

Biocontrol of pathogenic fungi growing in vitro by galvanic microcells

Spisak¹ Wojciech & Chlebicki^{2*} Andrzej

¹ Research & Development Centre ALCOR Ltd, Kępska 12, 45-130 Opole, Poland

^{2*} W. Szafer Institute of Botany, Polish Academy of Sciences, Lubicz 46, 31-512 Cracow, Poland

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Abstract: There are presented first research related to fungi growth within electric fields generated by galvanic cells with active metal electrodes. We used Al and Cu electrodes with 2 mm distance and fungal strains such as *Cladosporium cladosporoides*, *Exophiala sideris*, *Malbranchea* sp., *Oidiodendron truncatum*, *Paecilomyces fumosoroseus*, *Penicillium expansum*, *P. spinulosum* and *Phialophora sessilis*. In our experiments active electrodes buildup the gradients of pH which were indicated by eriochrome black T and bromothymol blue. Experiments showed that influence of galvanic current on fungal growth depends of fungal species, distance between galvanic electrodes and pH. Combination Cu-Zn appears the most promising for preventing of material biocorrosion.

Introduction

Expansion of fungi within the industrial space and in the human surroundings In city air a lot of particulates, mold spores and yeasts (air borne fungi) are present. Among them we can often find so called Black Yeasts. Another kind of dangerous fungi are yeasts, especially the ones belonging to the ascomycete genera of yeasts and some basidiomycete yeasts. There can be found also fungi from other groups, i. e. other ascomycete fungi and their anamorphs as *Arthroderma*, *Chaetomium*, *Cladosporium*, *Aspergillus* as well as basidiomycetous fungi as *Wallemia*, *Sporotrichum*, *Schizophyllum*, *Tilletiopsis* etc. Of them, Black Yeasts are not only dangerous pathogens to humans and animals, but they also are specially resistant to UV radiation, can decompose aromatic hydrocarbons (their development is increased by the presence of industrial particulates containing hydrocarbon molecules), are resistant to high temperature, their endospore are able to survive even 110° C. They can be often found in canteens, antique shops, greenhouses, saunas, farms, flower shops, summer cottages, hospitals, as well as inhabit biofilters, destroy the monuments and building surfaces. Toxic influence of fungi on human and animal health is considered to be much more dangerous than a materials biocorrosion (Anderson *et al.*, 1997; Fisher *et al.*, 2012). Diseases, which are a consequence of staying in moldy spaces are usually treated by biocides. However these biocides used to impregnate and eliminate fungi from various types of materials, may also have harmful influence on human health.

On the market, there are many preparations. However, chemical methods of fighting mold by using biocides are not commonly accepted with regard to relatively short protection time connected with immune mechanisms of microorganisms, and, therefore, it is necessary to reapply a protective layer (Russel and Chopra 1990). Toxicity and unfavorable influence of such preparations on environment and human health is also a factor (Tiano 1998).

Disruption of fungal colonies was earlier performed by many chemical agents such as antifungal drugs: imidazoles, triazoles, thiazoles, allylamines and recently, kind of phenol: triclosan (). Based on studies focused on the assessment of effectiveness of disinfection procedures in fighting against mold fungi so far described, it has been proved that using biocide to fight against moldy fungi on the building materials surface does not guarantee its long-term effectiveness. Such effect can be obtained only by repeatedly reapplying the disinfection procedure.

Electric field. Electricity and geomagnetic fields of environments exist very long time, perhaps as long as is Earth old. Some bacteria possess magnetosomes (composed of iron oxide or iron sulfide) that can orient magnetotactic bacteria in geomagnetic fields (Bazylnski et al. 1995). Also microorganisms named electricigens can transfer electron to electrodes when they oxidizing organic compounds to carbon dioxide and yield a small electrical current (Lovley 2006). The fungal hyphae generate electrical currents around their hyphal tips (Kropf 1986). The inward electrical current reflects local nutrient transport (Gow 1989).

