Immobilization of enzymes on aminated clays: A comparison using microwave radiation versus conventional methods

José Manuel Jiménez-Barrera (1), Fernando Madrid (1), Juan Vázquez-Cabello (2), Pablo Llombart (3), Tomás Undabeytia (1*)

(1) Institute of Natural Resources and Agrobiology (IRNAS-CSIC), Reina Mercedes 10. Sevilla 41012. Spain.
(2) Faculty of Chemistry, University of Seville, Prof. García González St., 41012 Sevilla. Spain.
(3) Faculty of Sciences, Autonomous University of Madrid, 28040, Madrid. Spain.
* corresponding author: undabeyt@irnase.csic.es

Key Words Enzyme, clay, covalent immobilization, MW radiation. **Palabras Clave:** Enzima, arcilla, inmobilización covalente. Radiación microondas.

INTRODUCTION

Enzymes are widely used in industrial processes due to their inherent properties: selectivity, low cost compared to other chemically catalyzed processes, less susceptible to inhibition by toxic substances, highly effective over a wide range of concentrations. The immobilization of enzymes onto solid surfaces permits to increase their thermostability and resistance to harsh conditions, and an easy separation from the reaction medium allowing its reuse; however, these processes are time-consuming. The application of microwave (MW) radiation in organic synthesis opens a new frontier in the field of enzyme immobilization. Although enzymes are considered thermosensitive molecules to the heat caused by the MW radiation, the current technology permits to use temperatures as low as 40ºC along the process, and shorten the entire course of the immobilization process with a very significant reduction of the synthesis time, *vs* conventional methods (Plagemann et al., 2014). In the present work, a ligninolytic enzyme, a laccase, will be immobilized on an aminated sepiolite by using MW radiation. The optimization of the experimental conditions for its immobilization will be studied. A comparison of the activity of the system obtained by using MW versus a conventional immobilization process will be investigated.

MATERIALS AND METHODS

Materials. Laccase from *Aspergillus* sp., 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid (ABTS) and 3-triethoxysilylpropilamine (APTES) were purchased from Sigma Chemical Co. (St. Louis,Missouri, USA). Sepiolite (Pangel S9) was kindly supplied by Tolsa S.A. (Madrid, Spain).

Methods. Laccase activity was determined spectrophotometrically at 25ºC and pH 5.0 by using a 0.6 mM ABTS solution (ε_{436}=29300 M^{-1} cm^{-1}). One unit of laccase is defined as the amount of enzyme required to oxidize 1 μmol of ABTS per minute. The kinetic constants Km and Vmax of the Michaelis equation, were determined for the free and immobilized laccase by measuring the initial reaction rates with ABTS concentrations ranging from 0.2 to 2.0 mM.

A sepiolite-APTES complex was prepared as in Undabeytia et al. (2019). The amount of APTES grafted on sepiolite surface amounted to 0.56 mmol/g, close to that of silanol sites on the external surface. One hundred milligrams of Sep-APTES were incubated for 1 hour with 15 mL of an enzyme solution of 0.8 g/L. After centrifugation, an aliquot was selected for the analysis of the protein content; the supernatant discarded and the complex was treated with a solution of glutaraldehyde (GLUT) in a commercial microwave reactor system. The protein was immobilized by formation of imines via nucleophilic addition (AN) with glutaraldehyde. The temperature in the reaction system is controlled by an optical fiber sensor. Several experimental conditions were tested: reaction time (5, 10, and 15 min), temperature (40, 50 and 60 ºC), cooling time (10, 20 and 30 min), and glutaraldehyde concentration (0.05, 0.1 and 0.5%). The amount of immobilized enzyme was determined by the measurement of the amount remaining in solution by the Bradford method. The set of conditions determined for a complex providing the largest amount of

immobilized enzyme, lower Km and higher Vmax, was tested. In parallel, non-MW assisted immobilization was also performed by using a conventional heater with stirring instead.

