Zeolite membranes to immobilize Catalase

A. Tavolaro[1], S. Catalano[2], P. Tavolaro[3]

[1]Research Institute on Membrane Technology (ITM-CNR), National Research Council of Italy c/o University of Calabria, I-87036 Arcavacata di Rende, ITALY
[2]Department of Physics, University of Calabria, I-87036 Arcavacata di Rende, ITALY
[2]Department of Pharmacy, Health and Nutritional Sciences University of Calabria, I-87036 Arcavacata di Rende, ITALY
*All authors contributed equally to this work.

***Abstract*—** *Processes based on immobilized enzymes have been studied extensively in the last few decades and today are also applied to the safeguard of environmental parameters. In this work, zeolite composite flat membranes with different chemical composition, transition metal, and microporous structures were prepared using in situ and secondary growth crystallization synthesis methods in/on stainless steel porous disks. All zeolite materials were been used in catalase adsorption to analyze the zeolite behavior andthe effect of chemical composition and structure on interaction with the enzyme. This study shows that the electrostatic type of interaction seems to be of the utmost importance in influencing immobilization, while the zeolite Brönsted acidity of the support is the subordinate parameter, which differentiates the adsorption performances of different zeolite structures (that distinct for chemical composition of the framework). Moreover, it permits to conclude that transition metal-containing membranes adsorb a higher percentage of the enzyme with respect to no-exchanged membranes and that, for all materials synthesized, the amount of catalase adsorbed onto the zeolite crystals and membranes increases with the temperature.*

***Keywords*— Catalase immobilization, Environmental application, Hydrothermal synthesis, Zeolite membranes.**

I. INTRODUCTION

Zeolite composite disk membranes provide an ideal support to immobilize enzymes for advanced applications such as membrane bioreactors, biosensors and disease diagnostics. In fact, they are composed of a zeolite selective film formed by inter-crystalline growth and, at the same time, they present a large number of zeolite crystals grown inside the meso- and macro-pores of inorganic support used in the hydrothermal synthesis [1]. Zeolite membranes have the advantage that the basic/acidic nature of the material can be modified by varying the Si/Al ratio or by introducing different metals (Me) into the crystalline framework. Furthermore, zeolite acidity can be adjusted by exchanging extra-framework metal cations with H^+[2]. Finally, zeolite membranes are known to be stable both in wet and dry conditions then normally compatible with biochemical analyses. We recently reported the application of zeolite crystals and membranes as adsorbent materials for the immobilization of hard and soft proteins (BSA and cytochrome c) and we observed that their amount adsorbed on zeolite materials increases when the zeolite crystals are inter-grown for forming a membrane [3], [4].

Here we report, the adsorption characteristics of catalase on different zeolite crystals synthesized in hydrothermal conditions. Catalase, present in the peroxisomes of nearly all aerobic cells, is a heme-containing metalloenzyme that is regarded as one of the most common enzymes in plant and animal tissues. It consists of four subunits, each of which contains a Fe^{3+} prosthetic heme group (protoporphyrin IX), which is exposed through a 26 Å long and 17 Å wide funnel shaped channel and is responsible for the its catalytic activity. This enzyme catalyzes the disproportion of hydrogen peroxide into water and oxygen (1):

$$2H_2O_2 \rightleftarrows O_2+2H_2O \quad (1)$$

Catalase has been used to eliminate of residual hydrogen peroxide in textile [5], food [6], semiconductor industries [7], and wastewater treatments [8], but the high cost of the enzyme has impeded its wide application. It is used in food technology [9] and was proposed as a therapeutic agent to be administered interperitoneally [10]. For these reasons and for its technological potentials, catalase was selected as model enzyme for this study. In all applications reported in the literature, catalase is largely preferred as immobilized enzyme, being more stable to proteolysis. Catalase films were immobilized by adsorption on a variety of polymeric surfaces such as carbon nanofibrous membranes [11], which were been studied using spectroscopic and electrochemical analyses. It was evident that major problems related to polymeric supports were been the inadequate resistance to extreme pH values of media, high temperatures, to bacterium presence, and degradation by proteolysis11 reaction. The novelty of the present work lies in the association of synthesis of zeolite membranes (which have superior physical-chemical characteristics such as thermal, pH and bacterial resistance) and the immobilization of enzyme.

Zeolite materials were characterized by powder X-ray diffraction (XRD), scanning electron microscopy (FESEM), ICP-MASS and single gas permeability. Owing to the high substrate surface area used, it was also possible to measure enzyme loss from the solution directly via UV spectroscopy. Catalytic tests were performed to verify the enzymatic activity after the adsorption on zeolite membranes.

