraction Time on Extraction Rate of PG

The ultrasound time is related to whether the cell is completely fragmented. Sun et al. [39] considered that the importance of the PG extraction time was second only to the solute–solvent ratio. The maximum extraction rate of PG was obtained at 30 min in this study (Figure 4c).

3.6.4. Effect of Extraction Temperature on Extraction Rate of PG

The temperature of extraction may affect both the fragmentation of the bacterium and the stability of the PG. Figure 4d illustrates that the maximum PG extraction rate was attained at 20 °C.

3.7. Response Surface Design to Optimize the Optimal Extraction Process

Based on the results of the single-factor experiment, the solvent/fermentation liquid ratio, extraction time, and extraction temperature were selected as parameters for the path of steepest ascent method. A three-factor, three-level Box–Behnken test was then conducted with PG as the response value, and a second-order polynomial equation was obtained using a multiple regression analysis as follows:

$$\begin{aligned} Y = \; & -1919.71098 + 567.9041A + 24.52029B + 71.07396C + 0.368883AB - \\ & 1.28697AC + 0.133345BC - 29.49559A^2 - 0.629907B^2 - 1.03392C^2. \end{aligned} \quad (2)$$

where Y denotes the production of PG (mg/L), A denotes solvent/fermentation liquid ratio, B denotes extraction time, and C denotes the extraction temperature, respectively.

The results showed that the F-value was 20.96 and $p = 0.0003$ for the model, indicating that the fitted model was more highly significant and statistically significant; the out-of-fit term $p > 0.05$ indicated that the out-of-fit term was not significant. Upon comparing the F-values of various factors, it can be discovered that the solvent/fermentation liquid ratio (1.60) had a greater impact on the extraction rate of PG than the extraction time (0.4333) or extraction temperature (0.1864). Therefore, it can be concluded that the solvent/fermentation liquid ratio had the most significant effect on the extraction rate of PG, while the extraction temperature had a relatively minor effect. Table 7 shows that the model's highly significant factors were A^2, B^2, and C^2, with *p*-values of less than 0.01. The *p*-value (>0.05) for the interaction terms AB, BC, and AC suggested that there was no significant interaction between the factors. Second-order polynomial equations were used to plot three-dimensional response surfaces, which visualize the influence of each factor on the response values. As shown in Figure 5a,c,e, combined with the analysis of the slopes of the response surfaces, the effects of the solvent/fermentation liquid ratio and extraction time (AC) on PG production were more significant, followed by the extraction temperature and extraction time (BC), and then the solvent/fermentation liquid ratio and extraction temperature (AB), which is in agreement with the results of the analysis of variance (ANOVA). According to the contour shapes in Figure 5b,d,f, the interaction between the factors was not significant, which is consistent with the data in Table 7.

Table 7. Analysis of variance (ANOVA) of the Box–Behnken design experiments for extraction process optimization.

Source	Sum of Square	df	Mean Square	F-Value	*p*-Value	Significant
Model	2.171×10^5	9	24,123.31	20.96	0.0003	**
A	1846.50	1	1846.50	1.60	0.2458	
B	214.52	1	214.52	0.1864	0.6789	
C	498.70	1	498.70	0.4333	0.5314	
AB	489.87	1	489.87	0.4256	0.5350	
AC	2650.09	1	2650.09	2.30	0.1730	
BC	1600.28	1	1600.28	1.39	0.2769	
A^2	58,609.86	1	58,609.86	50.92	0.0002	**
B^2	84,577.45	1	84,577.45	73.48	<0.0001	**
C^2	45,010.24	1	45,010.24	39.11	0.0004	**
Residual	8056.80	7	1150.97			
Lack of Fit	5122.69	3	1707.56	2.33	0.2161	

Table 7. *Cont.*

Source	Sum of Square	df	Mean Square	F-Value	*p*-Value	Significant
Pure Error	2934.11	4	733.53			
Cor Total	2.252×10^5	16				

