Phosphotriesterase-Like Lactonase Immobilized on Zeolites for Pesticides Degradation

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***Abstract*—** *In this study, a non commercial PTE was covalently immobilized on the NaX zeolite crystals and its ability to biodegrade the paraoxon to a less harmful compound was investigated. The immobilization method did not change the enzyme catalytic performance. In fact, the specific activity was the same of the free one. Besides, the process improved the stability of the enzyme.*

The obtained results are interesting because of usually the immobilization process increases the enzyme stability but causes a reduction of its catalytic activity. Therefore, this pioneer study of the immobilization of the PTE on zeolite particles seems to be an environmental-friendly solution to the problem of pesticides pollution.

Keywords*— *detoxification, enzyme immobilization, organophosphates, phosphotriesterase-like lactonase, specific activity, zeolite.

I. INTRODUCTION

Pesticides were and are extensively used in agriculture for controlling the spread of unwanted insects or weeds. The extensive use of pesticides causes serious environmental concerns; in fact, only 5% or less of the applied pesticides reach the target organisms which resulted in contamination of soil and water. Their continuous use brought an accumulation of pesticides and their residues in environment, endangering the populations by their varied toxicity [1]. Among the various groups of pesticides, organophosphates (OPs) are one of the most widely used types [2]. The current methods for the OPs compounds detoxification are harmful and determine serious environmental consequences. Therefore, the use of enzymes for the detection and decontamination of organophosphate agents has received considerable attention [2, 3]. Many enzymes catalyze the hydrolysis of the OPs among which the phosphotriesterases (PTE; EC 3.1.8.1) [4, 5] and PTE-like lactonases (PLL) [6]. Some of them are commercially available and were employed to purify OP-contaminated water. However, the main problem associated with the use of these enzymes is their low stability in solution [7].Usually, the enzymes are immobilized on inorganic or organic supports for improving their stability [8-10]. In addition, immobilization process facilitates the enzyme re-use and also leads to an easier separation of the enzyme from the reaction mixture, provides the control of the reaction time and reduces the enzyme loss in the product stream [11]. Among the various inorganic supports, zeolites have attracted significant attention for enzyme immobilization having remarkable properties: high surface area for high enzyme loadings, high chemical and mechanical stability [12]. Zeolites (alumino-silicate materials with well-defined pore size at molecular scale) are cost effective and non-toxic with respect to the health issues [13]. Considering these characteristics, they are suitable candidates for different applications such as water purification and softening, separation processes and sensors [14, 15] and for immobilization of enzymes [11, 16-18].

The topic of this work is the development of environmental-friendly materials for the treatment of waste water and for the remediation of contaminated sites. In particular, the study was focused on the covalent immobilization of a mutated version of the PLL from SsoPox namely *Sso*Pox W263F on the zeolite surface using the glutaraldehyde as cross linker. The immobilized enzyme was used for performing preliminary studies on the organophosphate pesticide (paraoxon ethyl) degradation.

II. MATERIAL AND METHOD

2.1 Materials

Glutaraldehyde (GLU), paraoxon ethyl and 4-nitrophenol were purchased from Sigma Adrich. Trizma Base ($C_4H_{11}NO_3$, ≥99.9%) and HCl, used for the preparation of TRIS/HCl buffer and HEPES sodium salt were also purchased from Sigma-Aldrich.

NaX zeolite particles (size ~ 2 µm; Sigma-Aldrich) were used as support for enzyme immobilization. Before the chemical modification, zeolite crystals were purified (*via* centrifugation) to enable the separation of the crystal fraction from the mother liquid. The solid phase was re-dispersed in distilled water and centrifuged again. The procedure was repeated for lowering the pH value from 10 to 7. Finally, the zeolite particles were heated at 500 °C for removing the amorphous organic materials and the water from the pores.

2.2 PTE production and purification

The enzyme SsoPox W263F, a mutated form of the wild type SsoPox from *Sulfolobus solfataricus*, was obtained, expressed in *E. coli* and characterized as previously reported [5]. The recombinant enzyme was produced in large scale (150 L) by coupling high cell density fermentation strategy with a galactose induction up to a 4660.0 $U{\cdot}L^{-1}$, as previously reported [19]. After extraction from the biomass by mechanical cell disruption, the enzyme was purified by coupling a thermo-precipitation step with a membrane-based ultra-filtration protocol. The final solution, containing the enzyme with a high purity grade, was freeze dried and preserved until it was used [19].

