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Biocolonization of Stone: Control and Preventive Methods

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New Environmentally Friendly Approaches against Biodeterioration of Outdoor Cultural Heritage

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ABSTRACT. Microbial biofilms have a role in the weathering of stone exposed to open air. It is a common practice to use toxic products to control microbial fouling. The aim of the present research was the development of an innovative, green, and cost-effective technology for the prevention of biodeterioration on outdoor cultural heritage surfaces. Therefore, the effects of a synthetic analogue of capsaicin (from chili peppers) and zosteric acid (from eelgrass, *Zostera marina*) in preventing the development of detrimental biofilms were studied. In particular, the use of zosteric acid was an effective biocide-free strategy against both bacterial and fungal biofilms. Zosteric acid caused a more than 90% reduction of *Escherichia coli* and *Bacillus cereus* adhesion, whereas coverage of *Aspergillus niger* and *Penicillium citrinum* was reduced by 57%. Under different environmental conditions, zosteric acid reduced *Candida albicans* (the model fungus for fungal biofilm studies) biofilm formation by at least 70%.

THE NATURE OF BIOFILMS

Bacteria have two modes of growth: planktonic and sessile. It is believed that planktonic cells suspended or growing in a fluid environment are important for rapid proliferation and spread into new habitats, whereas slow-growing sessile cells grow attached to a surface and act in a multicellular organized manner. Biofilms are defined as sessile microbial communities embedded in self-produced extracellular polymeric substances (EPS) on either organic or inorganic substrata (Costerton et al., 1995). Biofilms are ubiquitous and can be found on practically any surface, including those of cultural heritage, such as buildings and monuments.

Biofilms include bacteria, algae, and fungi that are responsible for the microbial deterioration of cultural heritage. A further problem associated with biofilm formation includes biofouling, the undesirable accumulation of microorganisms, plants, and animals on a surface (Whelan and Regan, 2006). Bacteria are generally the first organisms to foul surfaces exposed to different environments, through adhesion and subsequent biofilm formation.

The formation of microbial biofilms begins with the reversible adhesion of a small number of cells to a surface. On the abiotic surface, initial attachment between bacteria and the surface is governed by nonspecific interactions, such as electrostatic, hydrophobic, or van der Waals forces. Initial studies have proved that the rate of bacterial adhesion to a wide variety of substrata is responsive to some physical features, such as hydrophobicity. After binding to the surface through a glue-like matrix, bacterial cells begin the process of irreversible adhesion, proliferation, and accumulation as multilayered cell clusters. The attached cells synthesize new exopolysaccharide material in order to cement their adhesion to the surface and to other cells in the developing biofilm. These extracellular matrices, composed of a mixture of materials like polysaccharides, proteins, nucleic acids, and other substances, are considered to be essential in holding bacterial cells together in the biofilm structure, in helping to retain nutrients for cell growth, and in protecting cells from dehydration and other stresses. This phase is characterized by active binary division of attached cells and cell recruitment. Cell accumulation requires coordinated efforts from the microbial community to produce a well-organized structure. After having irreversibly attached to a substratum, bacterial cells undergo phenotypic changes. Mature biofilms typically consist of differentiated mushroom-like structures of cells embedded within the extracellular polymer matrix. This contains voids open to the bulk fluid to allow the transport of nutrients and oxygen from the interface to the inner parts of the biofilm and for the removal of metabolic wastes. Bacterial cells detached from the biofilm reenter the planktonic state and may start a new biofilm formation cycle.

