Optimization of Conditions for the Cleaning of overpaint from the Stone Carvings of the Potala Palace, Tibet using enzyme-based cleaning agents

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ABSTRACT

This work focuses on the study of the removal of overpaint from the stone carvings of the Potala Palace, Tibet. The removal of historical mortar and overpaint is a significant challenge for the conservation of stone carvings and requires careful optimization. Paints applied in the past were made of a mixture of hydraulic lime, water and milk, and are causing damage to the carvings of the Potala Palace. The protein content of lime-based paint samples collected from the Potala Palace was determined by enzyme-linked immunosorbent assay (ELISA). In order to optimize the removal of the overpaint without causing abrasion or other damage to the underlying stone carvings, we prepared a simulated paint with the same protein content. Removal rate and color difference were used to select the best cleaning agent from ionic surfactant, ethanol/aqueous solutions, five proteases and Twin-20 (non-ionic surfactant). With color difference and gloss as evaluation indexes, the influencing factors (temperature, pH, enzyme concentration, and enzyme hydrolysis time) were measured. Results suggest that alkaline protease was the most suitable cleaning agent for removing surface paint, and the best working condition is an enzymatic dosage of 1.92 mg/mL under 42℃, pH of 9.5, with a hydrolysis time of 5.4 min. This method generated the desired color difference of 2.25 and the minimal gloss (0.1±0.1 GU) on the underlying stone, with no significant variation from the predicted value ($P < 0.05$). Before and after cleaning, the change in surface roughness was less than 10%. This study provided a feasibilty plan for removing the paints from the outer wall of the Potala Palace by using enzymatic hydrolysis.

Key words: whitewash removal, the Potala Palace, Protein, Alkaline protease cleaning, Response surface methodology

1. Introduction

The Potala Palace is Tibet’s largest best-preserved palace-style fortress complex[1]. According to the local religious tradition, the walls of the Potala Palace are repainted with a lime-based paint every year. Locals typically combine milk, honey, and other materials with lime to manufacture paint, which is applied over the Potala Palace's outside walls to increase the consistency and stickiness of the paint[2]. Due to this tradition, layers of lime-based paint now covers the surface of originally unpainted stone carvings, which seriously affects the overall appearance of the Potala Palace[3]. The paint compromises both the legibility and the preservation of stone artifacts as paint obscures the surface of stones, and can create a barrier to moisture transport. Moreover, the paint covers up the cracks that easily expose the structural problems of the stone, resulting in missing the best restoration period. Therefore, an adequate cleaning strategy to remove the overpaint is necessary.

Lime-based mortars are primarily used as for masonry, jointing and plastering, while lime-based paints can be applied to stone as whitewash. Whitewash primarily consists of cementing materials (lime), water, and additives[4,5]. Several organic materials, such as milk[11-13], animal glue[14], egg white[15], and blood[16,17] are often added to lime in whitewash to improve the mechanical strength and adhesion of the paint.

Physical removal of overpaint from stone may be accomplished with dry cleaning (laser cleaning and particle injection) and wet cleaning (high-pressure water cleaning and steam cleaning)[18]. Despite the extensive applications of these cleaning technologies, obvious limitations still remain. For example, laser cleaning can remove micron-level adhesions on the stone carvings surface, but its effect is mostly confined to specific cases such as dirt accumulation, along with very low efficiency and high cost[19,20]. Abrasive blasting may cause surface damage. Dust contamination in the operating environment must be controlled[21,22]. High-pressure water spraying is challenging to control, resulting in damage to substrates that may result in discoloration, unevenness, flaking, cracks, etc[23]. The involvement of water will introduce moisture into the artifacts, resulting in the transfer of contaminants[24]. The high-temperature steam employed can cause uneven thermal expansion inside and outside the stone carvings artifacts and result in stress damage[25].

