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Forming of a functional biofilm on wood surfaces

Michael F. Sailer[a,1], Elke J. van Nieuwenhuijzen[a,*], Wieger Knol[b,2]

[a] Built Environment and Geosciences, Organization for Applied Scientific Research, van Mourik Broekmanweg 6, 2600 AA Delft, Netherlands
[b] Quality of Life, Organization for Applied Scientific Research, Utrechtseweg 48, 3704 HE Zeist, Netherlands

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ABSTRACT

The protecting and staining properties of biofilms grown on oil-treated surfaces of *Pinus sylvestris* L. sapwood were investigated with respect to their potential to create homogeneous coloured surfaces. Linseed oil pressure-treated blocks of *P. sylvestris* L. were evaluated after 36 months of outdoor exposure. The biofilm was characterized by colony counts and PCR cloning, the interactions with wood were assessed microscopically. The results show that a biofilm consisting of *Aureobasidium pullulans* has the potential to create protecting and staining functions on a wood surface. The conditions and factors which lead to a selective growth of *A. pullulans* are discussed with respect to the practical application of the formed biofilm in the field of environmental and civil engineering.

1. Introduction

Building elements, like timber or stone panelling, are frequently covered by discolouring biofilms. The effects of black discolouration, especially on water-repellent coatings, are usually considered negative (Horvarth et al., 1976; Williams and Feist, 1999) and therefore enormous efforts are put into understanding the growth and the control of film forming micro-organisms on materials (e.g. Whiteley, 1973; O'Neill, 1986). Related to civil engineering, microbial growth in biofilm on surfaces is frequently associated with aesthetical degradation, risk of biodeterioration (e.g. Krummbein et al., 1996; Kemmling et al., 2004; Gorbushina et al., 2004) or health risks caused by mycotoxins (Görs et al., 2007).

However, there are also reports mentioning positive functions of biofilms on building materials, for instance the conservation effect of a biofilm on stone (Pohl and Schneider, 2002) or controlled biomineralisation (e.g. Rodriguez-Navarro et al., 2003; Moroni et al., 2004).

Within the field of ecological engineering the cleaning properties of micro-organisms for water (e.g. Wu et al., 2006) or other effluents (Chaturvedi et al., 2006) are evident. The cleaning of soil or water from oil pollution is another important issue where biofilms contribute to the effects of bioremediation (Al-Awadhi et al., 2003).

Another broad field of research is the use of micro-organisms in the biological protection of materials. The application of antagonistic effects in relation to building or other materials looks quite promising. Research in this field has grown rapidly over the last 50 years, but reports referring to actual applications are still limited (Schoeman et al., 1999). Practical application principles to protect timber from pathogenic micro-organisms by antagonistic effects are published for example by Greaves (1970) or Varese et al. (2003).

This paper describes the creation and application potential of a biofilm in the built environment, which considers the basic concepts of ecological engineering, described by Mitsch and Jørgensen (2003). The idea of creating a decorative and/or conserving biofilm on wood with *Aureobasidium pullulans* is partly based on the principle on leaves and information found in literature concerning the growth of micro-organisms on water-repellent coatings. Some properties and functions of the formed biofilm and on a wood-based system and aspects of application will be discussed.

2. Materials and methods

2.1. Wood treatment

A serie of 48 pine sapwood (*Pinus sylvestris* L.) test specimens of 120 mm × 85 mm × 38 mm was prepared. The wood was climatised

* Corresponding author. Tel.: +31 15 2763458; fax: +31 15 2763016.
E-mail addresses: michael.sailer@tno.nl (M.F. Sailer), elke.vannieuwenhuijzen@tno.nl (E.J. van Nieuwenhuijzen), pim.knol@tno.nl (W. Knol).
[1] Tel.: +31 15 2763177; fax: +31 15 2763016.
[2] Tel.: +31 30 69 4 44 93; fax: +31 30 69 4 44 66.

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Table 1
Degradation after 36 months exposure (average of three samples).

