MATERIALE APATITICE SINTETIZATE PENTRU PROTECȚIA ARTEFACTELOR ÎMPOTRIVA BIODETERIORĂRII
SYNTHESIZED APATITIC MATERIALS FOR ARTEFACTS PROTECTION AGAINST BIODETERIORATION

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Most of the artefacts of cultural and / or historical value are exposed to fungal attack often causing irreversible damage. The need for new methods of antifungal protection led to the development of a recipe based on hydroxyapatite and barium hydroxide, with promising results. We synthesized and analytical characterized (trough energy dispersive X-ray fluorescence, X-ray diffraction and Fourier transform infrared spectroscopy) several materials based on hydroxyapatite. The efficiency of the synthesized materials was evaluated by diluted inoculums on the culture media technique and a modified Kirby-Bauer method, using simulated artefacts, previously reported to replicate the real artefacts.

Cele mai multe dintre artefactele de valoare culturală şi / sau istorice sunt expuse atacurilor fungice, rezultând daune ireversibile. Nevoia de noi metode de protecție antifungică a condus la dezvoltarea unei rețete bazate pe hidroxiapatită şi hidroxid de bariu, cu rezultate promițătoare. Am sintetizat şi caracterizat analitic (fluorescență de raze X, difracție de raze X şi spectroscopie IR cu transformata Fourier) mai multe materiale pe bază de hidroxiapatită. Eficiența materialelor sintetizate a fost evaluată prin tehnica inoculului diluat pe suprafața mediului de cultură şi o metodă Kirby-Bauer modificată, folosind artefacte simulate, raportate anterior a reproduce artefactele reale.

Keywords: *artefacts, biodeterioration, hydroxyapatite, X-ray methods*

1. Introduction

Evidences of past civilizations (collectively called artefacts) are part of the global heritage of humankind, which is to be preserved for future generations. Degree of concern about keeping them in good condition is an indicator of the level of civilization of every nation. When speaking of cultural heritage, there could be included in this definition the tangible evidence (as buildings, monuments, landscapes, books, artwork and other artefacts), intangible culture (folklore, traditions, language) and natural heritage (cultural landscapes and biodiversity) [1, 2].

Cultural heritage consists of almost all types of materials produced by nature and used by humans to perform various types of artefacts, from very simple single-component to complex structures integrating organic and inorganic materials. These artefacts, even if they are made of materials considered resistant (rock, metal), are influenced by environmental factors that can change their structure and composition. In addition, once introduced into the biosphere, they can be affected by biological mechanisms [3].

Among the many threats to the cultural heritage, in the present paper we will focus on biodeterioration. The artefacts colonization by harmful microorganisms is classified under the definition of *biodeterioration* [4].

From the biodeteriogens, the most encountered are the fungi, especially *Aspergillus Sp.* and *Penicillium Sp.* [5].

When fungal colonies appear indoor or outdoor, there are some treatments that can be applied. Peitzsch et al. describes in a recent paper some of these treatments and their efficiency [6]. The authors presents several commonly used remediation methods (Ozone, Peroxide, Hot air, Flaming, Steam, Boron-based chemicals, Ammonium chloride based chemicals, Sodium hypochlorite based chemical and Drying). The results of their study are that none were completely effective in removing all moulds, concluding that none of these methods which are widely used for removing biodeterioration is completely effective. Lin et. al. [7] presented the antibacterial effect of partially and totally strontium substituted hydroxy-

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apatite. Based on these data, our group has synthesized and analytical characterized (trough energy dispersive X-ray fluorescence - EDXRF, X-ray diffraction - XRD and Fourier transform infrared spectroscopy – FTIR) several materials based on hydroxyapatite. The efficiency of the synthesized materials was evaluated by the technique of the diluted inoculums on the surface of the culture media, using simulated artefacts [8] and a modified Kirby-Bauer technique [9, 10], compared with the efficiency of some previously reported materials [8, 11] obtained by our group.

