Inhibiting Effect of Sphagnum Moss Extract and Benzotriazole (BTA) on Conservation Waxes Fungal Degradation

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INTRODUCTION

Microcrystalline wax is a widely practiced conservation material used mainly as a protective coating for archaeological and modern bronze objects. It usually serves as an outer component of microcrystalline wax-acrylic compositions applied to the metal surface on the last stage of the conservation process. In the previous studies at Kaman-Kalehöyük the fast degradation of wax film by microbial consortium was shown (Zaitseva 2005). The tested composition (90% microcrystalline and 10% polyethylene waxes) with 20 μm thickness was completely degraded in 2 weeks. Two layered - wax + acrylic films with benzotriazole (BTA) added were degraded in 3 weeks. BTA didn't show any noticeable inhibiting effect regardless its relative toxicity. In these tests microbial consortium was represented by diversity of organisms including both bacteria and fungi. However, at the extreme conditions of the experiment (100% humidity, temperatures of 20-25°C, and high degree of bio contamination) bacteria were the main microbes participated in the wax disintegration.

Being hydrophilic organisms most of bacterial species cannot degrade wax at the recommended museum climate (Tumosa *et al*. 1996; Padfield 2005) i.e. temperature of 18-21°C and relative humidity between 35 and 65%. The optimal conditions for the growth are100% RH and 30°C for most of bacterial species (Prescott, Harley and Klein 2002). Fungi, on the contrary, are able to degrade petroleum waxes (e.g. microcrystalline wax) even at controlled museum conditions. Although fungi degrade hydrocarbons slower than bacteria they are able to penetrate into the wax film reaching digestive compounds of wax and hydroxilate a wide number of hydrocarbons transforming them into forms convenient for other microorganisms (Bennet, Wunch and Faison 2002).

Conservation petroleum waxes have not been studied as an subject for the fungal attack. However, the fungal degradation of a number of wax chemical composition representatives is widely studied respecting to the environmental problem of air and soil contamination by hydrocarbons. Hydrocarbon-degrading fungi are used for biofiltration of volatile organic air pollutants and for bioremediation of contaminated soils. Recently some fungi isolated from air biofilters exposed to hydrocarbon-polluted gas streams have been shown to assimilate volatile aromatic hydrocarbons such as toluene. Fungus *Scedosporium apiospermum* grown on mineral media in bioreactor at the 60% RH effectively converted toluene to CO_2 (García-Peña *et al*. 2001). Another toluene degrading fungus *Paecilomyces variotii* showed even higher elimination rate of toluene on bio filter than bacterial species (García-Peña *et al*. 2005).

Soil bioremediation experiments were focused on the ability of filamentous fungi, including wood-rotting fungi to degrade hydrocarbon pollutants in soil. Soil fungi metabolize normal alkanes and light polycyclic aromatic hydrocarbons –PAHs (Atlas 1981; Leahy and Colwell 1990; Pinto and Moore 2000). Recently potential of 64 filamentous fungi isolated from oil polluted soil (mostly *Aspergillus niger, Gliocladium cf. catenulatum, Penicillium cf. janthinellum, and Pseudallescheria boydii*) to degrade crude oil was tested (April, Foght and Currah 2000).These cultures were plated on the mineral media or introduced in the liquid media with addition of crude oil as a sole carbon source. Plates were incubated for 14 days at 22°C or

56 days at 5°C, liquid media samples were incubated for 14 days at 22°C. Crude oil (Norman Wells, NWT, Canada) was added to the solid mineral media and incubated for 14 days at 22°C and 5°C. As a result 5 species of *Aspergillus* and 18 species of *Penicillium* almost completely degraded $C_{12} - C_{26}$ normal alkanes and 6 of them degraded cyclic alkanes in the tested oil. Three from 64 species were able to grow at decreased temperatures (5°C). Some white rot fungi inhabited, have been shown to degrade polycyclic aromatic hydrocarbons in soil (Clemente and Durrant 2005). After 8 weeks of inoculation in the soil 65% of naphthalene, 82% of anthracene and 76% of pyrene were metabolized by two strains of *Aspergillus* and *Verticillium* sp. Fungi with the ability to metabolize BTEX components of gasoline (benzene, toluene, ethylbenzene, and the xylene isomers) were isolated from oil -contaminated soil and identified as black yeast-like fungi (*Aspergillus niger*, *Cladophialophora* sp., and *Exophiala* sp.), and allied fungi *Cladosporium sphaerospermu* (Prenafeta-Boldú *et al*. 2002).

