Electronic Journal of Biotechnology ISSN: 0717-3458
Vol.6 No.2, Issue of August 15, 2003

Received December 4, 2002 / Accepted April 24, 2003

TECHNICAL NOTE

Immobilized lysozyme protein on fibrous medium: Preliminary results for microfiltration applications

Odilio B. G. Assis*
Embrapa Agricultural Instrumentation Center
Caixa Postal 741, 13560-970
São Carlos, SP., Brazil
Tel: 55 16 2742477
Fax: 55 16 2725958
E-mail: odilio@cnpdia.embrapa.br

Luis C. Claro
PPG Interunidades - Universidade de São Paulo USP
Caixa Postal 369, 13560-970
São Carlos, SP., Brazil
Tel: 55 16 2742477
Fax: 55 16 2725958
E-mail: lclaro@cnpdia.embrapa.br

Financial support: Embrapa, FAPESP and CNPq.

Keywords: enzyme immobilization, packed bed column, surface analysis.

The protein lysozyme was deposited onto a permeable support comprising chemically functionalized glass fiber. The main objective of this study was to set a stable organic net with no effect on the medium bed permeability and a preliminary test the activity of this enzyme under immobilized conditions. The film formation is followed by atomic force microscopy (AFM) surface imaging. The effect on the bacteria *Escherichia coli* was tested using a simple microfiltration column. The filtration results pointed around 75% removal of bacteria in the effluent when compared to the influent concentration. The removal mechanism is assumed as being essentially due biointeraction. The surface polarity characteristics of the formed film were also considered as playing an important role, suggesting an electrostatic interaction mechanism in the microorganism removal.

Porous media alone is able to achieve some separation (by physical retention) for a broad range of ordinary contaminants present in liquids. However removal efficiency, especially in the case of wastewater pollutants is never totally effective when using a simple flow through a permeable medium. Numerous compounds such as chemical residues, organic matter, and fouling of tiny sizes are hardly retained by a filter medium, being, in most of cases, responsible for levels of toxicity commonly detected in drinking water (Mallevialle, 1996). A possible way to enhance removal effectiveness is to combine the separation process with the presence of living organisms, biopolymers or natural biofilms coatings in the filter cake, promoting interactions at molecular level (Bryers et al. 1990; Phelps et al. 1990; Weber-Shirk and Dick, 1997). The biopurification process is a very desirable clean-up technology employing active elements for in-situ removal, not only of bacteria and viruses but also for the degradation of chemical residues such as fertilizer and herbicide contaminants in water and soil (Bollag, 1996).

Some enzymes and biopolymers from animal-vegetable origin, such as the protein lysozyme have been suggested for developing trickling filters or bed reactors due to their specific activity of biocide upon bacterial agents (Moser et al. 1988). Lysozyme, also known as muramidase is a natural thermally stable enzyme found in colostrum, hen egg whites, and human nasal mucus and tears. It is characterized by multiples and complex structures and presents a distribution of charges on the molecular surface, defining hydrophobic and hydrophilic regions, according to the model constructed by Blake et al. 1965 and Kayushina et al. 1996.

A wide range of techniques for molecules fixation have been developed and are continuously improved. Amongst them, the covalent coupling on inorganic supports is one of the simplest and most used technique due to stability of enzyme attachment and existence of several strategies for improving the operational stability of the biocatalyst (Weetall, 1993). In particular, the self-assembled (SA) technique is a very simple andsuitable way to build up thin protein films onto solid supports in a controlled fashion (Fender, 1996). The SA methodology is based on the spontaneous adsorption of polar molecules dissolved in

*Corresponding author

water by the electrostatic attractions of oppositely charges between the molecules and the support (Whitesides, 1995; Lvov, 2000). In this work lysozyme films are self-assembled on a fibrous support (filter medium) made of glass fibers. Our goal is a preliminary evaluation of such immobilization procedure in forming a protein net in a permeable medium. We use the atomic force microscopy and the bacteria interaction in an aqueous medium to characterize de deposited medium.

MATERIALS AND METHODS

Filter medium preparation

The filter medium was prepared from commercially available glass fiber of 70-140 µm thickness. The fibers underwent chemical functionalization treatment, named 'piranha' method, that consists of a series of surface cleaning procedures in warm acid solutions using ultra-sonic baths. This treatment enhances the glass negative surface charges and the corresponding hydrophilicity support index. The cleaned fibers was tightly packed inside columns, one inch internal diameter by one and a half inches high, and filled with distillated water. An aqueous solution of lysozyme from hen egg-white protein (Sigma) was prepared (as delivered) at a concentration of 10^{-4} M (pH ~ 6.2) using ultrapure deionised water. The solution of protein was then poured into the columns where the solid surfaces were completely embedded for 15 min and then rinsed in distilled water. Sets of 10 columns were prepared for tests (5 with, and 5 without lysozyme immersed fiber). Figure 1 illustrates the fibrous medium aspect.