2. Experimental

2.1. Materials

All the experiments were performed on simulated artefacts, previously proven to develop the same fungal colonies as real artefacts (*Aspergillus Sp., Penicillium Sp.* and *Mucor Sp.*) [8].

The synthesized material are: strontium partially substituted hydroxyapatite (SrCaHAP), strontium totally substituted hydroxyapatite (SrHAP) and barium totally substituted hydroxyapatite (BaHAP).

Hydroxyapatite (HAP) was obtained as follows: 0.25 mol $Ca(NO_3)_2 \cdot 4H_2O$ (Merck KGaA, Germany) were dissolved in 250 ml distilled water; 0.25 mol of $(NH_4)_2HPO_4$ (Merck KGaA, Germany) were dissolved in 250 ml distilled water; the calcium containing solution was put into a flask and heated to the temperature of 80 °C. The phosphorus containing solution (with the pH adjusted to 10 with NH_4OH – Chimreactiv, Romania) was added into the calcium containing solution under vigorous stirring. The reaction was performed at 80 °C for 3 h, with the pH constantly kept at 10. After the reaction, the deposited mixtures were washed with distilled water, filtered, and rinsed with ethanol (Merck KGaA, Germany) in order to remove any unreacted precursors. The ethanol-containing gel was dried in a vacuum oven at 45 °C.

SrCaHAP powder was prepared by the same recipe, by using a mixture of $Ca(NO_3)_2 \cdot 4H_2O$ (0.125 mol) and $SrCl_2 \cdot 6H_2O$ (0.125 mol, Merck KGaA, Germany) to obtain a Ca/Sr solution instead of the Ca solution used for HAP synthesis.

For the SrHAP synthesis, 0.25 mol $SrCl_2 \cdot 6H_2O$ were dissolved in distilled water and the previously presented procedure was followed. For the synthesis of BaHAP, 0.25 mol $BaCl_2 \cdot 6H_2O$ were dissolved in distilled water and the previously presented procedure was followed.

2.2. Methods

Energy dispersive X-ray fluorescence analyses (EDXRF) were performed using a PW4025 MiniPal 2 spectrometer (PAnalytical). X-ray diffractions (XRD) were obtained by the use of a DRON UM1 diffractometer, operating at 32 kV and 25 mA, using Co K_α radiation (1.79021 Å). FTIR analyses were performed on a FT-IR GX (Perkin Elmer) spectrometer, in the region 4000-500 cm^{-1}. The analytic results were interpreted using a professional data analysis software (Origin 8.0).

The treatment of the samples was performed by spraying the samples with a suspension of the synthesized materials in isopropyl alcohol.

In order to evaluate the antifungal effect of the synthesized materials, we used the technique of the diluted inoculums on the surface of the culture media. For this type of analysis, samples are collected and suspended in sterile distilled water. The samples are inoculated at the surface of a solid growth medium in Petri dishes; the liquid is dispersed evenly on the surface of the plate (using a Drigalski rod, through tilt/rotation motions of the plate). The plates are incubated at 28 °C for several days. The culture media used was solid Sabouraud (SS) (produced by INCDMI Cantacuzino, Romania). The results were verified using a modified Kirby-Bauer method [9, 10]: sterile paper discs (5 mm 388 Filtrak paper) impregnated with the materials obtained (suspensions in isopropyl alcohol) were distributed directly in Petri dishes inoculated with spores obtained from the simulated artefacts; the antifungal effectiveness was evaluated as inhibited area, using the following formula *Antifungal efficiency= (diameter of the inhibited zone / diameter of the Petri dish) × 100*. The experiments were carried out in triplicate.

3. Results and discussions

3.1. Analytical characterization

The materials synthesized were analytical characterized by EDXRF (Figure 1), XRD (Figure 2) and FTIR (Figure 3).