It is clear that microcrystalline wax coatings in museums and museum storages are potentially vulnerable to fungal degradation, especially in partially controlled and uncontrolled climate. Spores of soil originated *Aspergillus* and *Penicillium* species that metabolize hydrocarbons are widely represented in museum air (Florian 2002). They are able to survive in poor nutrient conditions and relatively low temperatures. *Aspergillus* is mostly accommodated to museum environment due to its biochemical diversity, high reproducibility, and ability to grow in a dry climate (Florian 2002).

Although high molecular ingredients of microcrystalline wax are not digestible for microbes, degradation of even small amounts of low molecular ingredients may lead to depletion of a thin wax film. Both depletion of the wax protective film and accumulation of the acidic microbial metabolites provoke the corrosive processes on the surface of the metal (Zaitseva 2005).

Wax degradation rate depends on concentration of fungal spores in the interior of the facility and the spores ability to retain on the surface of wax. Natural aging of wax film caused by combined effects of ultraviolet radiation, oxygen, and moisture increases availability of wax film for a fungal growth.

Growing fungi increase the biomass of mycelia mat (Rudd *et al*. 1996). At the preferable conditions fungi metabolize hydrocarbons fast. For toluene degrading fungi the doubling time of biomass increasing was about 2-3 days (García-Peña *et al*. 2005). Oil degrading fungi metabolize CO_2, and volatile organic compounds – VOCs (April, Foght and Currah 2000). These volatile metabolites as well as airborne spores released into the air of museums may cause health problems. The black yeast-like fungi degraded toluene and other BTEX are opportunistic human pathogens causing severe mycoses, especially neurological infections (Prenafeta-Boldú, Summerbell and Sybren de Hoog 2006). The pathogenic influence of fungal metabolites (mycotoxines, glucanes, and VOCs) on human are reported in (Piecková and Jesenská 1999).

According to the written above, wax coating needs a special protection against mold even in controlled museum climate. One of the possible ways to protect wax is to involve microbial inhibitors in the treatment procedure, for example, to add them in a coating composition. Using natural bio inhibitors extracted from some medical plants looks promising in this respect due to their non toxicity for human. One of plants possessing microbial inhibiting properties is *Sphagnum* moss. Freshly harvested *Sphagnum* moss is widely practiced in Arctic archaeology as a wrapping material for artifacts to protect them from bio degradation (Sease 1988; Newton and Logan 1992).

Bio inhibiting properties of *Sphagnum* moss are caused by water soluble polysaccharide contained in leaves and steams of the plant (Painter 1991). Painter initially extracted this polysaccharide (called him Sphagnan) from *Sphagnum* moss and found its bacteria static properties (Børsheim, Christensen and Painter 2001). However, he never tested Sphagnan as an anti fungal agent.

Recently on the basis of Queen's University Art Conservation Department the influence of *Sphagnum* moss extracts on fungal spore germination was studied. Extracts showed the clear fungi static effect (Zaitseva, in process).

The present research project is an attempt to use the

Sphagnum moss extract to inhibit fungal growth on wax coatings. Fungi were isolated from the artifact storage facility on the archaeological site in Kaman-Kalehöyük. Extract was introduced into two conservation waxes exposed to a mature fungal lawn. The degree of wax bio degradation was estimated by weight loss of wax disks (disk test) Benzotriazole (BTA) as an additive to wax coating was also tested along with *Sphagnum* moss extract.

BTA is a well known corrosion inhibitor for copper alloys. It is widely used in the archaeological conservation practice. BTA is normally applied on the cleaned metal surface and also added into acrylic and wax coating films. Addition of the BTA into the coatings increases their protective properties against corrosion. Due to its toxicity BTA also was considered to possess antimicrobial properties. BTA was tested towards mature fungal lawn in the disk test (on the same way as *Sphagnum* moss extract) and towards fungal spores in the tube test. In tube test BTA was introduced into the fungal spore solution were the influence of BTA to the spore viability was determined depending on the time of spore exposure to BTA.

