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Response Surface Methodology-Based Optimization of Natural Pigment Production from *Penicillium purpurogenum*

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Abstract

Fungal pigments are increasingly recognized as environmentally sustainable alternatives to synthetic dyes. However, their commercial utilization requires effective optimization. This study aimed to optimize pigment production by *Penicillium purpurogenum* using Response Surface Methodology (RSM) with a Central Composite Design (CCD). Initial screening using a one-factor-at-a-time (OFAT) approach identified pH, incubation temperature, and incubation time as critical factors affecting pigment yields. Dextrose and yeast extract were selected as the optimal carbon and nitrogen sources. A three-factor CCD comprising 20 experimental trials was conducted to evaluate the individual, quadratic, and interactive effects of these variables on the pigment production. The experimental data were well represented by a quadratic polynomial model, which was statistically significant ($F = 13.49$, $p = 0.0002$) and exhibited a high adjusted coefficient of determination (adjusted $R^2 = 0.8554$). The analysis of variance revealed that pH exerted a significant linear effect on pigment production ($p = 0.0308$), whereas the quadratic terms pH^2 ($p < 0.0001$) and temperature^2 ($p = 0.0010$) were highly significant, indicating pronounced curvature effects. Response surface analysis demonstrated that the interaction between pH and temperature had the most substantial impact on pigment yield, whereas the interaction between temperature and incubation time had minimal effect. Numerical optimization indicated that maximum pigment production could be achieved under conditions of near-neutral pH, moderate incubation temperature, and intermediate incubation time, which was experimentally validated with results closely aligned with the predictions.

Keywords: Fungal pigment; Submerged fermentation; Response surface methodology; Central composite model; Optimization process

1. Introduction

Growing concerns regarding the adverse effects of synthetic dyes on human health and the environment have prompted a global movement towards the adoption of natural colorants [1]. Synthetic dyes, widely utilized in industries such as textiles, leather, food, and cosmetics, pose significant environmental and health challenges due to their chemical composition and production methods [2–4]. These dyes are generally derived from petrochemicals and involve intricate chemical reactions, resulting in high energy consumption and the production of waste effluents laden with hazardous substances, including heavy metals such as copper, arsenic, lead, cadmium, mercury, nickel, and cobalt, as well as carcinogenic compounds such as azo dyes and amines [5–7]. These toxic by-products cause environmental damage, affecting aquatic ecosystems by reducing sunlight penetration and increasing water turbidity. They also pose occupational hazards, including skin irritation, allergies, mutagenicity, and carcinogenicity [8–12].

Natural pigments sourced from plants, algae, and microorganisms are gaining prominence because of their environmentally friendly, compatible, and renewable properties [13,14]. Filamentous fungi are particularly notable for their capacity to produce a diverse array of stable pigments that are highly soluble, vividly colored, and have numerous

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industrial applications [15–17]. These fungi can proliferate rapidly on cost-effective substrates under controlled conditions, rendering them more suitable for large-scale pigment production than plant-based systems, which frequently rely on seasonal factors and require substantial land resources [18–20].

P. purpurogenum is a well-known fungus recognized for its ability to generate a diverse array of red polyketide pigments, primarily from the azaphilone group [21,22]. These pigments are characterized by their bright colors, heat resistance, and potential bioactive properties, making them suitable for applications in industries such as food, pharmaceuticals, textiles, cosmetics, and biotechnology [23]. However, the effective production of pigments from *P. purpurogenum* relies on specific fermentation conditions, including pH, temperature, carbon and nitrogen sources, and incubation duration [24,25]. Traditionally, the optimization of these conditions has been pursued using the one-factor-at-a-time (OFAT) approach, which fails to consider the interactions between variables and often results in suboptimal yields [26].

Statistical optimization techniques, particularly Response Surface Methodology (RSM), offer a robust multivariate approach for evaluating the combined effects of multiple factors [27]. RSM, particularly through the application of a Central Composite Design (CCD), facilitates a systematic analysis of various factors and their interactions, necessitating fewer experimental trials than conventional methods. The CCD aids in the construction of a second-order polynomial model, allowing for the precise prediction of optimal conditions and providing a comprehensive understanding of the interactions among variables influencing pigment synthesis [28].