Mechanochemical cleaning is based on mechanical cleaning with the addition of chemical reagents such as redox, organic solvents, gels and bioactive materials to clean the surface of the artifacts[26-28], which can be applied with adsorbent materials[29-32]. Chemical agents may endanger cultural relics and may lead to the development of secondary reactant that may damage the substrate[33,34]. By contrast, biological cleaning technologies have been proposed as safer for artifacts, the environment, and operators[35,36]. Using microorganisms and enzymes to target molecules within overpaint is an effective alternative to traditional cleaning technologies. By using enzymolysis to target protein in paint[37,38], Abdelaal et al. showed that protease can be safely used to remove unwanted substances in a controlled manner[39]. Proteases provide a powerful tool that enables many fragile artifacts to be cleaned and repaired efficiently and quickly[40].

Our study focuses on the optimization of the remove the overpaint from the surface of stone carvings by targeting the organic binder used in the paint mixture. Considering that the main component of the paint used in the Potala Palace is a protein-based compound, we selected protease as a cleaning agent because it can specifically bind proteins in milk. Influencing factors (temperature, pH, enzyme concentration, and enzymatic hydrolysis time) were optimized with the Box-Behnken Design to identify the best cleaning conditions. To evaluate the cleaning result, we compared the surface roughness values of the blank stones with the stones cleaned under ideal conditions.

2. Research aims

The Potala Palace has great significance, but is subject to the hazards of natural conditions and the past application of paint and mortar which greatly affects the preservation value of stone carvings. The aim of this study is to find a safe, efficient, inexpensive, and environmentally friendly cleaning method to remove overpaint from the surface of stone carvings, and to fill the gap in the cleaning technology of stone carvings.

3. Materials and methods

3.1 Materials and reagents

Milk with a protein concentration of 0.03 g/mL was procured by Inner Mongolia Mengniu Dairy (Group) Co., Ltd.(China). Hydrated lime powder was purchased from Shishun Limestone Mine Co., Ltd., Mingshan District, Benxi City (China). Enzyme-linked immunosorbent assay kit was bought from Shanghai Fusheng Industrial Co., Ltd.(China). Alkaline protease containing 2.0×10^5 U/g was manufactured by Novozymes Biotechnology Co., Ltd.(China). Papain enzyme-containing 3.0×10^3 U/g was manufactured by Sanland Chem International Co., Ltd.(China). The tryptic enzyme-containing 1.0×10^5 U/g was made by Shanghai Aladdin Biochemical Technology Co., Ltd. (China). Bromelain containing 1.7×10^3 U/g was obtained from Nanning Pangbo Bioengineering Co., Ltd.(China). Sodium hydroxide and hydrochloric acid (analytically pure) were purchased from Tianjin Benchmark Chemical Reagent Co., Ltd.(China). Sodium citrate, ethanol, and Tween 20 (analytically pure) were supplied by Shanghai Eon Chemical Technology Co., Ltd.(China).

3.2 Experimental design

3.2.1 Paint samples

The paint samples were taken from the surface of the stone sculptures at the Potala Palace. In Fig. 1, 5 g of samples were taken from each sampling point in the outer, middle, and bottom layers for the subsequent analysis. Fig. 2 shows the field images of the sampling points in H10-E39. (a: outer layer, b: middle layer, c: bottom layer). From Fig. 3, a total of 10 paint samples were acquired.

3.2.2 Determination of protein content of the paint

The protein crude extract was obtained by first mixing 4 mg of samples with 0.1 ml of encapsulation buffer and then extracted at 60°C for 10 min, followed by centrifugation at 3000 g for 10 min at 4°C. 50 μL of bovine casein was first placed into a 96-well ELISA plate, then 40 μL of sample dilution and 10 μL of the sample were added to the sample wells sequentially. The enzyme labeling plate was wrapped in sealing film and then warmed at 37°C for 30 min. The wash solution was then poured onto the enzyme labeling plate, which was then removed and left for 30 s before being repeated 5 times. Each well was spiked with 50 μL of enzyme standard reagent except for the blank. After repeated warming and washing operations, 50 μL each of chromogenic agents (A and B) were added to the enzyme labeling plate and left for 10 min at 37°C and protected from light. Finally, the stopping solution was applied to the enzyme labeling plate[41].