Microbial biofilms display discrete temporal and spatial organization (Pace et al., 2006). There are many microenvironments within a biofilm. The metabolic activities of the cells, together with diffusion processes, result in concentration gradients of nutrients, signaling compounds, and waste products within the biofilms. A variety of conditions are governed by local pH, oxidizing potential (redox), and other parameters. Cells close to the surface are exposed to high oxygen concentrations, whereas in deeper layers oxygen is hardly available. Gradients for either nutrients or metabolites from the biofilm create a heterogeneous environment even for a single-species biofilm. Within a biofilm community, cells with diverse genotypes and phenotypes follow distinct metabolic pathways, stress responses, and other specific biological activities, although they are located side by side (metabolic heterogeneity) (Stewart and Franklin, 2008). In order to develop preventive conservation strategies for biofilm control, it is necessary to fully

understand the mechanisms involved in initial attachment, development of the biofilm phenotype, maturation and detachment, and the related regulatory processes.

THE ACTION OF BIOCIDES

There are no current indexes of microbial biodegradation activity that are widely accepted by the scientific community involved in the conservation of stone objects or structures. Such indexes would be very helpful to plan a case-by-case conservation action compatible with the principle of minimal intervention (Gazzano et al., 2009). For decades, abatement of microbial growth has commonly been achieved by applying biocides to surfaces. Biocides are chemicals with an active, and in general toxic, effect on living organisms. European Community Directive 98/8/EC of the European Parliament addresses the issue of placing biocidal products on the market. In particular, it concerns the authorization for the marketing of biocidal products within the member states. At the European Community level this directive established a list of approved active substances that may be used in biocidal formulations. It aims to ensure that a high level of protection is provided for users, the public in general, and the environment since it has long been recognized that biocidal effects may extend beyond the target organism with resulting undesired adverse effects to man and the environment (Rasmussen et al., 1999). On the basis of the hazard assessment and risk characterization for human health, the European Commission has banned the use of some frequently used active substances, including some commonly used in conservation practice.

In recent years, the inherent resistance of biofilm microorganisms to biocides has been found to be generalized. Bacteria within a biofilm display a variety of phenotypes, providing the community as a whole with an enormous capability to resist biocides. Treatments with traditional doses of biocides are insufficient to destroy all of the biofilm population. Bacteria embedded in the biofilm matrix are remarkably more tolerant to biocides, up to 1,000-fold relative to planktonic cultures of the same bacterial strains, depending on the species-drug combination.

In addition, organic biocides might serve as nutrients; therefore, they must be used carefully in conservation practice (Krumbein and Gross, 1992; Leznicka, 1992; Warscheid, 2000). Dead cells may also provide nutrients for subsequent colonization; therefore, leaving dead cells on the surface is not advisable (Flemming, 1998).

Finally, after exposure to the killing effects of biocides, a small surviving population of persistent bacteria

can repopulate the surface immediately and can acquire enhanced resistance to further biocidal treatment.

A number of biofilm characteristics contribute to the biocide resistance of biofilm cells, such as the presence of a diffusion barrier provided by the EPS, the interaction with the exopolymers, the slow growth mode of sessile cells, the multicellular nature of the biofilm, and the possible genetic expression of certain resistance genes. A biofilm extrapolymeric matrix has the potential to reduce the penetration of biocides either by physically slowing their diffusion or chemically reacting with them. Sessile cells generally have low metabolic activity, thereby resulting in a reduction of the antimicrobial susceptibility. Genetic studies indicated that, in general, more genes are expressed in biofilms than by planktonic cells. Finally, it is thought that persisters, a subpopulation of extremely resistant cells, are responsible for biofilm regrowth when the treatment is discontinued (Pace et al., 2006).

