Study on Lipopeptide Antifungal Agents for Cultural Relics

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Abstract: In the field of cultural relics protection, the common physical and chemical anti-mildew methods are far from satisfaction. This article aims to testify the anti-fungi effects of lipopeptides from the fermentation liquid produced by Bacillus subtilis strains ⑤-1 and G61. The results of TLC chromatography proved that the anti-fungi activity resulted in fengycin and other lipopetides and we combined multivariable analysis with Oxford plate experiments to obtain the best fermentation formula. Lipopetide-mildew-inhibitor was confirmed both safe and effective by physical simulation and the anti-mildew tests on cultural relics, which proved lipopetide was an idealistic mildew-inhibitor in cultural relic protection.

1. Introduction

Cultural relics are the collective name of relics or relics with historical, cultural, aesthetic, scientific or primitive environmental value [1]. The collection of cultural relics can be divided into three categories according to the material: first, organic cultural relics, including bamboo, wood, lacquerware, silk fabrics, bone horns and building components. Second, inorganic non-metallic cultural relics, including masonry, glass, glaze, ceramics and so on. Third, inorganic metal cultural relics [2]. According to the different materials, the mechanism and repair methods of cultural relics are different. Therefore, cultural relics protection workers can determine the more effective cultural relic protection and restoration scheme by comprehensively analyzing the performance and destruction mechanism of cultural relic materials [3].

As an example, moulds are a kind of fungi that can form multicellular mycelia [4]. They have strong metabolic ability for many kinds of organic substances such as cellulose, lignin and protein. The reproductive capacity of fungi is very strong, which can be either truncated by mycelial fragments or sexually or asexually reproduced by spores. They are widely distributed in various habitats. If stored in humid, high temperature and poorly ventilated environment for a long time, fungi can easily grow on the surface of cultural relics, especially organic ones. The damage of fungi to cultural relics can be divided into three aspects. One is that the hydrolytic enzymes (such as proteases and cellulases) secreted in the metabolism of bacteria have a destructive effect on the material of cultural relics. Second, the organic acids or pigments secreted by mold growth damage the structure and color of the cultural relics. The third is that the mold causes localized moisture and heat during the metabolism of the surface of the cultural relics, further aggravating the mildew [5]. Considering that the management of cultural relics collection involves a wide range of knowledge and technical skills, in addition, local (city, county) museums have limited storage conditions and high cost of optimizing collection facilities [6]. so how to design safe, efficient and cheap cultural relics The agent is the focus of the current anti-mildew and restoration work.

Although there are many kinds of substances which have been proved to have fungicidal effects, considering the particularity of cultural relics protection, it is required that antifungal agents for cultural relics should not be strong oxidants and reductants with more active chemical properties. Commonly used antifungal agents for cultural relics include ethylene oxide, mildew enemies, p-nitrophenol, etc [7]. Ethylene oxide is flammable and explosive, and it is difficult to popularize in local museums because of the high cost of fumigation and disinfection and the difficulty of operation [8]. The mildew (the main component is 1, 2-benzisothiazolinone) is a moderately

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accumulating toxic substance, and long-term use is detrimental to the health of cultural relics protection personnel [9]. P-nitrophenol is easy to use and strong, but it will darken in the air due to oxidation, which will affect the color of cultural relics [10].

Protecting cultural relics through biotechnology, that is, using metabolites or induced synthetic products of some microorganisms, has the advantages of pollution, simple equipment, low cost and easy operation. It has played an important role in the field of wood products and silk reinforcement, cultural relics cleaning [11]. Therefore, the use of biotechnology for cultural relics is theoretically feasible.

Lipopeptide antibiotics are secondary metabolites synthesized by Gram-positive bacteria. They contain both peptide and aliphatic hydrocarbon chains in structure. At present, the research on lipopeptides produced by Bacillus subtilis has been more in-depth [12]. The main components of lipopeptides are surface active factors (Suefactins), Fengycins, Lichenysin and so on. It has been widely used in the fields of antimicrobial, antiviral and cancer therapy. Lipid peptide surfactants have high thermal stability and protease resistance. In actual production, cellulase and protease which can decompose organic matter in the extract can be inactivated or removed by purification, heating, etc. Peptide materials are used to lay the foundation for antifungal artifacts [13] [14].

