Antimicrobial Agent Effectiveness in Fish Glue Prepared by Heat Treatment and Enzymatic Hydrolysis of Swim Bladders

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Fish glue prepared from swim bladders has been used in China to glue wooden parts together since ancient times. It is also used as an important natural glue for wooden artifact and building restoration, musical instrument fabrication, as well as many other fields. Microorganism contamination is a major concern for fish glue preservation. In this research, fish glue was prepared from swim bladders using two methods, namely, heat treatment and enzymatic hydrolysis. Then, the molds that germinated in both samples were analyzed. Light microscope and scanning electron microscopy (SEM) observations of the molds' hypha morphology identified *Alternaria* in both glue samples. The antimicrobial efficiencies of borax, sodium diacetate, and Antim AL-D (an organic/inorganic composite antimicrobial) were then compared. The results demonstrated that all antimicrobial agents in the research effectively inhibited *Alternaria* germination in both fish glue samples. A 0.3% (by weight) solution of Antim AL-D was sufficient for preserving fish glue. As for borax and sodium diacetate, the addition of a minimum of 1.0% was adequate to inhibit *Alternaria* growth. Results also revealed that the addition of Antim AL-D minimally affected the shear strength of glued wooden parts.

Keywords: Natural adhesive; Swim bladder; Biomaterials; Wood bonding; Antimicrobial

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INTRODUCTION

The strength of adhesive bonding has always been a concern for the wood furniture industry. For centuries, nearly all furniture glues used for bonding wood parts have been obtained from nature (Lambuth 2003). Museum pieces dating back to Ancient Egypt confirm that animal or fish glues were used by furniture makers of that era (Tout 2000). In China, evidence of prehistoric bovine adhesive use comes from a ca. 3500-year-old bone inlaid wooden artifact collected from the Xiaohe Cemetery, Xinjiang, China (Rao *et al*. 2015). However, the earliest description of using animal glues as bonding materials, such as deer glue, ox glue, and fish glue, can be traced to the Chinese book of "*The Rites of Zhou· Dong Guan Kao Gong Ji*" (Warring States period 475 BC to 221 BC).

With the prevalence of synthetic adhesives since the mid-20th century, the market for natural adhesives was decreased. Recently, due to health-related concerns regarding carcinogenic formaldehyde released from some synthetic adhesives, natural alternatives are being pursued.

Among ancient animal glues, fish glue prepared from swim bladders is chiefly used as a wood gluing adhesive in China, while it is usually called isinglass and used for

clarifying beer and wine in Europe (Hickman *et al*. 2000). Fish glue is still used in paper conservation, musical instrument fabrication and repair, ancient wooden building conservation, and wooden artifacts restoration, as well as in the Chinese mahogany furniture industry (Pang 2002; Schellmann 2007; Petukhova 2013). The use of natural fish glue has been adopted due to the glue's superior properties: reversibility, water-solubility, nontoxic property, solidity, flexibility, and resistance to heat and solvents. Additionally, wooden joints bonded with fish glue are detachable, which is beneficial for the repair or replacement of valuable mahogany furniture parts. Indeed, benign fish glue is the best option for repairing antiques. With the popularity of repairing wooden buildings and a revival of the mahogany furniture industry (Yang *et al*. 2012), the demand for fish glue has risen.

However, a common drawback for fish glue is the presence of contaminating microorganisms, especially molds, during the preparation and storage periods. Microorganisms in fish glue are not only hazardous to valuable artifacts, but they also shorten fish glue's shelf life and weaken its bonding properties through the process of protein deterioration (Schellmann 2007). Although steam sterilization is an efficient treatment to inhibit the formation of microorganisms, it is inconvenient because most customers do not have the treatment equipment. Therefore, the use of antimicrobial agents is an easier and more operational alternative for fish glue preservation. Previous studies have been done on the antimicrobial properties of fish gelatin composites (Arfat *et al.* 2014; Shankar *et al*. 2015; Ravindranath *et al.* 2016). Nevertheless, to the authors' knowledge, no research has been conducted on the identification of microorganisms in fish glue and the efficiency of antimicrobials.