Fiber samples were randomly drawn from the medium in the column, in an as-activated state and after 5, 10 and 12 min of solution immersion. They were dried and then surface scanned by atomic force microscopy (TopoMetrix Discover) in non-contact mode in order to evaluate superficial features.

Interaction tests

Escherichia coli was used as the model microorganism for the filtration tests. *E. coli* is a gram negative, facultatively aerobic bacterium with simple nutritional requirements. The *E. coli* was cultured in minimum mineral medium at 37ºC for 24 hrs and then diluted in pure water. The bacteria concentration within the influent solution was 1008 col/100mL. The flux through columns was around 30 ml/min. The numerical evaluation of the removal was taken on the difference between colonies of bacteria measured in samples of feed and permeates streams. The data concerns an average of samples collected in 3-filtration sequences carried out for each column. The efficiency was performed by directly counting colonies in plaque-forming units, following Kawabata et al. 1996, relation:

$$\text{Removal (\%)} = (PFU_{inf} - PFU_{eff})/PFU_{inf} \times 100$$

Where PFU_{inf} and PFU_{eff} are the concentration of the specimens in the influent and affluent suspension.

RESULTS AND DISCUSSION

The expected net structure is a total covering of all fiber's faces. Since deposition proceeds under static conditions, the film formed due to electrostatic adsorption is believed to occur predominantly on the fiber surfaces immediately below the colloidal solution, *i.e.*, on the surfaces on which deposition may be favored by a gravitational action. Lysozyme deposition on flat horizontal surfaces is claimed to be propitious for the formation of stable films, despite the tendency to change conformation when adsorbed, as model proposed by Blomberg et al. 1994. Nevertheless most of its globular structure is preserved upon adsorption at the hydrophilic interfaces (Su et al. 1998).

In the packed fiber medium, the longitudinal directions are randomly distributed throughout the volume, and it is not precise to refer to a horizontal deposition in this situation, but rather to the dissimilar rates of aggregation along the circular faces of the fibers. Fibers in as-hydrophilic state were observed to present a certain surface roughness and then smooth as enzyme adsorption proceeds. Such a change permits an evaluation of the lysozyme deposition uniformity over immersion time by means of qualitative information obtained from AFM scanning. As shown in Figure 2, it is possible, based on the appearance of the deposited film, to assess the overall reduction in roughness intensity on the scanned surface. Numerical complementary roughness results can be found elsewhere (Assis et al. 2000).

After a 12 min immersion, excess formation of lysozyme can be observed. Previous studies imaging self-assembled lysozyme suggested that the assembly proceeds by nucleation and growth, while aggregation in solution may play an important role in the deposition features (Haggerty and Lenhoff, 1993; Wadu-Mesthrige et al. 2000).

It is clearly important that the immobilized enzyme should retain as much catalytic activity as possible. An interesting aspect regarding the SA technique is that the deposition takes place in the absent of intermediate coupling agent, that is, in the SA process most of the active sites remains unreacted during film formation. Another important feature is that the immobilized film do not exceeds the nanometer scale; consequently having no interference on the medium permeability. The medium density of the packing fibers measured along the columns was 0.55 gcm^{-3}. That indicates a high porosity, since a fully density glass is in the order of 3 gcm^{-3}. In addition, analysis by scanning electron microscopic showed that the void spaces in the fiber bed column lay in the range of 1-100 µm.

E. Coli is a bacterium of straight rod form with 0.5 µm in width by 2 µm in length, what is significantly shorter than

the average pore size of the packed medium and therefore just a small fraction is assumed as retained by simple mechanical way. The bacteria easily permeate the medium, concerning the ratio of the pores to the organism diameter. The removal fraction is than result of the enzymatic action of the lysozyme in immobilized condition, as indirectly assessed by the graphical result of the filtration tests as show in Figure 3.

Electrostatic interaction is also assumed as an important process in bacteria retention. The *E. coli* presents an electrokinetic mobility of –4 to –6 (μm/s)(V/cm), which corresponds to a zeta potential of –50 to –70 mV (Madaeni, 1997). That can be a decisive factor for optimizes microorganisms adsorption into lysozyme surface since predominant positive charge is distributed over the enzyme molecule volume. Although there is a lack in fundamental understanding of electrostatic interactions between irregular polymeric charged surfaces and colloidal distribution in a solution (Myers, 1991), it is presumed that the fibers medium configuration favors the surface attraction and thereby contact between the species. The initial adhesion of the bacteria proceeds on film surface (*i.e.* bacteria is not imbedded in the film matrix), and the elimination achieved by the proteolytic action subsequent to the electrostatic attachment.