Tian Jinying, Wu Wangting, Tang Huan and others sampled fungi from cultural relics in the museum. Although the fungi species of cultural relics vary slightly according to the material of cultural relics. However, most of them belong to Penicillium, Aspergillus, Trichoderma and Paecilomyces; therefore, we hope to explore the Penicillium glaucum and Aspergillus niger by exploring the lipopeptide-containing fermentation broth produced by Bacillus. The inhibitory effect is to find a lipopeptide antifungal formulation with the best antibacterial effect [15].

2. Materials and Methods

2.1 Source and Preservation of Materials

Penicillium flavum and Aspergillus niger were isolated and preserved in our laboratory. Bacillus subtilis G61 (CGMCC No. 16222), Bacillus subtilis Ordinary cow leather, bamboo slip, rice paper, natural silk (Founder Silk Co, Ltd.). African woodcarving (collection of the National Museum).

2.2 Preparation of Bacillus Antifungal Fermentation Broth

Inoculate strain G61 and 5-1 incubate in LB liquid medium at 37 ℃ and 250rpm for 12h with OD600≈4.5. 3% inoculum was added to 20mL of Landy medium (L- glutamic acid 5g, D- glucose 20g, Yeast Extract 1g, KCl 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, KH_2PO_4 1g, FeSO 4.7H2O 0.00015 g, CuSO 4.5H2O0.00016 g, $MnSO_4$ 0.005g, L- phenylalanine 0.002g, pH=7.0, H_2O 1000 mL) was cultured at 37 ℃ and 170rpm for different time gradients (12h, 24h, 30h, 36h, 38h, 42h, 48h). After the fermentation was completed, the fermentation broth was centrifuged at 4.degree. C. at 120min00rpm for 20 minutes to collect the supernatant, which was filtered by a 0.22. m u.m PVDF filter.

2.3 Crude Purification and Identification of Lipopeptide Antimicrobial Substances

The filtrate obtained in 1.2 was adjusted to pH=2.0 with 6mol L-1HCl, stirred with HCl and spent the night at 4 C. The precipitation was collected by centrifugation at 4 and 8000rpm for 20 minutes. The precipitation was dissolved in methanol, stirred for 2 hours, centrifuged for 20 minutes at 4 C and 8000rpm, supernatant was collected and concentrated by rotating evaporation in 30 mL water bath. Separation and identification of lipopeptide antibiotics by thin layer chromatography (TLC): the chromatographic solution (chloroform 13mL, methanol 5mL, ddH2O800μL) was immersed on a silica gel plate for 1h, scribbled with a capillary pipette, sealed and chromatographed for 30min. After drying, the surface was sprayed with a 0.5% aqueous solution of ninhydrin to develop an acid solution, and the obtained lipopeptide was extracted with an appropriate ratio of methanol, vortexed for 20 min, centrifuged at 12,000 rpm, and the supernatant was collected.

3. Determination of Antimicrobial Activity of Lipopeptides

3.1 Flat Plate Detection of Lipopeptides

Penicillium flavum and Aspergillus niger were inoculated on PDA medium. Lipopeptides purified by TLC and primary fermentation broth of different strains and different fermentation time were diluted according to specific concentration gradient. The inhibitory activity of the original fermentation broth and TLC purified product against Penicillium chrysogenum and Aspergillus niger was determined by the Oxford Cup method, and cultured at 37 ° C for 2 to 3 days to measure the diameter of the inhibition zone.

3.2 Antimicrobial Activity of Lipopeptides in Simulated Cultural Relics

Silk, leather or bamboo slips of the same size were sterilized by ultraviolet irradiation for 2 hours and then immersed in lipopeptides extracted by TLC for 5 minutes. The control group was immersed in the same amount of ddH2O for 5 min. After drying, the two groups were placed on PDA medium inoculated with P. chrysogenum or Aspergillus niger spores, and cultured at 37 °C for 2~3 days to observe the infection of the mold on the surface of the sample.

