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A biological cleaning agent for removing mold stains from paper artifacts

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Abstract

Efficient removal of mold stains becomes an important research topic for paper conservation. In this study, a cleaning scheme based on the combination of bioenzymes and biosurfactants was explored. Morphological and molecular biology identifications were first jointly applied to identify the dominant strains sampled from five ancient books that are stored in the same environment. Cellulolytic experiments were then conducted to evaluate the cellulose degradation ability of the strains according to the cellulolytic digestive index. Finally, paper Mockups for the ancient books were constructed to investigate the most effective combination of bioenzymes and biosurfactants in removing mold stains as well as its effect on the paper's physical properties. The result concluded that the combination of 3% papain, 7% of sophorolipid or 7% of betaine, and distilled water, achieved optimal stain removal effect with over 50% cleaning rate at 35 °C, after 30 min of infiltration. The maximum color difference of the paper material after cleaning was around 0.60, pH was between 7.45 and 7.79, and no significant changes in tensile strength were observed. At the same time, Sophorolipid and Betaine both have superior deacidification, anti-acidification, anti-aging, and reinforcement capabilities, which can provide extra support to the fibrous structure in addition to cleaning the paper materials. The microbial contamination cleaning agent proposed in this study shows promising application prospects in conserving mold-contaminated paper artifacts.

Keywords Paper artifacts, Mold stains, Bioenzymes, Biosurfactants, Cleaning agent

Introduction

Paper-based material, including ancient books, archives, paintings, calligraphy, historical documents, etc., is one of the most popular type of medium for information spreading throughout history. They are the carriers and embodiment of historical information [1, 2]. Thus, devising techniques to enforce long-term preservation of paper relics became an integral research topic in the field of cultural heritage conservation. However, paper artifacts, after surviving hundreds of years, are extremely vulnerable, especially during preservation and transportation. Exogenous factors [3], such as temperature, humidity, and microorganisms, can damage the fibrous structure of paper, which greatly reduces the durability of the material and results in various degrees of aging, acidification, mold contamination, etc. [4], causing incalculable and irreversible cultural losses.

Paper is rich in cellulose, hemicellulose, lignin-like substances [5], which can host a large number of microorganisms that feed on paper fibers as nutrients for growth and reproduction, such as: *Aspergillus niger*, *Aspergillus flavus*, etc. [6]. The process of mold contamination mainly consists of three stages: deterioration (initial), mildew (quality change), rotting (irreversible

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decay). Molds are aerobic, highly reproductive, metabolic, mutable, and adaptable, with optimal pH value of 4.0–5.8, optimal growth temperature of 24–30 °C. They can secrete extracellular enzymes and acidic metabolites that accelerate the destruction of paper fibers. Among them, extracellular enzymes can degrade cellulose and hemicellulose, which can lead to changes in the physical structure of paper, thus affecting paper's strength and toughness. Acidic metabolites can cause a decrease in the pH of pigments and paper, thus promoting aging and yellowing of the paper material and making it fragile and brittle. Colored metabolites left on the paper not only will affect the original appearance of paper artifacts, but also cause information loss due to content masking, affecting the reading and reproduction. Moreover, the spores and mycotoxins of some molds may even cause skin irritation or allergic reactions [7] when cultural and conservation workers come into contact with them. For example, *Aspergillus* secretes aflatoxin, one of the most toxic mycotoxins with strong carcinogenic and mutagenic activity [8], can affect human corneal epithelial cell biology activity, and, when exposed to low levels of aflatoxin, may cause respiratory diseases such as asthma and chronic airway inflammation [9]. Additionally, mycotoxins cause changes in mucin monosaccharide composition and intestinal mucin expression, which in turn affects mucin function, causing intestinal dysfunction and damage to intestinal mucosal immune barrier function [10, 11]. These effects seriously threaten the health of cultural preservation workers.

Given the common occurrence of mold contamination on paper-based relic surfaces, the development of effective mold stains removal technique is an important topic of research [12]. Aqueous solution method, chemical solution method, and mechanical method are prevalently applied in current conservation practices to remove mold contaminations on paper-based relic surfaces. Aqueous solution method [13] refers to treating the mold contaminated paper using distilled water without any chloride ions or transition metals, but the method can only remove spots that are formed most recently, and the removal rate is low. Chemical solution method [14] usually uses Potassium permanganate solution, hydrogen peroxide solution, oxalic acid solution, etc. for cleaning. However, the residual chemical reagents on the surface of the paper after treatment will accelerate the aging of paper, which induce secondary damage. Mechanical method [13] use soft brushes, cotton swabs, erasers and other small tools to clean the paper dust and solid pollutants from the surface. This method is usually used as the first step in the restoration of paper artifacts. The use of laser [15, 16] for cleaning paper-based artifacts selects the appropriate wavelength for cleaning depending on the degree of contamination, but the paper cleaned by a laser with 520 nm wavelength still showed fading after many years of natural aging. Liang et al. [17] used an electrochemical method to remove stains from the surface of paper artworks, which has excellent localized cleaning ability for stains formed by organic pollutants and has no significant effect on cellulose and the original mineral pigments on paper, providing an effective method for cleaning paper artwork. Mazzuca et al. [18] used a novel polyvinyl alcohol as a cross-linking agent to synthesize new chemical hydrogels that are biocompatible, transparent, stable, capable of absorbing degradation products that cause yellowing of paper, and do not leave residues after cleaning. It does not increase paper brittleness or change paper fiber properties, but the cleaning function of hydrogel can only be achieved at certain size. In addition, acidification is an important factor that promotes cellulose degradation in paper, so paper deacidification has also been a challenging task in paper protection. Baglioni [19], Cavallaro [20], Lisuzzo [21] reported successful strategies for paper deacidification by increasing the alkali reserves using calcium hydroxide or magnesium oxide, respectively. Despite the various forms of paper cleaning solutions for mold contamination, there is still a lack of a safe, effective and easy-to-use technology that allows effective cleaning while also providing protective features such as deacidification, which inspired this project.