Given the growing demand for natural colorants and the need for cost-effective production methods, it is essential to enhance the yield of fungal pigments using a comprehensive statistical approach. This study focused on extracting natural pigments from *P. purpurogenum*, employing a RSM model based on a CCD to identify and optimize the critical fermentation parameters affecting pigment yield. This study provides a robust framework for optimizing pigment production and contributes to the advancement of sustainable bioprocesses for developing natural colorants.

2. Materials and Methods

2.1. Chemicals and Reagents

Potato dextrose agar (PDA), potato dextrose broth (PDB), glucose, peptone, ethanol, methanol, and other analytical-grade reagents were purchased from HiMedia. All the solutions were prepared using double-distilled water.

2.2. Isolation and screening of pigment-producing fungus

Soil samples (n = 100) were collected from various locations in Mumbai, air-dried, and stored in sterile containers until analysis. Each soil sample (1 g) was suspended in 9 mL of sterile distilled water and serially diluted up to 10^{-4} using standard aseptic techniques. Aliquots (0.1 mL) from the appropriate dilutions were evenly spread onto PDA plates supplemented with streptomycin (50 µg/mL) to inhibit bacterial growth. The plates were incubated at 28 ± 2°C for 5–7 days. Following incubation, fungal colonies exhibiting visible extracellular or intracellular pigmentation were observed and recorded. Pigment-producing isolates were initially screened based on the intensity and stability of pigment diffusion in the medium. Among the screened isolates, fungi producing intense and stable pigmentation were selected for further study.

2.3. Identification of Pigment-Producing Fungus

2.3.1. Microscopic identification

The fungal isolate responsible for pigment production was identified using both microscopic and molecular methodologies. For morphological identification, the colony characteristics were assessed on PDA, and microscopic features were observed following staining with Lactophenol Cotton Blue (LCB). By examining the structure of the hyphae, conidiophores, phialides, and arrangement of conidia, the isolate was tentatively classified as belonging to the *genus Penicillium*.

2.3.2. Molecular identification

Molecular identification was conducted through sequencing of the Internal Transcribed Spacer (ITS) region of rDNA. Genomic DNA was extracted from fresh mycelia using the conventional phenol–chloroform extraction method (29). The ITS region was amplified using universal primers, ITS1 (5'-TCC GTA GGT GAA CCT GCG G -3') and ITS4 (5'-TCC TCC GCT TGA TAT GC -3') [30]. The amplified product was subsequently purified and sequenced using an ABI® 3730XL

automated DNA sequencer (Applied Biosystems, Foster City, CA, USA) in accordance with the manufacturer's protocol [31]. The sequence was then compared using BLAST against the NCBI database, which confirmed the isolate as *P. purpurogenum.* This identified strain was employed in all subsequent experiments.

2.4. Preparation of Inoculum

The inoculum was prepared by introducing 5 mm diameter agar plugs, extracted from the actively growing margins of a 7-day-old PDA culture, into 100 mL of sterile PDB contained within 250 mL Erlenmeyer flasks. The flasks were then incubated at 28 °C without agitation for 72 h to produce a homogeneous fungal suspension. This pre-culture served as the inoculum for all subsequent fermentation experiments.

2.5. Preliminary Screening of Process Variables (OFAT)

The initial evaluation of critical fermentation parameters affecting pigment production by *P. purpurogenum* was performed using the one-factor-at-a-time (OFAT) approach. This study examined the individual effects of pH (4–10), temperature (20–50 °C), and incubation duration (168–336 h). A range of carbon sources, including maltose, fructose, xylose, dextrose, sucrose, methanol, and glycerol, and nitrogen sources, such as peptone, soya peptone, malt extract, and yeast extract, were assessed. In each experiment, only one variable was modified while the others were held constant, and pigment production was quantified spectrophotometrically in terms of color value units (CVU). The most effective nutrient sources and significant process variables identified through OFAT were subsequently selected for further optimization using CCD-RSM analysis.