MATERIALS AND METHODS

Fungi were collected from the interior surface of biocontaminated polyethylene bags, used for storage of artifacts. The living spores were swabbed by sterilized tampon wetted in sterilized distilled water and inoculated into Petri plates with Sabouraud Maltose Agar media (SAB Maltose media).[1)] Plates were incubated at room temperature until the growth of fungal colonies occurred. The grown colonies were represented by diversity of fungal species. These species were isolated on separated Petri dishes and purified by a number of re-inoculations. Twelve species were distinguished and one of them was selected for further testing. The chosen culture was dominantly represented in polyethylene bags. It also possessed the best viability and highest consuming rate of wax films during preliminary tests. According to its morphology the selected culture belonged to *Aspergillus* genera.

Spore solutions of given concentrations were prepared to estimate inhibiting properties of *Sphagnum* moss extract and BTA. For accurate counting of colonies not more than 200 spores have to be plated on one Petri dish. To prepare *stock suspension* living spores were washed out from the fungal lawn with 1 % Tween 20 detergent in distilled water. Washes were suspended into sterilized plastic tube using Ependorf pipette. To prepare the *stock solution* with necessary concentration of spores, initial *stock suspension* was diluted from 10^{-1} to 10^{-9}. To estimate the concentration of spores in dilutions 0.1 ml aliquots from 10^{-6} to 10^{-9} dilutions were plated on Petri dishes with SAB Maltose media. Plates were incubated for 18 hours at the room temperature and day light. Colonies grown on plates were simply counted:

10^{-6} – too many;
10^{-7} – 199 col., 202 col., 172 col. (191 col. on average);
10^{-8} – 10 col., 23 col., 13 col.;
10^{-9} – 3 col., 9 col., 4 col.

The dilution of 10^{-7} was chosen for further experiments as a *stock solution*, 0.1 ml of which gave N_o=191 colonies grown per plate.

Sphagnum moss was freshly harvested from a peat bog located in Kingston area, Ontario (Canada) and identified as *Sphagnum* moss *palustre* on the Botanical Department of Queen's University. *Sphagnum* moss extract was prepared following the personal recommendations of Dr. Painter (see also Børsheim, Christensen and Painter 2001). The procedure included preparation of holocellulose by acetone removing the main chemical ingredients of the plant (waxes, steroids, triterpentoids, carotinoids, chlorophyll), bleaching of holocellulose to holocellulose chlorite, and final extraction of water soluble polysaccharide following by freeze drying of it to a solid *Sphagnum* moss extract. The obtained extract is most likely a water soluble polysaccharide Sphagnan described by Painter.

Waxes Be Square 195® and Polywax 2000® used for copper alloy protection at the Kaman-Kalehöyük archaeological site were selected for

[1)] Sabouraud Maltose Agar media and Sabouraud Maltose Broth media are used in medical mycology (Malloch 1997). They were designed to develop fungal and inhibit bacterial growth.

the present research. For the samples preparation, wax was melted slowly on a hot plate in a small aluminum pan. *Sphagnum* moss extract was introduced in wax as its 3% (w/v) solution in 1:1 Ethanol-Toluene mixture, 0.3 ml of extract in 8 g of wax. Benzatriazole (BTA) was introduced into wax as 0.5 % (w/v) solution in 1:1 Ethanol-Toluene mixture, 0.3 ml of extract in 8 g of wax. The solutions were carefully added by drops into melted wax. After the mixture was cooled to a soft mass, 10 mm disks were cut. The thickness of a disk was approximately 2 mm. The weight loss of wax samples was measured on Sartorius Analytical Balance with readability 0.1 mg.

RESULTS AND DISCUSSION

Weight loss test

The test was performed to evaluate the ability of *Sphagnum* moss extract and BTA to inhibit bio degradation of selected waxes. The degradation rate was determined as a weight loss of wax disks within given period of time.

Disks were weighted and exposed to the surface of mature fungal lawn grew on Petri dishes with the Sabouraud Maltose Agar media. There were 16 disks placed on each dish, 8 on one half of the dish, and 8 on another half, according to the table:

(8 disks Be Square+3% Extract+ 8 disks Be Square+0.5% BTA)	3 dishes=48 disks
(8 disks Polywax+3% Extract+ 8 disks Polywax+0.5% BTA)	3 dishes=48 disks
(8 disks Be Square–control+ 8 disks Polywax –control)	3 dishes=48 disks
	144 disks/9 dishes

Table 1 The weight loss of wax disks exposed to fungal lawn

Wax composition tested	W_{before}	W_{after}	_W, %
Be Square	8.1616	4.5666	44
Be Square+3% Extract	8.1123	7.7611	4
Be Square+0.5% BTA	8.8765	7.7235	13
Polywax	8.1120	7.1200	12
Polywax+3% Extract	8.1966	8.0112	2
Polywax+0.5% BTA	8.1241	7.2304	11

After 3 weeks of exposition at the room temperature and day light disks were gently taken off from the fungal lawn, washed with 1 % Tween 20 detergent in distilled water to remove mycelia, dried and weighted repeatedly. The weight loss $\_W = (W_{before}-W_{after})/W_{before} \_100\%$. Results are represented in the Table 1.

