Simple And Rapid Technique For Measure of Liquid Paints Antimicrobial Activity

Małgorzata Robak[1], Tomasz Koźlecki[2]

[1]*Department of Biotechnology and Food Microbiology,*
Wrocław University of Environmental and Life Sciences,
37 Chełmońskiego St., 51-630 Wrocław, Poland
[2] *Department of Chemical Engineering,*
Wrocław University of Technology, 4/6 Norwida St., 50-373 Wrocław, Poland

Abstract
Microbial invasion of building materials, including paints, is a manufacturer and future habitants problem. So, a need of antimicrobials protected materials, especially paints, is crucial for health of peoples and animals. The way of testing this antimicrobial activity in different kinds of paints is not always easy. The viscosity of emulsion and oil-paints makes the traditional microbiological technique very difficult or impossible. We propose to make serial decimal dilutions of bacteria and fungi suspension, performed a night incubation (10-16 hours) at room temperature with tested twice diluted paint(s), then special inoculation on agar medium (5 µL), incubation for 24-48 hours at 25-30°C and counting of developed colonies. Not killed by paint microorganisms have been going to growth as colony on agar. The technique was tested on four different emulsion paints and the results were clear and easy to interpret. Using this technique biocidal activity of silver nanoparticles at concentration of 6.25 mg/L was confirmed. This methodology could be applied for all liquid products involved in building materials, house cleaning and body hygiene.

Keywords — *biocidal activity, silver nanoparticles, AgNPs, paint*

I. INTRODUCTION

Filamentous fungi recovered from contaminated building materials, including paints, mainly belong to the following genera: *Aspergillus, Penicillium, Paecilomyces, Trichoderma* and *Chaetomium*. Beside deterioration of walls and wood in human location some of them produce mycotoxines, which were toxique or allergenic for people and animals [1-4]. It is why walls paints have to contain antimicrobial component(s). Known, since two centuries, antimicrobial chemicals added to paints and house cleaning products were based on chloral, ammonia, formaldehyde and phenol, all chemicals not indifferent for humans' health. Recently as antimicrobial agents were tested silver nanoparticles (AgNPs) of different radius and at different concentrations [5-11]. Studies of biocidal activity of AgNPs were numerous and ported on antifungal and antibacterial effects. The biocidal effects on bacteria were noted at concentration of 2-60 mg/L [12, 13]. According to Kim et al. [14] the concentration of 2 mg/L was sufficient to inhibited *C. albicans* yeasts and more effectively biocidal were AgNPs of 5 nm diameter. Near the same concentration (2.5 mg/L) of AgNPs was sufficient for inhibition of *Aureobasidium pullulans* and ten times higher concentration was needed for *Aspergillus niger* inhibition [15].

Testing of antimicrobial activity of paints and other building materials is not always simple and easy. Recommended procedures are based on disposal of paint on a piece of paper (or another support), placing small pieces of painted paper on agar and inoculated with bacteria or fungi [16, 17]. Observation of microbial invasion on paper (or support) has to be a prove of antimicrobial action but very often is not visible and convincing. Second type of technique which consist of the direct disposal of paints drops on inoculated with microbes agar is not a better procedure, because the diffusion of antimicrobial agents to the agar very often was not sufficient. So, a need of simple and reliable method emerged.

Aim of the study was to evaluate the antimicrobial activity of four paints against spores mixture of five fungi species: *Aspergillus brasiliensis, Penicillium pinophilum, Paecilomyces variotii, Trichoderma virens, Chaetomium globusom* and against two species of bacteria: *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

II. MATERIALS AND METHODS

A. Tested paints

Two different white emulsion paints (1313.1.Vm2 and 1355.1.Vm7) came from manufacturer (NN, Poland) and were prepared with standard antimicrobial biocide or with 13% addition of AgNPs - silver nanoparticles suspension (1313.1.Vm2+AgNPs and 1355.1.Vm7+AgNPs). AgNPs suspension of 20 nm Ø particles at concentration of 107.2±0.8 mg/L were prepared according to Koźlecki et al. [18] and mixed with paints by manufacturer as 13% additive. The final concentration of AgNPs in paint is 13.94 mg/L. In

aseptic conditions 45 g of paint was disposed in sterile Erlenmeyer containing 45 mL of physiological salt solution (0.9% NaCl). So, the paints were twice diluted and well mixed, so AgNPs concentration was 6.78 mg/L (6,78 ppm). For further analysis 1 mL of such dilutions was added to 0.1 mL of three serial dilutions of each microorganism and maintained 10-16h at room temperature. Samples of four twice diluted paints, added of 0.1 mL of 0.9% NaCl, maintained in the same conditions, were the controls of paints microbial purity.