The influence of electric field on microorganisms of our interest became a subject to research until the 1980's. For the first time, galvanotropism, which is a directional growth response of cells to externally applied electric field, was observed among fungi by Robinson (1985) and by Gow (1989). The response of fungi is also related to the electric field strength (Van Laere 1988) and medium composition (Cho *et al.*, 1991). Liu *et al.* (1993) investigated effect of electric current on bacteria colonizing intravenous catheters.

Also, the pH of environment had a significant effect on galvanotropism of mycelium (Roth and Theato 2008).

These observations indicate that observed galvanotropism of mycelium development may involve two processes: the electrophoretic redistribution of transmembrane proteins important to growth, and inducing mycelium growth by voltage-sensitive calcium ions in cell membranes (Olsson and Hansson 1995).

But there is another process recently discovered in nature: the electrical activity of microorganisms (electrode reducing microorganisms that have electron-transfer abilities). They do this by shutting electrons from the cells along tiny molecular wires. Such electrochemically active aerobic and anaerobic bacteria and yeasts have ability to produce electricity using device known as a microbial fuel cell (MFC), which is a kind of bioelectrochemical system or biological battery (Debabov 2008; Arends *et al.*, 2012). It is intriguing that within the mycelium of *Pleurotus ostreatus* and *Armillaria bulbosa* electric potentials, similar to neurons electric potentials, can be found spontaneously (Sionkowski and Kaczmarek 2010).

What is very interesting, frequency of generated electric potentials significantly changes when close to fresh wood particles, while they are not influenced at all by close presence of plastic particles. This unexplained phenomenon is still waiting to be further researched.

Metals It is commonly known that some metals and their salts have great biocidal and fungicidal properties. The most common example is silver, which is a subject to extensive research in the world (Olsson and Hansson 1995; Hemath *et al.*, 2010).

The technology of using silver and its salts as biocides may be concerned to be mature due to the many domestic and foreign patents (Olsson and Hansson 1995; Betbeze *et al.*, 2006; Ślusarczyk and Piotrkowska 2008).

Metals, which can be a base for biocides, may be presented in a series according to decreasing bioactivity (Nocuń-Wilczek 2011):

Ag>Hg>Cu>Cd>Pb>Co>Au>Zn>Fe>Mn>Mo>Sn

Some of them, e.g. mercury or cadmium, have very high toxicity and, naturally, cannot be used. Others, e.g. gold or silver face price limits or environmental limits in relations to silver nanoparticles. The most noteworthy metals

among the presented series are copper and zinc. Antimicrobial properties of copper, zinc and their salts are well documented. At the same time, these metals are microelements indispensable for life of many organisms including humans, which significantly differentiates them from other heavy metals with biocidal properties.

COPPER – biocidal activity of copper is inferior only to silver and mercury as far as metals are concerned. For many years now copper salts have been a basis for ecologically safe fungicides, historically known as Bordeaux mixture.

ZINC – zinc ions, even in low concentration, have strong antimicrobial properties¹. Antifungal activity of zinc sulfate is commonly used in medicine to fight dandruff. At the same time, zinc preparations, e.g. zinc oxide, are ingredients of dermatological medicines.

Effective biocidal or biostatic activity of particular metal or its salts requires direct contact with a cell of microorganism they fight against. According to de Rome and Gadd (1987) metal-binding capacity can be effected by pH, i. e. low pH restrict binding capacity of biomass for Zn and Cu.

It is well known that two different metals may form a galvanic cell when immersed in an electrolyte. Galvanic action between metals causes oxidation of anode metal to ion which diffuses in the surrounding medium as ion flux. It is believed, as yet unsubstantiated, that galvanic action offer high antimicrobial activity by one or more of several mechanisms. Electrodes may interact to form hydrogen peroxide from components of the water rich environments forming electrolyte. In a second mechanisms the metals may interact to release metal ions into surrounding environment and a significantly higher concentration of metal ions is released than occurs if the metals act independently without galvanic cell. We do not know about any research related to fungi growth within electric fields generated by galvanic cells with active metal electrodes.