3.2.3 Preparation of simulated stones

According to the protein content of the samples measured in 3.3.2, the simulated paint was made of milk, water and lime powder, in which the milk was 5% of the liquid, and the solid ratio of liquid (milk + water) was 0.61[42]. The paint was stirred until homogeneous and then applied to the surface of the original stones of equal size (5 × 5 × 2 cm), which were provided by the Potala Palace Administration and were made of the same material as the stone artifacts. Considering the temperature and UV intensity of Lhasa, the simulated stones were aged in a UV weathering test box (Foshan Huahe Zhensen Testing Machine Manufacturing Co., Ltd.(China)) in order to achieve the effect of sunlight exposure for one year in a shorter period of time. 80 samples were prepared by applying a paint (with protein content 0.86%) on unpainted stones. These were then placed in the ultraviolet aging box, with a UV exposure of 0.68 w/cm^2 and an irradiation time of 11 days. These stones are known as simulated stones.

3.2.4 Screening of cleaning agents

The optimal cleaning agent was selected from organic reagents (1 mol/L sodium citrate solution and 95% ethanol), distilled water, different types and concentrations of proteases (bromelain, neutral protease, papain, alkaline protease, and tryptic enzyme), and a non-ionic surfactant (Tween 20). To compare the effect of different cleaning agents on stones without paint, a simple corrosion test was carried out. Stones were soaked in cleaning agents 2 hours, and then washed in water and dried. The difference in mass was calculated by comparing the stone before and after soaking. [43]. The damage to the stone surface due to the corrosive action of the cleaning agent was calculated according to the Eq. (1):

$W(\%)=(m_1-m_2)/m_1\times100\%.(1)$

Where W is the corrosion rate; m_1 is the mass of the original stone; and m_2 is the mass of the cleaned stone.

3.2.5 Single factor experiments

The role of alkaline protease in cleaning the paint from stones was investigated using single factor experiments with four independent variables (temperature, pH, enzyme concentration, and enzymatic hydrolysis time). The simulated stones were wetted with paper towels and cling film for different times (3 min, 5 min, 9 min, and 15 min) at various temperatures (30°C, 35°C, 40°C, and 45°C), pH (7, 8, 9, and 10), and enzyme concentration (1.0 mg/mL, 1.5 mg/mL, 2.0 mg/mL, and 2.5 mg/mL) and a soft brush was used to remove the softened paint. After the simulated stones were dried, color difference and gloss were measured[29].

3.2.6 Optimization of alkaline protease for cleaning

Based on the results of the single factor experiments, we utilized a four-factor, three-level response surface analysis method using the Box-Behnken design. Table 1 shows the effect of temperature (A), pH (B), enzyme concentration (C), and enzymatic hydrolysis time (D) on the cleaning effect using color difference and gloss as assessment indices[44].

Table1 Experimental range and levels of independent variables

Independent variables	Coded levels		
	-1	0	1
A: Temperature(℃)	35	40	45
B: pH	8	9	10
C: Enzyme concentration(mg/mL)	1.0	1.5	2.0
D: Enzymatic hydrolysis time (min)	3	5	7

3.2.7 Determination of the color difference

A colorimeter (CM-700d, Konica Minolta Co., Ltd.(China)) was utilized to measure the standard samples, which were the original sample stones without paint, and the obtained values were recorded as L^*_1, a^*_1, and b^*_1. Then the simulated stones under different cleaning conditions were examined using the colorimeter, and the values obtained were recorded as L^*_2, a^*_2, and b^*_2. Each of simulated stone surface was measured at three points[45]. The color difference was calculated according to the Eq.(2):

$$\Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}.(2)$$

Where $\Delta L^*=L^*_2-L^*_1$ (brightness factor difference); $\Delta a^*=a^*_2-a^*_1$ (red-green Index Difference); $\Delta b^*=b^*_2-b^*_1$ (yellow-blue index difference). For this work, the cleaning effect is considered excellent ($\Delta E \leq 3$); the cleaning effect is general ($3<\Delta E \leq 5$); and cleaning experiments are failed ($\Delta E>5$)[29].