B. Microorganism

The antimicrobial activity of paints was tested against spores mixture of five filamentous fungi: *Aspergillus brasiliensis* DSMZ 1988, *Penicillium pinophilum DSMZ 1944*, *Paecilomyces variotii DSMZ 1961*, *Trichoderma virens* DSMZ 1963, *Chaetomium globusom* DSMZ 1962 and against two species of bacteria: *Staphylococcus aureus* DSMZ 799 and *Pseudomonas aeruginosa* DSMZ 939.

C. Media & reagents

Mixture of fungal spores was prepared according to PN-EN ISO 846 by spores recovery from separated cultures of each genus on wheat agar incubated for 72 h at 25°C. Spores were collected from agar by inundation with physiological solution (0.9% NaCl), washed and mixed. Bacterial suspensions were prepared from fresh liquid culture incubated for 24 h in 30°C, on appropriated media: Giolitti-Cantoni (Aldrich-Sigma) for *S. aureus* and nutrition broth (Aldrich-Sigma) for *P. aeruginosa*. *Evaluation of residual microbial growth was performed on Sabouraud medium (Aldrich-Sigma) for fungi and nutrient agar (Aldrich-Sigma) for bacteria.*

D. Serial dilutions

Concentration of bacteria and fungi spores in primary suspensions was measured with apparatus Moxi Z (Orflo, USA) and standardized to contain 10^5 cells/mL for bacteria and 1.5 x 10^5 spores/mL for fungi (witch 0.9% NaCl). Then, decimal dilution series were prepared: 1/10, 1/100 and 1/1000 for bacterial species and 1/10 and 1/100 for spores mixture. To 1 mL samples of twice diluted paint 0.1 mL of microorganism suspensions was added. For each tested paint and microorganism three serial dilution*s* were prepared for one replicate. All tests were performed in duplicate.

E. Inoculation of agar plates

From each tubes of incubated paints interaction on microorganism, 5 µL were disposed as radius and dispersed as presented (Fig.1) in separated Petri dishes, on the surface of appropriated agar. In microbiology research such procedure of inoculation was recommended for rapid obtaining of single colony suitable for identification process. Inoculated Petri dishes were incubated at 25°C for 24-48 h and the number of colonies appeared on agar was numbered and reported to 1 mL of initial bacterial or fungal suspension. All tests were performed in triplicate. Control of microbial growth potential was performed with suspensions not treated by paints. To easy reads of obtained results on agar plates numbers firstly attributed for tubes, were written on the top. For fungi growth tests the name of agar medium (Sabouraud) and paint were written on the top of Petri dishes.

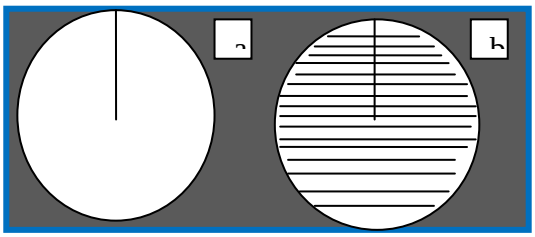

Fig. 1. Inoculation technique: 5 µL deposited as radius (a), strip separation and propagation of deposited material with new sterile utensil (b).

F. Enumeration

For each sample of paint the number of P. aeruginosa, S. aureus and fungal colonies was calculated on the base of all separated agar plate counts (3 dilutions, 2 replicates) and reported to 1 mL of suspension as colony forming units (cfu).