Results

First set of experiments. Recognition of optimal distance between electrodes

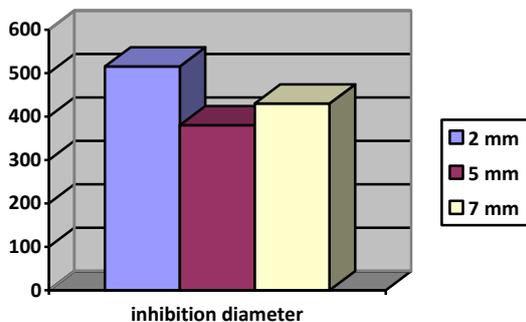


Fig. 1. Inhibition effect on growth of *Penicillium spinulosum*. Distance between Cu and Zn electrodes: 2 mm, 5 mm and 7 mm.

After one week colonies on PDA were measured. The bigger diameter of inhibition zone surrounding Cu and Zn electrodes has 2mm distance between them. It appeared that distance 2 mm is more effective.

2. We investigated inhibition effects of single metal electrode. We used: Ag, Bi, Cu, Fe, Mg, Ti and Zn electrodes. Of them Zn electrode has the highest inhibition effect on fungal growth (23 mm diam. of halo). Also Fe appeared toxic for fungal growth but in the lower level (7 mm diam. of halo). Influence of other investigated metals are not visible (Fig. 2).

diameter of inhibition zone

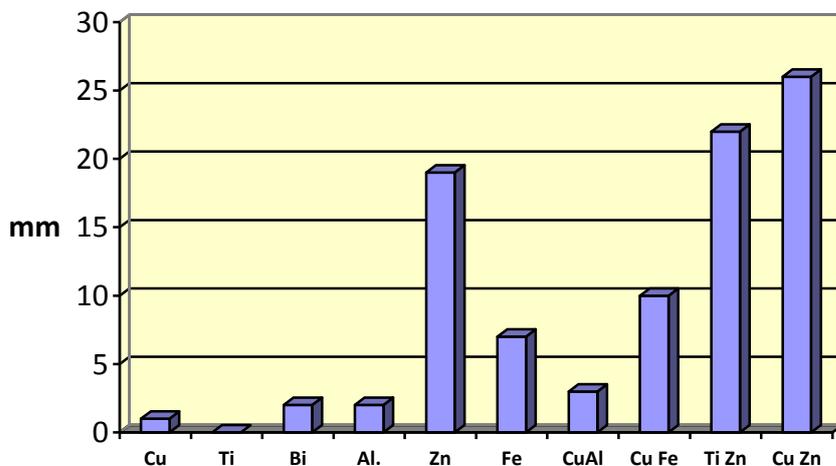


Fig. 2. Influence of metals on fungal growth (*Penicillium expansum*). Petri dishes with PDA medium in room temperature, day/nigh, after 3 days of incubation. Diameter of dishes = 90 mm.

3. We used metal electrodes, where ions movement was connected with (-) anode (Zn, Fe etc). Among used 16 combinations of electrodes the more promising appeared Ag-Zn, Cu-Zn and Ti-Zn (see Tab. 1). Optimal for medical using combinations of electrodes (Ti-Bi and Ti-TiO) posses distinctly lower range of voltage (Tab. 1).

Table 1. Electrical field between electrodes [V cm⁻¹]. Voltage of metal electrodes in V cm⁻¹

