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Antifungal activity of silver ions exchanged in mordenite

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ABSTRACT

We investigated the action of silver-exchanged mordenite (Ag–mordenite) against the growth of six fungi that are problematic in the food industry. The mould species studied were *Rhizopus oryzae*, *Mucor circinelloides*, *Geotrichum candidum*, and the yeasts were *Saccharomyces cerevisiae*, *Debaryomyces hansenii* and *Zygosaccharomyces rouxii*. Several instrumental methods (EPMA, XRD, XPS, TPR, AAS) were used for the characterization of Ag–mordenite in order to explain its antifungal activity. Results show that Ag–mordenite exerted an effective antifungal action due to a release of silver ions from the zeolite matrix, which acted directly on the walls of the microorganisms, being more effective than the free silver ions in solution. The yeasts were more sensitive than filamentous fungi, *S. cerevisiae* being the most susceptible specie whereas *G. candidum* was the more resistant.

1. Introduction

The antimicrobial capacity of silver particles is widely known but its effectiveness depends on many factors such as particle size and morphology. In general, since smaller particles have a more effective antimicrobial capacity, several methods have been developed to obtain silver nanoparticles, such as microemulsion, sonochemical reduction and photochemical synthesis [1–3]. On the other hand, nanoparticles tend to aggregate, which leads to a deterioration of their antimicrobial properties, also presenting the problem of their recovery and reuse. This has prompted the study of nanoparticle immobilization in various inorganic materials such as titania [4], silica [5], ceramics [6] or titanium phosphate [7], although this involves the intervention of new factors, namely the mechanical and chemical durability of the supports and their ability to release the nanoparticles. These active nanoparticles may also be silver ions hosted on inorganic matrices which act as vehicles to deliver the ions progressively, such as clays [8], titanosilicates [9] and zeolites [10]. These latter materials have a high exchange capacity, specific surface area, chemical inertness and are not toxic. In particular, mordenite is a zeolite of medium pore size with a 1-dimensional and linear system of channels, consisting of 12-membered rings with an elliptical pore aperture of 7×6.5 Å interconnected to another more closed channel system of 8 members (3.4×4.8 Å) running in the same direction [11]. We have selected this zeolite as a carrier because it has an appropriate Si/Al to allow a good ion exchange capacity and, moreover, because it has been reported that this framework can stabilize different types of cationic silver clusters [12]. In recent years, several types of zeolites containing Ag, Cu or Zn ions and/or nanoparticles applied mainly for antibacterial purposes have been studied [13–29]. In contrast, the literature on the action of Ag–zeolites against fungi is much more limited. We could mention the pioneering work of Ishitani [13] and Nikawa et al. [15], which employed a commercial product named Zeomic® [30], an zeolite A with 2.5% w/w Ag, for the growth control of *Saccharomyces cerevisiae* and *Candida albicans*, respectively. Ferreira et al. [14] and Malachová et al. [16] also showed the good efectiveness of Ag–zeolite Y and Ag–montmorillonite against *S. cerevisiae, C. albicans, Pycnoporus cinnabarinus* and *Pleurotus ostreatus* [16].

Fungi are problematic microorganisms that cause contamination especially in the food industry, provoking significant economic losses. In this context, the purpose of this work is to investigate the antifungal activity of silver-exchanged mordenite, against three yeasts and three molds which were isolated from contaminated food of industrial sources, in low oxygen tension conditions. The properties of the materials were characterized in order to correlate their physicochemical characteristics with their antifungal behavior.

2. Experimental

2.1. Preparation of Ag–mordenite

Na–mordenite (Zeolyst CBV 10A, Si/Al = 6.5) was employed as support, which was ion-exchanged with silver solutions of

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different concentrations. $AgNO_3$ (Sigma–Aldrich, 99.0% in distilled water) was employed to prepare exchange solutions with concentrations of 0.005, 0.01, 0.025 and 0.1 M. The general conditions were taken considering various ion-exchange procedures for zeolites [31], so as to obtain between 4% and 15% w/w Ag in mordenite. Briefly, 4 g of Na–mordenite (Na–mor) were suspended in the $AgNO_3$ solutions under stirring at room temperature for 24 h in darkness, to avoid reduction of ions to Ag^0. Then, the solids were filtered in darkness and washing twice with 250 ml of distilled water to remove the excess of silver solution. Afterwards, the solids were dried in an oven at 130 °C for 24 h and then stored in ermetic jars in a dry and dark place. Furthermore, $Ag(NO_3)$ solutions were also prepared in such a way as to obtain concentrations of 1670, 555, 170 and 55.4 μM in the culture medium. With these solutions, antifungal assays were performed in order to compare the action of free silver ions in solution with that provided for Ag–mordenite.

2.2. Microorganism isolation and microbiological assays

2.2.1. Isolation of fungi and preparation of inoculum suspension

The moulds employed were isolated from dairy products which presented alterations in conditions of low oxygen tension. The samples were obtained by the method of serial dilutions and seeded in malt extract agar (MEA) (malt extract 2.0%; agar; peptone bacteriological 0.1%, Glucose 2%, w/v in distilled water) with the addition of chloramphenicol (100 mg l^{-1}). The incubations were performed at 25 °C for 5 days and then the fungal flora was identified according to their macroscopic and microscopic characteristics [32,33]. The isolated moulds were *Rhizopus oryzae* Went and Prins. Geerl, *Mucor circinelloides* Tiegh and *Geotrichum candidum* Link: Fr., which were preserved in tubes containing glycerol-water (18% w/w) and stored at −20 °C until completion of the antimicrobial tests. The yeasts were isolated from altered fruit juices contained in plastic bottles, in a similar way as just described. The selected yeasts were *Saccharomyces cerevisiae* Meyen (S.c.) (LMFIQ-701); *Zygosaccharomyces rouxii* (Boutroux) Yarrow (Z.r.) (LMFIQ-702) y *Debaryomyces hansenii* (Zopf) Lodder & Kreger (D.h.) (LMFIQ-700).