2.3 Zeolite crystals characterization

Morphology and size of NaX zeolite crystals, used as support for the enzyme immobilization, were analyzed by scanning electron microscopy (SEM) using a Cambridge Zeiss LEO 400 microscope. The Si/Al ratio of the zeolite crystals was determined by energy dispersive X-ray (EDX) performed with EDAX-Phoenix in SUTW Detector, analyzer: Si/Li crystal).

2.4 Chemical activation of zeolite crystals

The NaX zeolite activation was carried out by immerging 60 mg of crystals in a GLU solution (0.05M; pH= 5.0, buffer phosphate) for 24 hours at 45°C [8]. The modified zeolite particles were recovered by vacuum filtration and washed with buffer phosphate different times to remove the organic material adsorbed on its surface. The process was stopped when organic molecules (not covalently bound) were not detected in the washing solution by spectrophotometric analysis. Subsequently, the activated zeolite crystals were dried overnight at room temperature and used for immobilizing the enzyme by covalent binding, *via* cross-linking.

2.5 Enzyme immobilization

The enzyme was covalently immobilized (formation of a Schiff's base) by immerging the activated zeolite crystals (30 mg) in the enzyme solution (30 mL, 20 mM HEPES buffer pH 8.5) at 25 °C, under gentle stirring for 24 hours.

At the beginning, the effect of different concentrations of GLU (5 w/v %, 10 w/v %, 15 w/v %, 25 w/v %) on the amount of the immobilized enzyme was evaluated, keeping constant the initial enzyme concentration (2×10^{-3} mg*mL^{-1}). Afterwards, the best GLU concentration was used for performing other immobilizations at different enzyme concentration (4×10^{-3} mg*mL^{-1}; 8×10^{-3} mg*mL^{-1}). The resulting immobilized enzyme was recovered by vacuum filtration and dried overnight at room temperature.

Some immobilizations were also carried out in absence of GLU to evaluate the percentage of the enzyme immobilised by physical adsorption on the zeolite surface.

The amount of the immobilised enzyme was determined by means of the equation of mass balance reported below:

$$m = C_i V_i - (C_f V_f + C_{cs} V_{cs}) \quad (1)$$

where m is the amount of immobilised enzyme, (C_iV_i) is the enzyme present in the initial solution, (C_fV_f) the enzyme present in the final solutions and ($C_{cs}V_{cs}$) that present in the cleaning solution. The enzyme concentration was measured using the Bradford's method [20]. A calibration curve constructed with BSA solutions of known concentration was used in the calculation of protein in the enzyme and cleaning solutions.

2.6 Phosphotriesterase activity for free and immobilized enzyme

For testing the enzyme activity some catalytic reactions were carried out on free and immobilized enzyme.

The catalytic activity of free enzyme was evaluated by monitoring the hydrolysis of the paraoxon-ethyl for producing 4-nitrophenol. In particular, the assay was performed at 25 °C in a mixture of Tris/HCl (0.4 M and pH=8.5) containing 1 mM of paraoxon as substrate. The enzyme concentration was 2×10^{-3}mg*mL^{-1}. The activity was measured by detecting the formation of 4-nitrophenol (reaction product) at 405 nm, in 1-cm path-length cell with a spectrophotometer equipped with a thermo-jacketed cell holder (UV-VIS Spectrophotometer lambda EZ 201, Perkin Elmer). The molar absorption coefficient used for 4-nitrophenol was 19920 M^{-1}cm^{-1} at 25 °C.

The catalytic activity of the immobilized enzyme was measured using a stirred batch reactor containing 30 mg of NaX-GLU-*Sso*Pox W263F particles and paraoxon (1mM) in 30 mL of Tris/HCl (0.4 M and pH= 8.5). Three different enzyme concentration were used (2×10^{-3} mg*mL^{-1}, 4×10^{-3} mg*mL^{-1} and 8×10^{-3} mg*mL^{-1}). Samples were collected in the time from the reaction mixture and analyzed with the spectrophotometer.

The stability of the immobilized PTE was investigated performing seven reactions at an initial enzyme concentration of 2×10^{-3} mg*mL^{-1}and at 25 °C. After each reaction, the PTE-zeolite particles were removed from the batch reactor, rinsed with the buffer solution and stored at 4 °C. After a certain period of time, the immobilized enzyme was immersed in a fresh substrate solution for carrying out a new reaction. For comparison, the stability of free enzyme was also investigated keeping constant the operating conditions.