Five ancient books that are stored in the same environment in the Special Collection room of Liaoning University were chosen as subject of research. Since the process of mold stains cleaning is irreversible and will cause irreparable damage if not handled properly, the composition of the cleaning agent needs to be chosen with caution. Based on the principle [22, 23] of maintaining the original appearance of paper artifacts with minimal intervention for cultural heritage conservation, modern biotechnology is introduced to the conservation practice of paper artifacts, and we are committed to researching a biological cleaning agent that can both preserve the original features of paper artifacts and effectively remove mold stains from their surfaces. The strains of mold attached to the antique books were sampled by sterile swab method, resuscitated, and cultured for morphological and molecular biological identification to determine the dominant strains causing the contamination on the antique books. Secondly, cellulose decomposition experiments [24] were conducted for the selected dominant strains. The colony diameters were recorded before staining, and the cellulose degradation ability of each strain was judged according to the ratio of cellulose degradation circle diameter, visualized by staining, to colony diameter. Finally, mockups for antique books were constructed for cleaning experiments. The composition of biological cleaning agent was carefully screened. Solvent [22, 25] was selected among deionized water, purified water, distilled water, tap water and hard water according to their characteristics. The solute is composed of bioenzymes and biosurfactants, which are all natural products that are generated by living organisms. In comparison to chemically synthesized enzymes and surfactants, bioenzymes and biosurfactants have milder reaction conditions, low toxicity, environmentally friendliness, and 100% biodegradability. Thus, the usage of bioenzymes and biosurfactants can avoid secondary damage to the paper, avoid pollution to the environment, and, more importantly, ensures the health and safety of cultural preservation workers. Among them, bioenzymes [26] serve as highly efficient and specific catalysts. They exist within all living organisms and are essential substances for maintaining normal biological functions, conducting substance metabolism, energy exchange, tissue repair, and other life activities. Their specificity can ensure that the structure of paper and pigments will not be damaged during the mold stains cleaning process, while their high efficiency can prevent negative side effects to the paper due to prolonged treatment. Bioenzymes are widely used to clean stubborn metabolic stains. For example, protease, a type of bioenzyme, can remove protein-based stains from silk and plant fiber materials [27]. They can efficiently decompose large protein molecules into small soluble peptides and can even further breakdown into amino acids, making the stain easily removable. As the protein stains are removed, the other stains that are closely adhering to the fibers and are highly stubborn due to the presence of the protein stains can then be removed relatively easily. Biosurfactants exhibit amphiphilic properties, consisting of a hydrophilic polar head and a hydrophobic non-polar tail [28], and can synergistically work with proteases to reduce the ability of stains to adhere to the surface of objects. They can reduce the adhesion of stains on the surface of objects and remove stains from fibrous surfaces at lower mechanical strength. They possess weak acidity and alkalinity, are naturally degradable, and their residues do not cause damage to paper. Compared to chemically synthesized surfactants, biosurfactants is a new type of natural cleaning agent that have high activity and stability [29]. At present, bioenzymes and biosurfactants as cleaning agents are mostly used on fabric cellulose, and there is very little research for their use on cleaning paper. We only found one literature [30] that compared four cleaning agents, including EDTA (ethylenediaminetetraacetic acid), oxalic acid, tween-80 and papain for mold removal from paper artifacts. Papain achieved the best result in their study, but the authors also suggested that a single concentration of a cleaning agent does not achieve a better removal of mold spots on Xuan paper, which is consistent with our previous results.

Thus, this study investigated the optimal proportion of bioenzymes and biosurfactants to be applied jointly to mold contaminated paper artifacts. The cleaning effect and the post-cleaning effect on paper properties were evaluated to determine the optimal cleaning agent composition that can maximize the removal of mold stains with minimal interference to original material.

Materials and methods

Sampling, purification, and morphological identification of the strains

The sampling was conducted on ancient books at the Special Collection room of Liaoning University (Fig. 1). The humidity was 48% RH and the temperature was 18 °C in the Special Collection room by the time of sampling. Book surface and spine with mold stains, and fading were selected as target sampling regions [31]. Strain No.1 was sampled from the 1/3 place of the book spine of “The True Interpretation of A Journey to the West” (Qing Dynasty), strain No. 2 was sampled from the 1/2 position from the fore-edge margin of “Imperial Edict of Yongzheng” (Qing Dynasty), strain No. 3 was sampled from the upper center of “Doctrine of the Mean and The Great Learning” (Qing Dynasty), strain No. 4 was sampled from the binding edge of the second page of “Veritable Records of the Qing Dynasty” (Qing Dynasty), strain No. 5 was sampled from the 1/3 place of the second page of “The True Interpretation of A Journey to the West” (Qing Dynasty), and strain No. 6 was sampled from the 1/3 position from the bottom of the spine of “Imperial Decree and Edict with Red Annotations” (Qing Dynasty). Non-invasive sterile swab sampling method [32] was used for strain sampling. The collected samples were

Fig. 1 Antiquarian sampling

then transferred to PDA and LB separately to isolate bacterial and fungal colonies. After colony formation, strains with different morphological structures were selected and purified for multiple rounds to obtain single colonies that consist only one strain. Preliminary identification of the isolated strains was conducted through observing morphological characteristics of the colonies and the microscopic structures of mycelium, conidial peduncle and conidia observed via optical microscope.

Purified cultures and molecular biology identification

The ascospores were collected using method referenced in [33–35] and incubated at 28 °C (fungi) or 37 °C (bacteria) for 2–4 days. After spore germination, the spores were transferred to PDA and LB for subsequent molecular biological identification after single colony formation. The DNA of the strains was extracted according to the procedures outlined by the bacterial and fungal DNA Mini Kits synthesized by Sangon Biotech (Shanghai) Co., Ltd. Then, the DNA concentration and purity were assessed by UV-absorptiometry, and the estimate for purity of nucleic acids was measured by the ratio of OD values at 260 nm and 280 nm (OD_{260}/OD_{280}) that were observed from the nucleic acid protein assay system. The molecular markers, 18S rDNA for fungal species and 16S rDNA for bacterial species, of the strains were amplified by polymerase chain reaction (PCR) method. The 18S rDNA gene fragment was amplified with the fungal DNA universal conserved primers [36] NS1 and NS8, where the upstream primer is NS1: 5′-GTAGTCATATGCTTGTCTC-3′ and the downstream primer is NS8: 5′-TCCGCAGGTTCACCTACG GA-3′. The 16S rDNA gene fragment is amplified with the bacterial universal conserved primers [37] 27F and 1492R, where the upstream primer is 27F: 5′-AGAGTT TGATCCTGGCTCAG-3 and the downstream primer is 1492R: 5′-AAGGAGGTGATCCAGCC-3′. All the primers mentioned above were synthesized by Sangon Biotech (Shanghai) Co., Ltd. PCR reactions were performed in a total volume of 50 μL containing 25 μL Taq PCR Master Mix, 1 μL DNA template, 2 μL upstream primer, 2 μL downstream primer, and 20 μL sterile water. The PCR reaction conditions for fungal species were: 94 °C pre-denaturation for 4 min, 94 °C denaturation for 30 s, 55 °C annealing for 30 s, 72 °C extension for 1 min, 72 °C termination for 10 min, 30 cycles in total; the PCR reaction conditions for bacterial species were: 94 °C pre-denaturation for 4 min, 94 °C denaturation for 30 s, 65 °C annealing for 30 s, 72 °C extension for 1 min, 72 °C termination for 10 min, 30 cycles in total. The PCR products then underwent 1% agarose gel electrophoresis. 5 μL of each of the PCR product were mixed with 1 μL of 6× Loading Buffer and subjected to 100 V constant pressure electrophoresis for 45 min while using DNA molecular weight marker (DL10000) as standard. The gel was then stained with nucleic acid dye for 15 min, and the purity of the amplified DNA of the strain was determined by gel imaging system. The obtained 18 S rDNA and 16S rDNA amplification products were also sent to Shanghai Majorbio Bio-Pharm Technology Co., Ltd for sequencing. The fungal 18S rDNA and bacterial 16S rDNA sequencing results were submitted to the GenBank database, and a homology sequence search was performed in the NCBI database and constructed corresponding phylogenetic trees.