EDXRF results presented in Figure 1 reveals the lack of impurities in the synthesized materials. The rhodium peak present in all the samples is a characteristic of the apparatus used for the determinations. The X-ray fluorescence spectra presented in Figure 1 reveals the reduction in calcium content in HAP (visible in both cases; the calcium specific peak is missing in the SrHAP and BaHAP spectra Figure 1a – black dashed line and, respectively Figure 1b grey line), as well as the apparition of the characteristic peaks of strontium (Figure 1a, grey line for SrCaHAP, peak that increases in intensity for SrHAP, Figure 1a black dashed line), and, respectively, barium (Figure 1b, grey line).

The XRD results (XRD patterns are shown in Figure 2) prove the successful isomorphic substitution. The patterns showed only the peaks

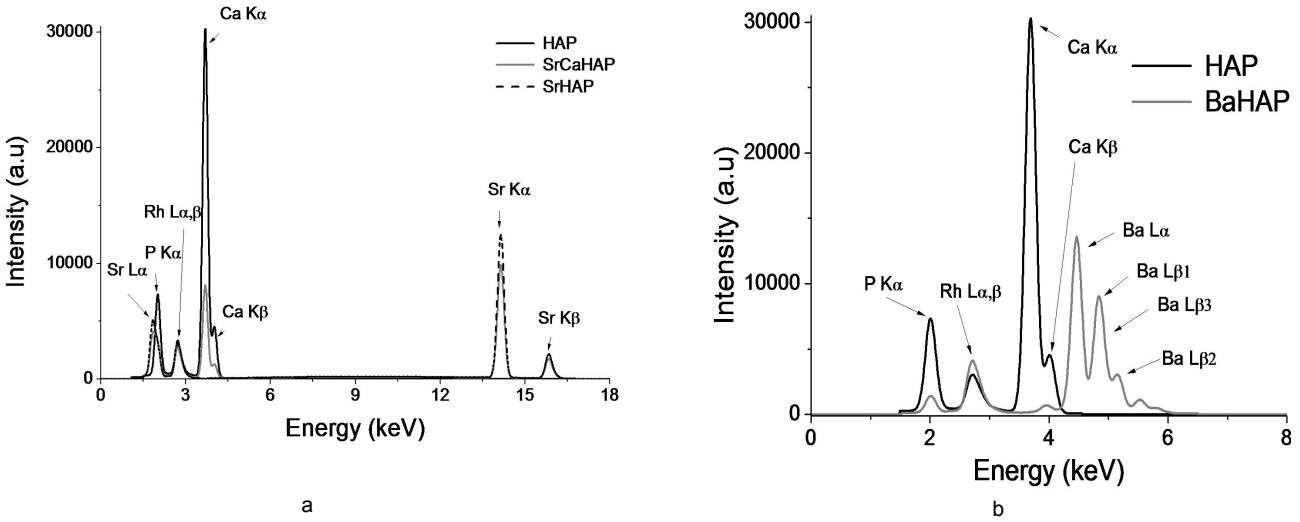

Fig. 1 - The EDXRF spectrum of the synthesized materials: a) HAP (black line)/SrCaHAP (grey line)/SrHAP (black dashed line); b) HAP (black line)/BaHAP (grey line). / *Spectrele EDXRF ale materialelor sintetizate: a) HAP (linie neagră)/SrCaHAP (linie gri)/SrHAP (linie neagră punctată); b) HAP (linie neagră)/BaHAP (linie gri).*