Traditionally, heat treatment has been used to prepare fish glue from swim bladders. However, the process of heat treatment is always resource consuming. Enzymatic hydrolysis has been shown to be an efficient and beneficial alternative in preparing protein adhesives, gelatin, or collagen (Hoque *et al.* 2011; Jiang 2012; Tavano 2013; Wang *et al.* 2013; Jain and Anal 2016). In this study, the authors prepared both kinds of fish glue and identified the molds that appeared. Moreover, the authors compared three antimicrobials: borax, sodium diacetate, and Antim AL-D, to examine their effectiveness. Borax and sodium diacetate are common food preservatives (Silva and Lidon 2016). Antim AL-D is a liquid antibacterial agent available on the market. All antimicrobials in this study were effective in inhibiting the formation of most fungi and bacteria. The authors' aim was to provide more information on the preparation and preservation of fish glue for wooden building conservation, mahogany furniture fabrication, and other uses.

EXPERIMENTAL

Materials

Approximately 5 cm long farmed yellow croaker swim bladders (*Larimichthys polyactis*) containing around 5% water were obtained from Gao Shun Hang Co., Ltd. (Guangdong, China).

The enzymatic hydrolysis was performed on swim bladders using Papain enzyme (Papain from papaya latex, P-3250, 0.5 units/mg to 2 units/mg solid, Sigma-Aldrich). Birch blocks were supplied by the Furniture Lab of Northeast Forestry University (Harbin, China).

The three antimicrobial agents used were $Na_2B_4O_7 \cdot 10H_2O$ (sodium tetraborate or borax, 99.5%, TianDa Chemical Reagent Co., Ltd., Tianjin, China), $C_4H_7NaO_4$ (sodium diacetate, 99.0%, Aladdin Industrial Corporation, Shanghai, China), and Antim AL-D (Beijing ChamGo Nano-Tech Co., Ltd., Beijing, China). Borax and sodium diacetate are common food preservatives, and Antim AL-D is a liquid organic/inorganic composite antimicrobial.

Methods

Heat treatment of swim bladders

Heat treatment is one of the traditional Chinese fish glue preparation methods. First, dried swim bladders (about 20 g) were soaked in distilled water (300 mL) at ambient temperature for 24 h. The softened swim bladders were then chopped into pieces that were less than 5 mm in length and width. The small pieces were steamed for 40 min and then crushed until a gelatinous thick paste was obtained. Subsequently, the paste was poured onto gauze and immersed in 200 mL distilled water (the beaker was placed in a water bath at 50 °C). Through repeated squeezing, hydrolyzed fish gelatin was dispersed in water. After filtering (filter mesh - 90 μm), the obtained fish gelatin was poured into a tray and dried in open air at a temperature below 25 °C.

Enzymatic hydrolysis of swim bladders

After being cleaned with distilled water, the swim bladders were softened in a water bath at 70 °C for 30 min and then were cut into fine pieces. The small pieces were then mashed to pulp. Next, the pulp was mixed with distilled water at a solid/solvent ratio of 1:15 (w/w). As the solution was stirred continuously, 0.4% papain enzyme was added, and enzymatic hydrolysis of the pulp was performed at 60 °C for 6 h. Next, a filter mesh (90 μm) was used to remove residues. The hydrolysate was dried under the same conditions described in the heat treatment.

Microorganism isolation and identification

Five fragments were cut from each fish glue sample and placed into potato dextrose agar (PDA) in Petri plates. The PDA was used as an isolation medium prepared as described in the previous report (Shen and Chen 2007). All media were autoclaved at 121 °C for 30 min before use.

To isolate microorganisms, the plates with fish glue segments were incubated at 25 °C to 28 °C, with a relative humidity of 85% with 12 h light/12 h dark. The incubated plates were periodically observed for fungal colonies growth. When the size of plaques in the culture medium reached around 2 mm in diameter, plaques with hyphal tips were separated into groups based on their morphology and pigmentation, and then cultivated for purification. The mycelium and spore suspension were prepared under aseptic conditions and observed with a microscope.

Morphology characterization

The fish glue samples were sputter-coated with gold and then observed with a scanning electron microscope (SEM; QUANTA200, FEI, Hillsboro, OR, USA). With the accelerating voltage of 5 kV, the SEM images were obtained at different zones on each sample. A light microscope (XSP-35TV, Shanghai Optical Instrument Factory, Shanghai, China) was adopted for observing the microorganisms in samples.