CELL-TO-CELL COMMUNICATION SYSTEMS

Cell-to-cell communication systems regulate the expression of a panel of genes, allowing microorganisms to adapt to modified environmental conditions. Bacteria organize themselves structurally through the synthesis of, and response to, intercellular signal compounds (small diffusible molecules). The ability to coordinate gene expression in accordance with population density and hence to act as a group is a process termed quorum sensing (QS) (Balaban, 2008). “Quorum” is the number of individual cells needed to induce an activity. In this process bacteria produce diffusible hormone-like molecules (autoinducers) that interact with specific receptors on themselves and on neighboring cells, which, in turn, regulate the expression of specific target genes. By integrating this with other environmental stimuli, bacteria are capable of exhibiting complex responses and take part in sophisticated interactions, allowing them to survive in adverse environments. A large number of bacterial species are known to possess this communication mechanism, through which bacteria can sense changes in their environment and coordinate gene expression in favor of the survival for the entire community. To date, several types of QS systems are known: one for gram-positive bacteria relying on polypeptides and another for gram-negative bacteria mediated by *N*-acyl homoserine lactone (AHL) derivatives. A third type of QS system, AI-2, has been proposed as a global signaling system common to all bacteria (Pace, 2006). Quorum

sensing in many bacteria regulates a number of physiological activities, including those that involve biofilm formation.

NEW STRATEGIES TO PREVENT BIOFILM FORMATION

Although it is evident that alternatives to the use of biocides for biofilm treatment are needed, very few studies in the conservation field have investigated them. The first step toward this direction was the EU project “Inhibitors of Biofilm Damage on Mineral Materials” (BIODAM). It evaluated the combination of biocides with (1) permeabilizers, (2) special slime (EPS) blockers, (3) pigment inhibitors, and (4) photodynamic treatments.

However, to the best of our knowledge, no study in the conservation field has investigated the prevention of bacterial cell adhesion to the substratum with nontoxic, environmentally friendly compounds and the disruption of cell-to-cell communication involved in biofilm formation through physical, chemical, and biological approaches, as proposed for other research areas (Flemming et al., 1996). The need for environmentally benign antifouling technologies has led to renewed interest in the ways that organisms protect themselves against predation. Therefore, natural nontoxic antifouling compounds represent an attractive strategy. These antifouling compounds have been isolated mainly from marine organisms that are not colonized by microorganisms. An interesting antifouling agent is zosteric acid (*p*-sulfoxy cinnamic acid), a natural extract from eelgrass (*Zostera marina*) that prevents biofouling by some organisms, such as algae, barnacles, and tubeworms, at nontoxic concentrations (Barrios et al., 2005). Most recently, with the further understanding of cell-to-cell communication in microorganisms, QS has emerged as one of the most important mechanisms for controlling the development of highly structured and cooperative biofilm consortia, on both organic and inorganic substrata. The QS systems offer three points of attack: (1) signal generation, (2) the molecule itself, and (3) the signal receptor. Steinberg et al. (1997) isolated halogenated furanones (secondary metabolites) from the Australian macroalga (seaweed) *Delisea pulchra*. These compounds interfered with bacterial signaling and displayed remarkable anticolonization activity (de Nys et al., 1993). Furanones protect the alga from colonization by both prokaryotes and eukaryotes and therefore are strong deterrents of the settlement and growth of both micro- and macrofouling (de Nys et al., 1993). Since this discovery, methods have been developed

to synthesize the natural antifouling molecules and their synthetic derivatives in laboratory.

The research goal proposed has been the development of this innovative, green, and cost-effective technology for the prevention of biodeterioration on outdoor cultural heritage surfaces. The results obtained so far indicate that this technology may be a powerful tool in the conservation field (Cappitelli et al., 2006; Cappitelli and Sorlini, 2008). In particular, effects of synthetic analogues to capsaicin (Villa et al., 2009) and zosteric acid in preventing the development of detrimental biofilms were studied. This is particularly relevant to materials in contact with water or exposed to outdoor conditions. Since antifouling agents play a strong and specific in situ effect against both micro- and macrofouling, one strategy considered for preventing biofilm formation is to coat cultural heritage surfaces with compounds capable of interfering with adhesion or signaling mechanisms. Antifouling agents affixed and immobilized on surfaces should inhibit bacterial attachment and retain activity for extended periods. Methods for binding antifoulants to surfaces of protectives or consolidants or for incorporating them into the products themselves are being investigated, as described in the next section.