2.6. Statistical Optimization Using Central Composite Design (CCD-RSM)

To optimize pigment production and evaluate the interactive effects of key variables, RSM employing CCD was applied. The experimental setup and statistical analyses were performed using the Design-Expert® software (version 13.0.5.0, Stat-Ease Inc., Minneapolis, MN, USA). Based on the OFAT results, three independent variables were selected: initial pH (A), incubation temperature (B), and incubation time (C). Each variable was assessed on five coded levels (−α, −1, 0, +1, and +α). The variable ranges were as follows: pH, 3.97–9.02; temperature, 21.59–38.40 °C; and incubation time, 143.45–264.54 h (Table 1). The experimental design comprised 20 runs, including 14 non-center points and 6 center points, with an alpha value of 1.68179 (Table 2).

Table 1 Independent variables and their corresponding levels for fungal pigment

Factor	Name	Type	Sub Type	Units	-α	-1	Mean	+1	+α	Std. Dev
A	pH	Numeric	Continuous	pH	3.977	5	6.50	8	9.022	1.27
B	Temp.	Numeric	Continuous	ºC	21.591	25	30	35	38.409	4.24
C	Incubation	Numeric	Continuous	hrs	143.455	168	204	240	264.545	30.52

(-α = Coded low, +α = Coded high, -1 = Minimum, +1 = Maximum)

2.7. Statistical Modeling and Data Analysis

The experimental response (pigment yield) was fitted to a second-order polynomial regression model to describe the relationship between the independent variables and the response (Eq. 1).

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad \text{..... (Eq. 1)}$$

where Y represents the predicted pigment yield, β_0 is the intercept, β_i are the linear coefficients, β_{ii} are the quadratic coefficients, and β_{ij} are the interaction coefficients. The adequacy of the model was evaluated using analysis of variance (ANOVA), coefficient of determination (R^2), adjusted R^2, predicted R^2, and lack-of-fit tests. Three-dimensional response surface plots and two-dimensional contour plots were generated to visualize the interaction effects of the variables and determine the optimal fermentation conditions.

2.8. Model Validation

To assess the predictive accuracy of the developed model, confirmatory experiments were performed under the optimal conditions identified by CCD-RSM. The experimentally determined pigment yield was then compared to the predicted value, and the model was deemed valid when a close correspondence between the predicted and experimental results was observed.

3. Results and discussion

3.1. Identification of Pigment-Producing Fungus

Microscopic examination of the pigment-producing fungal isolate stained with LCB revealed hyaline, septate hyphae with branched conidiophores exhibiting metulae and phialides arranged in a brush-like pattern (Fig. 1). Chains of globose to sub-globose conidia were observed at the termini of the phialides. These morphological characteristics are typical of fungi of the genus *Penicillium*.

Molecular identification through ITS rDNA sequencing further corroborated this, with NCBI BLAST showing a 99% match with *P. purpurogenum*. The integration of microscopic and molecular analyses conclusively identified the pigment-producing fungus as *P. purpurogenum*, which was subsequently used for RSM-CCD optimization studies.