III. RESULTS

A. Antibacterial activity

A very important reduction in bacterial cells number was noted for the four tested paints (Table 1). An example of colonies growth was shown on Fig. 2.
Antibacterial effect was higher for the paints 1313.1.Vm2 (without or with nanosilver) than for 1355.1.Vm7 (without or with nanosilver). Biocidal effect of paints without AgNPs, measured as folds of reduction in cfu/mL number in comparison to bacterial suspension, was 13 times higher in the case of paint 1313.1 Vm2 against *S.aureus* and nearly 9 times against *P.aeruginosa* than paint 1355.1.Vm7. Comparing paints with AgNPs the antibacterial activity was 56 times higher in the case of 1313.1.Vm2 against *S. aureus* and nearly 6 times against *P. aeruginosa* than the action of paint 1355.1.Vm7. The paints with 13% addition of AgNPs presented more pronounced bactericidal effect, from nearly two thousand (1355.1.Vm7+AgNP) to more than one hundred thousand (1313.1.Vm2+AgNP) against *S. aureus* and against *P. aeruginosa* from nearly three thousand to nearly seventy thousand, respectively. Noteworthy is that AgNPs at

concentration 6.25mg/L highly enforced antibacterial properties of paints.

TABLE 1
Bacteria cells number (cfu/mL) in primary suspension after 16 h treatment with twice diluted paint

Paint	CFU/mL		Folds of cells number reduction	
	S. aureus	*P. aerug.*	*S. aureus*	*P. aerug.*
1313.1.Vm2	43 667	20 000	850	848
1313.1.Vm2 +AgNP	333	1000	111 111	16 950
1355.1.Vm7	567667	177 333	65	96
1355.1.Vm7 +AgNP	18 767	40 000	1972	2924
(Control*)	37 x10^6	16. 95 x10^6	1	1

* Bacterial suspension without incubation with paint

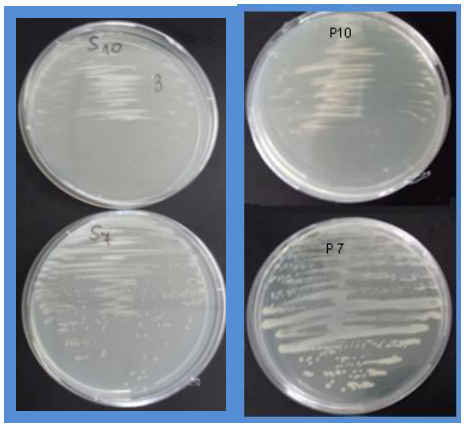

Fig.2. Photo of bacteria growth from 5 µL of 10 diluted suspension incubated with 1 mL twice diluted paint 1355.1.Vm7 (S7, P7) and 1355.1.Vm7 with AgNPs (S10, P10); S7 and S10 *Staphylococcus aureus;* P7 and P10 *Pseudomonas aeruginosa*

B. Antifungal activity

A very important reduction in fungi cells number was noted for the four tested twice diluted paints (Table 2). Biocidal action of AgNPs on fungi was stronger than on bacteria, because a total inhibition of fungi growth by paints with AgNPs occurred. However, both paints without AgNPs showed an antifungal activity. For the paints 1313.1.Vm2 the growth of fungi decreased by a factor 6.5 and for 1355.1.Vm7 decreased 2.5 times. The paints with addition of AgNPs presented more pronounced antifungal effect (zero colonies on agar plates). Noteworthy is that AgNPs in paints was at low concentration (6.25 mg/L).

Example of fungi colonies growth, resulting from residual presence of fungal spores in serial dilutions samples interacting with twice diluted paints was shown on Fig. 3. On this photo a clear decrease in the fungal colonies number was observed. So, the incubation of spores mixture with twice diluted paints engendered important damage of spores, destroying their capacity to growth.

TABLE 2
Fungi spores number (cfu/mL) in primary suspension after 16h treatment with twice diluted paint

Paint	CFU/mL	Folds of reduction of spores number
1313.1.Vm2	550	6.5
1313.1.Vm2 +AgNP	0	∞
1355.1. Vm7	1392	2.6
1355.1. Vm7 +AgNP	0	∞
Control*	3605	1

* Fungi suspension without incubation with paint

Example of fungi colonies growth, resulting from residual presence of fungal spores in serial dilutions samples interacting with twice diluted paints was shown on Fig. 3. On this photo a clear decrease in the fungal colonies number was observed. So, the incubation of spores mixture with twice diluted paints engendered important damage of spores, destroying their capacity to growth.