Cellulolytic activity

Strains previously isolated from paper were tested for cellulolytic activities. The cellulose degradation ability assay was conducted by cultivating the strains on carboxy methylcellulose (CMC)—agar (1%) [38]. Plates were incubated at 37 °C, for bacterial strains, or at 28 °C, for fungal strains, and the incubation time was adjusted depending on the growth rate of each strain. The diameter of the colonies was measured using the crossover method before staining, and the staining time was determined according to the growth rate of the colonies. The CMC-agar plates, inoculated with fungi and bacteria, were stained by Lugol's iodine [24, 39] (10 mL/plate, 10 mg/mL) for 10 min and washed with distilled water to allow visualization and measurement of the diameter of the hydrolytic halo [40]. Cellulolytic activity was evaluated according to the cellulolytic index proposed by Menicucci et al. [31] by the following formula.

$$\begin{aligned}&\text{Cellulose degradation index (CI)}\\&=\text{hydrolysis halo diameter }(\varnothing\text{h})/\\&\quad\text{colony diameter }(\varnothing\text{c}).\end{aligned}$$

Screening of biological cleaning agent

Preparation of mold-stained paper mockup samples

The identified strains were purified and inoculated into Erlenmeyer flasks containing PDB to make suspensions. Chinese Xuan paper, glassware, distilled water and PDB, underwent sterilization in high pressure sterilizer at 121 °C for 15 min; sprayer and constant temperature incubator were treated with 75% ethanol. After sterilization, Chinese Xuan paper was laid flat on culture dishes of the same size, and the suspension was sprayed evenly on the surface of the paper with a sprayer. The inoculated samples were placed in a constant temperature incubator (28 °C) for 10–12 days. During the period, a certain amount of distilled water and PDB were sprayed every

12 h to accelerate mycelium multiplication. After the formation of visible mycelium on the surface, the paper was dried at room temperature for 2 months. The mycelium on the surface of the paper were then gently brushed off with a soft brush.

Screening of biological cleaning agent composition and cleaning conditions

The bioenzymes components in the microbial cleaner were screened by assessing the content and composition of amino acids in the mold stains paper mockup samples and the characteristics of the bioenzymes candidates. Trypsin [41] and papain [42] were selected as potential candidates for further validation (see Additional file 1: Bioenzyme selection, for more details).

Distilled water was selected as solvent after weighing the characteristics of different types of water, pH changes after treatment, cleaning quality, and preparation costs (see Additional file 1: Selection of solvent, for more details).

After considering whiteness, tension strength, and pH, of mold-stained paper mockup samples in response to different treatment conditions, 30 min treatment using 3% protease at 35 °C is identified as the optimal condition (see Additional file 1: Application condition (enzyme concentration, temperature, and time duration) selection, for more details).

Different biosurfactants have varying effects on the bioenzymes activity. A biosurfactant that has no inhibitory effect on the bioenzyme activity during the cleaning process is the optimal choice. In this experiment, Saponin [43], Tea saponin [44], Sophorolipid [45] and Betaine [46] with antibacterial ability were selected as the biosurfactants to be screened.

(1) Effect of biosurfactants on bioenzymes activity

Folin method was used to detect the activity of protease. Four biosurfactants, Saponin, Tea saponin, Sophorolipid, and Betaine, at 2% concentration and two bioenzymes, Trypsin and Papain, at 10 μg/mL concentration were pairwise mixed with volume ratio of 1:10 to make the solutions, which will be tested against the control group (Ctrl) that only contains protease to evaluate the effect on bioenzymes activity. The control group was constructed by replacing biosurfactants with distilled water. The OD_{680} value from Folin method reflects the activity of bioenzymes. The greater the OD_{680} value, the greater the bioenzymes activity. If the OD_{680} values from testing groups are greater than that of the Ctrl group, it indicates that the biosurfactants had no effects on enzyme activity.

(2) Effect of biosurfactants on color difference

Distilled water was used to dissolve biosurfactants to create solutions with concentrations of 1%, 3%, 5% and 7%. The Chinese Xuan paper was infiltrated in the biosurfactant solution for 30 min, transferred to distilled water and infiltrated for 5 min, dehumidified using filter paper, and then dried under room temperature for color difference detection.

Cleaning rate and paper mechanical properties evaluation

Using the bioenzymes and biosurfactants selected from the previous steps to prepare biological cleaning agent. 3% concentration bioenzymes and biosurfactants at four levels of concentrations, 1%, 3% 5%, 7%, were dissolved and mixed in distilled water. Mold-stained paper mockup samples were placed between two glass slides in a container containing the biological cleaning agent for 30 min at 35 °C. Then, the siphon device was used to take advantage of the fluidity of water to wash down the stains from paper fiber as well as residual from cleaning agent. The siphon device set up and mechanism are shown in Fig. 2. The cleaned subjects were dried using filter paper and split into two groups. One group was placed under 25 °C, 35% humidity environment to naturally dry for 16 h [22], and calculated the cleaning rate. The other group was placed in dark and dry environment at room temperature for 2 months before testing the physical and chemical properties of the paper material itself. Paper was cut according to specifications before testing and equilibrated for 48 h at (23 ± 1) °C, (50 ± 2)% RH under constant temperature and humidity. The cleaning rate was calculated using the following formula:

$$\text{Cleaning rate} = (m_1 - m_2)/(m_1 - m_0) \times 100\%;$$

m_0 is untreated paper quality; m_1 is the quality of the mold spots pattern before cleaning; m_2 is the quality of the mold spots pattern after cleaning, the unit is g. All the

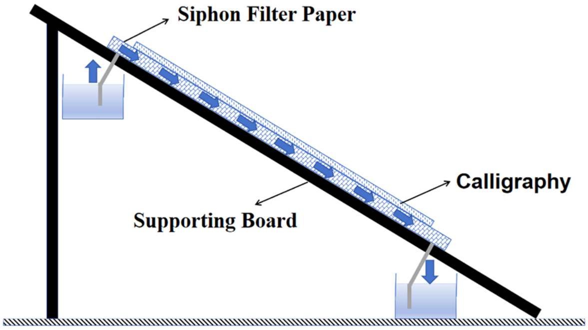

Fig. 2 Siphon device

samples were dried at 25 °C and 35% RH for 16 h before measurement to achieve the most suitable condition for the detection of various performance parameters.