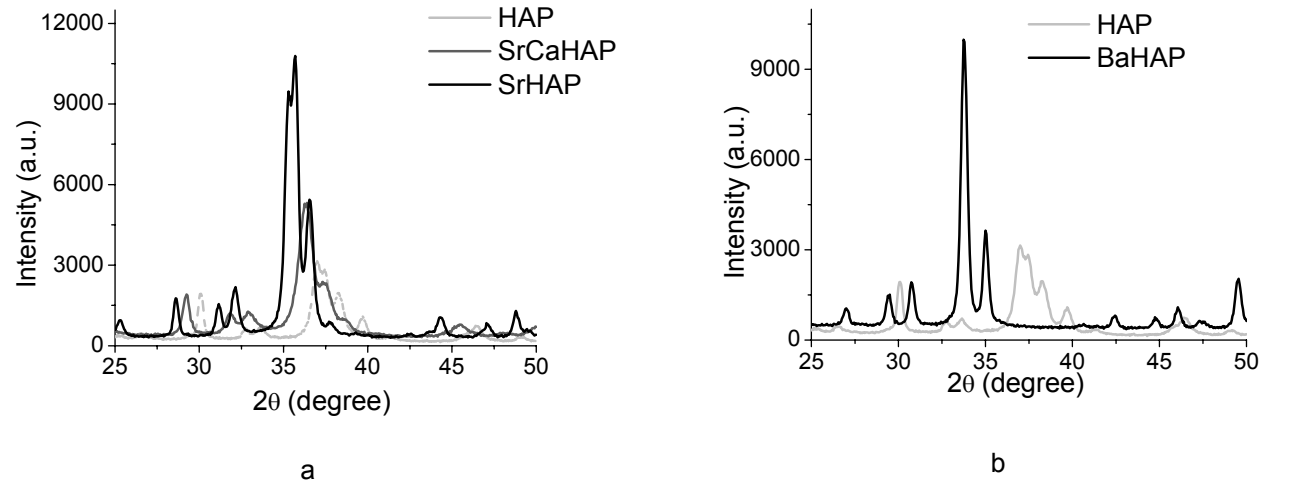

Fig. 2 - XRD patterns of the synthesized materials: a) HAP (light grey), SrCaHAP (dark grey), SrHAP (black); b) HAP (light grey), BaHAP (black) / *Difractogramele materialelor sintetizate: a) HAP (gri deschis), SrCaHAP (gri închis), SrHAP (negru); b) HAP (gri deschis), BaHAP (negru).*

characteristic to the synthesized materials with no obvious evidences on the presence of other additional phases [12, 13]. The diffractograms shows that all XRD peaks were shifted toward lower diffraction angles. These shifts were indicative to the increase in unit cell dimensions which was due to the replacement of Ca^{2+} (ionic radius 0.99 Å) with Sr^{2+} (ionic radius 1.16 Å – Figure 2a) and respectively Ba^{2+} (ionic radius 1.35 Å – Figure 2b), into the cell lattice of apatite crystals [14].

Crystallite size was calculated from the XRD data, using the Scherrer equation [15]:

$$D = \frac{K\lambda}{h_{1/2}\cos\theta} \quad (1)$$

Where: D is the crystallite size, $h_{1/2}$ is the peak width at half height, λ is the wavelength of CoKα radiation, K is the shape factor and chosen as 0.9, and θ corresponds to the peak position.

Using the equation (1), the results obtained for the crystallite size were: 12.65 nm for HAP, 9.67 nm for SrCaHAP, 16.67 nm for SrHAP and 20.35 nm for BaHAP.

The FTIR spectra presented in Figure 3a to 3d are similar for all the synthesized samples, being characterized by broad and not intense bands around 3400 cm^{-1} assigned to the stretching and liberation mode of OH group vibration. The broadness of the O–H stretching band in the region 3550–3400 cm^{-1} was caused by the H-bonding between the adsorbed H_2O and the OH group of the hydroxyapatite [12]. The bands at 1645, 1420 and 873 cm^{-1} characterize the incorporation of a small amount of CO_3^{2-} ions in the modified hydroxyapatite crystal [16]. The FTIR spectrum presents strong bands around 1027, 960 and 600 cm^{-1} (characteristic for hydroxyapatite structure), attributed to the PO_4^{3-} group. [12, 16, 17].

