Microbiological prevention using functionalized materials as ecological additives in hygienic paints

Romina Arrechea, Katerine Igala, Natalia Bellotti, Patricia Vázquez*

*Corresponding author. Tel.: +54-221-210711 ext 122; fax: +54-221-211353 ext 125. E-mail address: vazquez@quimica.unlp.edu.ar

International Congress of Science and Technology of Metallurgy and Materials, SAM–CONAMET 2014

Abstract

At the present time, one of the environmental challenges is the microbiological control in the indoor environment. The microorganisms that form biofilms on substrates located inside buildings and houses contribute to the formation of bio-aerosols when they are, partial or totally, dispersed and transported by the air. An example is the poisoning by certain fungal products as the mycotoxins (carcinogenic) that comes from fungi that can generally be in the interiors of buildings. Therefore, it is of vital importance to contribute avoiding and/or minimizing the microbiological growth in the indoor environments. In such sense the evaluation of new antimicrobial agents is of fundamental importance and can be applied in diverse areas of development of protective coating and construction materials. The global efforts to reduce the dangerous remainders and technologically clean the chemical processes are being integrated with modern progresses as much in science as in the industry. In this investigation we will work with remainders of seeds of sunflower functionalized with 3-aminopropyltriethoxysilane (APS), as support of additives, to a compound of Mo and K, that will act as antimicrobial, to be used in hygienic paintings. The originating solid of the sunflower seeds will be functionalized by means of reactions between organosilanes and the superficial groups of the mentioned solid. The characterization of synthesized solids will be made by means of SEM-EDS, DRX, FT-IR, textural properties, potentiometric titration, among others. These solids were microbiologically evaluated in front of fungi and bacteria, giving promissory results for novel ecological additives in hygienic paints.
1. Introduction

During the period 2012/2013, Argentina integrated the select group of the four main producers of sunflower seed, with a production of 3,100,000 tons. Our country produces about 9% of the mentioned seed world production, and is located on the fourth place. Most domestic production is destined for its industrialization in order to obtain oil and flour sunflower, both for export and for use in the domestic market (Calzada, 2012). The main macronutrients of sunflower seed are lipids, carbohydrates and proteins; on the shell there is a high content of lignin and cellulose-hemicellulose. For the purpose of producing oil the seeds are hulled mechanically. The shells are abundant agro-industrial wastes which have been marketed for special purposes, such as making firewood for home and other products with high fiber content. But these markets are limited.

There were also attempts to use shells as material fodder for ruminants such as cows and sheep, but the high lignin content made it unsuitable for such purpose. Furthermore, it established the practice of laying the shells rest on the floor, which is dangerous for the healing of the grounds due to the content of *Sclerotinia sclerotium*, a fungal pathogen to plants. Therefore, shells are often burned in processing plants (Curvetto et al., 2005).

In this work, were evaluated for antimicrobial activity of ashes of sunflower seed shells functionalized with different concentrations of 3-aminopropyltriethoxysilane (APS) and, subsequently, impregnated with the Lindqvist [V₂Mo₄O₁₉]K₄ (KMo) type polyoxometalate, for use as antimicrobial agents or biocides in hygienic paints. In addition, the samples were characterized by different techniques as Fourier Transform Infrared (FTIR) spectroscopy, potentiometric titration with n-butylamine, Scanning Electron Microscopy with Energy Dispersive X-ray Spectroscopy (SEM-EDS), X-ray diffraction (XRD), and structural properties (Sᵃₑᵗₑ).

2. Experimental procedure

2.1. Washing ash of sunflower seed shell

The ashes of outer sunflower seeds (CG) was used as solid to be functionalized and subsequently to support the Lindqvist-type phase [V₂Mo₄O₁₉]K₄. These were washed with hot water to remove excess powder (dust) which might come mixed.

2.2. Functionalization of the CG washed

The method used for the functionalization of pure and clean CG with 3-aminopropyltriethoxysilane (APS), was intended to. The solid obtained was dried at 100 °C for 2 h, obtaining the following concentrations 0.5% (w/w), 1.5% (w/w), and 3.0% (w/w) of APS, respectively.

2.3. Synthesis of compound type Lindqvist [V₂Mo₄O₁₉]K₄

For obtaining the synthesis of compound type Lindqvist different reactive compound as MoO₃, K(OH), NH₄VO₃, HCl and potassium acetate, were used. The mixture was stirred at 50 °C, yielding an orange solution which was filtered in hot. The obtained solution was placed in a crystallizer to form crystals of the Lindqvist phase. After this refer as KMo.