In order to prepare the inoculum suspensions, mould spores and yeasts were collected in the late exponential growth phase, poured in sterile tubes containing 5 ml of malt extract broth (MEB) and adjusting the concentrations to 10^4–10^5 spores ml^{-1}.

2.2.2. Fungicidal tests

Assays were performed by suspending 300, 100, 60, 30 or 10 mg of Ag–mordenite containing 5.6% w/w Ag in 100 ml MEB. The suspensions were sonicated for 10 min to homogenize the culture medium and remove the air entrapped in the porosity of the zeolite particles. Then, 9 ml of suspensions were distributed into tubes of 10 ml of capacity with screwcaps, seeded with 1 ml of the fungal inoculum and incubated at 28 °C. Tests were conducted in triplicate under low oxygen tension conditions. Error bars were displayed later in the respective curves. For all the strains, control tests were also conducted without Ag–mordenite or with the addition of Na–mordenite. After the incubation of the microorganisms at different times (0, 1, 2, 4, 6, 8, 12, 24 and 48 h), counts (CFU ml^{-1}) using decimal dilutions were conducted. For this, 1 ml of each suspension was seeded in depth in MEA, the plates were incubated and the counts were obtained in duplicate. For tests with silver solutions we proceeded in the same way as described above, working with concentrations of 1670, 555, 170 and 55.4 mM contained in 9 ml of MEB. The incubations were carried out in darkness.

2.3. Physicochemical characterizations

The X-ray diffraction (XRD) of the solids was performed with a Shimadzu XD-D1 equipment at 2° min^{-1} between $2\theta = 5°$ and 50° using Cu Kα radiation (λ = 1.5418 Å, 30 kV, 40 mA). The relative crystallinity was estimated considering the integrated area below the main peaks and taking 100% crystallinity for Na–mordenite before ion exchange.

MEB media containing Ag–mordenite were incubated and then filtered with 0.45 μm Sartorius membrane filter to recover the liquid phase. The amounts of silver released into the culture medium were determined by atomic absorption spectroscopy (AAS) with a Perkin Elmer 800 AAnalyst with flame atomization. The amount silver present in Ag–mordenite was also determined by AAS, the solid being previously digested by treating 100 mg of the sample with 10 ml $HClO_4$ + 2 ml HNO_3 on a heating plate for 8 h. Then 2 ml of HF were added and digested for 1 h, diluted and filtered. Temperature-programmed reductions with H_2 (H_2-TPR) were performed with an Ohkura TP-2002S to evaluate the amount and type of silver species. Previously, in situ pretreatments to dry the sample were performed, flowing N_2 at 300 °C and maintaining at this temperature for 30 min. Then, the sample was allowed to cool and changed to a stream of H_2 diluted in He, after which a temperature ramp to 900 °C at 5 °C min^{-1} was applied. H_2 consumption vs sample temperature was recorded and the profiles were deconvoluted to separate individual peaks.

Elemental probe microanalysis (EPMA) was performed to determine the Na/Al and Ag/Al molar ratios in the zeolite with an energy dispersive equipment EDAX coupled to a SEM JEOL JSM-35C. The sample was coated with graphite and X-ray spectra were obtained with an accelerating voltage of 20 kV. Semiquantitative analyses were obtained using the SEMIQ method, which does not require the use of standards.

The oxidation state of the silver surface species was examined with a module Multitechnique Specs equipped with a dual X-ray source Mg/Al and hemispherical analyzer 150 Phoibos in fixed analyzer transmission mode (FAT). We analyzed the binding energies (BE) of Ag 3d, Si 2p, Al 2p, C 1s and O 1s core-levels. The kinetic energy (KE) in the region of the Ag M_4VV Auger transitions was also analyzed and thereby determined the modified Auger parameter ($\acute{\alpha}$), defined as $\acute{\alpha}$ = KE (Ag M_4VV) − KE (Ag $3d_{5/2}$) + 1253.6 eV. The spectra were obtained with a pass energy of 30 eV with a Mg anode operated at 200 W. The pressure during the measurement was less than 2.10^{-8} mbar. The samples were ground, pressed, supported on the sample holder, subjected to vacuum dehydration at 300 °C for 20 min and finally evacuated under vacuum prior to the readings. The Si 2p peak of the zeolite at 102.4 eV binding energy (BE) was taken as internal reference. Data processing and peak deconvolution were performed using the Casa XPS software.

3. Results and discussion

3.1. Physicochemical characterizations

3.1.1. Compositional studies by AAS and EPMA

Table 1 shows the different Ag–mordenite samples obtained after the ion- exchange processes. By AAS it was determined that solids containing 3.9%, 5.6%, 10.3% and 13.6% w/w of silver were obtained, which were designated as Ag(4)Z, Ag(6)Z, Ag(10)Z, Ag(14)Z, respectively. It is observed that the silver loading in the zeolite increased and, at the same time, the sodium content decreased as the concentration of the exchange solution was higher (indicated between brackets). Table 1 also shows that the Ag/Al and Na/Al atomic ratios, as determined by EPMA, exhibited the