** represents $p < 0.01$.

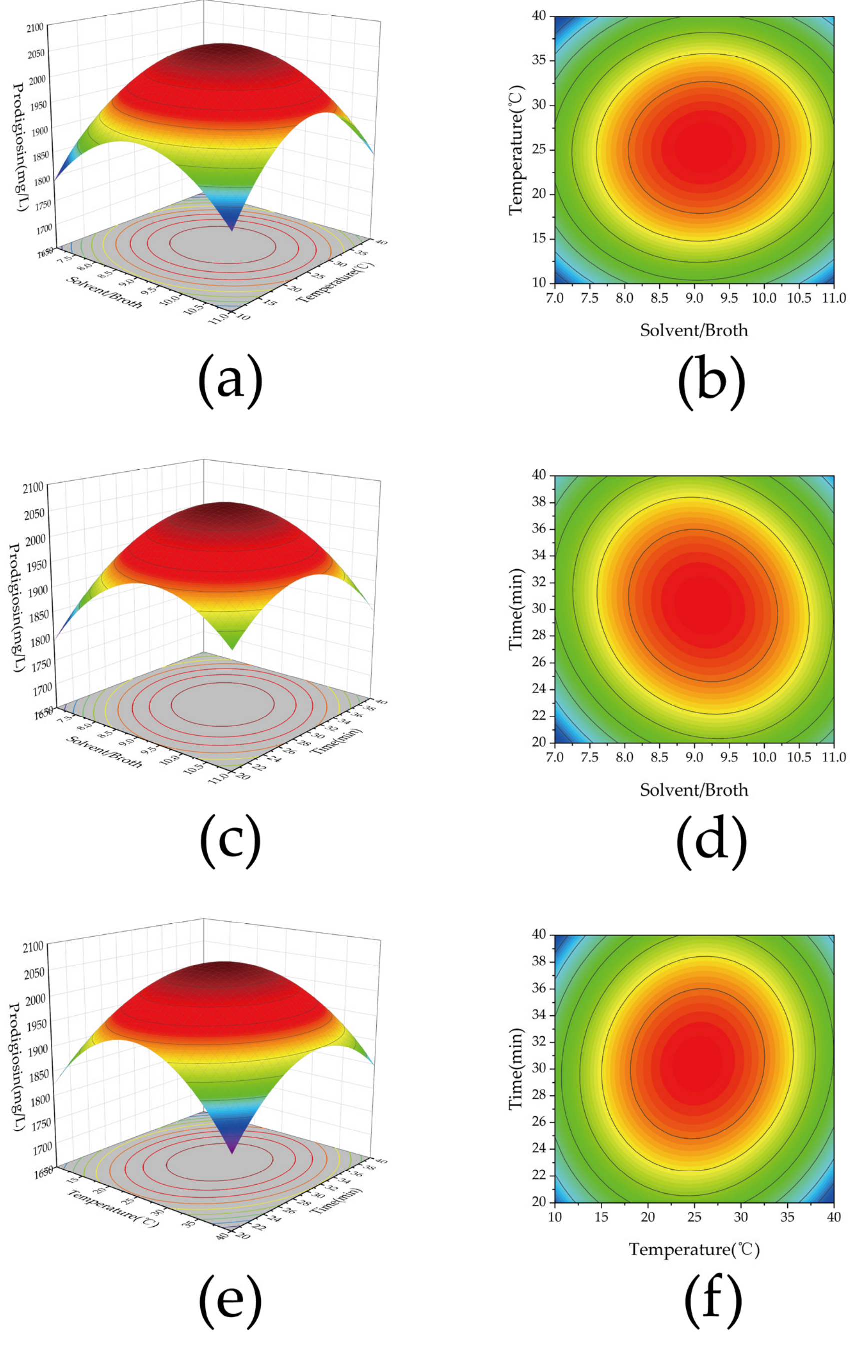

Figure 5. Three-dimensional plots and corresponding contour plots of the three variables for the second-order polynomial function model response (PG (mg/L) production). ((**a**,**b**) Effect of solvent/fermentation liquid ratio and extraction temperature; (**c**,**d**) effect of solvent/fermentation liquid ratio and extraction time; (**e**,**f**) effect of extraction time and extraction temperature).

3.8. Validation of the Optimal Extraction Process

Based on the second-order polynomial model obtained from the regression analysis, the extraction process of PG was optimized, and the best-predicted extraction conditions were as follows: a solvent/fermentation liquid ratio of 9.12:1, an extraction temperature of 25.35 °C, an extraction time of 30.33 min, and a predicted production amount of 2059.47 mg/L. The solvent chosen for the extraction was 100% methanol with a pH of 2. The PG production was measured using high-performance liquid chromatography (HPLC). The production amount obtained was 2142.75 ± 12.55 mg/L, which is in agreement with the model's prediction.

4. Discussion

It is well known that PG is economically valuable for its anticancer, antimalarial, and other bioactivities. While the inability to produce it in large quantities limits its widespread application, until today, most known reports focused on improving the production of PG based on high-quality fermentation media, and few of them took into account the loss of PG during the extraction process. However, the optimization of PG extraction conditions is equally important to solve the problem of PG yield.