Metal electrodes	1 day [V cm ⁻¹]	2 day [V cm ⁻¹]	4 day [V cm ⁻¹]	9 day [V cm ⁻¹]
Cu	0,1280	0,2144	0,0626	0,0224
Ti	0,1744	0,9204	0,4300	0,4604
Bi	0,0260	0,0838	0,2240	0,2098
Al	1,0276	0,9380	1,0694	1,1050
Zn	1,5740	1,5868	1,6494	1,6476
Fe	0,8570	0,8740	0,4723	0,5550
Ag-Zn	1,9440	2,0826	2,6780	1,0640
Ag-Cu	0,1872	0,2314	0,3102	0,2562
Cu-Al.	1,0460	1,0916	1,2128	1,2040
Cu-Fe	1,0200	0,9878	0,9016	0,8692
Cu/Zn	1,8052	1,7254	1,7760	1,7124
Bi-Cu	0,2104	0,0316	0,2154	0,2116
Bi-Fe	0,7660	0,8704	0,8134	0,8654
Fe-Al	0,3082	0,2098	0,4812	0,8840
Ti-Bi	0,0168	0,2284	0,1266	0,1800
Ti-Cu	0,1470	0,0864	0,4360	0,4418
Ti-Zn	1,4290	1,6682	1,9560	1,6862
Ti-Fe	1,0010	1,1260	1,4412	1,1822
Ti-Al	0,7640	0,9256	1,5258	1,4902
Ti/TiO	0,2040	0,2694	0,0770	0,5840
Zn-Al	0,4514	0,5688	0,4896	0,5746

It is interesting that voltage of metal electrodes changing with time for Cu, Ti, Fe but is on the same level for Zn and Al (Tab. 1). Zn possess highest values for single metal electrode, whereas for galvanic cells combination Ag-Zn (Tab. 1).

4. For next experiment we used four fungal strains: *Cladosporium cladosporoides*, *Oidiodendron truncatum*, *Penicillium spinulosum* and *Penicillium expansum* and three combinations of electrodes: Cu-Zn (where Zn is anode), Mg-Zn (where Mg is anode) and Ag-Cu where Ag is anode (Fig. 3).

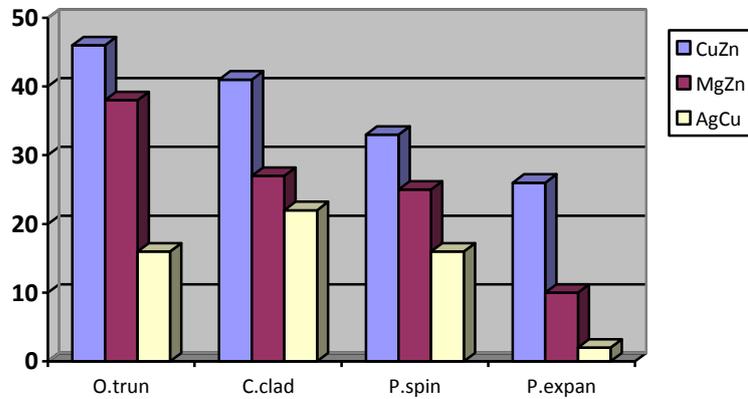


Fig. 3. Inhibition of fungal growth by metal electrodes: Cu-Zn, Mg-Zn and Ag-Cu. Used strains of fungi (from the top): *Cladosporium cladosporoides*, *Oidiodendron truncatum*, *Penicillium spinulosum*, *Penicillium expansum*. Petri dishes with PDA, in room temperature, day/night, after 6 days.

There is visible strongly Zn inhibition effect on fungal mycelia. The widest halo were observed in the Cu-Zn electrodes (Fig. 3). *Penicillium expansum* appeared the most resistant fungus on metal ions influence. Our results indicate that inhibition effect on fungal growth depend on fungal species and used electrodes (Fig. 3).

5. Vertical styrene net with galvanic microcells ca 70 um diam. of Cu and Zn was plated between two parts of Petri plate. Between next two parts of the same Petri plate a pure styrene net was plated as a control.