3.3 Anti-mildew Test of Lipopeptides in Cultural Relics

Some African woodcarving and other cultural relics that have been breeding mould in the National Museum were treated. First, 75% ethanol was smeared to remove the breeding mould. Then, lipopeptide substances were dipped in degreased cotton and smeared on the breeding mould parts twice. The processed cultural relics were stored in a constant temperature (20℃), constant humidity (50%) warehouse for 6 months, and the mold growth at the same site was observed.

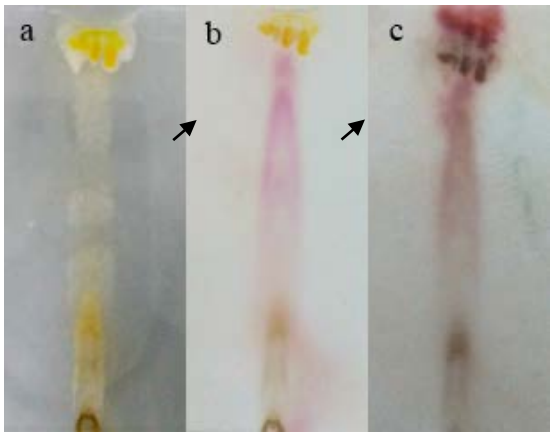
3.4 Prevention of Pollution

All bacteriostasis experiments were completed in a special ultra-clean platform. Before each experiment, the laboratory was irradiated by ultraviolet light for a long time, and the ultra-clean platform was sterilized by ultraviolet light for more than 30 minutes. In the anti-mildew experiment, the reagents were filtered by 0.22 μm PVDF filter in advance, boiled for 10 minutes, and then applied after fully cooling. During the above experiment, the experimental personnel uniformly wear the special experimental clothes after disinfection, wearing masks, disposable gloves and hats. All the experimental consumables are autoclaved to avoid contamination by bacteria.

4. Result

4.1 Isolation and Identification of Lipopeptides

The crude lipopeptide extracts of Bacillus subtilis strain 5-1 and G61 were analyzed by TLC. Lipopeptides sprayed directly with ninhydrin showed orange at the corresponding position of the sample and purple red at the edge of the in situ acidolysis band (see Fig. 1). It is indicated that the lipopeptides of Bacillus sp. strains 5-1 and G61 contain fengycin-like cyclic lipopeptides. In Figure 1, the color distribution of the dyed strips is uneven and there is a tailing phenomenon, indicating that the lipopeptides in the extracts of the two B. subtilis fermentation broths are not single. It is consistent with the fact that lipopeptides often coexist in the form of multiple homologs [11]. The lipopeptides on the silica gel plate were manually recovered and extracted with methanol for later use.

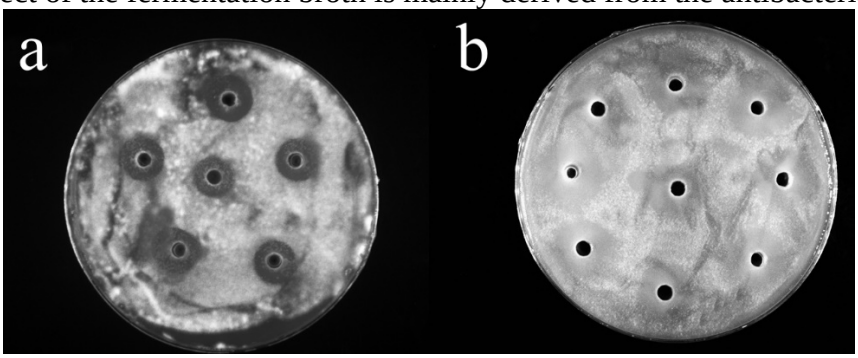


a: Lipopeptide extract b: Lipopeptide extract + ninhydrin c: Lipopeptide extract + ninhydrin + in situ acidolysis