3.2.8 Determination of gloss

The change in surface morphology of the simulated stones before and after cleaning under different conditions was presented in terms of gloss. The glossiness instrument (3nh Co., Ltd.(China)) was used to measure the original samples, and the obtained values were noted as GS_1 (GU). The simulated stones under different cleaning conditions were examined and the values obtained were noted as GS_2 (GU)[46]. Gloss was calculated according to the Eq.(3):

$GS=GS_1-GS_2$. (3)

Where cleaning effect is excellent when GS is very small.

3.2.9 Determination of surface roughness

The surface roughness was used to check whether the cleaning affected the stone surface[47]. As the control group of three original stones, nine measuring points were selected on the surface of each original stone in three rows and three columns. The values of the original stones measured by the TR 200 surface analyzer (Beijing Jitai Keyi Testing Equipment Co., Ltd.(China)) were averaged and then recorded as Ra_1. The blank stones were coated with protein-containing paint, and treated under optimal cleaning conditions. Nine measuring points were selected on the surface of each painted stone. The values measured for the painted stones were averaged and then noted as Ra_2. The surface roughness was calculated using the Eq. (4):

$\Delta Ra=（Ra_1-Ra_2)\times100\%$. (4)

In this work, cleaning is considered to be excellent ($\Delta Ra \leq 10\%$); cleaning effect is general ($10\% < \Delta R \leq 15\%$); and cleaning experiments are deemed to have failed ($\Delta R > 15\%$)[48].

3.3 In-situ experiment

During cleaning of stone carvings, the first step was to remove surface dirt from the paint. This was followed by the application of an alkaline protease under the best conditions to wet the stone carvings. The paint was removed using a soft brush dipped in clean water.

3.4 Statistical analyses

The data obtained from the experiment were described by the mean and standard deviation. The experimental data were analyzed using Excel 2019 and IBM SPSS Statistics 25 software. Box-Behnken Design and variance analysis were completed by Design-Expert. V8.0.6 software. All experiments were performed three times in parallel.

4. Results and discussion

4.1 Results and analysis of protein content in samples

4.1.1 Standard curve of casein

We calculated casein content in the paint samples by ELISA. The standard curve is plotted in Fig. 4. The standard curve's determination coefficient (R^2) was 0.9965, implying that the model was well fitted. The linear equation of the standard curve for the determination using ELISA was as follows:

$Y=00142X+0.0097$. (5)

Where Y is the absorbance value of casein, and X is the concentration of casein solution.

4.1.2 Protein content in paint samples

The results of protein identification of 10 paint samples determined by ELISA are presented in Table 2, where some of the samples showed significant differences in protein content ($P < 0.05$). After calculation, the protein percentage was estimated as 0.68%.

Table 2 Protein content in paint samples

Run	Name of samples	Protein concentration (μg/mL)
1	H10-E37 stone carving outer paint layer	206.75±5.12^{b}
2	H10-E39 stone carving middle layer	181.85±3.82^{de}
3	H10-E11 stone carving outside the double paint layer	183.20±1.39^{de}
4	West side lower road outer paint layer	271.20±4.69^{a}
5	H10-E11 stone carving bottom paint layer	177.09±5.51^{e}
6	H10-E11 stone carving middle paint layer	178.95±4.40^{e}
7	H10-E39 stone carving bottom paint layer	179.49±3.61^{e}
8	H10-E39 stone carving outer paint layer	195.13±3.51^{c}
9	H10-E37 stone carving bottom paint layer	188.62±2.49^{cd}
10	H10-E11 stone carving outer paint layer	193.55±4.49^{c}

Notes: Different letters within a column indicate significant differences at $P < 0.05$

4.2 Analysis of cleaning agents

According to Fig. 5, we found that sodium citrate solution (1 mol/L) and ethanol (95%) were more corrosive to the surface of simulated stones[25], while distilled water, alkaline protease (1 mg/mL), and Tween 20 were less corrosive. In Fig. 6, although the cleaning effects of distilled water and Tween 20 (0.3%) were not as great as alkaline protease, Tween 20 (0.3%) could achieve the purpose of cleaning the paint from simulated stones[49]. Therefore, we studied the cleaning effect by the combination of alkaline protease (1 mg/mL) with Tween 20 (0.3%) as compared to alkaline protease (1 mg/mL) alone. The experimental results showed no significant difference ($P < 0.05$) in the cleaning effect of the two cleaning agents. In order to reduce the cost and achieve a better cleaning effect, alkaline protease was selected as the cleaning agent for simulated stones in this study.