RESULTS AND DISCUSSION

In the optimization of the preparation of an enzyme-clay based complex by using MW irradiation, a multiple linear regression (MLR) model with stepwise-regression method was performed. At first, 20 tests were carried out considering a confidence level of 95% and a study power of 80%, by using a general model; both the magnitude and the direction of the effects of the reaction variables on the activity (Km, Vmax) and the amount of immobilized enzyme of the complex obtained were analyzed. The four explanatory (input) variables at three levels in duplicate used were the reaction time, the reaction temperatura, the cooling time, and the glutaraldehyde concentration.

The outcome of the multiple lineal regression showed accuracies that were low for Km (20.7%) and moderate for Vmax 57.1%) (data not shown). As seen in Figure 1, the input variable that was able to explain the model with Km was the reaction time. A more complex model was obtained for Vmax. In that case, the input variables with significant p-values at a 0.05 level were the glutaraldehyde concentration and the cooling time as occurred previously with the content of adsorbed protein, but also it does cover signficance the reaction temperature. These three predictors were of approximately equal importance (not shown). According to the data obtained in this statistical study, an estimate of the experimental conditions that will result in an optimal complex, i.e. with a high percentage of protein content, and low Km and high Vmax values, will be the use of a glutaraldehyde concentration of 0.05%, a reaction temperature of 40°C, a reaction time of 5 or 10 min and a cooling time of 20 min. Effectively, by using these experimental conditions a complex with a Km value in the range of those observed in Figure 1 (214 μM) but higher immobilization percent (6.8% enzyme content) and Vmax, of 1375 U/g was obtained. The use of a conventional procedure using these operational variables showed higher Km (460 μM) and lower Vmax (775 U/g).

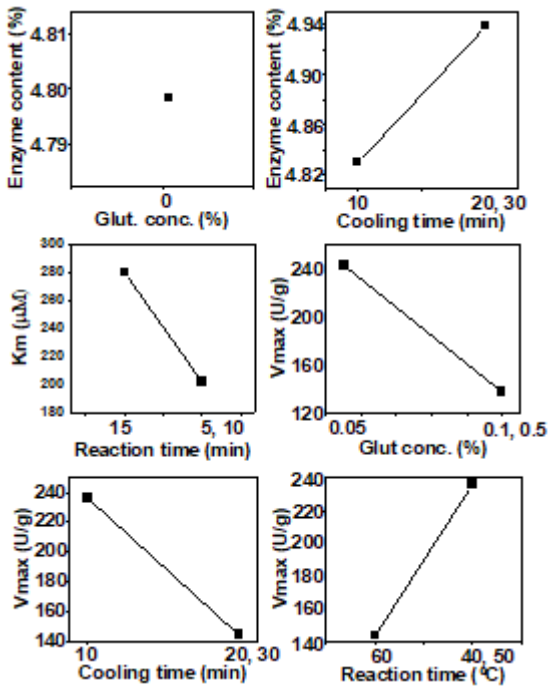

Fig. 1. *Plots of the relationship between the independent variables with the significant input variables according to the MLR model.*

CONCLUSIONS

The new techniques in the use of microwave radiation, widely used in the field of organic synthesis, allow extending its use to thermosensitive biological systems such as enzymes. Its use in processes of covalent immobilization of enzymes on surfaces allows obtaining complexes with higher activity than when using conventional procedures.

ACKNOWLEDGMENTS

J.M. Jiménez-Barrera thanks a contract as Technical Support Personnel Funded by the European Union "Next Generation EU", within the Recovery, Transformation and Resilience Plan (PRTR), Government of Spain.

REFERENCES

Plagemann, R., Langermann, J. von, Kragl, U. (2014). Microwave-assisted covalent immobilization of enzymes on inorganic surfaces. Eng. Life Sci. **14**, 493-499.

Undabeytia, T., Madrid, F., Vázquez; J., Pérez-Martínez, J.I. (2018). Grafted sepiolites for the removal pharmaceuticals in water treatment. Clays Clay Miner. **67**, 173-182.