The preparation of insoluble supports to adsorb enzymes is a primary aim of current research because immobilized enzymes possess several advantages over the free enzyme, for example an increase of biomolecule stability and the facility of its re-use and separation from the reaction media with spin off in advanced applications in various fields such as biotechnology, bio-processing [12],[13], filtration, biosensors, diagnostic products, food manufacturing, cell culture, drug delivery, dentistry and medical engineering.

II. MATERIAL AND METHOD

2.1 Materials

Zeolite materials were prepared by using tetraethyl orthosilicate (JANSSEN), tetrabutylammonium bromide, iron and aluminium nitrate nonahydrate (ALDRICH), Silica fumed (SIGMA), tetrapropylammonium bromide (JANSSEN) and cobalt sulphate (BAKER).The crystals obtained were used, as seeds, to synthesize new zeolite composite membranes: Sil-2, Fe-MFI, Fe-ZSM-5, Co-MFI, Co-ZSM-5. All the samples were characterized by XRD, SEM and EDX analyses. Finally, the catalase has been adsorbed on these materials, using different amount of enzyme, pH and temperature values.

2.2 Adsorption Studies

Batch adsorption experiments were carried out using the same amount of the different zeolites synthesized with the enzyme solution. The adsorbent crystals and enzyme solution were vigorously shaken at different temperatures. The balanced samples were centrifuged for 10 min at 13,000 rpm, after which the supernatant was submitted to analysis. Enzyme concentrations before and after immobilization were determined using the Bradford method [14] which employs a UV spectrophotometer (Shimazdu UV-160 A). The test kit was purchased from Biorad (Munich, Germany). The experiments were carried out at selected solution pH values using appropriate buffer. A mass balance was then applied to calculate the enzyme adsorbed on the zeolite crystals and membranes. Defined calculation of catalase immobilized in percentage is as follows:

$$\%\text{adsorbed catalase} = \text{amount of catalase adsorbed} / \text{amount of initial catalase} \times 100 \quad (2)$$

where the amount of catalase adsorbed = total amount of catalase in solution before immobilization – total amount of catalase in solution (free) after immobilization.

The isothermal adsorption experiments were performed using a starting solution concentration of 1mg/ml. The percentage of enzyme solution used in contact with the zeolite crystals ranged from 0.4 to 4%.

III. RESULTS AND DISCUSSION

Zeolite crystal adsorbents were synthesized with Silicalite-2 (Sil-2, MEL-type), Fe-Silicalite-1 (Fe-MFI), Fe-ZSM-5 (Fe-ZSM-5), Co-Silicalite-1 (Co-MFI), and Co-ZSM-5 (Co-ZSM-5) (MFI-type) zeolite structures. In order to adjust the surface acidity of the MFI structure, aluminum, iron and cobalt atoms were incorporated into the crystalline framework and the Si/Al, Si/Fe or Si/Co ratios were varied by changing the chemical composition of precursor reaction gels. Silicalite-2 crystals synthesized in fluoride and alkaline medium were used for comparison.

The equilibrium and kinetic characteristics of the enzyme on these materials were studied by varying incubation temperature and contact time, enzyme concentration and pH values. Lastly, in order to examine the influence of the membrane configuration on the enzyme adsorption, five zeolite composite membranes, with the studied crystals having an identical chemical composition, were prepared by different crystal growth by using stainless steel supports in hydrothermal syntheses. Fig. 1a and b show the X-ray diffractogram of scrapped film and FESEM image of nanocrystalline composite membrane of silicalite-2 zeolite structure, respectively. Fig. 2a and b exhibit the same analysis for the Fe-ZSM-5 zeolite structure, respectively. Fig. 3a and b reveal the EDX analysis of scrapped film and FESEM image of nanocrystal composite membrane of Co-ZSM-5.XRD analyses reveal the preparation of pure silicalite-2, and Fe- and-Co- ZSM-5 type zeolite structures and FESEM microphotographs of zeolite membranes evidence the different crystalline morphologies prepared.