CONCLUDING REMARKS

Lysozyme enzymatic film grown on fibrous permeable medium is potentially suitable for applications in biofiltration systems. The enzyme is easily immobilized on permeable glass medium not interfering with the permeability of the system. The efficiency in removing *E. coli* was approximately high as 50% greater than the same medium without the film. Two removal mechanisms, enzymatic and physical attachment action are expected to occur during the bacteria interaction. Despite removal capability here demonstrated by means of preliminary tests, a broad or industrial application of lysozyme filtration system still requires further and deep investigations. Evaluation of the durability of system efficiency, regeneration aspects and a pilot-scale testing should be carried out to be than adequately compared to the existing approaches such as porous membrane conventional filtrations.

ACKNOWLEDGMENTS

We are grateful for support received from the Fundação de Amparo à Pesquisa do Estado de São Paulo – FAPESP, Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq and Embrapa.

REFERENCES

ASSIS, O.B.G.; VIEIRA, D.C. and BERNARDES-FILHO, R. AFM characterization of protein net formation on a fibrous medium. *Brazilian Journal of Chemical Engineering*, 2000, vol. 17, no. 1, p. 245-249.

BRYERS, J.D. and CHARACKLIS, W.G. Biofilms in water and waste water treatment. In: CHARACKLIS, W.G. and MARSHALL, K.C. eds. *Biofilms*. Wiley, NY, 1990, p. 671-696.

BLAKE, C.C.; KOENIG, D.F.; MAIR, G.A.; NORTH, A.C.; PHILLIPS, D.C. and SARMA, V.R. Structure of hen egg-white lysozyme: A three-dimensional Fourier synthesis at 2 Ångstrom resolution. *Nature*, 1965, vol. 206, p. 757-761.

BOLLAG, J.M. Bioremediation of contaminated environmental sites. In: *Proceedings of the International Workshop on Biodegradation*. (14th – 16th October, 1996, Campinas, Brazil). 1996, p. 40-51.

BLOMBERG, E.; CLAESSON, P.M.; FRÖBERG, J.C. and TILTON, R.D. The interaction between adsorbed layers of lysozyme studied with the surface force technique. *Langmuir*, 1994, vol. 10, p. 2325-2334.

FENDER, J.H. Self-assembled nanostructured materials. *Chemical Materials*, 1996, vol. 8, p. 1616-1624.

HAGGERTY, L. and LENHOFF, A.M. Analysis of ordered arrays of adsorbed lysozyme by scanning tunneling microscopy**.** *Biophysics Journal*, 1993, vol. 64, p. 886-895.

KAYUSHINA, R.; STEPINA, N.; BELYAEV, V. and KHURGIN, Y. X-ray reflectivity study of self-assembly of ordered lysozyme films. *Crystallography Reports*, 1996, vol. 41, no. 1, p. 146-150.

KAWABATA, N.; FUJITA, I. and Inoue, T. Removal of virus from water by filtration using microporous membranes made of poly(N-benzyl-4-vinylpyridinium chloride). *Journal of Applied Polymer Science*, 1996, vol. 60, p. 911-917.

LVOV, Y. Eletrostatic layer-by-layer assembly of proteins and polyions. In: LVOV, Y. and MÖHWALD, H. eds. *Protein Archicture: Interfacing molecular assemblies and immobilization biotechnology*. Marcel Dekker Inc., NY, 2000, p. 125-167.

MADAENI, S.S. Mechanism of virus removal using membranes. *Filtration & Separation*, Jan/Feb 1997, p. 61-65.

MALLEVIALLE, J. Why is natural organic matter problematic? In: *Proceedings of Natural Organic Matter: Influence of Natural Organic Matter Characteristics on Dinking Water Treatment and Quality Conference*. (18th – 19th September, 1996, Poitiers, France). 1996, p. I1-I16.

MYERS, D. *Surfaces, Interfaces and Colloids*. VCH Publishers Inc., NY, 1991. 432 p. ISBN 1-56081-033-5.

MOSER, I.; DWORSKY, P. and PITTNER, F. Degradation of bacterial cell walls by immobilized lysozyme. *Applied Biochemistry and Biotechnology*, 1988, vol. 19, p. 234-238.