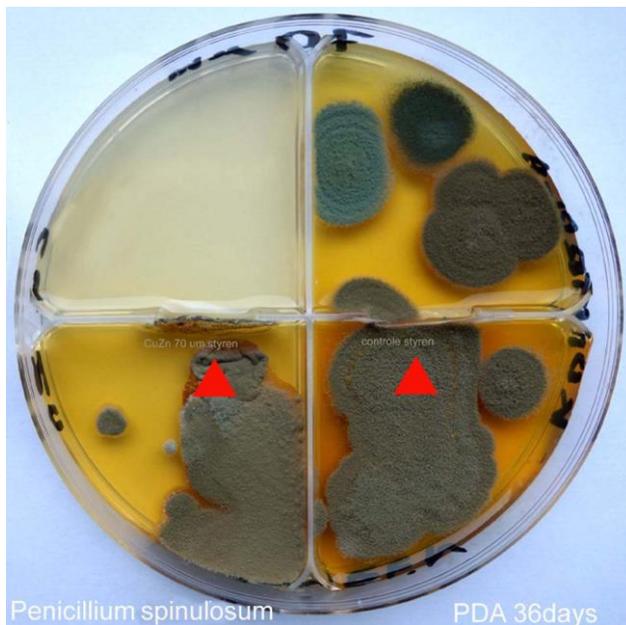


Fig. 4. Mobility of fungal mycelia through plates with galvanic microcells, Cu and Zn 70 um diam., PDA medium, day/night, room temperature, 36 days of incubation.

Fungal mycelia cannot across the nets with galvanic microcells (Cu-Zn), but styrene nets are not a barrier for them (Fig. 4).

Second set of experiments

1. Because the rate of zinc dissolution is greatest at lower pH we will start measurement of pH distribution in the Petri plates with electrodes and fungal strains.

We used following pigments: phenolphthalein, azobenzenecarboxylic acid, Congo red and dibromothymolsulfonophthalein (Fig. 5).