Material	Mass change based on dry weight	Macroscopic observations
Untreated pine sapwood	−5%	Degradation of cell walls
Pine sapwood (25% oil concentration)	0%	No degradation noticed
Pine sap wood (75% oil concentration)	0%	No degradation noticed

at 20 °C and 65% relative humidity resulting in moisture content of 12%. The specimens were impregnated with chemically refined pure linseed oil with concentrations of 25% and 75%. In order to achieve this different concentration, the linseed oil was dissolved in acetone. The impregnation was carried out using a vacuum period of 12 min at -8×10^3 Pa followed by 1.5 h pressure of 8×10^5 Pa. Thereafter the specimens were cured at increasing temperatures up to 103 °C in an oven over a period of 260 h. This treatment led to an oil film on the surface. The oil retention was determined by comparison of the weight of the sample before and after the treatment at 20 °C and 65% relative humidity.

2.2. Exposure

The development of a homogeneous biofilm took place under natural conditions. The treated specimens together with untreated references were exposed in racks 1 m above the ground in an outdoor test field. After 36 months exposure the biofilm was evaluated using microscopic and microbiological methods. The mass loss of the samples was calculated by comparison of the oven dry weight before and after exposure.

After exposure one wood block was cut into pieces (20 mm × 10 mm × 10 mm) in order to assess the degradation pattern. Micro-slices were cut with a thickness of 30 μm and stained with Sudan IV and Astra blue. Other samples were used to isolate and identify the main micro-organisms forming the biofilm.

2.3. Isolation and identification of micro-organisms

After cleaning the surface to remove loose particles, biofilm material on the specimen was scratched from a surface area of approximately 40 cm^2 and suspended in sterile physiological salt solution. From here two methods were followed. In the first method plate counts of cfu (colony forming units) of yeast and moulds and bacteria were carried out in the suspension in order to quantify the growth of micro-organisms. An oxytetracycline–glucose–yeast extract–agar and tryptone–soya–agar with pimaricine, respectively, were used as a selective medium. Pure colonies on the plates were subcultered for further research. In the second method DNA was extracted from the suspension by transferring 2 ml into screw cap eppendorf tubes with zirconium–silica beads. Then phenol was added and the samples homogenized with a Mini beadbeater. DNA was extracted with the AGOWA mag Mini DNA Isolation Kit (AGOWA, Berlin, Germany), in accordance with the manufacturer's recommendations.

Using specific primers 16S, 18S and ITS fragments were amplified by PCR and cloned. Of each PCR-product 94 clones were sequenced. The resulting sequences were used to screen the non-redundant NCBI database, using the program BLASTN 2.2.19+ (Zhang et al., 2000).

3. Results

3.1. Wood treatment

The wood impregnation with linseed oil at a concentration of 25% led to an average retention of 170 kg/m^3. Using an oil concentration of 75% an average of 330 kg/m^3 was determined. The specimens with an oil retention around 330 kg/m^3 were covered by an oil film, which was completely homegenously distributed on the surface. On samples with a lower retention (around 170 kg/m^3) a slightly unequally oil distribution was noticed.

Fig. 1. Reference wood without additional treatment.

3.2. Exposure

After 36 months exposure all the linseed oil-treated wooden specimens were covered with a homogenous dark film. The top and the sides of the samples were completely covered, the bottom of the sample to a large degree. The specimens with oil retention of 330 kg/m^3 appeared to be darker than the specimens with retention of 170 kg/m^3. Compared to the untreated reference in Fig. 1 all the oil treated samples (Fig. 2) showed a much darker surface. The reference wooden blocks showed also a change in colour which was lighter and inhomogeneous.

In Table 1 the mass change of the three categories of specimens and macroscopic observations are shown.

Fig. 2. The wood piece is covered with a uniformly coloured biofilm.