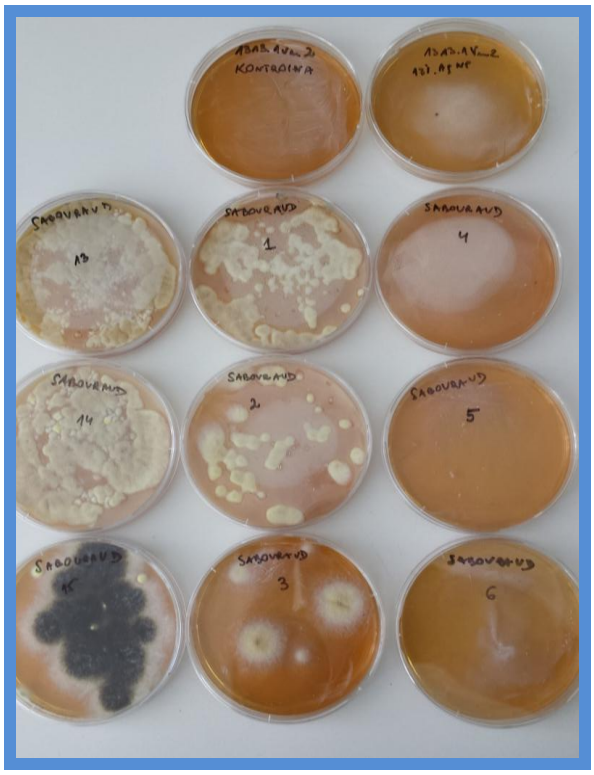

Fig. 3: Photo of fungi growth (72h at 25°C) from spores mixture treated with twice diluted paints. Medium colon – paint 1313.1.Vm2 without AgNPs, right colon - paint 1313.1.Vm2 with of AgNPs. Mixture of spores: not diluted – nr. 1, 4, 13; diluted 1/10 - nr. 2, 5, 14 and diluted 1/100 – nr. 3, 6, 15. On the top - medium and right colons – agar inoculated with paints without fungi spores addition, showing that paints were not contaminated; and on left colon - control growth of fungi spores mixture at different dilution.

IV. DISCUSSION

Direct inhibition of growth by AgNPs of filamentous fungi: *A. brasiliensis*, *P. pinophilum*, *P. variotii*, *T. virens*, *C. globusom*, bacteria: *E. coli*, *P. fluorescens*, *P. aeruginosa*, *S. aureus* and yeasts: *Saccharomyces cerevisiae*, *Candida albicans* and *Yarrowia lipolytica* was reported by Koźlecki et al. [19] and Żarowska et al. [20] in Bioscreen C study. Technique described above allowed determining that AgNPs significantly enforced antifungal and antibacterial properties of paints. Fungi spore are totally destroyed in the presence of AgNPs at the concentration of 6.25 mg/L (twice diluted paints was diluted 1.1 in interactions tubes) and bacterial cells number diminished by factor of two thousand to one hundred thousand. Effective microbiocide action of AgNPs was reported by Kożlecki et al. [19] and the used concentration (9-10.7 mg/L) was more than twenty to two thousand times lower in comparison to active compounds tested on wood preservation by Koziróg et al. [21]. This biocidal properties of AgNPs are recognized and described in the literature [5, 7-9, 22, 23]. However, some abnormalities concerning interaction with medium components during microbial analysis were reported also [24]. It is why a need of technique without the use of medium at the first step-interference of AgNPs and microbes - emerged as more adequate performance. Proposed technique is very simple and the effect of antimicrobial action is easily interpreted. The incubation of microorganisms suspensions with twice diluted paints during 10-16h at room temperature–just in physiological salt solution (0.9% NaCl)- was sufficient to kill the majority of cells and spores. This killing activity of microbial cells and spores was easily detected by growth tests on appropriated agar media, where the influence of AgNPs don't take place. According to Wikipedia page [https://en.wikipedia.org/wiki/Disinfectant#cite_note-Sandle-35] a similar technique for measurements of effectiveness of disinfectants was described by Sandle in 2012[25].

Finally, antibacterial action of tested building related products was stronger than presented here because paints were twice diluted in the tests.

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