Fig.1. the TLC color result of lipopeptides extraction

4.2 Detection of Antibacterial Activity of Lipopeptides

The antimicrobial activities of lipopeptides extracted from fermentation broth of Bacillus subtilis 5-1 and G61 against Penicillium spp. and Aspergillus niger were determined by Oxford cup method (see figure 2). The results showed that the fermentation broth of Bacillus subtilis and lipopeptide extract had strong antibacterial activity against Penicillium and Aspergillus niger, and the lipopeptide extract could produce a larger and more regular transparent inhibition zone. The bacteriostatic effect of the fermentation broth is mainly derived from the antibacterial lipopeptide.



a: Inhibitory effects of lipopeptides extracted from fermentation broth of Bacillus subtilis 5-1 on Penicillium sp.

b: Inhibition of Penicillium spp. by Bacillus subtilis 5-1fermentation broth

Fig.2. the anti-fungi effects of lipopeptides and fermentation liquid generated by Bacillus subtilis

The Oxford cup antifungal experiment of Bacillus subtilis strain 5-1 and G61 fermentation broth and its crude lipopeptide extract was carried out. The results showed that the inhibitory effect of 5-1 fermentation broth and lipopeptide on Penicillium (left) was not obvious, but on Aspergillus Niger (right) was better. The optimum fermentation time was 30 hours (fig. 3). The fermentation broth and crude extract of G61 strain had obvious effects on Penicillium (left) and Aspergillus niger (right), and the optimal fermentation time was 38h (Fig. 4). For the specific mold, the inhibition curve of the fermentation broth of each Bacillus strain is basically consistent with the general trend of the antibacterial curve of the crude extract, which further confirms that the antifungal activity in the fermentation broth of Bacillus is derived from lipopeptide.

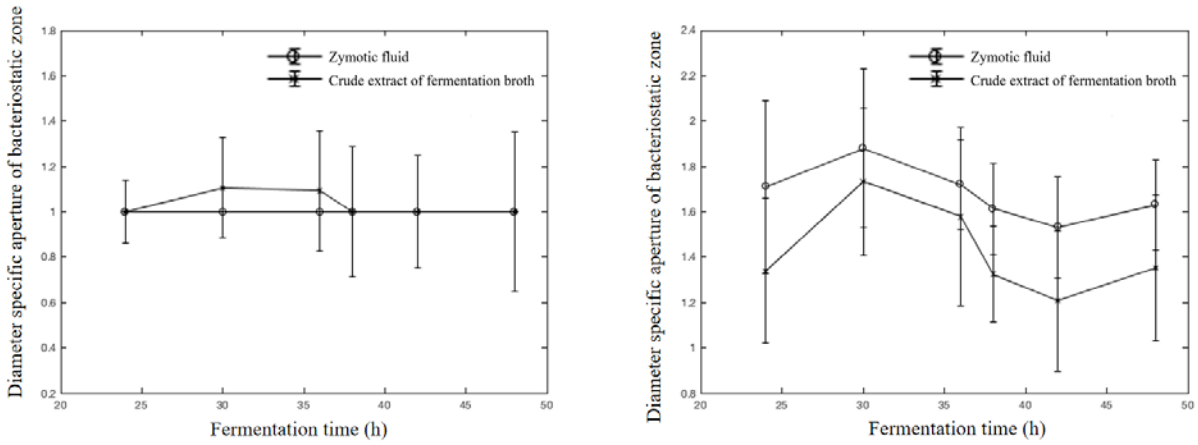


Fig.3. the anti-fungi effects of fermentation liquid and lipopeptides of *B. subtilis* strains 5-1 with different fermentation time on *P. glaucum*, error bars representing standard error.