METHODOLOGY TO SELECT POTENTIAL ANTIFOULING AGENTS

Bacteria and fungi used in the experiments were either pure or mixed cultures. It is advisable to employ microbial isolates from biofilms, especially those growing on a cultural heritage substrate (Figures 1 and 2).

All organisms require a source of carbon and energy for growth. A good candidate for an environmentally friendly antifouling agent should not be a carbon and/or energy source for the microorganisms tested. Mineral media that would not provide a source of carbon and energy were therefore prepared with the antifoulant added at different concentrations. Furthermore, the antifouling molecule was used at a concentration below the minimal biocidal concentration. To complement these studies, toxicity experiments were conducted in a rich medium to which the antifoulant was added at different concentrations. The specific growth rates with or without the antifoulant were determined via cultivation or by measuring the optical density spectrophotometrically. Alternatively, the viability of microbes was measured by staining with a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Figure 3).

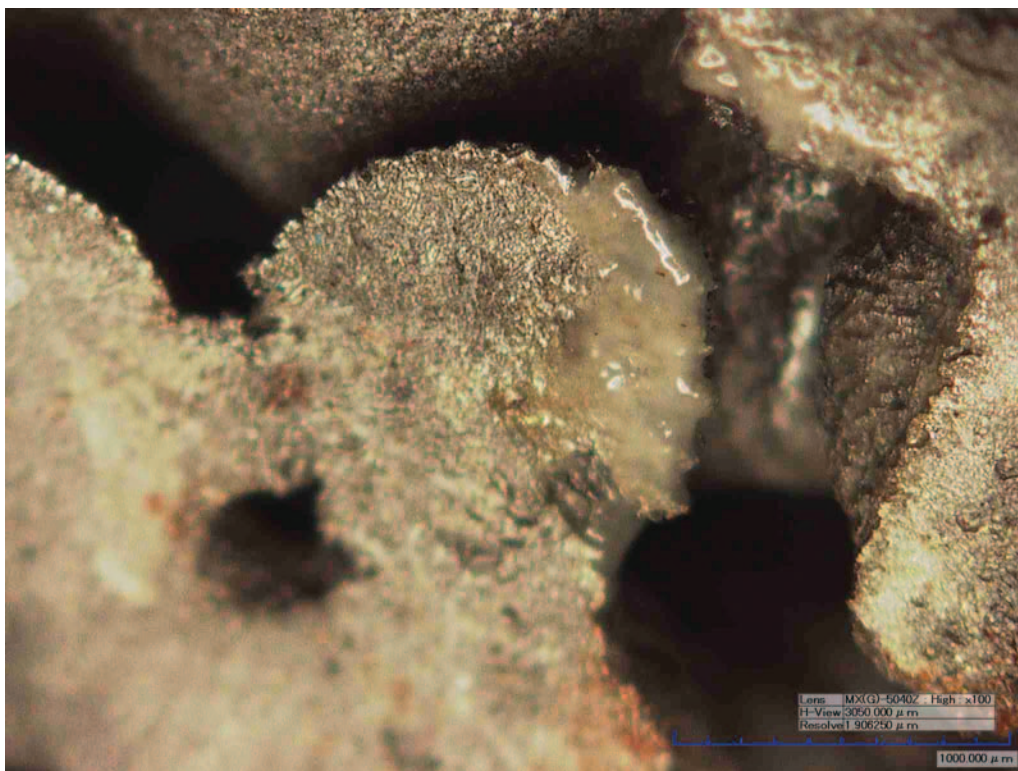


FIGURE 1. Bacterial biofilm on a metal surface (image taken with a 3D HIROX KH-7700 video-microscope).