Efficiency of antimicrobial agents

The following amounts of each antimicrobial were added to the fish glue at four levels: 0.3%, 0.5%, 0.7%, and 1.0% (weight ratio to fish glue solid content). The cultured samples included one control group (fish glue without antimicrobial) and three experimental groups. Every group had three replicates.

The fish glue with the specific amount of each antimicrobial agent was stirred adequately and then poured into Petri dishes. Then, the mold culture media were spread evenly on the surface of samples. All samples were cultured in a light incubator (HPG-400PX, Harbin Donglian Electronic & Technology Development Co., Ltd., Harbin, China) at 37 °C with a relative humidity of 78% and observed daily across a ten-day period.

Bonding strength determination

Freshly cut birch blocks were used to test the compression shear strength. Two glue samples were prepared, with and without the optimal antimicrobial, and applied to the wood blocks. Two wood blocks with dimensions of 10 × 25 × 30 mm^3 (thickness × width × length) were glued together along the grain direction with an overlapping area of 25 × 25 mm^2. At an ambient temperature of 23 °C ± 2 °C, the samples were held under static pressures of 0.5 MPa to 1.0 MPa for 24 h. Shear strength experiments were conducted according to the Chinese national standard GB/T 17517 (1998).

After being conditioned for 72 h, the compression shear strength was evaluated with a universal testing machine (AG-A10T, Changchun Kexin Instruments Co., Changchun, China). Ten replicates were carried out for each test and the averages of the ten were reported.

RESULTS AND DISCUSSION

Identification of Microorganism

Abundant hyphae on the surface of fish glue samples are shown in Figs. 1(a) and (b). Conidiophores are short, straight or flexures, and germinated in single or bushy heads, as shown in Figs. 1(b) and (c). Figure 1(d) exhibits the olive-brown ellipsoidal conidia with typically vertical and transverse septa. Conidia were catenated. *Alternaria* was identified in the present study in accordance with existing descriptions of the fungi (Wei 1979).

Protein products have been shown to be susceptible to molds. Previous research has isolated *Alternaria*, *Aspergillus*, *Penicillium*, and other fungal species from fresh and frozen fish (Iqbal *et al*. 2012; Oranusi *et al*. 2014). Although treatment at high temperature could inhibit the formation of some contaminants, foodborne *Alternaria* contamination, likely resulting from imperfections in the drying process or preparation procedures, was still identified in the fish glue samples.

Airborne *Alternaria* spores, a major allergen, have received increasing recognition as a risk factor for asthma syndromes (Gergen and Turkeltaub 1992; Bush and Prochnau 2004; Tham *et al*. 2017). In addition, Duncan's research revealed that *Alternaria*, a wood-attacking fungus, caused a soft rot type of decay (Duncan 1960). *Alternaria* not only degrades the properties of wood, it is also hazardous to human health. Therefore, it is critical to inhibit *Alternaria* spore germination during the preparation of fish glue.