4.3 Optimization of the cleaning conditions with single-factor experiments

4.3.1 Temperature

We evaluated the color difference and gloss of painted stones at different temperatures ranging from 30°C to 45°C to observe how temperature affected the cleaning result. The results in Fig. 7a indicated that from 30°C to 40°C, both the color difference and gloss decreased sharply with increasing temperature. This phenomenon was due to the activation of the active gene of alkaline protease at lower temperature, resulting in the most efficient hydrolysis of casein in milk. The degree of casein hydrolysis reached a maximum value at the ideal temperature[50,51]. Above the ideal temperature, the removal of paint is compromised due to enzyme inactivation. Based on the cleaning effect, we set the optimal cleaning temperature at 40°C for subsequent tests.

4.3.2 pH

We tested the color difference and gloss in the pH range of 7 to 10 to find the best pH for cleaning paint from stones with alkaline protease. The results in Fig. 7b showed that when pH was 7-9, the color difference and gloss had a substantial negative association, but showed no significant changes when pH was further increased to 10. The negative correlation could be explained by the most rapid hydrolysis rate of alkaline protease when working under the optimum pH range, which led to changes in color and gloss[52,53]. When exceeding the optimum pH range, changes in color and gloss were reduced due to the loss of enzyme activity. Considering the cleaning effect, we selected pH (9) as the most suitable starting point for subsequent tests.

4.3.3 Enzyme concentration

We investigated the color difference and gloss of painted stones with enzyme concentrations ranging from 1.0 mg/mL to 2.5 mg/mL to acquire adequate parameters to understand the influence of enzyme concentration on cleaning performance. Fig. 7c illustrates that the color difference and gloss gradually decreased through increasing the enzyme concentration. However, increasing enzyme concentration beyond 1.5 mg/mL eliminated the negative correlation and showed a smooth trend. The explanation was that as the enzyme concentration increased so did the hydrolysis of the paint binder. Later, when the enzyme concentration surpassed 1.5 mg/mL, a part of the alkaline protease could no longer bind to the proteins due to the saturation of most substrates[54]. According to the cleaning performance, we chose 1.5 mg/mL as the starting point for subsequent tests.

4.3.4 Application time

We conducted experiments ranging from 3 min to 15 min to test how enzymatic hydrolysis time impacted cleaning efficiency. In Fig. 7d, both the color difference and gloss decreased steadily with increased enzymatic hydrolysis time from 3 min to 5 min. The two indexes gradually stabilized when the enzymatic hydrolysis time surpassed 5 min. This phenomenon could be ascribed to the fact that the catalytic site of alkaline protease decreased as the hydrolysis reaction proceeded[55], which led to a declined protein hydrolysis level in the paint that generally maintained the color difference and the gloss of the simulated stones. Given the cleaning effect, we selected 5 min as the starting point for subsequent tests.

4.4 Optimization of the cleaning conditions

After carrying out single-factor experiments, we used four factors with three levels and 29 different runs to compare the results of the combined treatments and the data obtained are recorded in Table 3.