III. RESULTS AND DISCUSSION

The morphology of the NaX zeolite crystals is shown in Figure 1. The size of the crystals was about 2 µm.

The Si/Al ratio for the NaX zeolite, determined by EDX analysis, was 1.37 (see Figure 2) indicating a hydrophilic nature of this zeolite.

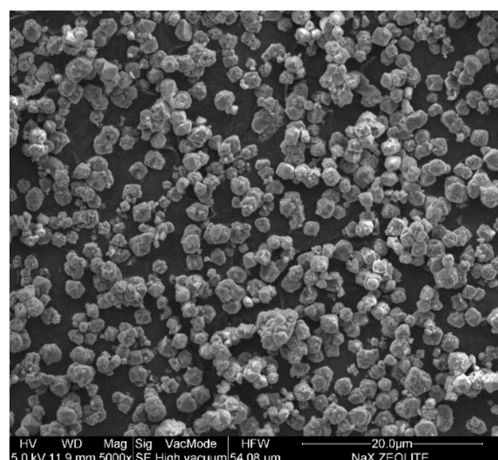

FIGURE 1. SEM image of the NaX zeolite.

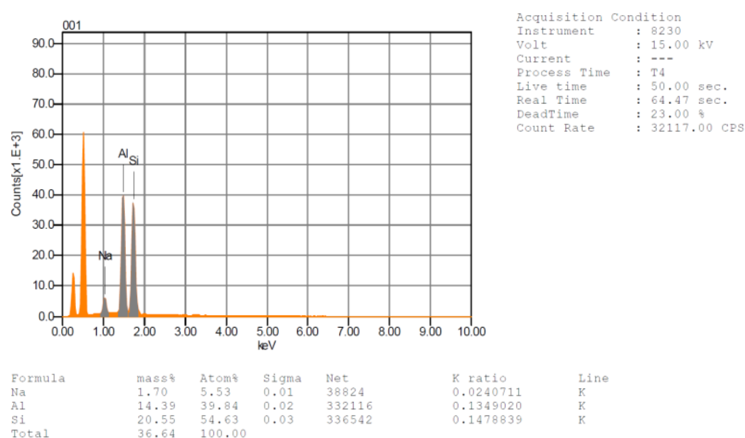

FIGURE 2. EDX analysis of the NaX zeolite.

Glutaraldehyde was used for the NaX zeolite activation [24]. Subsequently, the enzyme was immobilized on the NaX-GLU particles by means of covalent binding, *via* cross-linking. The chemical reaction allowed the formation of a Schiff's base between the enzyme amine and the free group of the glutaraldehyde (see Figure 3).