PHELPS, T.J.; NIEDZIELSJI, J.J.; SCHRAM, R.M.; HERBES, S.E. and WHITE, D.C. Biodegradation of trichloro-ethylene in continuous-recycle expanded-bed bioreactors. *Applied Environmental Microbiology*, 1990, vol. 56, p. 1702-1709.

SU, T.J.; LU, J.R.; THOMAS, R.K.; CUI, Z.F. and PENFOLD, J. The adsorption of lysozyme at the silica-water interface: A neutron reflection study. *Journal of Colloid and Interface Science*, 1998, vol. 203, p. 419-429.

WADU-MESTHRIGE, K.; AMRO, N.A. and LIU, G. Immobilization of proteins on self-assembled monolayers. *Scanning*, 2000, vol. 22, p. 380-388.

WEBER-SHIRK, M. and DICK, R.I. Physical-chemical mechanisms in slow sand filters. *Journal American Water Works Association*, 1997, vol. 89, no. 1, p. 87-100.

WEETALL, H.H. Preparation of immobilized proteins covalently coupled through silane coupling agents to inorganic supports. *Applied Biochemistry and Biotechnology*, 1993, vol. 41, p. 157-188.

WHITESIDES, G.M. Self-assembling materials. *Scientific American*, September 1995, p. 146-149.

APPENDIX

Figures

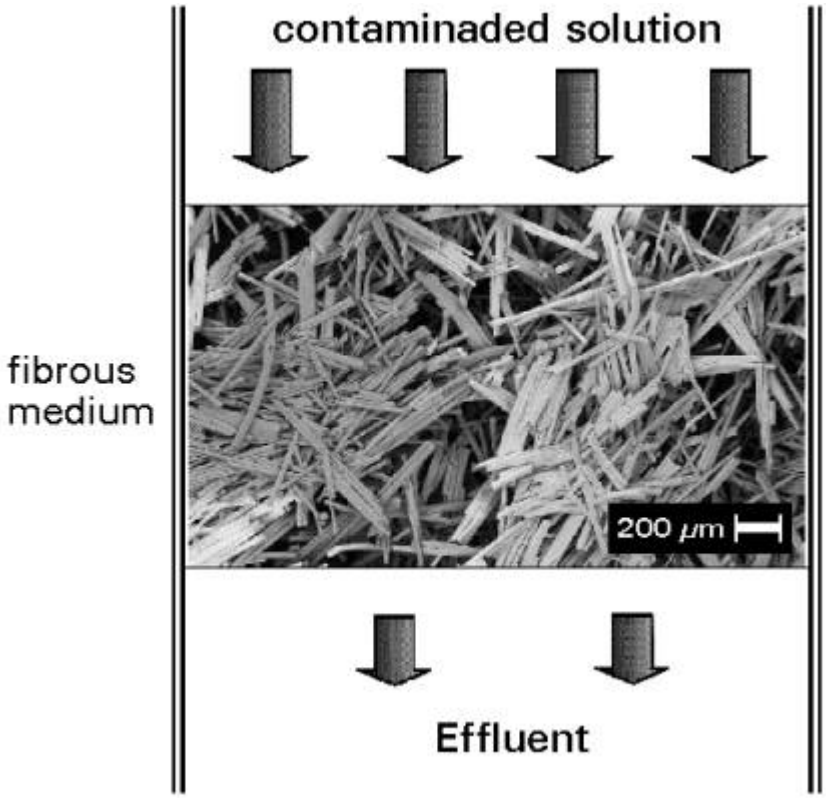

Figure 1. Column set up with real aspect of the fibrous medium (SEM photomicrograph).