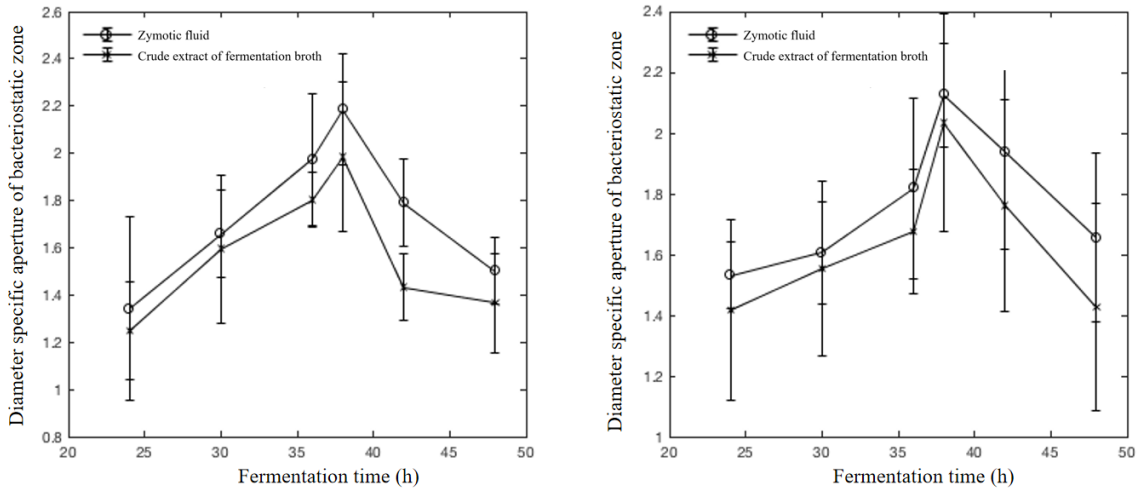


Fig.4. the anti-fungi effects of fermentation liquid and lipopeptides of *B. subtilis* strains G61 with different fermentation time on P. glaucum, error bars representing standard error.

4.3 Screening of the Optimum Production Process of Lipopeptide Antifungal Agents

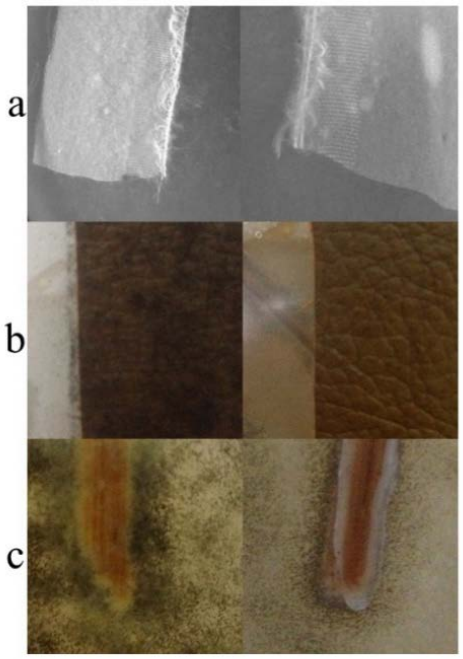
Bacillus subtilis strain 5-1 and G61 mixed with fermentation broth obtained under the above optimal fermentation conditions showed that the fermentation broth of strain 5-1 and strain G61 mixed with Penicillium or Aspergillus Niger at a volume ratio of 3:5 showed the best bacteriostatic effect (Table 1). Therefore, we determined this mixed fermentation broth as the optimal formulation of the lipopeptide anti-mold agent, and used this formula to carry out the physical antibacterial test.

Table 1 Synergistic Inhibition of Penicillium and Aspergillus Niger by 5-1 and G61 Fermentation Broth

5-1:G61 Volume ratio	Diameter Specific Pore Diameter of Bacteriostasis Circle for Penicillium spp. (measured value)	Diameter Specific Pore Diameter of Bacteriostasis Circle for Aspergillus Niger (measured value)
08	1.559	2.941
17	1.706	3.256
26	1.735	4.086
35	1.794	4.441
44	1.647	4.029
53	1.529	4.294
62	1.647	3.535
71	1.529	3.725
80	1.559	3.700

4.4 Identification of in-kind bacteriostasis of lipopeptide antifungal agents

Lipopeptide fungicides were applied to silk, leather, bamboo slips and other organic matter samples. Penicillium Niger and Aspergillus Niger grew rapidly in the control dish. After 2 days of cultivation, the dish was covered with fungi. Serious fungi grew on the surface of the sample. In the Petri dishes soaked with lipopeptide antifungal agents, Penicillium Niger and Aspergillus Niger hardly grew after 2 days or formed transparent inhibitory rings on the surface of samples (Fig. 5). After 7 days of continuous culture, the mold basically covered the surface of the physical sample, but the growth condition was significantly inhibited. The lipopeptide antifungal agent prepared from the fermentation broth of Bacillus subtilis strains 5-1 and G61 can effectively inhibit the growth of Penicillium and Aspergillus niger.