Color difference evaluation method: in reference to "The Uniform Color Space and Color Difference Formula" (GB/T 7921-2008). Paper samples were cut into 5 cm × 5 cm pieces and are measured for color difference using WR-10 colorimeter. The measurement aperture was Ø4 mm, the short-term repeatability was $\Delta E \leq 0.03$, the operating temperature was 23 °C and the humidity was 50% RH. Each sample was averaged using a 5-point sampling method. The evaluation formula was:

$$\Delta \mathrm{E} = (\Delta \mathrm{L}^2 + \Delta \mathrm{a}^2 + \Delta \mathrm{b}^2)^{1/2}.$$

ΔE values for color difference change; ΔL for brightness changes; Δa for red and green differences; Δb for yellow and blue differences.

pH value evaluation method: in reference to "The Surface pH Measurement of Paper" (TAPPIT 529 om-04).

Tensile strength measurement method: cut mockup papers in 5 cm × 10 cm pieces, measure their mechanical stretching properties using the ZQ-21 tensile testing machine. The operating temperature is 23 °C, the humidity is 50% RH, the pulling speed is 20 mm/min, the test stroke is 200 mm, and the maximum load is 200 N.

Biosurfactants for paper protection experiment

To evaluate the protective performance of biosurfactants on paper, acidification simulation experiments and aging simulation experiments were conducted. Depending on the characteristic of biosurfactants being measured, paper was treated with biosurfactants either prior to or after the simulation experiments. Changes in pH value and tensile strength of the paper were measured to analyze the ability of biosurfactants to protect paper from acidification and aging.

Biosurfactants treatment method: Chinese Xuan paper was infiltrate in a 7% biosurfactants solution for 30 min, excess moisture was removed using filter paper, and the paper was air dried at room temperature.

Acidification simulation method: Aspirate 0.025 mL of 0.6 g/L concentration alum solution using pipettor and dispense on the Chinese Xuan paper at the center of each 1 cm × 1 cm square region. The solution can be naturally absorbed by the paper through capillary activity, brush was used to dissipate any bubbles and ensure 0.025 mL/cm^2 distribution on the surface. The paper was dried at room temperature to simulate the acidification process.

Aging simulation method: Chinese Xuan paper was placed in a dry heat aging box at an environmental temperature of (105 ± 2) °C. The paper was subjected to dry heat aging for 72 h, followed by 24 h of light-free storage to simulate the aging process.

Raman spectroscopy detection: The DXR 2xi micro-Raman imaging spectrometer [47] was used to detect spectral changes in paper fibers under different treatments. A 785 nm laser with 20 mW power was used for excitation. The spectral range was 50–3200 cm^{-1}. A 785 nm semiconductor laser served as the light source. The exposure time was 10 s, and the spectral resolution was set to 1 cm^{-1}. Raman spectroscopies were collected after a 1 min equilibration.

Protection ability in terms of acidification was categorized as follows: Blank group (Ctrl), representing untreated normal paper; Acidification treated group (Acidification), representing paper treated using the acidification simulation method; Biosurfactant treated group (Biosurfactant), representing paper treated with the selected biosurfactants. By comparing the Biosurfactant group with Ctrl group and the Acidification group, we can determine if biosurfactant can induce acidification. Normal paper treated with biosurfactant followed by acidification treatment (Biosurfactant + Acidification) was compared to the Ctrl and Acidification group to determine the biosurfactant's anti-acidification ability. Normal paper was first acidified and then treated with biosurfactant (Acidification + Biosurfactant) was compared to the Ctrl and Acidification group to determine the biosurfactant's deacidification ability.

Protection ability in terms of aging was categorized as follows: Blank group (Ctrl), representing untreated normal paper; Aging treated group (Aging), representing paper treated using the aging simulation method; Biosurfactant treated group (Biosurfactant), representing paper treated with the selected biosurfactants. By comparing the Biosurfactant group with Ctrl group and aged group, we can determine the effect of biosurfactant on paper aging. Normal paper treated with biosurfactant followed by aging treatment group (Biosurfactant + Aging) was compared to the Ctrl and Aging groups to determine the biosurfactant's anti-aging ability. Normal paper was first aged and then treated with biosurfactant (Aging + Biosurfactant) was compared to the Ctrl and Aging groups to determine the biosurfactant's ability to strengthen.

Since two biosurfactants (betaine and sophorolipid) were identified to have superior cleaning effects in the previous experiment, the protective performance testing regarding acidification and aging was conducted of both types of biosurfactants.

Data statistics and analysis

The amplified sequences were compared using Custal X software, and MEGA7.0 software was used to construct an N-J style molecular phylogenetic tree, while the bootstrap number was 1000 times statistical tests Data was processed using SPSS 20.0, diagrams were plotted in Prism8.0. And Raman spectroscopy was rendered using Origin 2017. Experiment for each group was repeated for three times and the mean ± standard deviation value of each group was recorded.

Experimental results and analysis

Results of sampling, purification, and morphological identification of the strains

The morphological and microscopic features of the colony of six mold strains isolated and purified from the samples taken from the ancient books were observed and recorded (Fig. 3).

Strain No. 1: colonies appear green color in the center, surrounded by white fluffy substance, with some difference in color between the front and the back, the back being blood red and the colonies growing extremely densely opaque. Strain No. 2: aerial mycelium and growing rapidly, colonies becoming larger in diameter after 3 days, with a small amount of exudate, mycelium dark black velutinous, raised in the center, with radial grooves. Strain No. 3: aerial mycelium is well developed, growing rapidly, after 3 days of culture, the mycelium is dark to black, and there is white mycelium at the edge, after 6 days the colony is dark green fluffy, with the increase of culture time, the white mycelium at the edge gradually disappears, and finally the colony is all dark green. Strain No. 4: white, transparent, and smooth colonies emerge after 3 days of incubation, later radially wrinkled, white fluffy, colonies are golden yellow, located in the center of the body or slightly off, the edge of the white mycelium gradually becomes lighter, the surface has a small amount of yellowish material exuding and soluble pigment, the back of the colony is light yellowish brown. Strain No. 5: after 2 days of incubation, the surface of the colony begins to appear slimy, opaque, creamy white in color, white in the middle and yellow at the edges on the back, and when later placed in the PDB for growth, the surface of the medium forms a wrinkled mold. Strain No. 6: slow growing, yellowish slimy surface, oval on the front, flat on the back, white opaque edges, creamy surface, white on the reverse, no fluffy mycelium. The microscopic examination shows that Fig. 3A has no branching, and the conidial peduncle is brown; Fig. 3B, D have the same structural characteristics: the microscopic mycelium is brown, with conidia, the spore area is black, with septum, and the terminal capsule produces two layers of small peduncles; Fig. 3C Conidia inverted rod-shaped, light brown, short conidial peduncle, solitary or in clusters, mostly unbranched, similar to the nutrient mycelium; Fig. 3E, F have the same structural characteristics, the ellipsoidal budding makes the spore capsule expanded, and the budding mesophyll to telogen.