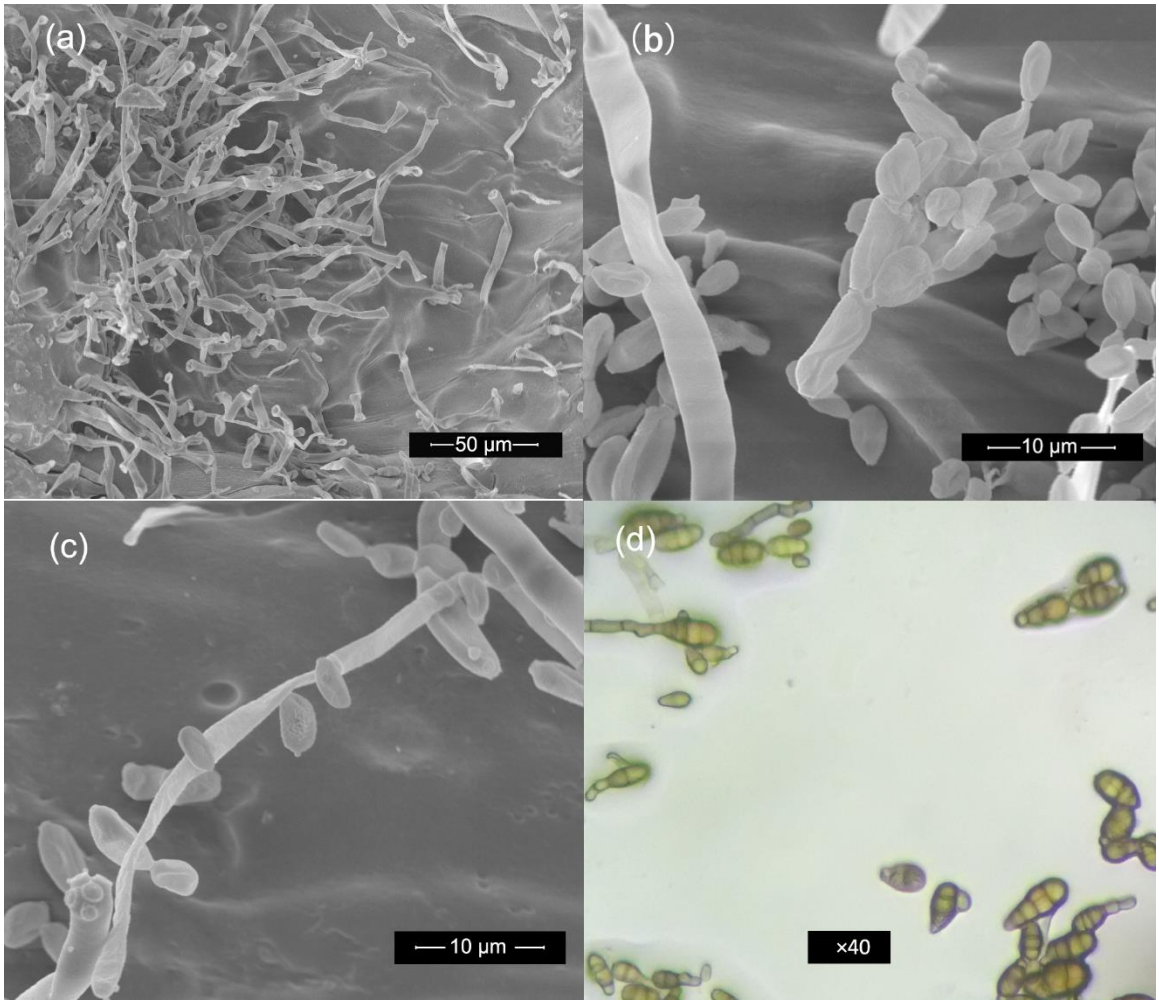

Fig. 1. SEM photos of hyphae and conidia of *Alternaria*: (a); (b); (c); and (d) photomicrograph of spores

Efficiency of Antimicrobials

All of the antimicrobials tested in this research were effective in inhibiting the growth of *Alternaria* in both the heat treatment and enzymatic hydrolysis fish glue samples. As shown in Table 1, *Alternaria* colonies were observed in all of the control samples on the second day.

The addition of antimicrobials inhibited *Alternaria* growth. Among the three antimicrobials, Antim AL-D was the most effective in preventing the growth of *Alternaria*. During a ten-day observation period, there were no *Alternaria* colonies observed when a 0.3% weight ratio of Antim AL-D was added.

As for borax and sodium diacetate, a 0.3% addition was insufficient in inhibiting *Alternaria* germination. However, increased addition of borax and sodium diacetate contributed to the enhancement of their effectiveness. To inhibit *Alternaria*, the addition of no less than 1.0% of borax or sodium diacetate into fish glue samples produced the most desirable outcomes.

Table 1. Effectiveness of Antimicrobials on *Alternaria* Colony during a Ten-day Observation Period

Amount of Antimicrobial Addition (w/w %)	Colony Observed Time (day)					
	Heat Treatment			Enzymatic Hydrolysis		
	$Na_2B_4O_7$	$C_4H_7NaO_4$	Antim AL-D	$Na_2B_4O_7$	$C_4H_7NaO_4$	Antim AL-D
0	2	2	2	2	2	2
0.3	4	3	N/A*	3	2	N/A*
0.5	4	5	N/A*	3	3	N/A*
0.7	9	7	N/A*	5	5	N/A*
1.0	N/A*	N/A*	N/A*	N/A*	N/A*	N/A*

*No colonies were observed on the tenth observation day

Shear Strength

In comparison to borax and sodium diacetate, Antim AL-D was the most efficient antimicrobial. Additionally, the liquid Antim AL-D was dispersed in fish glue easily without changing the glue's appearance or viscosity. Thus, the effect of Antim AL-D on the shear strength of glued samples was examined.