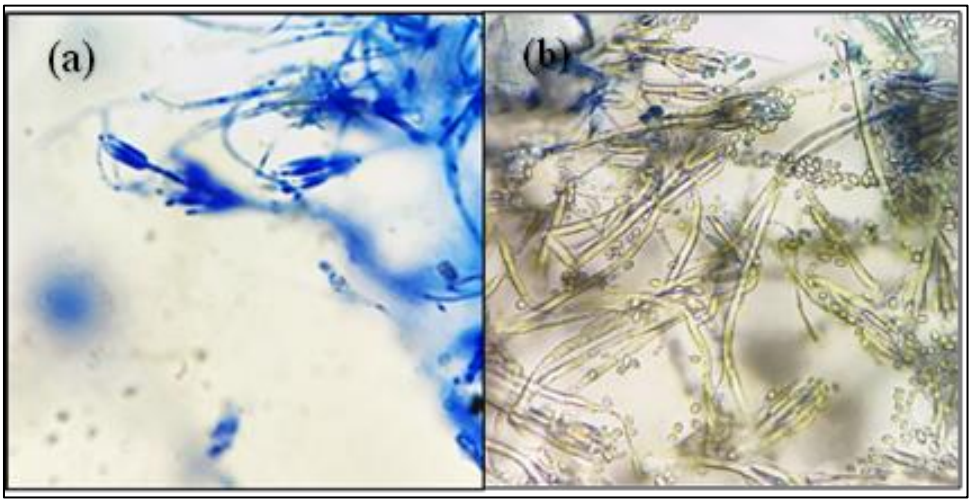

Figure 1 Microscopic morphology of the pigment-producing isolate: (a) septate hyphae and (b) conidiophores with conidia, typical of *P. purpurogenum*

3.2. Preliminary Screening of Process Variables (OFAT)

Preliminary screening employing the OFAT methodology revealed that pH, temperature, incubation duration, and nutrient source significantly influenced the pigment production by *P. purpurogenum*. The pigment yield increased with pH levels up to 7-9 (Fig. 2), beyond which a decline was observed. Similarly, temperature positively affected pigment synthesis up to 30-40 °C (Fig. 3a), after which pigment production decreased. The duration of incubation had a significant impact on pigment production, with the maximum pigment yield being observed at 216 h, after which it reached a plateau (Fig. 3b). Among the evaluated carbon sources, dextrose produced the highest pigment yield, as indicated by the color value units (CVU). Yeast extract was identified as the most effective nitrogen source, resulting in the highest pigment intensity (Fig. 3c). Based on the findings of the OFAT analysis, pH, temperature, and incubation time were identified as the most critical factors. These factors were selected for further optimization using CCD-RSM. Concurrently, the carbon (dextrose) and nitrogen (yeast extract) sources were maintained at constant levels for subsequent experiments.

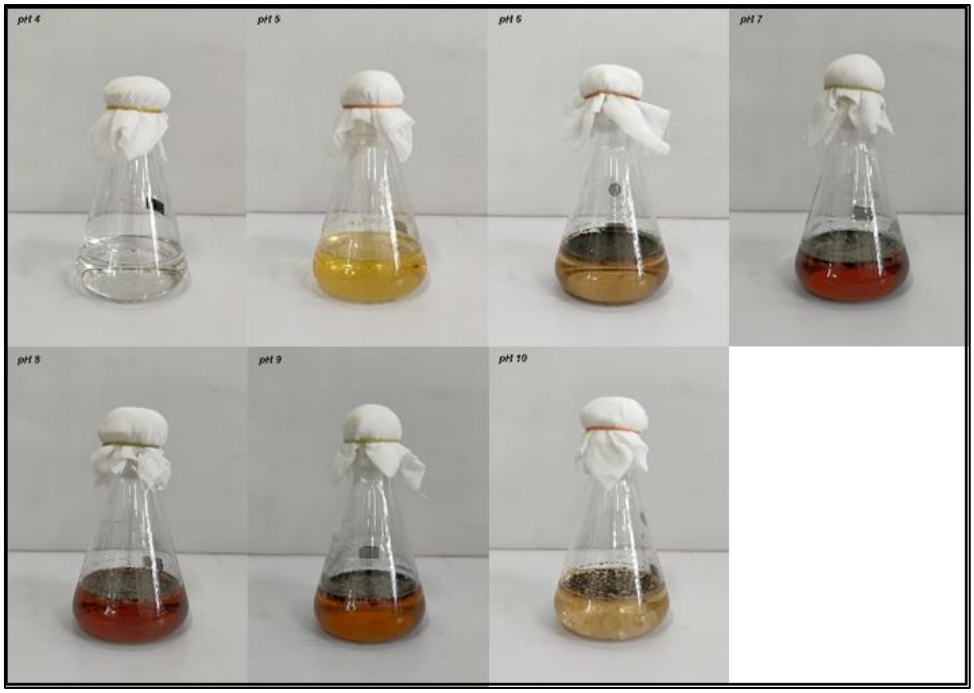

Figure 2 Visual assessment of pigment production by *P. purpurogenum* at different initial pH levels (4–10) during preliminary OFAT screening