Molecular biology results of the strains

The sequence length after PCR reaction for the six strains and strain identification results are summarized in Table 1. The 18S rDNA fragment lengths of fungi No. 1,

Table 1 Strain sequence length and accession number

Name	Sequence length (bp)	Strain name	Accession number
No. 1	1713	*Cladosporium*	KP997210
No. 2	1713	*Aspergillus niger*	GQ338836
No. 3	556	*Alternaria*	KC146356
No. 4	1711	*Aspergillus ochraceus* gene	AB008405
No. 5	1443	*Bacillus subtilis*	MF136610
No. 6	1454	*Paenibacillus polymyxa*	KC692186

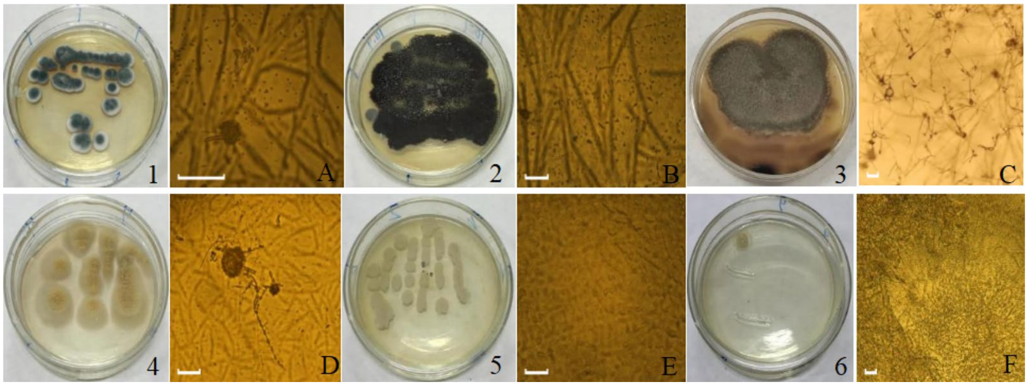

Fig. 3 Colony morphology (**1**–**6**) and microstructure diagrams of the strains (**A**–**F**). Scale bars = 30 µm

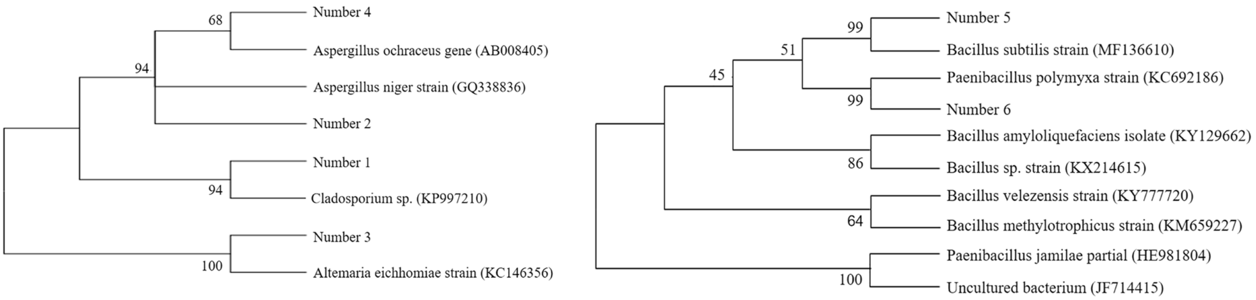

Fig. 4 Phylogenetic tree of 4 species of fungi and 2 species of bacteria. GenBank registry numbers in parentheses; numbers on branch points are percentages of spreading values

Table 2 Statistics of cellulose decomposition index of strains

Strain	Øc (cm)	Øh (cm)	CI
Cladosporium	0.80	1.20	1.50
Aspergillus niger	2.90	3.30	1.14
Aspergillus ochraceus gene	5.30	5.30	1.00
Alternaria	1.20	2.10	1.75
Bacillus subtilis	0.50	2.80	5.60
Paenibacillus polymyxa	0.50	1.30	2.60

No. 2 and No. 4 were all around 1800 bp, the 18S rDNA fragment length of fungus No. 3 was around 600 bp, and the 16S rDNA fragments of the two bacterial species were both around 1500 bp. The sequencing results were submitted to the Genbank database of NCBI for homology sequence search and construction of phylogenetic trees (Fig. 4).

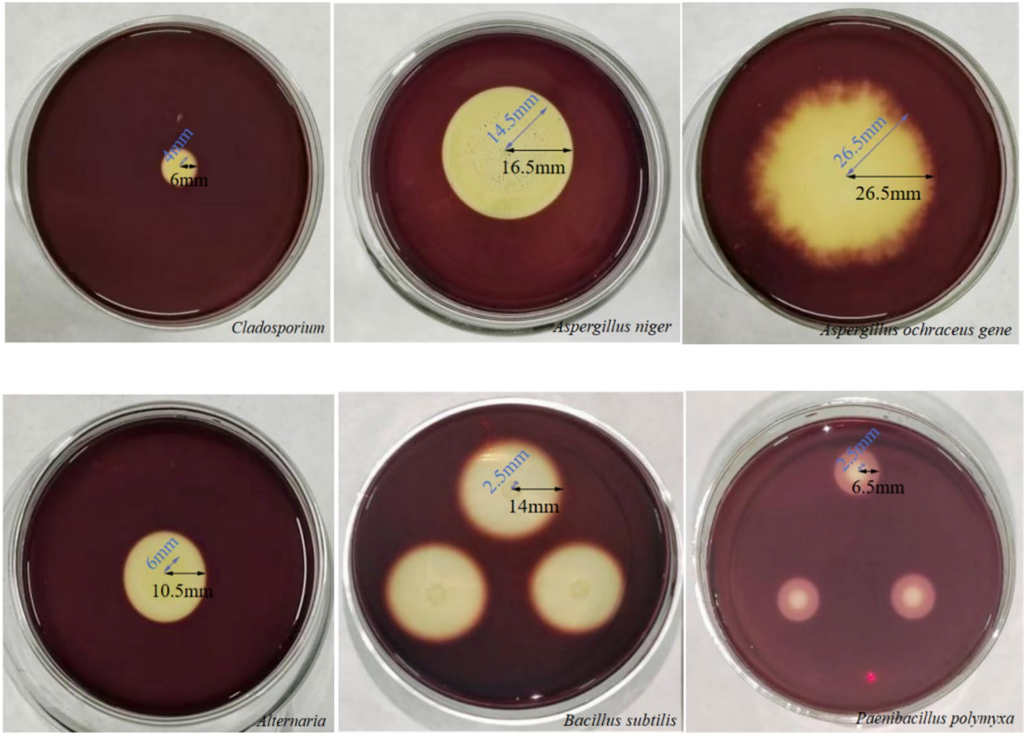

Fig. 5 Staining results of 6 strains

Cellulolytic activity results

The six strains isolated from the five ancient books were tested for their cellulose degradation ability using the Lugol's iodine staining method. The results showed (Table 2) that all six strains have cellulose degradation ability, and their respective cellulose degradation ability can be assessed by the diameter of the produced hydrolysis halo (Øh) and the diameter of the colony (Øc). The larger the ratio of Øh to Øc, the higher the cellulase activity produced by the strain or the greater the cellulase content. According to the staining results of the six strains (Fig. 5), the cellulolytic index of *Alternaria* (CI: 1.75) and *Cladosporium* sp. (CI: 1.50) was larger compared to *Aspergillus niger* (CI: 1.14) and *Aspergillus ochraceus gene* (CI: 1.00). It has been reported in the literature [31] that *Cladosporium* sp. has strong cellulose hydrolyzing activity, and the results of this paper are consistent with it. In the bacterial category, the colony diameter was relatively smaller than that of fungal strains, about 0.50 cm, but both produced a larger hydrolysis halo. In particular, *Bacillus subtilis*, with a hydrolytic halo diameter of about 2.80 cm and a cellulolytic index of 5.60. Overall, the cellulose degradation ability of bacteria was significantly higher than that of fungi.