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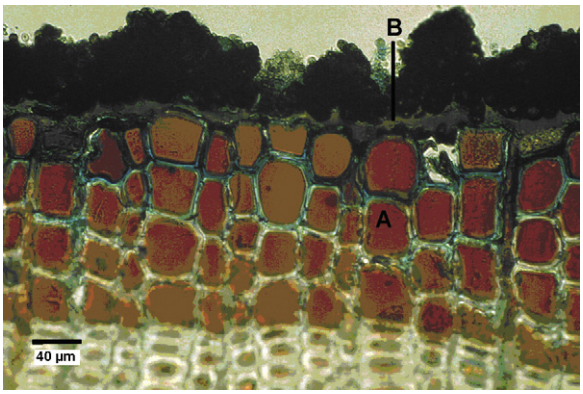

Fig. 3. Biofilm on treated wood after 36 months exposure (magnification 100×).

From a few specimens the biofilm formed on the surface was partly removed in order to get an impression of the wood condition under the film. On the oil treated samples no visible UV- and biological-degradation of the wood was noticed. In case of the reference specimens, the wood surface appeared soft and fibres could easily removed from the surface.

Microscopic examination of the stained slice of oil-treated wood (Fig. 3) shows red-filled lumina (A) indicating that linseed oil is present. The surface of the wood is covering a biofilm consisting of polymerized linseed oil and does not show the red colour (B). On top of this layer a 10–50 μm thick biofilm of black-stained substance is visible. Further it is remarkable that the dark colour is present in the cell walls close to the surface, indicating that the micro-organisms are growing into the cell walls close to the surface. Fig. 3 shows an intense interaction of the biofilm between the wood and the surface of the polymerized fatty acids.

By microscopic observation of a slice of untreated sapwood a degradation of the cell walls can clearly be recognized (Fig. 4). The darker soft rot-like degradation pattern in the cell walls indicates degradation caused by Basidiomycedes.

3.3. Isolation and identification of organisms

On the agar plates selective for yeasts and moulds only yeast-like, colonies were found, being slimy and coloured pink to black. Pure colonies on the agar plates were subcultered for further research. Per cm^2 area of biofilm on the wood specimen the cfu number of yeast-like organisms amounted to 1000, and the cfu number of aerobic bacteria was 3.

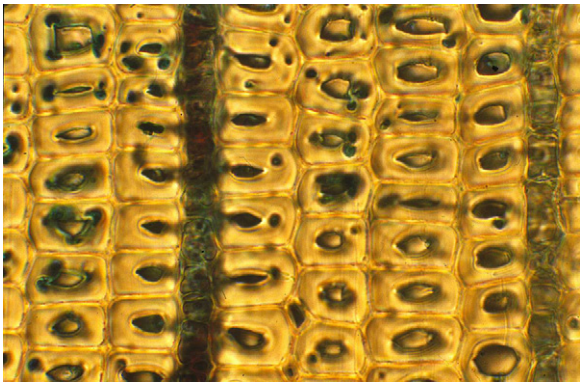

Fig. 4. Degradation pattern of untreated pine sapwood reference after 36 months exposure (magnification 100×).

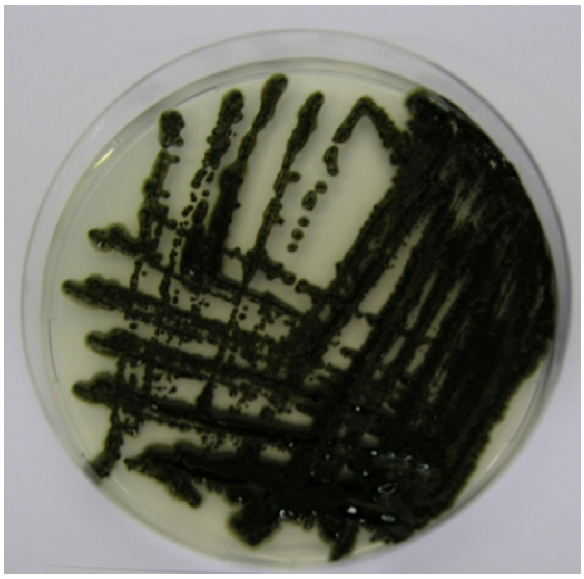

Fig. 5. Yeast-like colonies on oxytetracycline–glucose–yeast extract–agar.