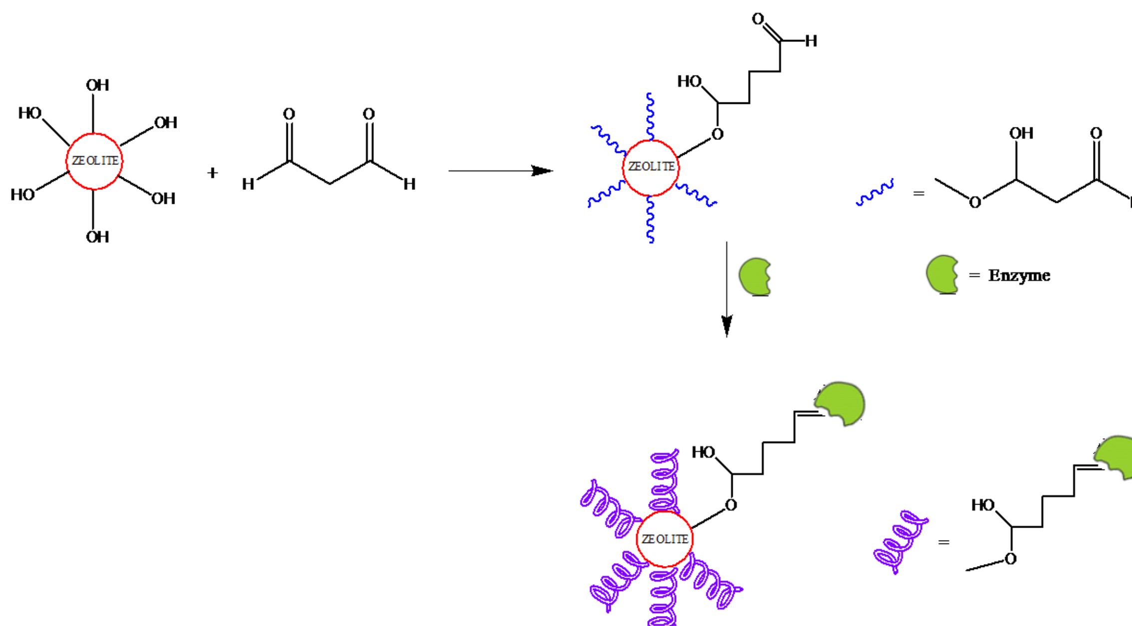

FIGURE 3. Reaction scheme of zeolite activation and enzyme immobilization via cross-linking.

The effect of different GLU concentrations (5 w/v %, 10 w/v %, 15 w/v %, 25 w/v %) on the amount of the immobilized enzyme was evaluated keeping constant the initial enzyme concentration (2×10^{-3} mg*mL^{-1}). The results showed that increasing the GLU content (from 5wt/v % to 10 wt/v%) the amount of immobilized enzyme also increased (see Figure 4). Moving the GLU concentration from 15 w/v % to 25 w/v % the immobilized SsoPox W263F amount reached a plateau. These results can be explained considering that an excessive increase of the GLU concentration leads to a steric hindrance, avoiding a further enzyme immobilization [25]. Other experiments, performed with inactivated zeolite crystals (physical adsorption), indicated a very low quantity of immobilized SsoPox W263F (about 10 %).

The effect of initial enzyme concentrations (2×10^{-3} mg*mL^{-1}, 4×10^{-3} mg*mL^{-1} and 8×10^{-3} mg*mL^{-1}) on the immobilization process was also evaluated by keeping constant the GLU concentration (10 w/v %). As it can be seen in Figure 5, an increase of the initial enzyme concentration permitted to enhance also the immobilized SsoPox W263F amount.

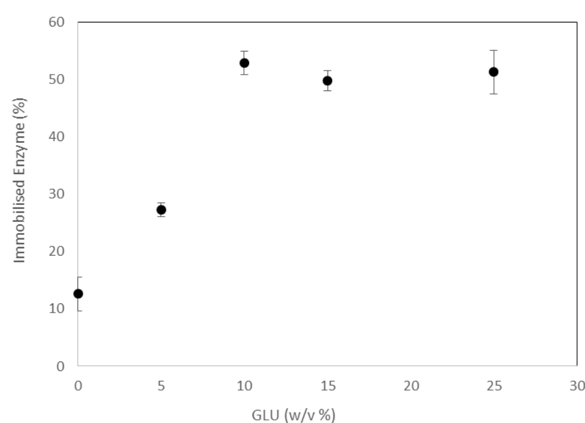

FIGURE 4. Effect of GLU concentration on the amount of immobilized enzyme.

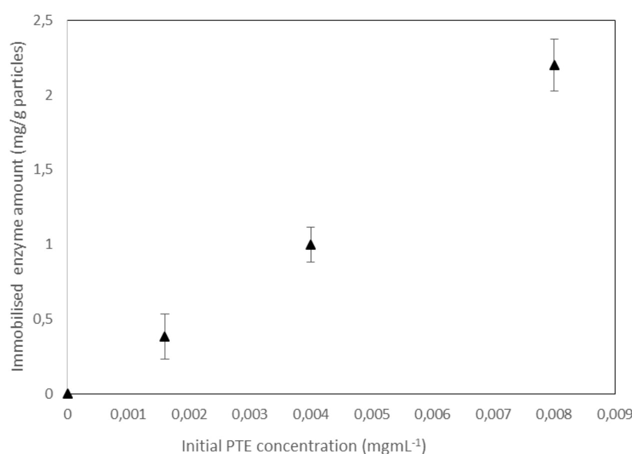

FIGURE 5. Effect of initial enzyme concentration on the immobilized enzyme amount (Operating conditions: pH= 8.5; 60 mg of NaX-GLU particles in 30 mL solution; T=25 °C).

Figure 6 shows that the specific activity of the immobilized *Sso*Pox W263F was independent of the initial enzyme amount and its value was the same of that the free one. This means that the immobilization process did not modify the enzyme properties.