Results of biological cleaning agent screening

Results of the biosurfactants selection

(1) Effect of biosurfactants on bioenzymes activity

In of the experimental groups with papain as the bioenzymes component (Fig. 6a), all four kinds of biosurfactant's OD_{680} values were higher than that of the Ctrl group. Saponin and tea saponin had a synergistic effect on the activity of papain, while sophorolipid and betaine had almost no effect; In of the experiment group with trypsin as the bioenzymes component (Fig. 6b), all four kinds of biosurfactants OD_{680} values were higher than that of the Ctrl group. Saponin and tea saponin had a synergistic effect on the activity of trypsin, while sophorolipid and betaine had almost no effect. Therefore, all four biosurfactants did not inhibit papain and trypsin activity.

(2) The color difference test results

In the cleaning process of precious paper-based material, the golden standard is the cleaning agent itself does not interfere with the color of the paper, with the acceptable color difference value below 1.50 [48]. Results show that the color difference increases as the concentration of each of the four biosurfactants increases, with the greatest difference occurring at 7% concentration. In general (Fig. 7), the color difference of saponin and tea saponin is much greater than that of sophorolipid and betaine. Color differences of saponin and tea saponin all exceed 1.5 unit when concentration is greater

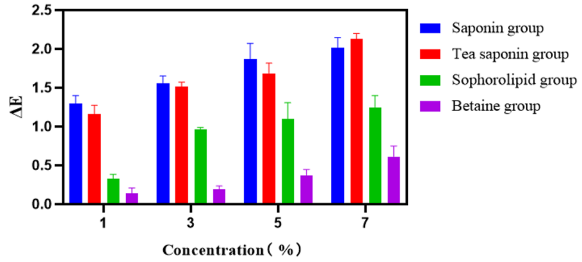

Fig. 7 Color difference values test results

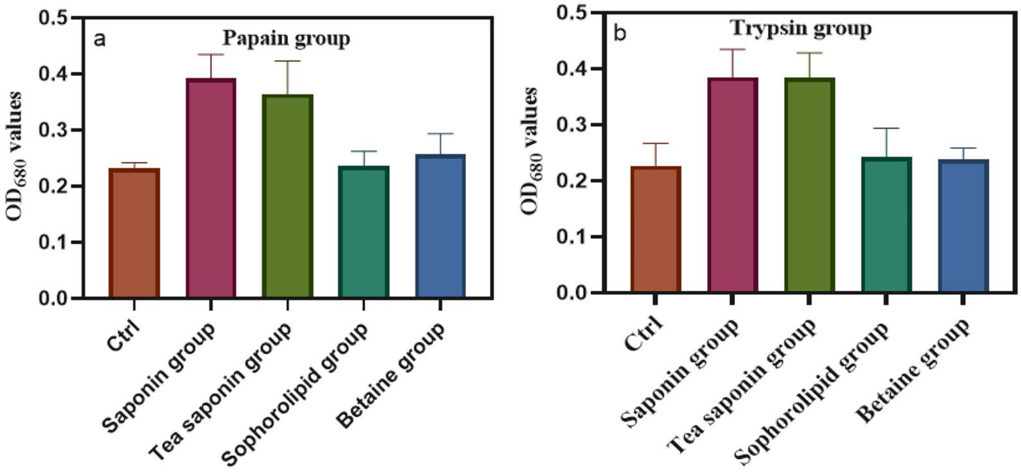

Fig. 6 OD_{680} values results

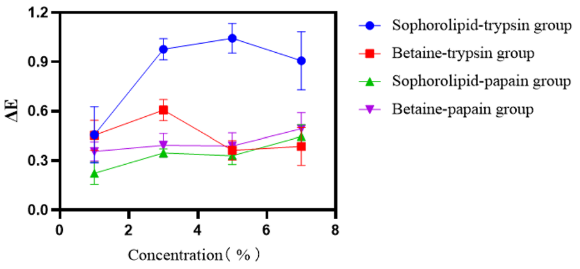

Fig. 8 Color difference test results

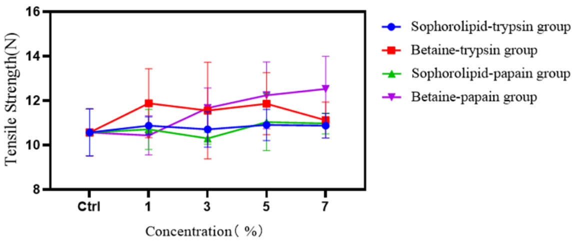

Fig. 10 Tensile strength test results

than or equal to 3%. On the other hand, the maximum color differences of sophorolipid and betaine, at 7% concentration, were around 0.30 and 0.14, which were well below 1.50. Thus, sophorolipid and betaine are more suitable as cleaning agents due to reasonable color difference.

Post-cleaning paper properties tests results

The selected biosurfactants (sophorolipid and betaine) and bioenzymes (papain and trypsin) were separately mixed to create four types of biological cleaning agents, which were then applied to mold stains paper mockup samples. In terms of color difference detection, as shown in Fig. 8, the ΔE values of all groups were less than 1.5, which is imperceptible to the human eye and does not affect visual appearance, meeting the requirements of cultural relic preservation. Among them, the combination of sophorolipid-papain exhibited the smallest ΔE, followed by betaine-papain. In terms of pH detection, as depicted in Fig. 9, the pH values of the treatment groups were as follows: betaine–papain (7.00 to 7.45) less than betaine–trypsin (7.11 to 7.65) less than sophorolipid–trypsin (7.33 to 7.65) less than sophorolipid–papain (7.48 to 7.79). All values showed a tendency toward weak alkalinity, adhering to the ideal pH requirements for paper preservation. Zhang [30] reported that cleaning by 3% papain does not cause paper acidification, and the pH of the paper rises from about 6.40 to 6.75 after cleaning. The results of this paper also proved that the cleaning of papain and biosurfactant compound does not cause paper acidification, and shows a better tendency to be weakly alkaline, and the pH is above 7.0 in all cases. Regarding the tensile strength test shown in Fig. 10, compared to the Ctrl group, after treatment with betaine–papain, the tensile strength gradually increased with increasing betaine concentration, reaching a maximum value of 14.4 N at a betaine concentration of 7%. After treatment with betaine–trypsin, the tensile strength fluctuated between 9.5 and 13.9 N. The tensile strength values of this group were higher than those of sophorolipid–trypsin, while the ranges of tensile strength values for sophorolipid–trypsin were quite similar, ranging from 9.4 to 11.5 N and 9.8 to 12.5 N, respectively. It can be observed that the tensile strength of each group remained within a reasonable range, without affecting the mechanical properties of the paper, in compliance with cleaning standards.