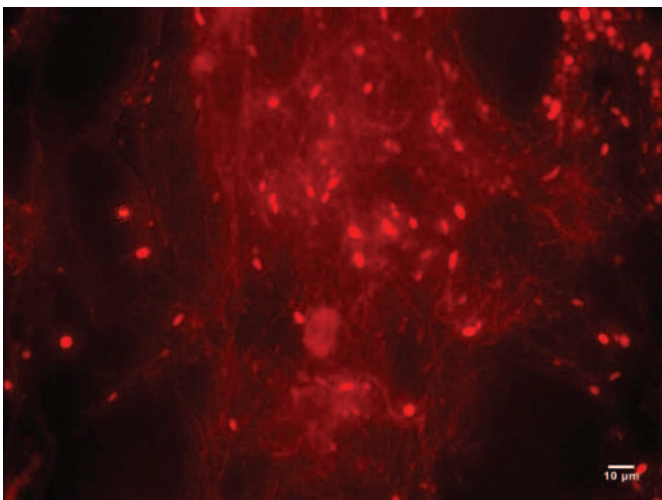
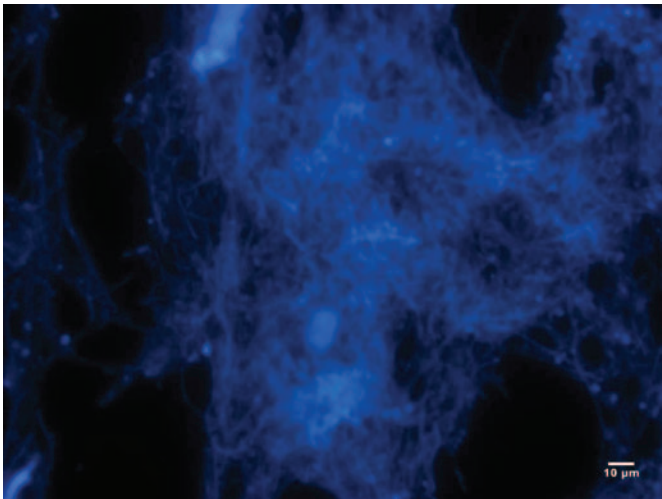
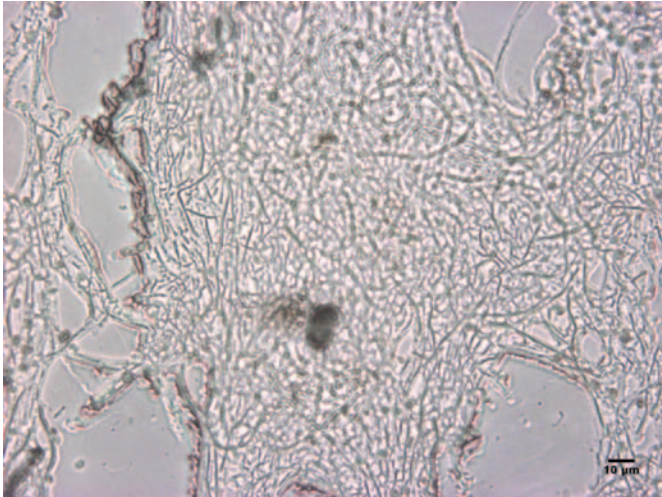


FIGURE 2. (Top) Phase contrast and epifluorescence images of biofilm on adhesive tape strips sampled from an outdoor wall showing (middle) DAPI staining and (bottom) hybridization with the universal Eukarya probe EUK 516.