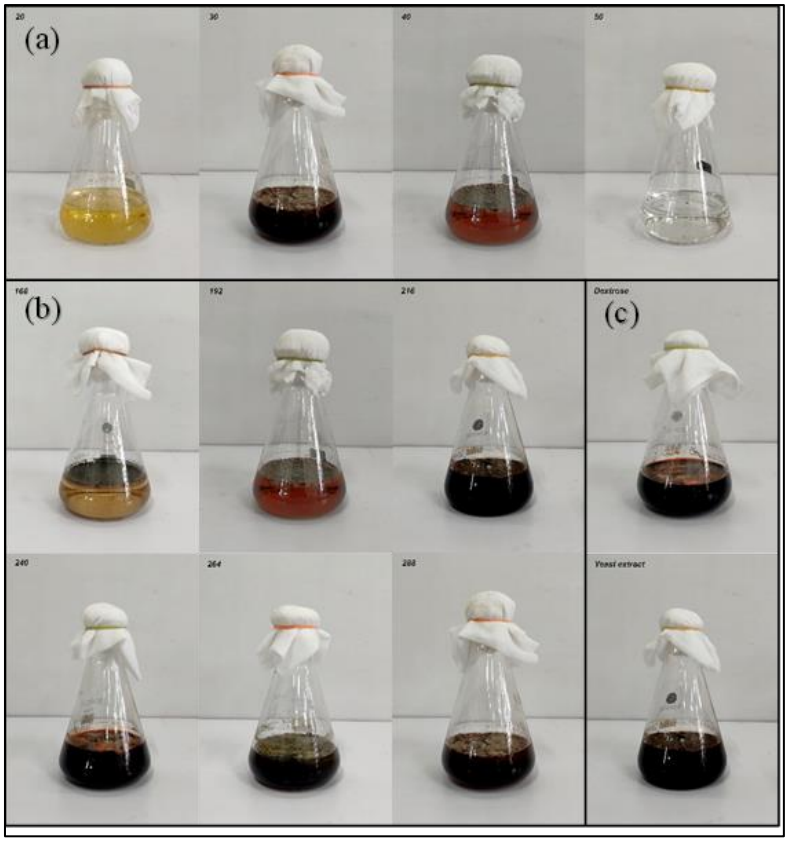

Figure 3 Visual assessment of pigment production by *P. purpurogenum* under different parameters: (a) Temperature (20-50 °C), (b) incubation time (168-288hrs); and (c) carbon source (dextrose) and nitrogen source (yeast extract)

3.3. CCD–RSM Model Fitting, ANOVA, and Process Optimization

The experimental data obtained from the CCD were analyzed using RSM to evaluate the combined effects of pH (A), temperature (B), and incubation time (C) on the production of fungal pigments (Table 1). A quadratic polynomial model was found to best describe the relationship between the independent variables and the response, as indicated by the sequential model sum of squares and fit statistics (Table 2).

Table 2 CCD Experimental Runs and Pigment Yield

Std	Run	Space type	Factor 1 pH	Factor 2 Temp.	Factor Incubation	Pigment yield
12	5	Axial	6.5	38.40	204	22.1
10	6	Axial	9.02	30	204	15.8
13	9	Axial	6.5	30	143.45	24.2
11	13	Axial	6.5	21.59	204	20.9
14	16	Axial	6.5	30	264.54	27.4
9	17	Axial	3.97	30	204	14.8
17	1	Center	6.5	30	204	26.8
19	4	Center	6.5	30	204	26.6
18	14	Center	6.5	30	204	26.8
20	18	Center	6.5	30	204	26.9
15	19	Center	6.5	30	204	26.8
16	20	Center	6.5	30	204	26.7
1	2	Factorial	5	25	168	17.4
7	3	Factorial	5	35	240	20.2
4	7	Factorial	8	35	168	25.9
3	8	Factorial	5	35	168	14.7
6	10	Factorial	8	25	240	19.5
5	11	Factorial	5	25	240	18.0
8	12	Factorial	8	35	240	21.4
2	15	Factorial	8	25	168	17.9