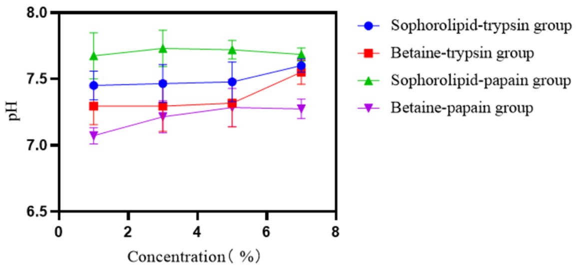

Fig. 9 pH test results

From the above experimental results, sophorolipid and betaine, at all four concentration levels, when mixed with 3% papain and trypsin, achieved color difference, pH and tensile strength of the paper samples within reasonable range, which met the cleaning standard.

The cleaning agent cleaning results

The study found that the cleaning rate increased gradually as the concentration of the biosurfactants increased, and there were still observable stain residues on the paper surface at 1%, 3% and 5% biosurfactants concentrations. At 7% biosurfactants concentration, all four biosurfactants achieved satisfactory cleaning result, the cleaning effect was particularly better in the sophorolipid–papain group and the betaine-papain group, with over 50% cleaning rates (Table 3) and almost no conspicuous stain residue. In accordance with the principle “minimum intervention”, experimental results showed that biological cleaning agent composed of 7% of sophorolipid or betaine with 3% papain applied at 35 °C for 30 min is the best treatment scheme.

Mold-stained paper mockup samples contaminated with *Aspergillus niger* were cleaned by applying sophorolipid–papain and betaine–papain (Fig. 11). Black

Table 3 Cleaning rate results

Biosurfactant concentration	Quality of mold-stained paper mockup samples before and after cleaning	Group (average across three groups)			
		Sophorolipid–papain	Betaine–papain	Sophorolipid–trypsin	Betaine–trypsin
1%	Before cleaning (g)	0.0869	0.0898	0.0878	0.0854
	After cleaning (g)	0.0851	0.0881	0.0858	0.0834
	Cleaning rate	26.09%	19.32%	20.41%	23.81%
3%	Before cleaning (g)	0.0871	0.0868	0.0898	0.0907
	After cleaning (g)	0.0841	0.0857	0.0866	0.0872
	Cleaning rate	35.81%	18.97%	27.12%	25.55%
5%	Before cleaning (g)	0.0881	0.0862	0.0878	0.0848
	After cleaning (g)	0.0852	0.0846	0.0856	0.0819
	Cleaning rate	42.25%	30.77%	22.45%	37.18%
7%	Before cleaning (g)	0.0883	0.0907	0.0848	0.0820
	After cleaning (g)	0.0838	0.0853	0.0815	0.0796
	Cleaning rate	54.22%	55.67%	48.53%	48.00%

"Weight before or after cleaning (g)" refers to the constant weight after drying for 16 h at 25 °C and 35% RH humidity

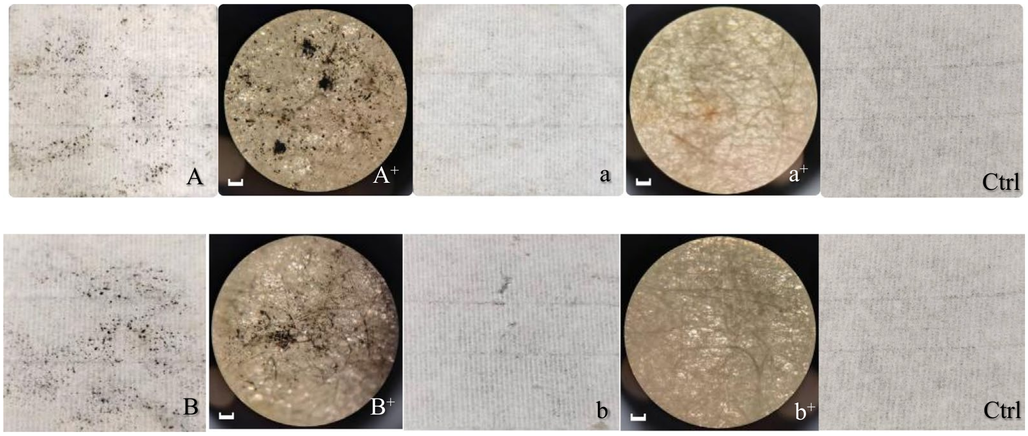

Fig. 11 Cleaning effect of mold contaminated paper. **A** and **B** are biological contamination mockups; **A+** and **B+** are ×10 micrographs of biological contamination mockups; **a** and **a+** are paper after cleaning with 7% sophorolipids and 3% papain and ×10 micrographs after cleaning; **b** and **b+** are paper after cleaning with 7% betaine and 3% papain and ×10 micrographs after cleaning; Ctrl is paper without biological contamination (scale bars = 30 µm)

mold stains in the form of blotches with varying sizes were observed on the surface of the paper before cleaning. Microscopically, a large number of mycelia was present between the paper fibers and wrapped around the outside of the cellulose in a dense and complex distribution pattern. After cleaning, the dark mold stains faded significantly, light-colored stains are also almost completely removed, only a small number of stubborn stains remain in the fiber. At the same time, the use of sophorolipid and betaine in combination with papain can effectively remove yellowing stains due to prolonged contamination and natural aging and bring the cleaned paper closer to its original appearance.

Biosurfactants paper protective results

Screening showed that Betaine and Sophorolipid are good candidates for the biosurfactants component of a biological cleaning agent, thus their protective ability were investigated. The pH of the blank group (Ctrl) in Fig. 12a was around 7.45 and the pH of the acidification treatment group (Acidification) was around 6.12. In comparison to the blank group (Ctrl), no acidification of the paper was observed in the sophorolipid and betaine treated groups. All groups showed significant protective effects compared to the acidification treatment group, with paper pH ranging from 7.15 to 7.54 after deacidification. In Fig. 12b, the blank group (Ctrl)