2.4. Impregnation of solid CG functionalized with APS

In Table 1 the nomenclature of synthesized samples can be observed. Their synthesis was performed as follows:
1 g of support (CG-0.5APS, CG-1.5APS, CG-3.0APS) was contacted with 4 ml solution of \([V_2Mo_4O_{19}]K_4\), during 24 h, to functionalize solids, in the amount of 5% (w/w), expressed in% (w/w) of K.

| Sample       | Functionalizing APS | Functionalizing KMo |
|--------------|---------------------|---------------------|
| CG           |                     |                     |
| CG-0.5APS    | 0.5                 |                     |
| CG-1.5APS    | 1.5                 |                     |
| CG-3.0APS    | 3                   |                     |
| CG-0.5APS-KMo| 0.5                 | 5                   |
| CG-1.5APS-KMo| 1.5                 | 5                   |
| CG-3.0APS-KMo| 3                   | 5                   |

2.5. Characterization of the synthesized samples

The textural properties, as surface area of solid (S_BET) were determined using a Micromeritics 2100 Accusorb, with N_2 as absorbable gas. Acid properties of material were performed by potentiometric titration with n-butylamine. It was carried out in a pH/mV/°C based on a microprocessor 211 Hanna Instruments pH, using a combined pH electrode: 0.025 ml/min of n-butylamine solution in acetonitrile (0.05 N) to a known amount (0.05g) of the solid of interest, previously suspended in acetonitrile (90 ml), and stirred for a period of 3 h. X-ray diagrams (XRD) were performed with a Philips model PW-1390 (control channel) and PW-1394 (motor control) chart recorder with built-sweep. Cu Kα radiation was used (λ=1.5417 Å), nickel filter, 20 mA and 40 kV high voltage source, scanning angle (2ş) between 5° and 60°, scanning rate of 2°/min and amplitude of the vertical scale 2000 counts/sec. Scanning Electron Microscopy and Energy Dispersive (SEM-EDS), SEM was performed to obtain solid micrographs using a Philips Model 505, working at a potential of 15 kV, on samples supporting on graphite and gold. The images were obtained with a ADDAII acquirer with Soft Imaging System. FT-IR spectra were obtained using a Bruker IFS 66 and the sample include in KBr. Measurements were made in a range between 400 and 4000 cm⁻¹. Digital Photography were taken.

2.6. Antifungal activity

Antifungal activity of the solid was measured using a variation of the agar diffusion and Kirby Bauer technique called "cut-plug" (Kenawy et al. 2006, Alamri et al. 2012). The evaluation of the antimicrobial activity through this type of procedure is determined by contacting of solid surface assessed through it’s inoculated with microorganisms (Morello et al. 2003). The agent studied diffuses radially making a gradient of concentration. At the end of the incubation period the solids are surrounded by a zone of inhibition. In each case, the measured diameter of the inhibition zone, which is related to a greater or smaller susceptibility of microorganisms used for testing against the target agent.

The fungi Alternaria alternata and Chaetomium globosum and Penicillium sp. and Aspergillus fumigatus isolated from previously painted substrates bio-deteriorated (Prescott et al.,1999) were used as biomarkers to determine the antifungal activity of the ashes of sunflower seeds with APS and APS-KMo. Subcultures were isolates and performed in Petri dishes. The composition of the agarized culture medium (MCA) was used: 1.5 g agar, 1 g dextrose, 0.5 g proteose peptone, 0.1 g KH_2PO_4, 0.05 g MgSO_4·7H_2O and distilled water. The plates were incubated in an oven at 25 °C between 15-25 days, depending on the species used. The above fungi were used in this work, not only for their ability to grow paintings and films, also by the negative effects that they have on human health (Vagui et al. 2005, Cooley et al. 2004). From subcultures, the inoculums were obtained by removing pores with the aid of a loop solution and 5 ml of 0.85% (w/v) NaCl and Tween 20 0.005% (w/v). The concentration of spores (0.3-
0.5x10^6 spores/ml) in the inoculum was adjusted through a Neubauer chamber.

MCA plates inoculated with 200 µl of spore suspension were prepared. Then, holes with 7mm diameter were made, on which 20 mg of each of the solid evaluated (CG with APS and APS-KMo) and their respective controls were introduced (CG and without solid). The procedure was performed three times for each solid. Finally, plates were incubated for 48 h at 25 °C. Photographic record was taken of each sample. It was considered whether or not it grew on the solid and the degree of inhibition around it. Therefore, diameters larger than 7 mm indicated a greater susceptibility of the organism against the tested product. In this regard, it was taken into account whether the inhibition was complete (no growth of mycelium) or had a decrease in mycelial growth around the solids studied.