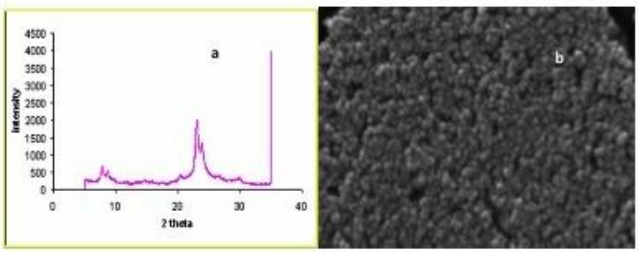

FIGURE 1. (a) XRD pattern of zeolite film prepared by hydrothermal synthesis, (b) FESEM microphotographs of silicalite-2 zeolite membrane.

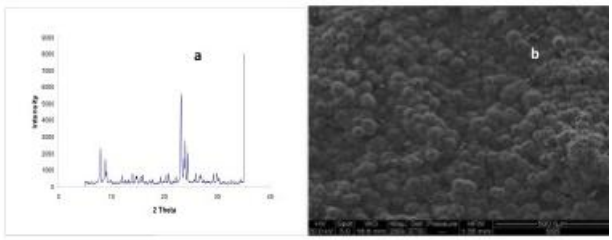

FIGURE 2. (a) XRD pattern of zeolite film prepared by hydrothermal synthesis and (b) FESEM microphotograph of Fe-ZSM-5 zeolite membrane surface

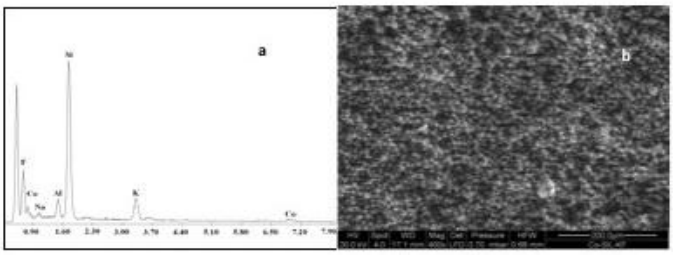

FIGURE 3. (a) EDX analysis and (b) FESEM microphotograph of Co-ZSM-5 zeolite membrane surface

In order to rationalize interactions between catalase and the zeolite support we analyzed the effect of various experimental parameters such as the incubation temperature and time, the concentration of enzyme solution and the pH value by using zeolite samples synthesized with different structures and chemical compositions.

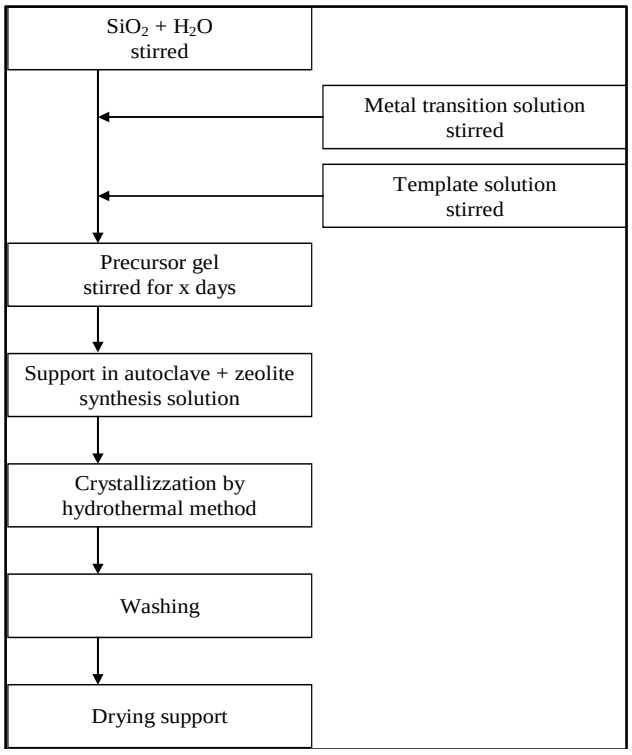

FIGURE 4. Sketch of the experimental method used to prepare the isomorphous substituted zeolite composite membranes

The zeolite composite membrane set that was prepared includes a wide range of Brönsted acidity: from the rather basic form of Sil-2 to the acidic zeolite Fe-ZSM-5 according the synthetic method reported in Figure 4.An interface that is formed between two different phases usually has a higher standard free energy than the bulk phase. As a result, it is apt to be thermodynamically stabilized by adsorbing any chemical species that are different from the solvent molecules. Adsorption profits from interactions between the surface of the support and the outer shell of biological molecules. In this paper, the

amount of adsorbed enzyme was measured by using the so-called depletion method, based on the decrease of the enzyme concentration in the solution after immobilization. It is well-known that the adsorbed amount of enzyme is influenced by several factors such as the polypeptide characteristics, the solid support surface and the environmental conditions. As regards the enzyme, its charge, its structure stability (hard or soft behavior), its amino acid composition and its steric conformation need to be taken into consideration.