Table 3 Box-Behnken response surface design and experimental results

Run	Factors				Responses	
	A(°C)	B	C(mg/mL)	D(min)	ΔE	GS (GU)
1	-1(35)	-1(8)	0(1.5)	0(5)	7.15	1.1
2	1(45)	-1(8)	0(1.5)	0(5)	4.59	0.6
3	-1(35)	1(10)	0(1.5)	0(5)	6.29	1.0
4	145()	1(10)	0(1.5)	0(5)	3.07	0.3
5	0(40)	0(9)	-1(1.0)	-1(3)	5.66	0.8
6	0(40)	0(9)	1(2.0)	-1(3)	4.39	0.6
7	0(40)	0(9)	-1(1.0)	1(7)	4.67	0.6
8	0(40)	0(9)	1(2.0)	1(7)	2.86	0.2
9	-1(35)	0(9)	0(1.5)	-1(3)	6.60	1.0
10	1(45)	0(9)	0(1.5)	-1(3)	5.66	0.8
11	-1(35)	0(9)	0(1.5)	1(7)	5.38	0.8
12	1(45)	0(9)	0(1.5)	1(7)	3.07	0.3
13	0(40)	-1(8)	-1(1.0)	0(5)	6.38	0.9
14	0(40)	1(10)	-1(1.0)	0(5)	5.38	0.8
15	0(40)	-1(8)	1(2.0)	0(5)	4.67	0.6
16	0(40)	1(10)	1(2.0)	0(5)	2.75	0.4
17	-1(35)	0(9)	-1(1.0)	0(5)	6.21	0.9
18	1(45)	0(9)	-1(1.0)	0(5)	5.22	0.7
19	-1(35)	0(9)	1(2.0)	0(5)	5.66	0.8
20	1(45)	0(9)	1(2.0)	0(5)	4.06	0.5
21	0(40)	-1(8)	0(1.5)	-1(3)	6.05	0.9
22	0(40)	1(10)	0(1.5)	-1(3)	5.22	0.7
23	0(40)	-1(8)	0(1.5)	1(7)	4.03	0.5
24	0(40)	1(10)	0(1.5)	1(7)	2.35	0.2
25	0(40)	0(9)	0(1.5)	0(5)	2.90	0.3
26	0(40)	0(9)	0(1.5)	0(5)	2.90	0.2
27	0(40)	0(9)	0(1.5)	0(5)	3.23	0.2
28	0(40)	0(9)	0(1.5)	0(5)	2.90	0.3
29	0(40)	0(9)	0(1.5)	0(5)	2.56	0.4

Notes: A temperature; B pH; C enzyme dosage; D enzymatic hydrolysis time.

4.4.1 Model fitting and statistical analysis

The results of the four factors and three levels are shown in Table 3. The results in Table 4 and regression equations were obtained by multiple linear regression analysis and quadratic multinomial fitting of the data in Table 3 with Design-Expert. V8.0.6 software.

$Y_{\Delta E}$=2.90-0.97A-0.65B-0.76C-0.94D-0.17AB-0.15AC-0.34AD-0.23BC-0.21BD-0.13CD+1.53A^2+0.90B^2+0.90C^2+0.65D^2. (6)

Y_{GS}=0.28-0.20A-0.100B-0.13C-0.18D-0.050AB-0.025AC-0.075AD-0.025BC-0.025BD-0.050CD+0.29A^2+0.19B^2+0.17C^2+0.12D^2. (7)

In Equation (6), the constant term was unrelated to the factors, while the quadratic terms of A^2, B^2, C^2, and D^2 positively affected the color difference, and consequently, the color difference value increased as these terms increased. The primary terms A, B, C, and D, as well as the interaction terms AB, AC, AD, BC, BD, and CD, did not have a significant effect on the color difference, therefore when these terms increased, the color difference decreased. Similarly, Equation (7) was consistent with the above analysis. The constructed model's dependability and importance were confirmed by the F-values (16.27 and 13.93). From the F-values, the effects of each factor on the color difference and gloss were established as follows: temperature (A) > enzymatic hydrolysis time (D) > enzyme concentration (C) > pH (B). The interaction between the independent variables can be understood by observing the P-value. From Table 4, the P-values (<0.0001) verified that the regression equations established with color difference and gloss as response values were significant. The linear terms' impacts on color difference and gloss were extremely significant ($P < 0.01$), whereas interaction terms' effects on color difference and gloss were not substantial enough ($P > 0.05$) to alter them. The quadratic terms (A^2, B^2, and C^2) exhibited an extremely significant ($P < 0.01$) influence on the color difference and gloss. However, the effects of D^2 on the color difference and gloss were respectively highly and generally significant. In addition, R^2 (0.9421 and 0.9330) and adj-R^2 (0.8842 and 0.8660) values indicated that the model fitted the experimental data well and minor changes (less than 0.1) cannot be explained by the models. The lack of fitted values for the response surface models (0.0558 and 0.3288) were not significant, indicating that the models were meaningful for the optimization of the cleaning conditions. Therefore, the models could be used to analyze and predict the effect of alkaline protease to remove paint.