The samples and Aspergillus niger were cultured at 37 ~C for 2 days.
A silk sample plate bioassay; B leather sample plate bioassay; C bamboo slip sample plate bioassay

Fig.5. Anti-fungi activity against *Aspergillus nigerv* on organic samples of lipopeptide-mildew-inhibitor

In order to verify the feasibility and practicability of the application of lipopeptide antifungal agents in the field of cultural relics protection, the mouldy African woodcarving collected in the National Museum was selected for antifungal treatment. The surface mould was isolated and cultured in the laboratory and proved to be Penicillium. The wood carvings after anti-mildew treatment did not show any obvious mildew growth after six months. There is also no visible change in the surface material or a perceptible change in the surface (Fig. 6). The lipopeptide antifungal agent after high temperature treatment does not contain active cellulase or protease, so the lipopeptide antifungal agent is safe for cultural relics.

Fig.6. Anti-fungi activity against *Aspergillus nigerv* on cultural relics of lipopeptide-mildew-inhibitor

5. Discuss

Traditional anti-mildew methods are mainly confined to physical (control environment to prevent mildew breeding) and chemical (chemical agents to prevent or kill mildew). Although good anti-mildew and anti-mildew effect can be achieved, the cost and operation difficulty are not satisfactory, and there are problems such as easy residue and environmental pollution. The antifungal mechanism of lipopeptides is to block the synthesis of fungal cell wall or inhibit the respiratory metabolism of fungi, so it will not have a greater impact on the organic cultural relics themselves. Since lipopeptides have high heat resistance, residual bio-enzymes can be inactivated by high-temperature treatment without impairing the antibacterial activity of lipopeptides. In addition, as a biological agent, lipopeptides have the natural advantages of environmental protection, degradability and low toxicity, and are an ideal anti-mold substance for cultural relics.

On the other hand, there are many studies on lipopeptides produced by Bacillus, but previous reports focused on the analysis of the properties and components of lipopeptides. There is little research on the effective fermentation technology and extended application of antimycoprotein in academia. In this experiment, the lipopeptides extracted from the fermentation broth of Bacillus strains 5-1 and G61 were used. After repeated experiments, the correlation curves between the antifungal activity of lipopeptides and fermentation time were explored, and the two antifungal peptides were obtained. Good synergistic formula.

In the anti-mildew experiment, by comparing the differences of mold growth, surface gloss, hand feeling and appearance before and after the anti-mildew treatment of physical samples and cultural relics woodcarving, it is basically proved that the new antimildew agent of lipopeptides is safe and effective. It proves the feasibility of applying lipopeptides to the anti-mildew work of organic cultural relics, and also proves the practicability and reference of the anti-mildew activity curve data obtained from the Oxford Cup experiment. In the actual work, according to the main types of mold on the surface of the cultural relics, refer to the preparation and application method of the anti-fungal agent in this study. Combined with the preservation of cultural relics, targeted and safe selection of safe and efficient mold removal strategies will help the development and implementation of cultural relics prevention programs.

6. Conclusion

This experiment focuses on the optimization of production strategy and practical application of lipopeptide from Bacillus. In the early stage, comprehensive and reliable experimental data were obtained by Oxford Cup method. The aperture ratio of bacteriostasis circle was used as an important index in the experiment, and the change of bacteriostasis activity under different fermentation time and concentration combinations was analyzed by means of multi-factor analysis. The purpose of screening the optimum production process and formulation of lipopeptide antifungal agents was achieved, and a new field of antifungal agents for biological cultural relics was opened up. In addition, the results of this study will also evaluate the performance of other new anti-fungal agents and their application in the protection of organic cultural relics. And further research on biological, physical and chemical comprehensive cultural relics prevention strategies have a good reference and reference value.

Acknowledgement

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