2.7. Antibacterial activity

The bacteria *Escherichia coli* and *Staphylococcus aureus* were used as bio-indicators of the antibacterial activity of CG with APS and APS-KMo with the corresponding controls. Subcultures were performed in slant agar tubes for bacteria (BVAC). The composition of the culture medium used was: 0.1 g yeast extract, 0.1 g proteose peptone, 0.1 g starch, 0.1 g dextrose, 0.1 g casamino, 0.05 g pyruvic acid, 0.06 g KH₂PO₄, 0.01 g MgSO₄ and 1.5 g agar to 100 ml of solution. The tubes were incubated for 24 h in an oven at 37 °C. The antibacterial activity of the solid was evaluated by the "cut-plug" method, previously detailed. After a 24 h cultivation, saline suspensions were obtained by adjusting the turbidity of McFarland 0.5 (1.5x10^8 Ufc/ml) scale. Dilution is then performed to obtain a bacterial suspension of 1.5x10⁶. BVAC plates inoculated with 500 µl of the bacterial suspension were prepared. Then, three holes were made where 20 mg of each studied solid was added, respectively. The plates were incubated for 24 h at 37 °C. At the end of the test, the hales inhibition were measured and results recorded by digital photographs.

3. Results and Discussion

Regarding the potentiometric titration, it was observed that the initial potential (E_i), the initial acidity indicators solid synthesized decreased coinciding with the steep fall of the impregnated samples (Fig. 2). This behavior indicates that all samples have a high basicity. The predominant property Lindqvist type phases, in this case the KMo, basic.

![Fig. 2. Potentiometric curves samples: (A) CG-0.5APS, CG-1.5APS and CG-3.0APS; (B) CG-3.0APS and CG-3.0APS-KMo.](image)

Lindqvist structure, published in 1950, is the most symmetrical of isopolyanions and involves the merger of a six octahedral sharing a common vertex, which is an oxygen atom bonded to the six metal centers (Fig. 3).
With respect to FTIR spectra (Fig. 4), changes in the spectra of the husk ash functionalized sunflower seeds are not perceptible with respect to the ashes sunflower, but there is a variation in the three visible solids impregnated with different amounts of APS, which may be attributable to the groups containing Mo and V, in KMo phase.

The presence in XRD of KMo phase has a signal in accordance with $2\theta = 28.2^\circ$, observed (Fig. 5). The ashes of sunflower have a characteristic signal at $2\theta = 30^\circ$ and the previously mentioned signal (Fig. 6 A and B).
Besides, it was observed that the samples tested have low or no surface area. This characteristic makes the agar diffusion test more difficult, as the average should contact the porous material so that an inhibition on the growth of microorganisms occurs. If the surface area is low there is no sufficient porosity to make it happen.

The samples were characterized by SEM-EDS and results obtained by EDS corresponding with potentiometric titration. Numerous cations were found and to make evident the presence of some of them, such as Ca, a comparative mapping was performed. It can be concluded that the low acidity is due in part to the presence of these cations (Ca) on the surface in contact with the APS and in the pores of the solid (ashes).

Table 2 shows the results obtained from agar diffusion test with solid tested and corresponding controls to determine its antifungal activity.

Table 2. Assess agar diffusion. Diameter of inhibition zone (mm) average, after 48 h.

| Sample            | Alternaria alternata | Chaetomium globosum | Penicillium sp. | Aspergillus fumigatus |
|-------------------|----------------------|---------------------|-----------------|-----------------------|
| Control           | --                   | --                  | --              | --                    |
| CG                | IS                   | 23.1* ± 3.1         | IS              | 17* ± 1.2             |
| CG-0.5APS         | IS                   | 23.4* ± 2.3         | IS              | 24.5* ± 1.3           |
| CG-1.5APS         | IS                   | 24.4* ± 1.7         | IS              | 20* ± 3.3             |
| CG-3.0APS         | IS                   | 23.3* ± 1.4         | IS              | 22.0* ± 3.0           |
| CG-0.5APS-KMo     | IS                   | 27.3* ± 0.3         | IS              | 30.3* ± 2.0           |
| CG-1.5APS-KMo     | IS                   | 32.2* ± 0.5         | IS              | 29.7* ± 1.7           |
| CG-3.0APS-KMo     | IS                   | 8.9 ± 0.8           | IS              | 27.9* ± 1.5           |

*a Average data from triplicate. * Indicates lower growth around the solid. IS: inhibition in solid (7mm in diameter). –Uninhibited.

Antifungal activity was observed in cultures of C. globosum (Fig. 7) and A. fumigatus (Fig. 8), the first showing greater susceptibility to solid-CG-3.0APS-KMo presented as an inhibition average of 8.9 mm without mycelium growth (Fig. 4 A, B). Notably, among the species used to perform the assay C. globosum is the greater water content required for its germinating spores and mycelium to develop [10]. Therefore, increased hydrophobicity of the agent under study could further affect normal development.
Fig. 7. Photographs of fungal culture of *C. globosum* with solid 
(A, B) CG-3.0APS-KMo; (C, D) CG, representing 
(→) the halo of inhibition.