Table 4 ANOVA of response surface model for the color difference and gloss.

Source	Color difference			Gloss		
	Mean square	F Value	Prob > F	Mean square	F Value	Prob > F
Model	54.71	16.27	<0.0001**	2.02	13.93	<0.0001**
A	11.25	46.85	<0.0001**	0.48	46.45	<0.0001**
B	5.08	21.17	0.0004**	0.12	11.61	0.0042**
C	6.95	28.93	<0.0001**	0.21	20.65	0.0005**
D	10.49	43.68	<0.0001**	0.40	39.03	<0.0001**
AB	0.11	0.45	0.5117	0.010	0.97	0.3419
AC	0.093	0.39	0.5437	2.5×10^{-3}	0.24	0.6304
AD	0.47	1.95	0.1839	0.022	2.18	0.1622
BC	0.21	0.88	0.3638	2.5×10^{-3}	0.24	0.6304
BD	0.18	0.75	0.4004	2.5×10^{-3}	0.24	0.6304
CD	0.073	0.30	0.5903	0.010	0.97	0.3419
A^2	15.20	63.28	<0.0001**	0.56	54.01	<0.0001**
B^2	5.28	21.97	0.0003**	0.24	23.46	0.0003**
C^2	5.25	21.85	0.0004**	0.18	17.79	0.0009**
D^2	2.77	11.52	0.0044**	0.091	8.79	0.0102*
Residual	3.36			0.14		

Lack of fit	3.14	5.59	0.0558	0.12	1.67	0.3288
Pure error	0.22			0.028		
Cor total	58.07			2.16		

Notes: ** highly significant ($P < 0.01$); * significant ($P < 0.05$).

4.4.2 Analysis of surface responses

In order to optimize the key factors for optimal cleaning conditions, response surface (3D) and contour plots (2D) of the interaction between the four factors were produced in Figs. 7 and 8.

4.4.2.1 Effect of independent variables on color difference

Six pairs of parameters influenced the color difference in Fig. 8. It can be observed in the 3D plots that when one variable was fixed, the color difference decreased and tended to stabilize as the other variable increased. The interactions of temperature with pH and enzyme concentration had no significant effect on the color difference due to the circularity of the contours in Figs. 7a and b. Given the elliptical shape of the contours in Figs. 7c and f, interactions concerning enzymatic hydrolysis time (temperature, pH, and enzyme concentration) and the interaction of pH with enzyme concentration on the color difference were significant. However, 3D surfaces of pH (Fig. 8a), enzyme concentration (Fig. 8b) and enzyme hydrolysis time (Fig. 8c) were more flat than temperature, indicating that the effect of temperature on the color difference was the most obvious. The 3D surface of enzymatic hydrolysis time was steeper than pH (Fig. 8e) and enzyme concentration(Fig. 8f), while the 3D surface of pH was flatter than that of enzyme concentration (Fig. 8d), which indicated that the magnitude of the effect of three factors on gloss is enzymatic hydrolysis time > enzyme concentration > pH.