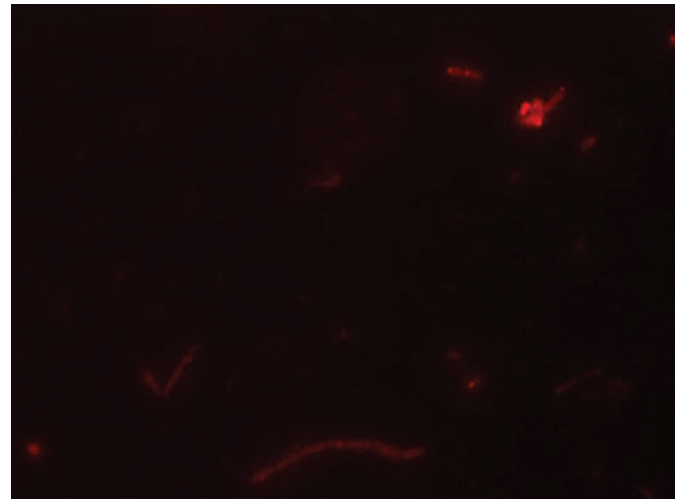
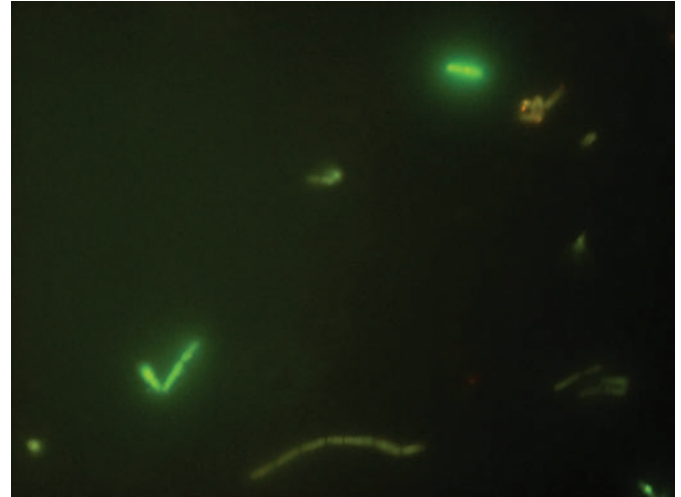


FIGURE 3. A LIVE/DEAD® BacLight™ Bacterial Viability Kit was applied to estimate both viable and total counts of bacteria. BacLight is composed of two nucleic-acid-binding stains: SYTO 9™ and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells green (top), whereas propidium iodide only penetrates cells with damaged membranes, producing red fluorescent cells (bottom). The pictures show that the tested molecule acted as a biocide, killing all cells.

To statistically validate the investigation, biofilm tests were carried out in 96-well microtiter plates. There are different kinds of microtiter plates in terms of their hydrophobic or hydrophilic character (Figure 4a).

Adhesion can be assessed quantitatively using fluorochrome-labeled cells incubated on various black microtiter plate test substrata. Fluorescence was measured using a plate reader (Figure 4b). For each experiment, a standard fluorescence intensity curve versus cell number (as measured by direct counting) and microorganism type was prepared.