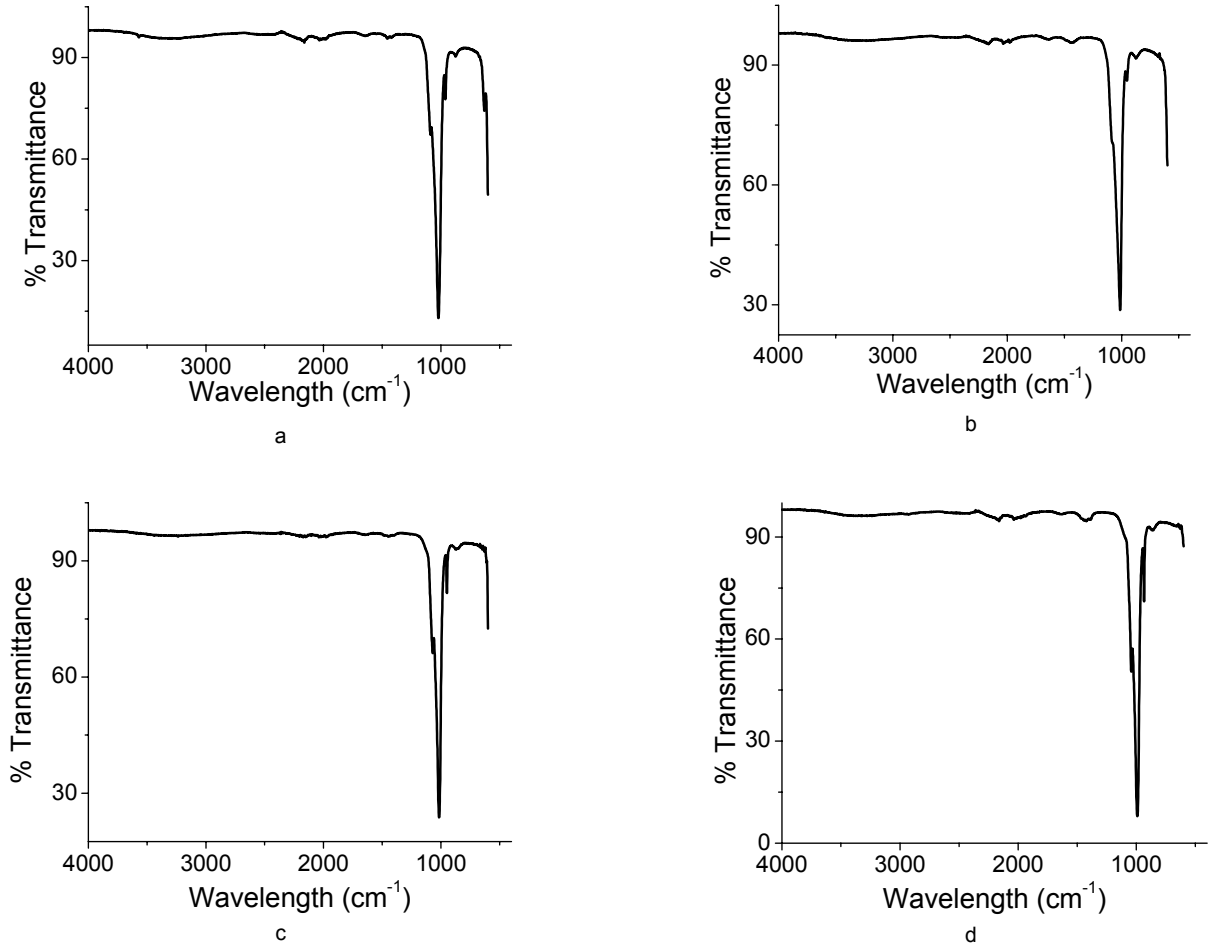

Fig. 3 - FTIR results obtained for the synthesized samples: a) HAP; b) SrCaHAP; c) SrHAP; d) BaHAP / *Rezultatele FTIR ale probelor sintetizate: a) HAP; b) SrCaHAP; c) SrHAP; d) BaHAP.*

3.2. Antifungal activity

Previous presented results [8] allowed us to use simulated artefacts, in order to avoid the over-use of real artefacts. The hydroxyapatite, strontium-substituted hydroxyapatite (half and totally) and barium totally substituted hydroxyapatite were pulverized on the simulated artefacts as alcoholic suspension (in isopropyl alcohol). The artificial artefacts were kept for 15 days in dark and humid environment. After this period, samples were collected from the surface of the simulated artefacts in order to determine the efficiency of the treatment using the synthesized materials. The results obtained are presented in Figure 4.