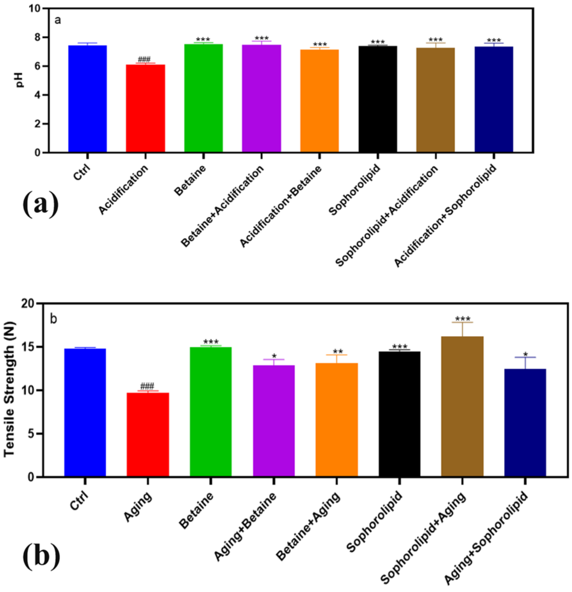

Fig. 12 Biosurfactants on paper the influence of **a** deacidification, anti-acidification, **b** anti-aging and reinforcement abilities. $^{\#}$Compared with the blank group difference, *compared with acidification or aging group difference, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, *in the same way

had a tensile strength of around 14.76 N and the aging treatment group (Aging) had a tensile strength of around 9.73 N. In comparison to the blank group (Ctrl), no aging of the paper was observed in the sophorolipid and betaine treated groups. Compared to the aging treatment groups, the (Aging + Sophorolipid) and (Aging + Betaine) groups were significantly more protective, with tensions ranging from 12.43 to 12.83 N after treatment, while the other treatment groups all achieved significant protection effects, with tensions ranging from 13.15 to 16.16 N after treatment. Literatures have reported that biosurfactant has the advantage of acid and salt resistance [49] can increase the degree of paper polymerization [50], and the above experiments also proved that the two biosurfactants are resistant to acidic environment and can provide reinforcement to the paper fiber structure. The results indicate that sophorolipid and betaine can assist with deacidification, anti-acidification, anti-aging, and reinforcement of paper.

According to the Raman spectroscopy, under acidification condition (Fig. 13a), the absorption intensity of paper at 280–480, 847–1164, 2825–2970 cm^{-1} decreased, indicating that the paper fiber structure changed significantly during the acidification process. Compared to the acidification treatment group, several of the above absorption peaks appeared enhanced to varying degrees in the other treatment groups, indicating that the use of betaine and sophorolipid reagents can effectively resist the damage to paper fiber due to acidification, which may be due to the quaternary ammonium groups in the two biosurfactants containing basic nitrogen atoms, along with the hydroxyl cationic and anionic groups are acid resistant and less prone to acidification. In the aging condition (Fig. 13b), The absorption intensity of the paper at 232–480, 835–1198, 2813–2970 cm^{-1} was significantly enhanced, indicating that the fiber structure of the paper changed significantly during the aging process, which is consistent with the literature reports [51]. Compared with the aging treatment group, the above absorption peaks of the other treatment groups decreased, falling in the range between the control group and the aging group, indicating that

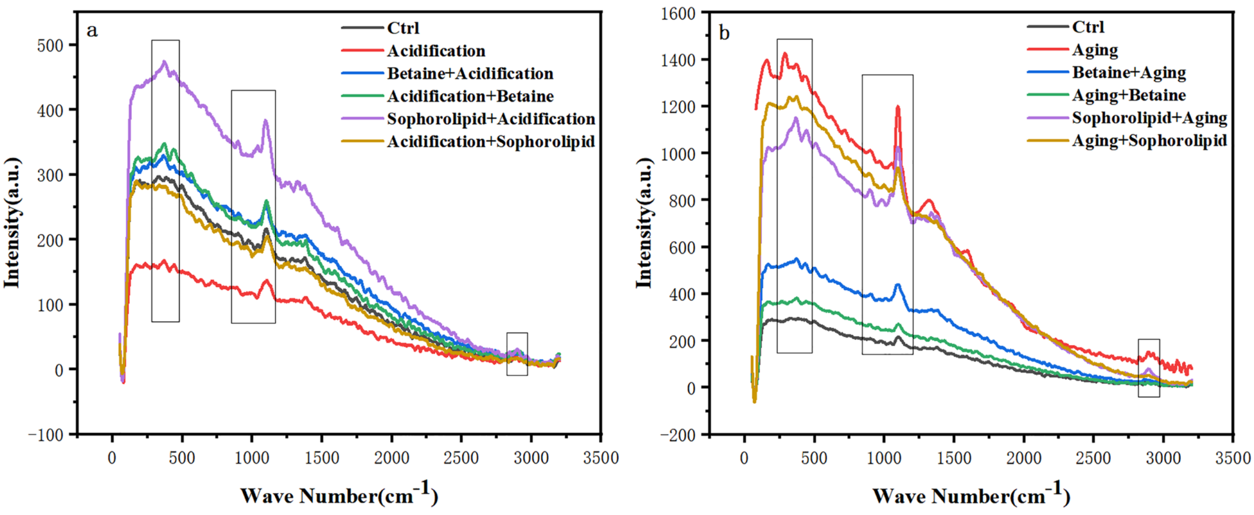

Fig. 13 Under the 780 nm paper acidification condition (**a**) and aging condition (**b**) study of Raman spectrum

the use of sophorolipid and betaine reagents were effective in resisting the damaging effects of dry heat aging on paper fibers, probably due to the presence of a large number of glycosidic bonds and long chain hydroxyl groups in both, which promoted the formation of hydrogen bonds and van der Waals forces in the paper, Meng [50] also reported that the polymerization of paper after Sophorolipid cleaning was higher than that of the control group, which may be the reason for providing protection for paper fibers.

Conclusion

This study revived and identified six contaminating mold strains from five ancient books, such as the "The True Interpretation of A Journey to the West" (Qing Dynasty). Research was conducted to investigate their harmful effects and remedy approaches. Several strains exhibited strong cellulose degradation activity, leading to the development of a biological cleaning agent by combining bioenzymes and biosurfactants. Papain, with high efficiency and specificity, can effectively decomposed large protein molecules in mold stains, while the biosurfactants sophorolipid and betaine can effectively reduce the adhesion of mold stains to paper fibers. By combining these two components in a scientifically determined ratio, notable results were achieved in cleaning mold contaminated paper samples. Using a cleaning scheme of 3% papain, 7% biosurfactants (sophorolipid or betaine), and distilled water as the solvent, carried out at 35 °C for 30 min, a cleaning rate of over 50% for paper mold stains was achieved. The impact on paper color difference, pH value, and tensile strength remained within reasonable ranges. Additionally, sophorolipid and betaine exhibited properties such as deacidification, anti-acidification, anti-aging, and reinforcement for paper. The biological cleaning agent not only can effectively remove mold stains but is also low-cost and environmentally friendly. Moreover, the agent is non-toxic and harmless to personnel's health. The biological cleaning agent proposed in this study holds the potential to provide a green, safe, and efficient cleaning solution for mold-contamination treatment for paper products.

Supplementary Information

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Additional file 1. 1. Bioenzyme Selection. 2. Selection of Solvent. 3. Application Condition (enzyme concentration, temperature, and time duration) Selection.

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Author contributions

The research was designed by SB and CL. Experiments were conducted by QM, XL, and JG. Data were processed and interpreted by QM and CL. All authors wrote and revised the main text and contributed to the study conceptualization. All authors have read and agreed to the published version of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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