As regards the solid support, the zeolite properties that can influence the enzymatic adsorption are the zeolite structure, the chemical composition of the crystalline framework, the crystal morphology and size, the Brönsted acidity, the number and the distribution of defect and hydroxyl groups. These groups can be seen as silanol groups whose acidity can be enhanced by the interaction with a strong Lewis acid center (Al^{3+}) or decreased by the presence of fluoride ions in the framework. Moreover, in order to study the influence of transition metal atoms on catalase adsorption, we prepared Ag-, Fe-, and Co-zeolite materials by ion exchange procedures. Since the zeolite structures have micropores, which are too small with respect to the kinetic diameter of the catalase, the adsorption occurs solely on the external crystalline surface. There is no systematic study in the literature, to our knowledge, about the adsorption of catalase on zeolite materials that rationalize its performance on different involved crystalline structures. The effect of pH on the catalase adsorption was investigated at three different pH conditions: 4.8, 6.4 and 11.8. As can be seen from Fig. 5, for zeolite crystals synthesized, the adsorption capacity of the enzyme decreased with increasing pH. The pH value that gave the highest adsorption, among those tested, was found to be 4.8, very close to the beef catalase isoelectric point that is 5.4 [15]. So, when the pH 4.8, there is no net charge on the surface of catalase. In this case, electrostatic repulsion between the enzyme and the surface of the material is minimal. Fig. 6 shows the adsorption isotherms obtained by using the zeolite composite membranes prepared and it reveals the dynamic evolution of adsorbed enzyme. The initial trend of the curves obtained for the microporous zeolite crystals has a very fast adsorption. When the time is prolonged, the amount of adsorbed enzyme increases slowly. This behavior was observed, for the same structure, for all the temperatures tested. Since all the experimental parameters (temperature, weight and enzyme/zeolite ratio) were kept identical for all the experiments, the observed variations of catalase adsorption come only from the surface crystalline interactions with enzyme molecules.

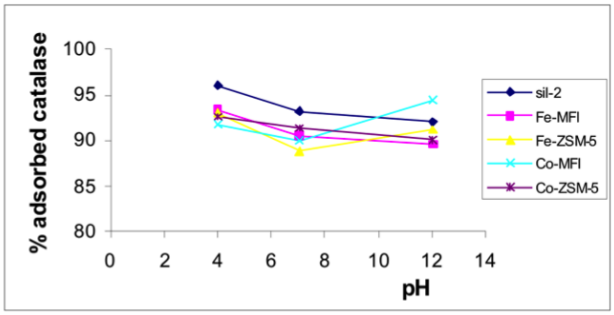

FIGURE 5. Influence of pH on catalase adsorbed onto different zeolite supports

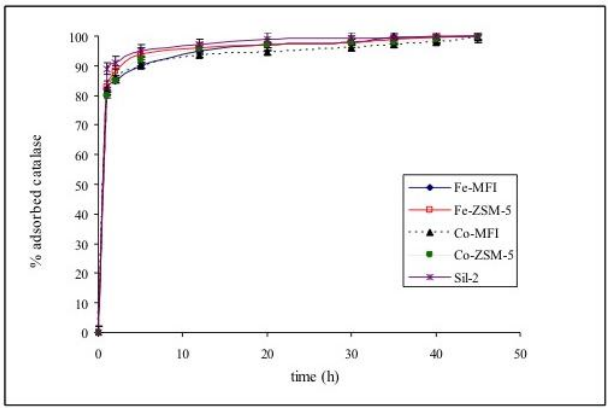

FIGURE 6. Dynamic behaviour of Catalase adsorbed onto different zeolite membranes

IV. CONCLUSION

Zeolite composite membranes were prepared by *in situ* and secondary growth crystallization hydrothermal synthesis methods and characterized in order to apply in adsorption of catalase. In this work, the adsorption of catalase on various zeolite structures was studied, because these inorganic materials could have tremendous potential applications in biotechnological industrial processes, but their interactions with biological species in the literature have been inadequate up to now. Although the adsorption of enzymes on inorganic surfaces is a very simple phenomenon at first glance, this behavior needs further elucidation because there are many factors that affect the process. The interaction between polypeptides and surfaces is also complicated since forces such as hydrophilic and hydrophobic, electrostatic and structural interactions are involved to a greater or lesser extent.