According to the existing reports, we concluded that the main factors to improve PG production are the medium composition of *S. marcescens* metabolism and the extraction conditions of PG. Therefore, a two-step optimization method was attempted in this study. Firstly, the fermentation medium of *S. marcescens* was optimized, and then the extraction conditions of PG were optimized. The results showed that components of the optimized fermentation medium were low-cost and readily available, which would lay the foundation for industrial production. In addition, the optimized extraction conditions resulted in a significant increase in the production of PG and a significant reduction in the loss of PG. Finally, the obtained accurate fermentation medium and extraction conditions simplified the whole experimental process and saved materials and cost of labor.

We conducted two response surface optimizations in this study. The optimized fermentation medium (experimental group 1) resulted in a 6507.9% increase in PG production compared to the initial medium (the control). It was speculated that sucrose can better promote the respiratory metabolism of the bacteria by affecting the metabolic pathways of *S. marcescens*, which have an impact on the production of PG after optimization. Peptone produces large proteins after hydrolysis, which can stimulate protein expression and promote product metabolism. The addition of proline supplemented the requirements for PG synthesis and further increased production. Additionally, the amount of PG produced with the optimized extraction conditions (experimental group 2) resulted in an 8460.7% increase compared to the initial medium (the control) (Table 8). It was hypothesized that an appropriate sonication time and temperature played important roles in maintaining the stability of PG, while an appropriate solvent/fermentation liquid ratio allowed for a more adequate fragmentation of the bacterium, and allowed more PG to be extracted by the solvent, thereby improving the extraction rate of PG. Therefore, the method obtained from two-step optimization in this study is efficient and rapid for producing PG.

Table 8. Comparison of PG production before and after optimization.

Parameters	Control Group	Experimental Group 1	Experimental Group 2
Prodigiosin (mg/L)	25.03 ± 2.13	1653.95 ± 32.12	2142.75 ± 12.55
Comparison (%)	0	6507.9%	8460.7%

There were also other protocols that were reported to increase PG production. Salas-Villalobos et al. [40] reported that the recovery of PG by extraction after fermentation with low-cost media can reduce the inhibition of PG in the final product. El-Bialy and Abou El-Nour [41] induced mutations in *S. marcescens* using ethyl methanesulfonate (EMS) and

UV irradiation. The mutated *S. marcescens* exhibited an 8-fold increase in PG production. Sun et al. [42] found that mutant strains increased the transcript levels of the pig gene cluster and genes related to the PG precursor pathway, such as proline, pyruvate, serine, methionine, and S-adenosyl methionine, which, in turn, led to increased PG production. A transcriptional regulator of the OmpR family from *S. marcescens* JNB5-1, consisting of proline, serine, and methionine genes proC, serC, and metH; a fusion fragment inserted into the CpxR gene; and a transcriptional regulator of the OmpR family in *S. marcescens* JNB5-1 were combined to obtain a 41. 9% increase in PG production through the newly engineered bacterium compared to the original strain. Pan et al. [43] obtained the recombinant strain PG-6 by inserting promoter P17 into *S. marcescens* JNB5-1 to efficiently express PG synthesis activators OmpR and PsrA. This resulted in a 1.62-fold increase in PG production compared to the original strain. These studies suggested that there was untapped potential for PG production through the fermentation of *S. marcescens*. The prospect of PG biosynthesis through various fermentation methods is very broad and promising.

5. Conclusions

In summary, improving PG production requires not only a suitable medium composition, but also excellent extraction process technology. This study aimed to optimize the medium components of *S. marcescens* using response surface methodology. The optimal medium composition was found to be sucrose, 16.29 g/L; peptone, 11.76 g/L; tween 80, 2.64 g/L; $MgSO_4$ and $FeSO_4$, 2 g/L; and proline, 1 g/L (pH 7.2–7.4), resulting in a 65-fold increase in PG production with a production amount of 1653.95 ± 32.12 mg/L. On this basis, we conducted a response surface optimization of the PG extraction process. The extraction conditions were optimized to obtain a higher production of PG. The solvent/fermentation liquid ratio was 9.12:1, the extraction temperature was 25.35 °C, and the extraction time was 30.33 min. The solvent used was 100% methanol (pH = 2), resulting in a final PG production amount of PG of 2142.75 ± 12.55 mg/L. This is 29.6% higher than that obtained from the initial extraction conditions. Two-step optimization resulted in an 84-fold increase in PG production. The optimal fermentation medium and extraction process, obtained through two-step optimization, can provide a basis and reference for the large-scale production of PG in the future.

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