Analysis of DNA, extracted from the biofilm material, showed that all sequences derived from eukaryotic-specific 18S and ITS fragments scored fully for the yeast-like fungus *A. pullulans*. Sequences derived from prokaryotic-specific 16S fragments pointed to *Pseudomonas* sp. Considering the relatively low number of bacteria in the biofilm and the obtained low yield of PCR-products from 16S fragments, bacteria do not significantly contribute to the biofilm. In conclusion, *A. pullulans* was dominantly present in the biofilm (Fig. 5).

4. Discussion

The growth of *A. pullulans* on surfaces treated with oil-derived products is well known and frequently described in the literature under different names like mildew, blue stain, black stain or discolouration (e.g. Goll and Coffey, 1948; Whiteley, 1973; Bardage, 1998; Williams and Feist, 1999). This fungus is one of the typical micro-organisms associated with mildew (Jakubowsky et al., 1983).

A. pullulans is frequently found on building surfaces but usually not mentioned as a micro-organism forming a closed homogeneous biofilm. Water-repellent coatings covering a wood surface obviously create optimal conditions for *A. pullulans* to grow intensively at outdoor conditions (Sell, 1968; Horvarth et al., 1976; Bjurman and Herder, 1992). At conditions with enough moisture on the surface and UV-radiation it is successfully occupying a wood paint niche (Sharpe and Dickinson, 1993).

4.1. Selective conditions

The dominant growth of *A. pullulans* during the exposure time of 36 months in our experiment indicates that the conditions of the exposed timber treated with water-repellent fatty acids containing linseed oil are probably very selective, i.e. favourable to enable growth of *A. pullulans* and unfavourable for other micro-organisms.

The micro-organisms forming the film were determined by staining in combination of microscopy, selective plate counts and DNA isolation. DNA sequencing revealed that the biofilm is consisting almost entirely of the fungus *A. pullulans*. This indicates that the conditions created by the oil treatment and the exposure conditions were quite selective and supported the growth of *A. pullulans* only.

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4.2. Moisture and temperature

Generally it is assumed that the optimum growth conditions for moulds on wood are above 95% RH and a temperature between 25 and 40 °C (Viitanen, 1997). In wood in which the water is homogeneously distributed, free water will only be available above the fibre saturation point (f.s.p.). Usually, depending on the wood specie, this value lies between 20% and 35% moisture content. In practice however the moisture is usually not homogeneously distributed in the wood. Therefore, even at lower wood moisture contents, very locally and temporarily the moisture content of the material can be above f.s.p. If a hydrophobic coating is applied on the wood, liquid water is frequently temporarily available on the surface, which enables the growth of *A. pullulans*. On the other hand, the water can evaporate as soon as the temperature rises. Since the water is not absorbed by the wood this can cause an extremely dry surface on which *A. pullulans* may survive and provide negative conditions for other micro-organisms. One of the selecting factors is probably the influence of the temperature. On the surface of dark material used outside and above the ground, the temperature can rise during the day above 70 °C (Castenmiller, 2004). Although temperatures in this range can be fatal to micro-organisms, *A. pullulans* is able to survive somehow under these conditions.

4.3. Nutrients

It is discussed if *A. pullulans* is actively degrading macropolymers like wood or coatings (Horvarth et al., 1976). Most authors state that *A. pullulans* has a preference for metabolizing simple sugars (Kudanga and Mwenje, 2005).

Intensive assessment of the growth of moulds on wood was carried out by Schoeman and Dickinson (1996). They found that certain strains of *A. pullulans* were able to use breakdown products of lignin as nutrients. Some authors assume that UV-radiation causes degradation products of wood and coatings. This may explain the supply of nutrients for *A. pullulans* since substances formed by the degradation processes can be metabolized by *A. pullulans* (Crang and Pechak, 1979; Sharpe and Dickinson, 1992; Chedgy et al., 2007). The intensive growth on completely oil covered wood surface suggests that linseed oil derivates have on the long-term a positive influence on the growth of *A. pullulans*.

Otherwise, also dust and rain may also provide nutrients for microbial growth.