4.4.2.2 Effect of independent variables on gloss

Fig. 9 shows 3D and 2D plots for gloss as a function of the four independent factors. In the 3D plots, it can be noticed that for one variable to be stable, the gloss decreased and smoothed out with the increase of the other variable. By the tight circularity of the contours in Figs. 9a, b, and e, the interactions of temperature with pH and enzyme concentration, as well as the interaction between pH and enzymatic hydrolysis time, were irrelevant. From the elliptical contours in Figs. 9d, f, and c, the interactive effects of enzyme concentration with pH and enzymatic hydrolysis time, as well as temperature and enzymatic hydrolysis time, had a significant effect on gloss. The 3D surfaces of pH (Fig. 9a), enzyme concentration (Fig. 9b), and enzymatic hydrolysis time (Fig. 9c) were more flat than temperature, hence temperature had a more substantial impact on gloss than the other three factors. The 3D surface of enzyme concentration, both steeper than pH and flatter than enzymatic hydrolysis time, revealed that the order of their effects on glossiness was enzymatic hydrolysis time > enzyme concentration > pH.

4.4.3 Determination and experimental validation of optimal cleaning conditions

The Design-Expert software's numerical optimization results revealed the most effective cleaning conditions: temperature: 42°C; pH of 9.5; enzyme concentration: 1.92 mg/mL; and enzymatic hydrolysis time: 5.4 min. Three parallel experiments were conducted under optimal cleaning conditions to validate the developed model's dependability and accuracy. The results showed no significant differences ($P > 0.05$) between the color difference (2.25 ± 0.26) and gloss (0.1 ± 0.1 GU) obtained by parallel tests and the color difference (2.27) and gloss (0.2 GU) predicted by the models. As a result, the model was ideal and it can be used to remove surface paint using alkaline protease.

4.5 Validation experiments and analysis of surface roughness

According to the roughness measurements, the results indicated that the surface roughness values of blank stones and painted stones cleaned under optimal conditions were 2.61 ± 0.13 μm (Ra_1) and 2.53 ± 0.13 μm (Ra_2), respectively. The difference between the experimental results and the blank value was 8% (less than 10%), which indicated that the optimal cleaning conditions obtained from the model comply with the requirements for cleaning[56].

4.6 Practical cleaning test of stone carvings in the Potala Palace

The surface paint from stone relics H10-E09 of the Potala Palace was cleaned with alkaline protease under the optimum cleaning conditions. The unclean stone carvings were covered with white paint to conceal the information in Fig. 10a. It was found that after cleaning by alkaline protease that most of the paint was removed, indicating that alkaline protease had a good cleaning effect (Fig. 10b). The results were in good agreement with those of the simulated cleaning test.

5. Conclusion

We successfully selected the best cleaning agent from nine available biologically active options. The results show that alkaline protease has a good cleaning effect in terms of the removal of whitewash containing protein as a binder. This study provided a theoretical basis for the enzymatic cleaning of stone carvings and plays an essential role in protecting and repairing the Potala Palace stone carvings.

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FIGURE LEGENDS

Fig. 1 Sampling location map of Potala Palace: the bottom left side of H10-E11 stone carving (①), right side of stone carving E37-E38 (②), and right side of H10-E39 stone carving (③) were sampling points, respectively.

Fig. 2 The field images of the sampling points in H10-E39. (a: outer layer, b: middle layer, c: bottom layer).

Fig. 3 Images of the powdered paint samples used in the experiments.

Fig. 4 Standard curve of casein.

Fig. 5 Corrosion of simulated stones by different cleaning agents. Different lowercase letters after the data in the same column indicate that one-way ANOVA of the mean values differs significantly ($P < 0.05$).

Fig. 6 The color difference of simulated stones after being cleaned by different cleaning agents. Different lowercase letters after the data in the same column indicate that one-way ANOVA of the mean values differs significantly ($P < 0.05$).

Fig. 7 Influence of various factors on color difference and luster of simulated stone. Different lowercase letters after the data in the same column indicate that one-way ANOVA of the mean values differs significantly ($P < 0.05$).

Fig. 8 Response surface plots (3D and 2D) for the effect of temperature, pH, enzyme concentration, and enzymatic hydrolysis time on the color difference.

Fig. 9 Response surface plots (3D and 2D) for the effect of temperature, pH, enzyme concentration, and enzymatic hydrolysis time on gloss.

Fig. 10 The Potala Palace stone carvings practical cleaning application.