As can be seen from Figure 4, the best results are obtained when using totally substituted strontium hydroxyapatite, results superior to those obtained using natural extracts or other chemicals, previously reported by our group [8, 11]. Nevertheless, all tested materials present some antifungal activity, if compared with the untreated sample and the blank sample (treated with isopropyl alcohol).

The funguses (cultivable on the selected culture media) affecting the untreated sample were previously identified by their characteristics as *Asspergillus Sp., Penicillum Sp*. and *Mucor Sp.* [8].

In order to quantify the effectiveness of the materials, antifungal activity was measured using a variation of the Kirby-Bauer method (typically used to determine the antibacterial response to different antibiotics) [9]. Sterile paper discs were impregnated with the alcoholic suspensions containing the synthesized materials and were distributed directly in Petri dishes inoculated with spores obtained from the simulated artefacts; the antifungal effectiveness was evaluated as inhibited area, using the following formula *Antifungal efficiency = (diameter of the inhibited zone / diameter of the Petri dish) × 100* [10]. The results obtained are presented in Figure 5. The results are reported as the average of three experiments and are presented as mean ± standard deviation (SD).

The results obtained confirm the previous observations, showing a very good antifungal efficiency for the SrHAP.

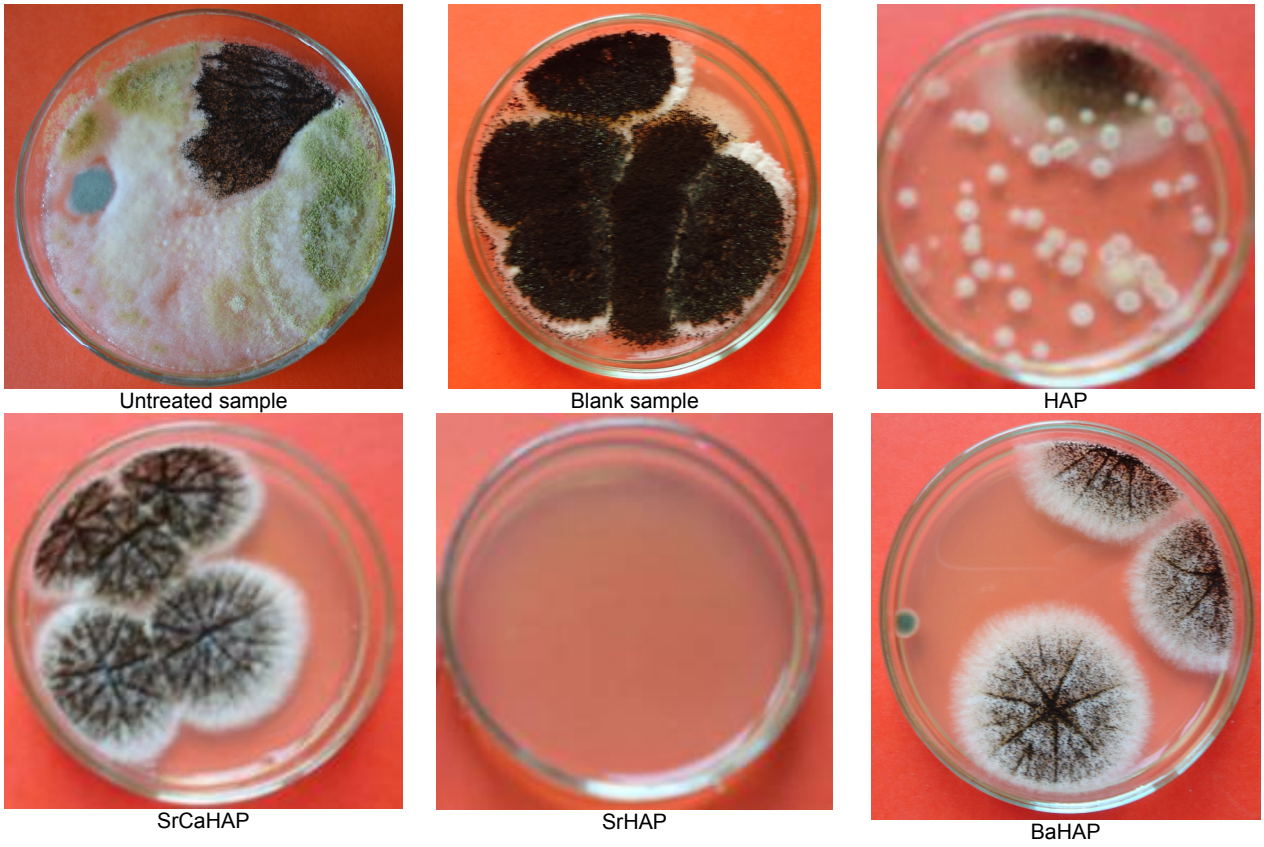

Fig. 4 - Results for the fungal growth inhibition of the synthesized materials; the culture media use was solid Sabouraud and incubation time 144h, except for blank and untreated samples (96 h) and SrHAP (216h) / *Rezultatele inhibării dezvoltării fungice folosind materialele sintetizate; mediul de cultură folosit – Sabouraud solid, timp de incubare 144h, cu excepția probei martor şi a probei netratate (96h) şi SrHAP (216h).*

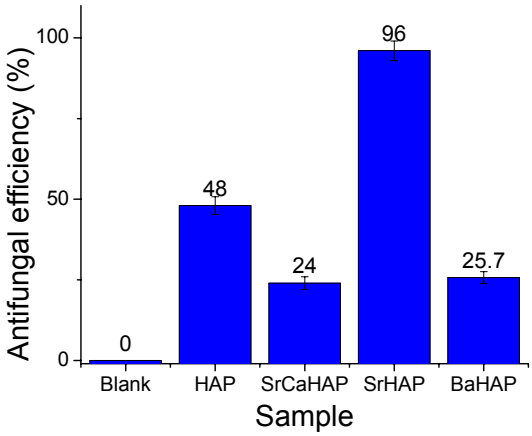