A potential source of nutrients in this respect is the accumulation of carbohydrates from the air. Wittmaak et al. (2005) give an overview of bioaerosols measured in South Germany. Components of the bioaerosols, e.g. brochosomes, pollen or other particles of biogenic origin, could be used by fungi as nutrients and therefore contribute to the long-term survival on non-organic materials (Pitzurra et al., 2003; Moroni and Pitzurra, 2008).

4.4. Biofilm application

The properties of a functional, induced biofilm are expected to be comparable to existing coatings. Additional functions of a biofilm such as a prolonged service-life, with self-healing effects and the continuous formation of a pigment provide an interesting and considerable added value in the civil engineering area.

The homogenous growth of *A. pullulans* is giving a positive esthetical picture of the biofilm. Melanin is the colour-giving pigment of *A. pullulans* (Ritschkoff et al., 1997), which is able to transform UV-radiation into harmless temperature increase. Besides radiation a strong influence of the nutrition on the pigment formation of *A. pullulans* was observed by several authors (e.g. Seviour et al., 1984; Yang, 1999). Sugar improves the forming of melanin, while a high amount of nitrogen slows down the production of melanin (Ritschkoff et al., 1997).

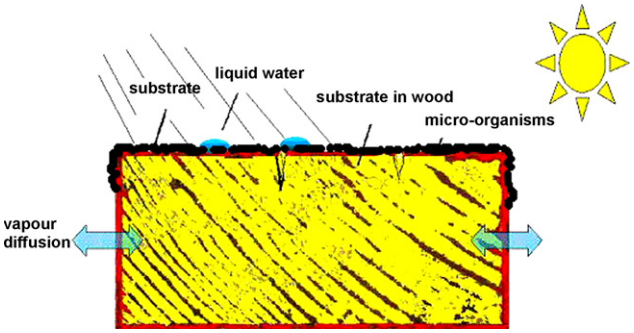

Fig. 6. Conditions supporting the creation of a biofilm.

4.5. Protection

The combination of oil impregnation, biofilm and wood is creating a system that is obviously able to protect wood (Fig. 6) at exterior conditions. Additionally, laboratory tests according to EN 113 (unpublished results) clearly showed clearly the protective effects. The combination of hydrophobation of the oil and biofilm which is covering the surface reduces is obviously preventing any wood degrading organisms to penetrate into the samples.

4.6. A stable system

The long-term stability of the biofilm during its functional lifetime is an important factor for its application in the built environment. Since the intensive interaction between the biofilm, oil and wood is contributing to a good attachment, peeling is avoided. The micro-organisms are able to close small cracks or grow over the spots where the biofilm was removed, which is in fact a self-healing system. The question of whether the nutrients provided by the oil and the wood, together with substances from dust and rain, will be enough to protect the wood for a period of several years cannot be answered yet.

An important aspect of the practical application of a biofilms, containing living cells of *A. pullullans* is the potential emission to the environment of biofilm particles such as cells and metabolites. However, *A. pullulans*, is a very common component of both natural and agro-ecosystems and is generally not considered to be pathogenic or toxic for humans. In many cases where *A. pullulans* is mentioned in relation to clinical infections *A. pullulans* was considered a contaminant, although invasive infections are mentioned as well (Hawkes et al., 2005). Growth experiments with the isolated *A. pullulans*, showed very slow growth at temperatures of 35–37 °C (not published results). Melanin and pullulan, important metabolites of *A. pullulans*, are not considered as toxic (Fogarty and Tobin, 1996; Kimoto et al., 1997).

5. Conclusions

The selective biofilm formation in general can be described as creating appropriate stable conditions to let micro-organisms form a biofilm. The tests carried out with wood samples treated with a water-repellent substrate indicate that it is possible to create a biofilm on a building material with desired functions, such as on timber. The combination of temporary high temperatures, UV-radiation and low water availability in combination with a water-repellent layer provide conditions that produce a relatively homogeneous biofilm which is able to conserve the wood and can

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create a self-healing "coating". The results achieved up to now indicate that the biofilm is able to grow and maintain itself over a period of 36 months without destroying the wood. The effects on the environment of such a condensed agglomeration of *A. pullulans* on larger surfaces are not known yet, but will be assessed in further research.

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