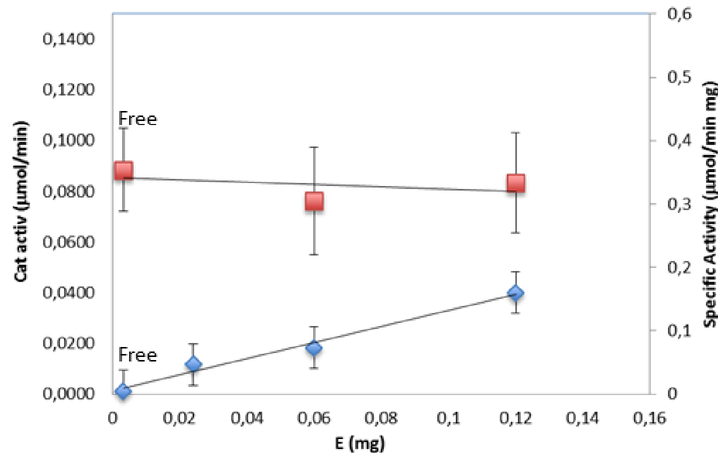

FIGURE 6. Catalytic activity ◆) and specific activity (■) *vs* the enzyme amount for immobilized and free SsoPox W263F.

The stability of free and immobilized enzyme was also investigated for about two months. The *Sso*Pox W263F in its free (re-suspended in a buffer solution from lyophilized lots) exhibited a specific activity of 0.34 μmol*min^{-1*}mg^{-1} and this value was lost in about two months (see Figure 7). On the other hand, the immobilized form exhibited a slower decay indicating as the immobilization process tends to stabilize the enzyme [26].

An important aspect that must be highlighted is the possibility to recovery and reuse the zeolite crystals for other immobilizations after burning the organic material by thermal treatment at 550°C [9].

This study demonstrated that zeolites can be used as a support for the covalent immobilization of a non-commercial PTE. In particular, it was assessed the possibility to increase the enzyme stability and maintaining the same catalytic activity of its free form. This results is interesting considering that, usually, the immobilization process increases the enzyme stability but causes a reduction of its catalytic activity. Therefore, the NaX-GLU-SsoPox W263F provides an environmental-friendly solution to the problem of pesticides degradation.

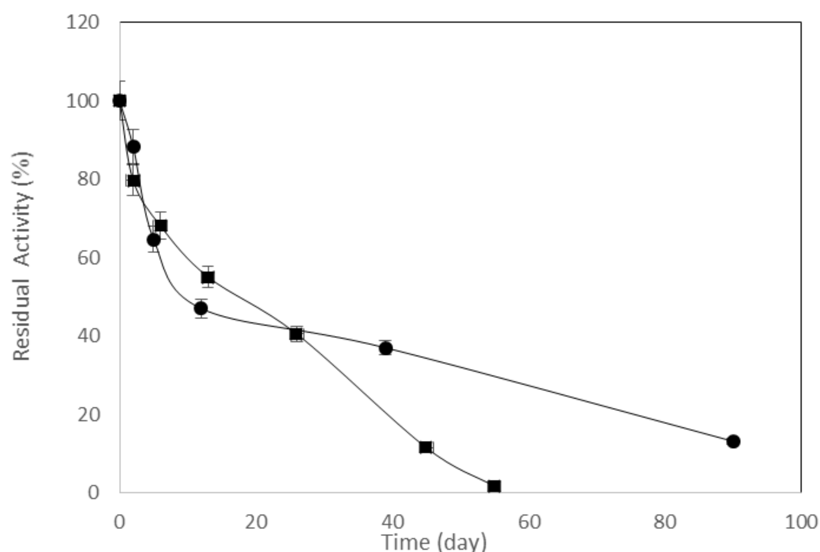

FIGURE 7. Stability of free (■) and immobilized (●) SsoPox W263F *vs* the time.

IV. CONCLUSION

Owing to the toxicity of organophosphate pesticides used in agriculture, is very important to perform the remediation of polluted sites. In particular, the degradation of pesticides with specific enzymes is environmental and socially acceptable. In

this scenario, the present study was focused on the paraoxon degradation by using a PTE-like lactonase covalently immobilized on the NaX crystals. The immobilization process did not change the catalytic properties of the enzyme in terms of specific activity. Besides, the process improved the stability of the enzyme. Considering the peculiar characteristics of the zeolites and the performance of the enzyme immobilized on its surface, this system seems to be a promising functionalized material for application in environmental decontamination.

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