3.3.1. Model Selection and Adequacy

The model fitting results indicated that the quadratic model was the most appropriate for describing the pigment yield, as evidenced by a highly significant sequential model sum of squares ($p < 0.0001$). The quadratic model exhibited a high adjusted R^2 value of 0.8554, indicating that over 85% of the variability in pigment production could be explained by this model. The predicted R^2 (0.3630) was lower than the adjusted R^2, indicating reduced predictive performance outside the experimental domain; however, the model was considered adequate for optimization within the studied factor ranges (Table 3).

Table 3 Fit summary, Response 1: Pigment yield

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.6866	< 0.0001	-0.0854	-0.4327	
2FI	0.8200	< 0.0001	-0.2476	-1.4908	
Quadratic	< 0.0001	< 0.0001	0.8554	0.3630	Suggested
Cubic	0.0096	< 0.0001	0.9666	-1.2961	Aliased

The model F-value of 13.49 further confirmed the statistical significance of the quadratic model (p = 0.0002), suggesting that the observed response variations were not due to random variations.

3.3.2. Analysis of Variance (ANOVA)

ANOVA for the quadratic model indicated that the initial pH (A) had a notable linear impact on the pigment yield, with a p-value of 0.0308. Among the quadratic components, A^2 (pH^2) and B^2 ($temperature^2$) were highly significant, with p-values of less than 0.0001 and 0.0010, respectively. This suggests strong curvature effects and highlights the existence of optimal operating ranges for these variables.

In contrast, the linear effects of temperature (B) and incubation time (C) and the interaction terms (AB, AC, and BC) were not statistically significant (p > 0.05). Nevertheless, these terms were retained in the model to preserve its hierarchy. The significance of the quadratic terms suggests that pigment production is predominantly influenced by nonlinear responses rather than by simple linear patterns.

The lack-of-fit test was found to be significant (p < 0.0001), likely attributable to the minimal pure error resulting from the high reproducibility at the center points, which is a common occurrence in well-regulated fermentation experiments [32,33]. Consequently, the model was deemed suitable for conducting response surface analyses and numerical optimization.

3.3.3. Regression Equation and Effect of Variables

The final quadratic regression model, expressed in terms of coded variables, is as follows:

$$Y=26.80+1.18A+0.84B+0.63C+1.30AB-1.12AC-0.15BC-4.29A^2-2.10B^2-0.58C^2$$

where *Y* represents the pigment yield, and *A*, *B*, and *C* correspond to pH, temperature, and incubation time, respectively.

The positive linear coefficient associated with pH indicates that pigment synthesis is enhanced near neutral pH. Conversely, the significant negative coefficient of A^2 suggests a marked decrease in the pigment yield at extreme pH values. Similarly, the pronounced negative quadratic coefficient for temperature (B^2) highlights the sensitivity of pigment production to deviations from the optimal temperature.

Table 4 Regression coefficients of the quadratic model for pigment production by *Talaromyces australis*

Factor	Coefficients Estimate	Df	Standard error	95% Cl low	95% Cl high	VIF
Intercept	26.80	1	0.7063	25.23	28.38	
A-pH	1.18	1	0.4686	0.1334	2.22	1.0000
B-Temperature	0.8361	1	0.4686	-0.2081	1.88	1.0000
C-Incubation time	0.6284	1	0.4686	-0.4158	1.67	1.0000
AB	1.30	1	0.6123	-0.0643	2.66	1.0000
AC	-1.12	1	0.6123	-2.49	0.2393	1.0000

BC	-0.1500	1	0.6123	-1.51	1.21	1.0000
A^2	-4.29	1	0.4562	-5.31	-3.28	1.02
B^2	-2.10	1	0.4562	-3.12	-1.08	1.02
C2	-0.5810	1	0.4562	-1.60	0.4355	1.02

3.3.4. Response Surface Analysis and Optimization

To illustrate the interaction of the selected variables in influencing pigment production, three-dimensional response surface and contour plots were generated (Fig. 4). These visual representations identified a distinct optimal region characterized by moderate pH and temperature levels, highlighting the significance of quadratic effects in regulating pigment production.