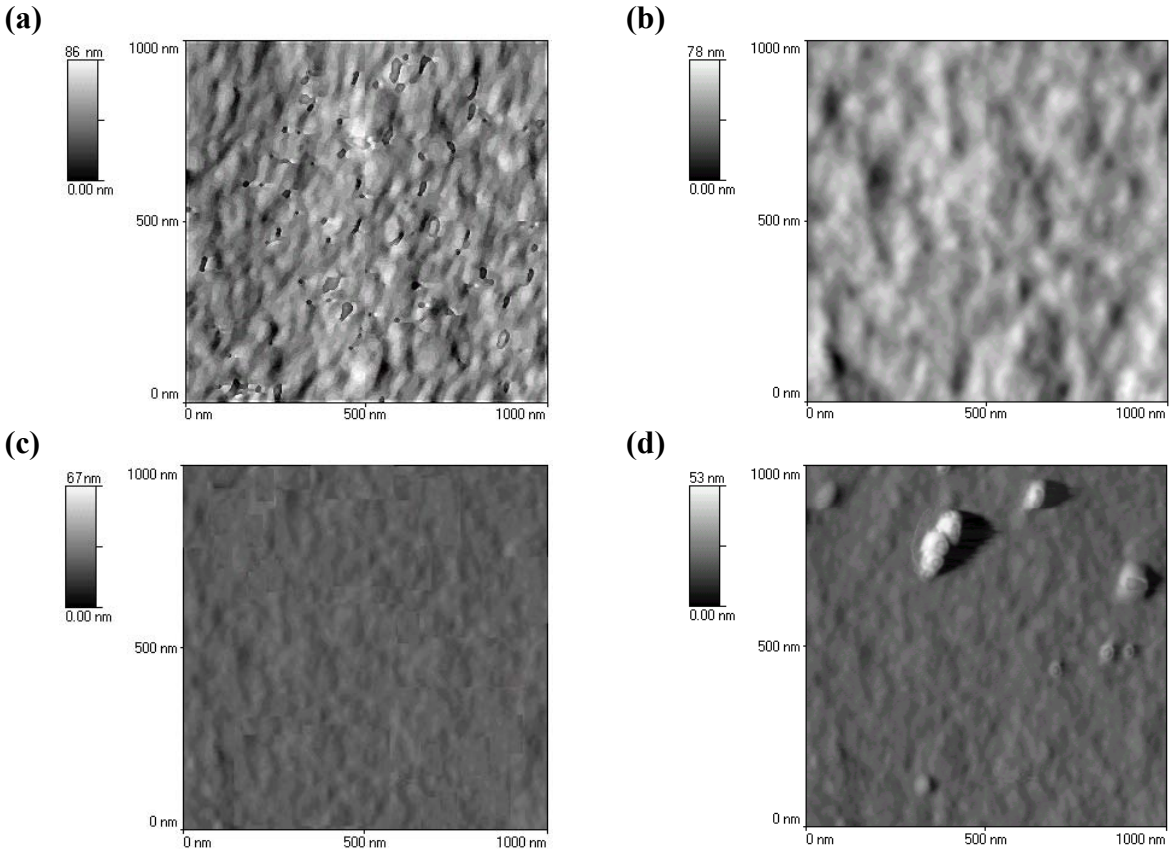

Figure 2. AFM aspects of the scanned surfaces where changes in topographical features can be observed:

(a) as-hydrophilic fiber.

(b) 5-minute surfactant immersion.

(c) 10-minute immersion.

(d) 12-minute immersion.

The decrease in superficial roughness indicates lysozyme deposition. Ridges observed after 12 min indicate excess of deposited enzyme.

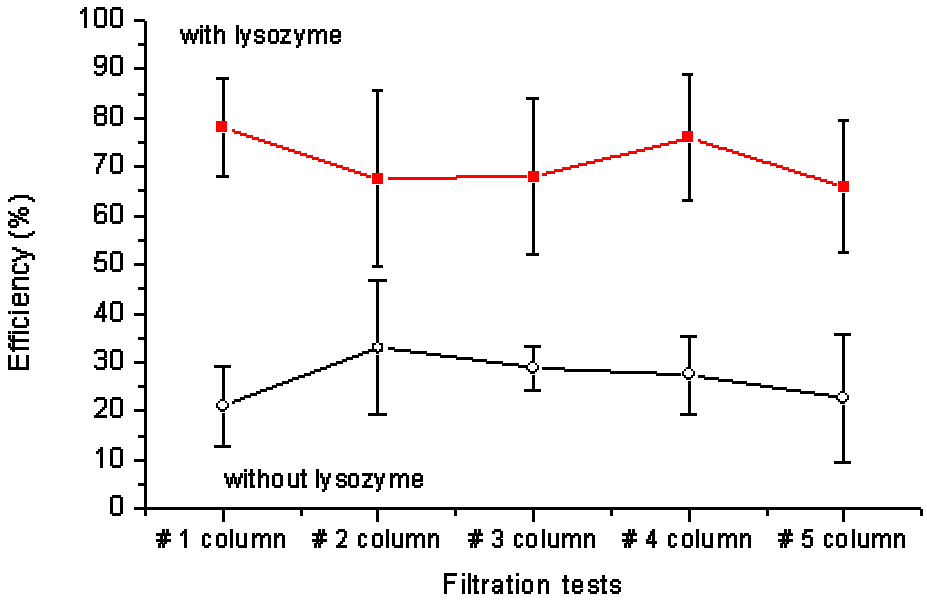

Figure 3. Efficiency of the fiberglass medium, with and without immobilized lysozyme. Each point represents the average of three analyses.