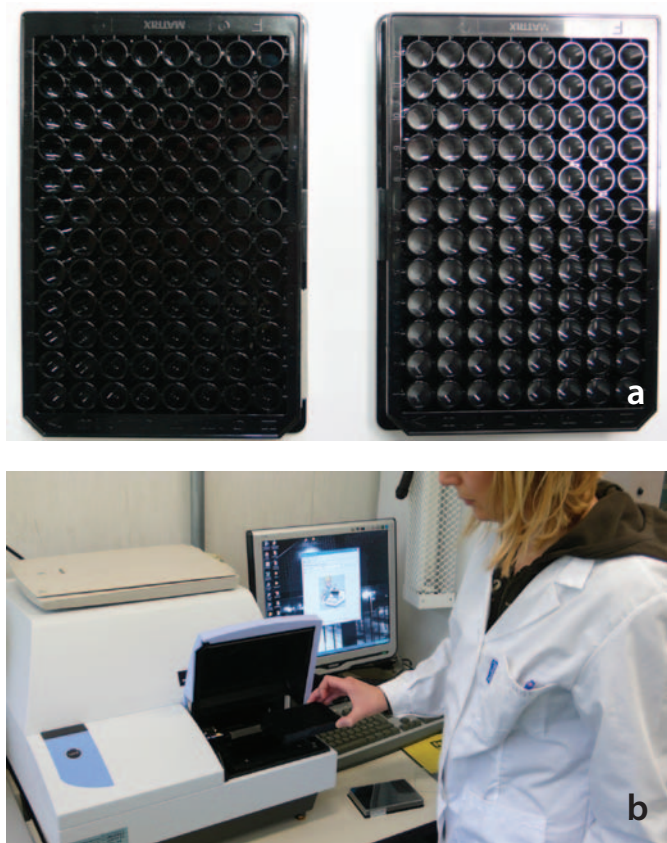


FIGURE 4. (a) The 96-well plates for (left) hydrophobic surfaces and (right) hydrophilic surfaces. (b) Multilabel plate readers by Perkin Elmer.

There are several parameters that should be taken into account to evaluate the efficiency of an antifouling molecule. The most important are the following: type of microorganism, type of surface (hydrophobic or hydrophilic), antifoulant concentration, pH, time, and temperature. In the past, the assay optimization process has typically taken between 4 and 12 months, using traditional one-factor-at-a-time experiments (Altekar et al., 2006). However, statistically designed experiments are a powerful tool for improving the efficiency of experimentation. The technique of design of experiments (DOE) is an important link between the experimental and the modeling world. It requires only a small set of experiments where all the variables are analyzed simultaneously within fixed ranges (selected so they do not inhibit cell growth) according to rigorously formulated mathematical protocols. Besides identifying the variables that most influence the results, DOE serves to identify possible interactions and synergies among factors. With this multivariate approach it is

possible to obtain a model describing the performance of potential antifoulants in complex scenarios.

Although the microplate assay demonstrates the antifouling properties of a new compound, it does not predict its impact on biofilm architecture. Laboratory-scale biofilm reactors combined with microscopic techniques are extremely valuable for discovering the fundamental characteristics of biofilms (e.g., the spatial and temporal patterns of microbial colonization, specific biofilm components, interactions, and cellular activities), providing useful insights for developing effective antifouling technologies.

Once a compound has been proven to be effective against bacterial attachment, a delivery system must be found. This could be a protective or a consolidant, e.g., a synthetic polymer (e.g., Villa et al., 2009).

RESULTS

Using the above-mentioned techniques, the antifouling potential of *N*-vanillylnonanamide and zosteric acid was investigated for both bacteria and fungi. Before the adhesion tests, it was proved that neither the synthetic capsaicin nor zosteric acid was toxic or represented a carbon and energy source for all the tested microorganisms. Microbial attachment assays on glass and polysine slides (special glass microscope slides with a permanent adhesive) were carried out with *N*-vanillylnonanamide in dispersion and were applied onto the surfaces using a polymer coating (Villa et al., 2009). It required 205 μM ($\mu\text{mol/L}$) of *N*-vanillylnonanamide to inhibit *Bacillus* adhesion by 48% on glass slides. The same compound blended into or sprayed onto a polyurethane coating at 205 $\mu\text{mol/kg}$ showed that this capsaicinoid did not have any substantial impact on preventing adhesion.

As the results from *N*-vanillylnonanamide investigations were not completely satisfactory, attention was focused on the new potential antifoulant zosteric acid. For the studies, zosteric acid was successfully synthesized by Domenico Albanese (Dipartimento di Chimica Organica e Industriale, Università degli Studi di Milano). Using the DOE method, it could be shown that the antibiofilm activity of zosteric acid is species specific, causing a more than 90% reduction of *Escherichia coli* and *Bacillus cereus* adhesion, whereas *Aspergillus niger* and *Penicillium citrinum* coverage was affected by 57% (Villa et al., 2010). Zosteric acid reduced *Candida albicans* (the model fungus for fungal biofilm studies) adhesion and subsequent biofilm formation by at least 70%. In addition, the mathematical models revealed that the molecule successfully