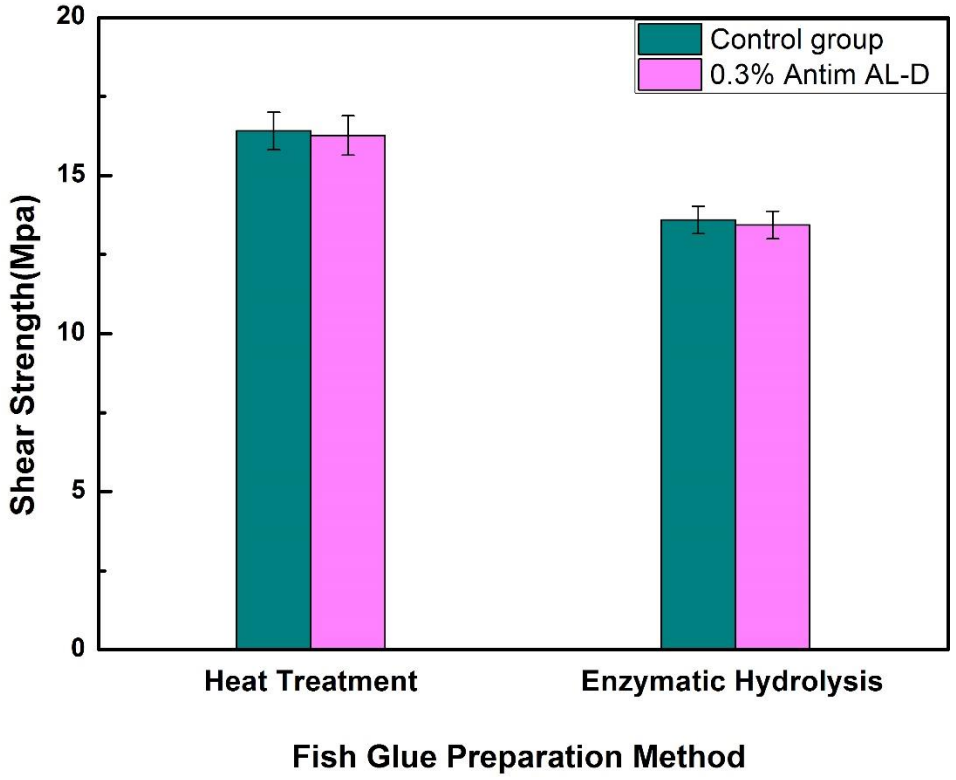

Fig. 2. Antim AL-D influence upon shear strength

Figure 2 shows that the shear strength slightly decreased when 0.3% of Antim AL-D was added. Although the dry shear strengths of the fish glues prepared by the heat treatment and enzymatic hydrolysis were reduced by 0.8% and 1.4%, respectively, the samples still met the requirements of the standard for polyvinyl acetate (PVAc) emulsion adhesives for wood (HG/T 2727 2010). Currently, PVAc emulsion is a very common chemical adhesive used for gluing furniture parts, conservation of ancient wooden buildings, and wooden artifacts repair. The results demonstrated that the heat treatment and enzymatic hydrolysis of fish glues with 0.3% Antim AL-D were strong enough to be used in wooden product conservation and mahogany furniture fabrication.

CONCLUSIONS

1. *Alternaria* contaminated fish glue samples were prepared using the methods of heat treatment and enzymatic hydrolysis. It was found that the addition of an antimicrobial agent was necessary to protect fish glue from contaminants, especially when sterilization procedures were inadequate.
2. All antimicrobials in this research were effective in inhibiting the formation of *Alternaria*. Antim AL-D was the most efficient antimicrobial, causing a slight decrease in shear strength. Addition of a 0.3% solution of Antim AL-D was sufficient to prevent *Alternaria* growth in fish glue.

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