Among the various interactions examined, the combination of pH and temperature (Fig. 4a–b) exhibited the most pronounced curvature, indicating a strong synergistic effect on the pigment yield. The interaction between pH and incubation time (Fig. 4e–f) demonstrated a moderate impact, with increased pigment production observed at mid-range pH levels and incubation periods. In contrast, the interaction between temperature and incubation time (Fig. 4c–d) exerted the least influence on pigment yield, as evidenced by the relatively flat response surface and broader contour area.

Utilizing numerical optimization via the desirability function, it was anticipated that maximal pigment production would be achieved at a nearly neutral pH, accompanied by a moderate incubation temperature and medium incubation duration. These optimal conditions were subsequently corroborated through experimental validation, which demonstrated a strong correlation between the predicted and actual pigment yields.

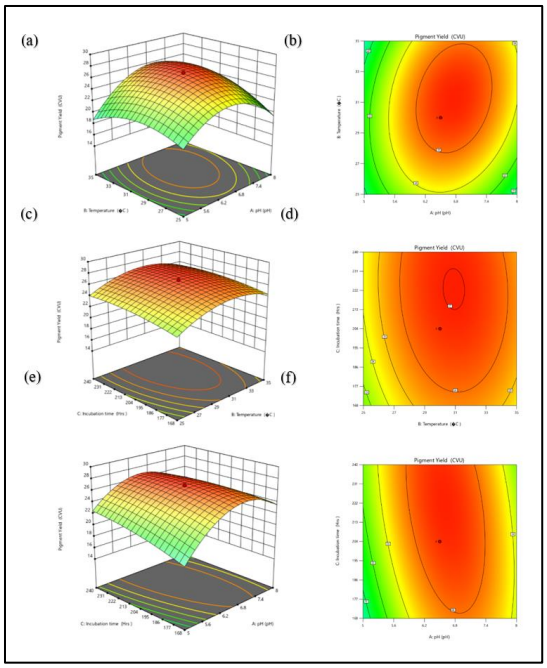

Figure 4 Three-dimensional response surface and contour plots illustrating the interactive effects of pH, temperature, and incubation period on pigment production by *P. purpurogenum*

3.4. Validation of Optimized Conditions

To evaluate the adequacy and predictive precision of the developed CCD–RSM model, experiments were conducted under conditions recommended by the numerical desirability function. The optimization predicted that the maximum pigment production would occur at a nearly neutral pH, moderate incubation temperature, and medium incubation duration.

Three validation experiments were conducted under these optimized conditions, and the experimentally obtained pigment yield closely approximated the value predicted by the model used. The minimal discrepancy between the predicted and actual results underscores the high accuracy of the developed quadratic model.

The strong concordance between the experimental and predicted pigment yields substantiates the efficacy and reliability of the CCD–RSM method for optimizing pigment production by *P. purpurogenum*. These results further demonstrate that the selected process variables and their optimized levels significantly enhanced the pigment yield within the experimental parameters studied.

4. Conclusion

RSM was effectively employed to enhance the pigment production by *P. purpurogenum* using a CCD. The experimental data were accurately represented by a quadratic model, which identified pH as the most significant factor, followed by temperature. RSM revealed a strong interaction between pH and temperature, whereas the effect of incubation time was relatively moderate. Numerical optimization indicated that the highest pigment yield could be achieved at nearly neutral pH, moderate incubation temperature, and intermediate incubation period, which was experimentally confirmed with results closely aligning with the predictions. This study confirms that CCD–RSM is a reliable and efficient method for optimizing fungal pigment production processes.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors declare that they have no conflict of interest or competing interests related to this manuscript.

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