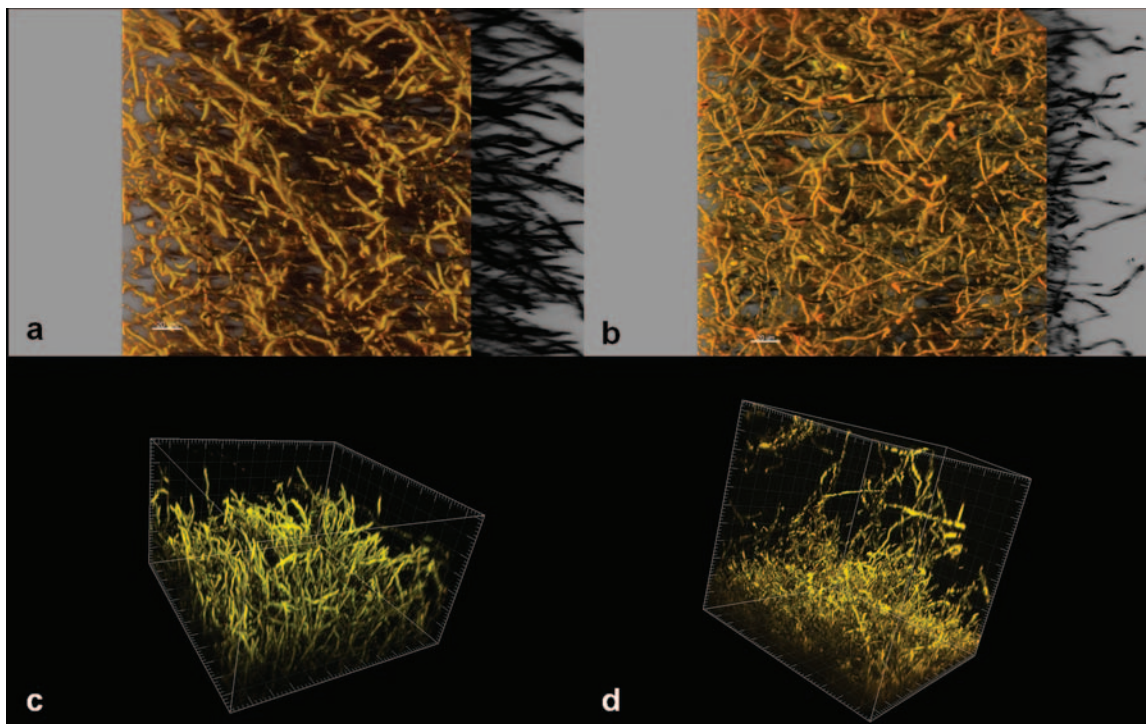


FIGURE 5. Three-dimensional projection of *C. albicans* biofilm (a, c) without and (b, d) with zosteric acid. The biofilm was visualized by confocal laser scanning microscopy after staining with the fluorescence probe FUN1. Images were analyzed with IMARIS software. Top is the view as seen from above; bottom is the view as seen sideways.

counteracted the effects of some colonization-promoting factors, such as time and temperature (Villa et al., 2010).

Experiments on *Candida albicans* showed that zosteric acid strongly impacted biofilm thickness and morphology, resulting from the inability of fungal cells to form filamentous structures while maintaining metabolically active cells (Figure 5). Finally, zosteric acid enhanced their sensitivity to different traditional biocidal treatments, causing a decrease in biofilm resistance of 0.5–8 log units.

By virtue of its ability to reduce microbial adhesion, modify the shape of fungal biofilm architecture, and enhance the performance of antimicrobial agents, zosteric acid should be further tested and developed as a means of preventing biofilm formation, by itself or integrated into a more complex protective system.

CONCLUSIONS

Although more research is still needed, environmentally friendly alternatives to toxic biocides to prevent biofilm formation represent a very promising method to

control biocolonization. It is hoped that conservators will be eager to use these alternatives to biocides when they are technically available.

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