Fig. 5. - Results for the modified Kirby-Bauer test; the culture media use was solid Sabouraud and incubation time 144h / *Rezultatele folosind testul Kirby-Bauer modificat; mediu de cultură Sabouraud solid, timp de incubare 144h.*

From the two antifungal tests performed (a qualitative one, performed on simulated artefacts, and the Kirby-Bauer test), the antifungal efficiency of the synthesized materials can be summarized as follows:

SrHAP>HAP>BaHAP>SrCaHAP>Blank

4. Conclusions

Artefacts in general and especially paper artefacts are subjected to the fungal attack. The classical preventive measures (special conditions for their storage) can often prove to be expensive and/or inapplicable.

The paper describes a study regarding the use of synthesized materials for the protection of the artefacts against biodeterioration. The materials synthesized were characterized using modern analytical techniques (Energy-dispersive X-ray Fluorescence, X-ray Diffraction, Fourier transform infrared spectroscopy), as well as regarding their antifungal efficiency, using two different techniques (the diluted inoculums on the surface of the culture media and a modified Kirby-Bauer method). The obtained results prove to be superior to those obtained when using other chemical compounds, previously reported by our group (HAP-barium hydroxide mixture) [11].

These preliminary results presented encourage us to hope that a novel, less toxic or even non-toxic method can be developed, to be applied on the restoration/conservation of cultural heritage artefacts affected by biodeterioration.

Acknowledgement

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNCS – UEFISCDI, project number PN-II-RU-PD-2011-3-0023

This work was supported by a grant of the Romanian National Authority for Scientific Research, CNDI-UEFISCDI, project number 222/2012.

The authors gratefully acknowledge the help received from their colleague Sanda Maria Doncea in characterizing the materials.

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