Fig. 8. Photograph of fungal culture of *A. fumigatus* with solid (A, 
B) CG-3.0APS-KMo; (C, D) CG. The arrow (→) represents the 
halo of lower growth.

Regarding growing of *A. fumigatus* shown in Fig. 5, it can be observed that there is less growth of mycelium with 
CG-3.0APS-KMo solid with respect to control, since the greenish hue is an observed characteristic of conidia 
production.

In cultures of *A. alternata* and *Penicillium sp.* there was no growth on solid but no change either was observed 
with respect to controls with CG.

In Table 3 the results obtained from the agar diffusion test with solid and corresponding controls tested for 
antibacterial activity thereof are presented. Such results show increased susceptibility of *S. aureus* against CG-
3.0APS-KMo showing a halo appreciably relative to control with the solid support alone (Fig. 9) average inhibition 
(9.8 mm). In the case of *E. coli* with the results obtained functionalized solid showed no difference with the controls 
(Fig. 10).

Table 3. Evaluation of antibacterial activity by agar diffusion assay. Diameter of inhibition zone (mm) after 24 h.

| Sample          | *Escherichia coli* | *Staphylococcus aureus* |
|-----------------|--------------------|-------------------------|
| Control         | --                 | --                      |
| CG              | --                 | --                      |
| CG-0.5APS       | --                 | 7.9 ± 0.2               |
| CG-1.5APS       | --                 | 10.7 ± 1.5              |
| CG-3.0APS       | --                 | 10.7 ± 1.0              |
| CG-0.5APS-KMo   | --                 | --                      |
| CG-1.5APS-KMo   | --                 | --                      |
| CG-3.0APS-KMo   | --                 | 9.8 ± 0.7               |
Fig. 9. Pictures of *S. aureus* bacterial culture with the solids (A, B) CG 3.0 APS- KMo; (C, D) CG, representing the inhibition zone.

Fig. 10. Photos of *E. coli* bacterial culture with the solids (A) CG 3.0 APS- KMo; (B) CG.

4. Conclusion

Tested solid CG-3,0APS-KMohas presented the highest antimicrobial activity because it proved effective against a larger number of fungal isolates and also to inhibit the growth of one of the bacterial strains used. These preliminary results allow us to think about the next stage, in which they could evaluate the efficiency of the integrated solid bioactive formulation paint hygienic nature. The testing of new compounds related to recyclables should be continued, in order to help the rise of eco-friendly additives.

Acknowledgements

The authors thank L. Osiglio, M. Theiller, G. Valle, E. Soto for their experimental contribution. UNLP and CONICET for the financial support.

References

Alamri, A., El-Newehy, M. H., Al-Deyab, S. S., 2012. Biocidal polymers: synthesis and antimicrobial properties of benzaldehyde derivatives immobilized onto amine-terminated polyacrylonitrile. Chemistry Central Journal 6, 111-124.

Calzada, J., 2014. Argentina entre los 4 principales productores de semilla de girasol [online] Mitre y el campo, 8 de enero 2014, Disponible en http://secciones.cienradios.com.ar/radiomitre/2014/01/08/argentina-entre-los-4-principales-productores-de-semilla-de-girasol/http://secciones.cienradios.com.ar/radiomitre/2014/01/08/argentina-entre-los-4-principales-productores-de-semilla-de-girasol/.

Carrillo, L., 2003. Los hongos de los alimentos y forrajes. Universidad Nacional de Salta, Área Micología.

Cooley J.D., Wong W.C., Jumper, C.A., Straus, D.C., 2004. Fungi and the Indoor Environment: Their Impact on Human Health. Advances in Applied Microbiology 55, 3-30.

Curvetto, N.R., Figlas, D., González Matute, R., Delmastro, S., 2005. Cultivo de Shiitake en Bolsas, Shiitake, 127-133.

Kenawy, E., Abdel-Hay, F. I., El-Magd, A. A., Mahmoud, Y., 2006. Reactive & Functional Polymers 66, 419-429.

Morello, J.A., Granato, P.A, Mizer, H.E, 2003. Antimicrobial agent susceptibility testing and resistance. Laboratory manual and workbook, Ed. McGraw-Hill, 95-105.

Prescott, L., Harley, J., Klein, D., 1999. Microbiologia, (4°edicion), McGraw-Hill Interamericana, 38-67.

Vagui, E., Simándi, B., Suhajda, A.,Héthelyi, É., 2005. Essential oils composition and antimicrobial activity of Origanum majorana L. extract obtained with ethyl alcohol and supercritical carbon dioxide. Food research international 38, 51-57.