Surface of the disks contacted with the fungal growth was completely covered with filaments. On the upper surface of disks filaments were located along the edge of the disk. Disks were appeared much thinner, suppressed by mycelium from the periphery to the center of disk, concaved, and cracked. Results show a quite fast degradation of microcrystalline wax, 44% in 3 weeks. On the contrary, polyethylene wax disks were almost intact. They lost only 12% of their weight.

The measured rate of microcrystalline wax fungal degradation is higher than those for bacterial degradation reported by (American Petroleum Institute 2003). According to their results two microcrystalline waxes incubated for 4 weeks at 20-25˚C in nutrition rich liquid media (oil-contaminated soil; domestic sewage sludge mixed with soil) were degraded for 21and 27% accordingly. The conditions of experiments were optimal for active bacterial growth. Our results confirm that at the preferable conditions degradation of microcrystalline wax by fungi is even faster than by bacteria (44%/3 weeks vs. 27%/4 weeks).

Sphagnum moss extract showed a significant inhibiting effect. The 0.3 ml of 3% i.e. 0.1% (w/w) extract added to 8 g of wax almost completely protected both waxes for 3 weeks. Only 4% of microcrystalline and 2% of polyethylene wax was consumed.

BTA addition in wax showed 3 times weaker inhibiting influence on fungal growth- 13% weight loss for 3 weeks. For polyethylene wax BTA didn't show distinguished protective influence respecting to the given fungal specie. The relatively weaker effect of BTA can be primarily explained by its 5 times lower concentration introduced into waxes – only 0.02% (w/w). Solubility of BTA in most of solvents is very limited. The concentration of BTA in Ethanol-Toluene mixture 0.02% (w/w) was maximal for the temperature of experiment.

Table 2 Number of colonies (N) grown out of spores exposed to BTA and control spores (not exposed). Number of colonies listed is an average for 3 plates.

Exposition time, t	5 min	5 h	8 h	11 h	15 h	20 h	24 h	28 h
N_{Broth}, col/plate	151	138	142	147	152	148	138	140
$N_{Broth+BTA}$, col/plate	142	114	116	116	100	3	2	2

Tube test

In this experiment the influence of BTA to spore germination in the liquid media- SAB Maltose media Broth was tested. In each of 16 test tubes contained 1.0 ml of Broth 0.1 ml of *stock solution* was added. Tubes were divided in two sets of 8/8 tubes. Into each tube of the first set 40µl of 0.5 % v/w BTA were added. Into the second set of 8 tubes no BTA was added and they were considered as controls. Both sets were incubated at the room temperature and day light for 5 min, 5, 8, 11, 15, 20, 24, and 28 hours (8 intervals). To check the ability of spores to germinate after exposure to BTA, 0.1 ml aliquots from tubes with Broth and with Broth+BTA were taken and plated on 3 Petri dishes with SAB Maltose media according to the time table (6 plates per one time interval). Plates were incubated during 18 hours at room temperature and day light until colonies occurred. The difference between numbers of colonies grown from spores exposed to BTA and control spores characterize the inhibiting influence of BTA (Table 2.).

Plates with grown colonies are shown on Fig.1. Colonies on control plates (above) are stronger than colonies grown from spores treated with BTA for each exposition time.

Results show that the number of colonies grown from control spores was stable ($\bar{N}$=145 colonies/plate) during the whole time of the exposition. Viability of spores on controls estimated as $\bar{N}$ / N_o_100%= 76% or rather high. (N_o =191 col/plate).

The number of colonies grown from spores treated with BTA didn't change significantly during the first 11 hours of exposition. After 15 hours of the exposition viability of the treated spores fell down almost to zero showing clear inhibiting effect of BTA on spore germination in Broth. As an additive to microcrystalline wax, BTA also shows inhibiting influence on the selected fungal culture. At the same time, BTA addition in wax didn't show any protection against microbial consortium included both bacteria and fungi (Zaitseva 2005). The difference in the degree of BTA antimicrobial effect in liquid media and in wax can be primarily explained by not resembled experimental conditions of these tests and microbial species participated. Another reason of such different results could be caused by mechanisms of BTA influence on mature fungi and spores.