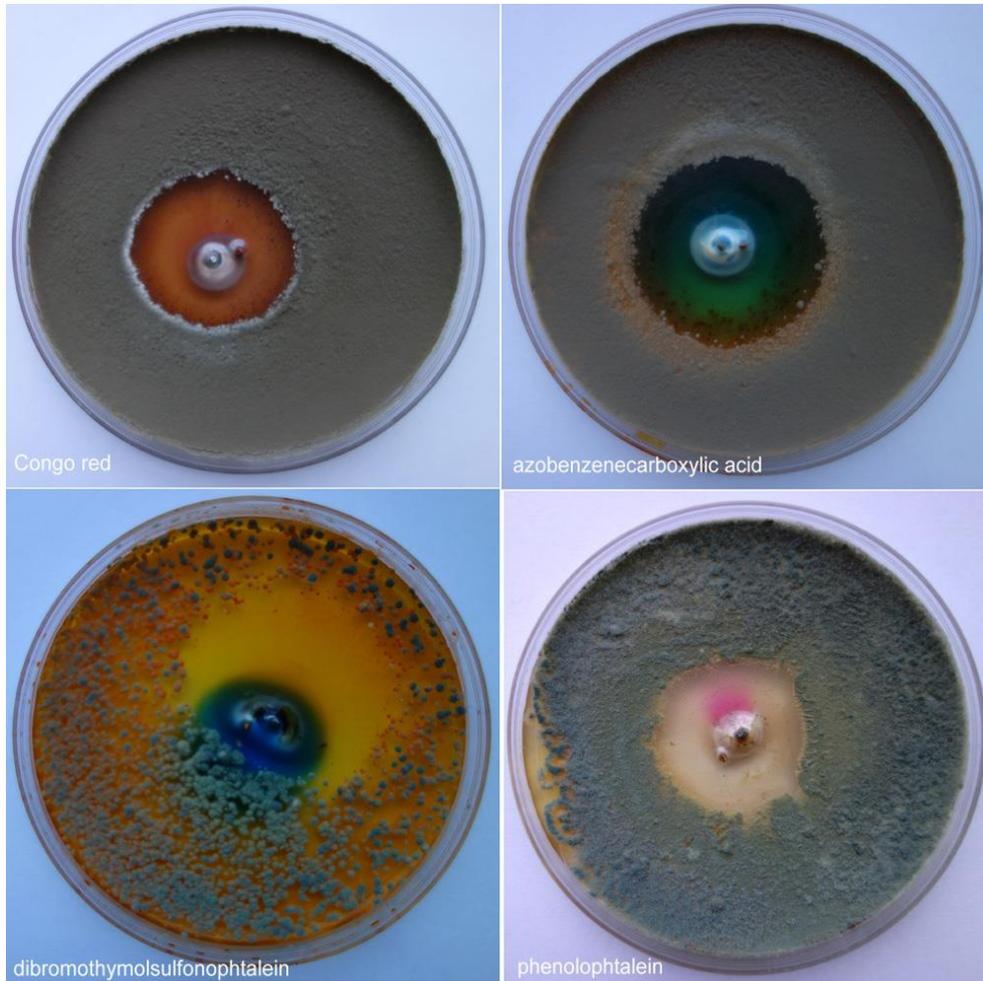


Fig. 5. pH changes in Petri plates with fungi: *Penicillium spinulosum* (upper line) and *Penicillium expansum* (lower line). Used pH indicators: phenolphthalein, azobenzenecarboxylic acid, Congo red and dibromothymolsulfonophthalein. Petri dishes with PDA, in room temperature day/night, 10 days incubation.

Dibromothymolsulfonophthalein appeared the more appropriate for our investigation. Anode (zinc) form alkaline area whereas catode (copper) form acid area (Fig. 6).

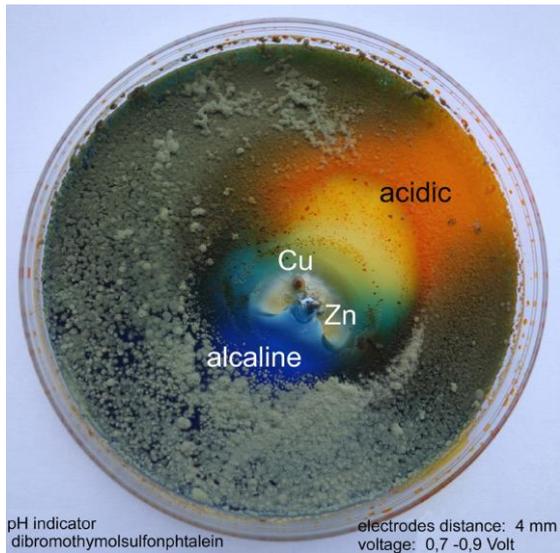


Fig. 6. Distribution of pH with Cu-Zn electrodes and fungal strain of *Penicillium expansum*

We observed asymmetrical inhibition zone surrounding electrodes in many cultures (Fig. 6).

2. We inoculated fungal propagules on medium with dibromothymolsulfonphthalein such species as: *Aspergillus nidulans*, *Exophiala sideris*, *Malbranchea* sp., *Paecilomyces fumosoroseus*, *Phialophora sessilis* and *Penicillium polonicum*. It appears that mycelia can change pH of used media (Fig. 7).