This study leads to interesting conclusions permitting to evidencing that transition metal cation-containing zeolite membranes adsorb a higher percentage of catalase. Moreover, since the amount of catalase adsorbed on the zeolite crystals and membranes increases with the temperature for all samples, we suppose that the acidity of the surface hydroxyl groups

plays an important role in this interaction. Moreover, the catalytic activity of the catalase-zeolite system was preserved and it constitutes an undeniable advantage of this technique for future applications.

ACKNOWLEDGEMENTS

This work was supported by "PON R&C (Programma Operativo Nazionale Ricerca e Competitività 2007-2013) project PON01_01 00293 Spread Bio-Oil".

REFERENCES

[1] J. Coronas and J. Santamaria, "State-of-the-Art in Zeolite Membrane Reactors," Top. Catal., vol. 29, no. 1/2, pp. 29–44, May 2004.

[2] A. Tavolaro, P. Tavolaro, and E. Drioli, "Influence of synthesis parameters on vanadium-silicalite-1 crystal growth prepared with fluoride-containing media," J. Cryst. Growth, vol. 289, no. 2, 2006.

[3] A. Tavolaro, P. Tavolaro, and E. Drioli, "Zeolite inorganic supports for BSA immobilization: Comparative study of several zeolite crystals and composite membranes," Colloids Surfaces B Biointerfaces, vol. 55, no. 1, 2007.

[4] P. Tavolaro, A. Tavolaro, and G. Martino, "Influence of zeolite PZC and pH on the immobilization of cytochrome c: A preliminary study regarding the preparation of new biomaterials," Colloids Surfaces B Biointerfaces, vol. 70, no. 1, 2009.

[5] S. A. Costa, T. Tzanov, F. Carneiro, G. M. Gübitz, and A. Cavaco-Paulo, "Recycling of textile bleaching effluents for dyeing using immobilized catalase," Biotechnol. Lett., vol. 24, no. 3, pp. 173–176, 2002.

[6] L. Tarhan, "Use of immobilised catalase to remove H2O2 used in the sterilisation of milk," Process Biochem., vol. 30, no. 7, pp. 623–628, Jan. 1995.

[7] S.-H. Oh, H.-J. Yu, M.-S. Kim, S. So, and H.-J. Suh, "Biodegradation of Hydrogen Peroxide in Semiconductor Industrial Wastewater with Catalase from Micrococcus sp.," Prev. Nutr. Food Sci., vol. 7, no. 1, pp. 33–36, Mar. 2002.

[8] D. S. Yoon et al., "Continuous removal of hydrogen peroxide with immobilised catalase for wastewater reuse," Water Sci. Technol., vol. 55, no. 1–2, p. 27, Jan. 2007.

[9] E. Akertek and L. Tarhan, "Characterization of immobilized catalases and their application in pasteurization of milk with H2O2," Appl. Biochem. Biotechnol., vol. 50, no. 3, pp. 291–303, Mar. 1995.

[10] M.-E. E. van Rossen, W. Sluiter, F. Bonthuis, H. Jeekel, R. L. Marquet, and C. H. J van Eijck, "Scavenging of Reactive Oxygen Species Leads to Diminished Peritoneal Tumor Recurrence," CANCER Res., vol. 60, pp. 5625–5629, 2000.

[11] L.-S. Wan, B.-B. Ke, and Z.-K. Xu, "Electrospun nanofibrous membranes filled with carbon nanotubes for redox enzyme immobilization," Enzyme Microb. Technol., vol. 42, no. 4, pp. 332–339, Mar. 2008.

[12] Y. Fan, F. Su, K. Li, C. Ke, and Y. Yan, "Carbon nanotube filled with magnetic iron oxide and modified with polyamidoamine dendrimers for immobilizing lipase toward application in biodiesel production," Nat. Publ. Gr., 2017.

[13] H. K. Hustoft et al., "Integrated enzyme reactor and high resolving chromatography in 'sub-chip' dimensions for sensitive protein mass spectrometry," Sci. Rep., vol. 3, no. 1, p. 3511, Dec. 2013.

[14] M. M. Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding," Anal. Biochem., vol. 72, no. 1–2, pp. 248–254, May 1976.

[15] T. Samejima, T. Miyahara, A. Takeda, A. Hachimori, and K. Hirano, "On the Acid Denaturation of Porcine Erythrocyte Catalase in Relation to Its Subunit Structure1," J. Biochem, vol. 89, pp. 1325–1332, 1981.