In the tube test BTA is uniformly distributed in the liquid media and effectively envelope spores. Being added into the wax BTA functions differently and affects mature organisms, not spores. It can be considered that BTA is not uniformly incorporated into the wax crystal lattice. The growing hypae of filamentous fungi such as *Aspergillus* can pass by BTA decreasing the inhibiting influence of the latter. In case of wax exposition to a diversity of microorganisms including fungi and bacteria combined digesting mechanisms with the more effective consumption of wax are realized. It leads to the fast and complete degradation of the wax film.

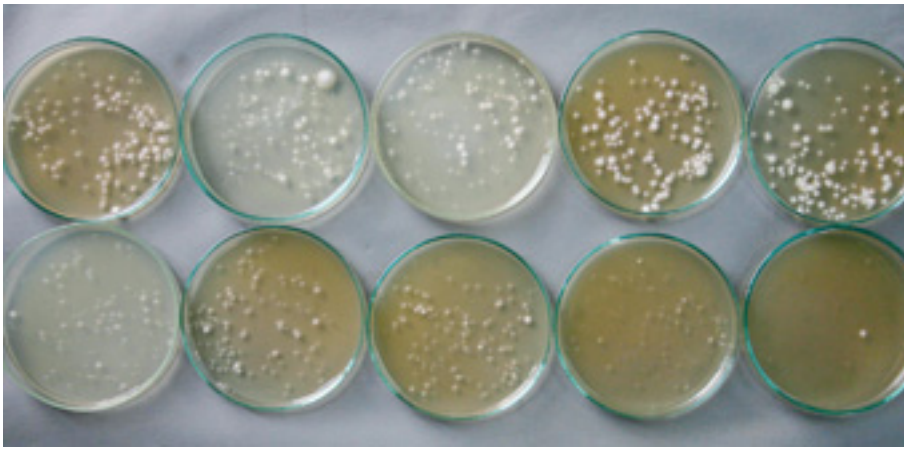

Fig.1 Above-control plates with colonies grown from spores untreated with BTA. Below-colonies grown from spores treated with BTA. Plates are arranged according to their exposition time: 5, 8, 11, 15, and 20 hours. The number of colonies drastically decreased between 11 and 15 hours of spore exposition to BTA.

CONCLUSION

The dominant fungal culture inhabited in Kaman-Kalehöyük artifact storage facility was isolated and identified as *Aspergillus*. Some *Aspergillus* species survive and develop even in controlled museum climate and degrade petroleum waxes chemical composition.

The ability of *Sphagnum* moss extract and BTA added in microcrystalline and polyethylene waxes to inhibit growth of selected fungi on wax substrate was tested. Wax disks weight loss test showed considerable degradation rate of microcrystalline wax by selected specie - 44% in three weeks. Being the main ingredient of wax coating composition practiced at the Kaman-Kalehöyük archaeological site (90% of microcrystalline wax and 10% of polyethylene wax) microcrystalline wax determines degradation of whole such a composition. Polyethylene wax showed much lower degradation rate -only 12% in three weeks.

Sphagnum moss extract showed clear inhibiting effect towards the selected fungal specie. Addition of 0.1% (w/w) of the extract to tested waxes almost stopped their degradation. The weight loss of wax disks was 4% for microcrystalline wax and 2% for polyethylene wax in three weeks. Using of *Sphagnum* moss extract as a fungistatic ingredient of wax coating looks promising and needs a further study.

BTA showed a three times less wax protection rate. The weight loss of both waxes was about 12% in three weeks. The relatively weaker effect of BTA can be primarily explained by its 5 times lower concentration in waxes – 0.02% (w/w). Being more soluble, *Sphagnum* moss extract can be introduced in wax in a greater concentration and cause the better inhibiting effect than BTA.

In liquid SAB Maltose media with 0.02% (w/v) of BTA the fungal spore germination was decreased to zero after 15 hours of exposition. The difference in the inhibiting effect of BTA in wax and in liquid media is more likely explained by different experimental conditions and different mechanism of BTA influence on mature fungi in wax and their spores in Broth.

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