3. Changes in pH by fungal mycelia.

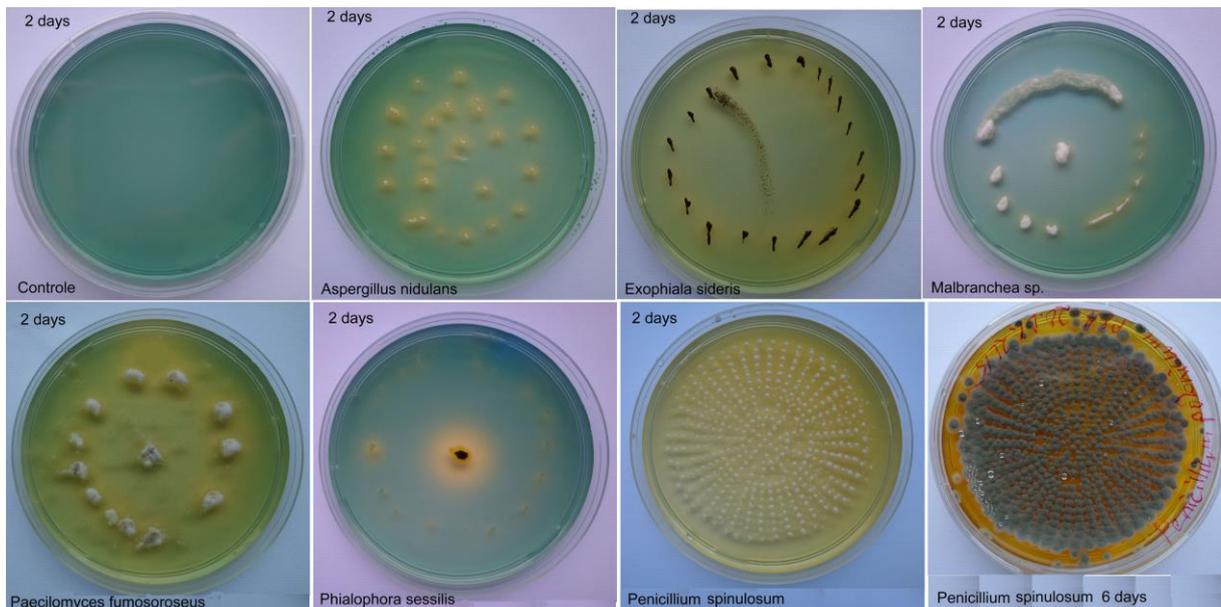


Fig. 7. Influence of fungal mycelia on pH of medium (PDA, room temperature, day/night, 2 days); pH indicator: dibromothymolsulfonphthalein

Obtained results indicate that almost all fungi produced acids. Only *Malbranchea* sp. (anamorph of *Amauroascus kuehnii*) produced very small amount of acids.

DISCUSSION

Galvanic cells create electric fields from 0,01 V cm⁻¹ up to 2,678 V cm⁻¹ between electrodes according to electrodes materials system. Electric fields from these range are source of galvanotropism itself for some fungi

as reported by Kropf (1986) and Gow (1989). Moreover the electric field enable moving of toxic metal ions. Acidification of medium surrounding mycelium enable more effective activity of these ions. Asymmetric inhibition zone is connected with changes of pH surrounding electrodes. Also McGillivray and Gow (1986) applied electrical field that dramatically affect the polarity of growth of filamentous fungi. But we used galvanic electrodes that create own electrical current. In our experiments active electrodes buildup the gradient of pH which were indicated by eriochrome black T and bromothymol blue. Gradient of pH is probably correlated with gradients of oxygen, metals ions, nutrients and electrolytic by-products. Combination Cu-Zn appears the most promising for preventing of material biocorrosion.

Material and Methods

Fungal growth was performed on MEA (Malt extract agar, Merck KGaA) and PDA (Potato dextrose agar, Merck KGaA); preparation according to description added to the containers. Inoculated media on Petri dishes were preserved in room temperature, in the dark.

The morphological characters of the living fungi were examined in water and lactophenol blue solution (cotton blue in lactophenol, Merck) using light microscopy (Nikon SMZ 1500, Nikon Eclipse 80i). Microphotographs were taken with these microscopes equipped with a digital camera Nikon DS R1. For scanning electron microscope (SEM) studies, mycelium was fixed in 3% buffered glutaraldehyde (pH 7), washed 2 times in buffer for 10 min. and after dehydrated in ten graded series of ETOH and acetone, for 10 min in each serie. Fixed samples were coated with gold, and photographed using a scanning electron microscope (SEM). Fungus-species nomenclature follows Kirk et al. (2001).

Galvanic cells were formed from 4 mm long and 1 mm diam. wires of Bi, Ag, Al, Cu, Fe, Ti, Zn, putted in distance 2-7 mm. For next experiments were used only electrodes Cu and Zn with distance 2mm.

The current generated by galvanic cells was detected by using true-rms Industrial Logging Multimeter with TrendCapture model 289, by Fluke.

Measurement of electric fields generated by galvanic cells was be provided by using true RMS Industrial Logging Multimeter with Trend Capture, Fluke 289. For experiment with pH indicators we used PDA medium with 20 drops dibromothymolsulfonophthalein and 10 drops of 10% KOH. Such media were green at the beginning (pH = 7).

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