Practical use of immobilized lysozyme for the remediation process of *Escherichia coli* in aqueous solution

**Carluicio R. Alves**
Environmental Biotechnology Group
State University of Ceará
60.640-020 Fortaleza, Ceará, Brazil
Tel: 55 85 3101 9822
Fax: 55 85 3299 2500
E-mail: alvescr@yahoo.com

**Maria Gardenny R. Pimenta**
Environmental Biotechnology Group
State University of Ceará
60.640-020 Fortaleza, Ceará, Brazil
Tel: 55 85 3101 9822
Fax: 55 85 3299 2500
E-mail: gardenny@yahoo.com.br

**Regine H.S.F. Vieira**
Science Institute - Labomar
Federal University of Ceará
60.165-081 Fortaleza, Ceará, Brazil
Tel: 55 85 3101 9822
Fax: 55 85 3299 2500
E-mail: regino@labomar.ufc.br

**Roselayne F. Furtado**
Environmental Biotechnology Group
State University of Ceará
60.640-020 Fortaleza, Ceará, Brazil
Tel: 55 85 3101 9822
Fax: 55 85 3299 2500
E-mail: roselayneff@hotmail.com

**Maria Izabel F. Guedes**
Environmental Biotechnology Group
State University of Ceará
60.640-020 Fortaleza, Ceará, Brazil
Tel: 55 85 3101 9822
Fax: 55 85 3299 2500
E-mail: florinfg@terra.com.br

**Rui C.B. Silva**
Environmental Biotechnology Group
State University of Ceará
60.640-020 Fortaleza, Ceará, Brazil
Tel: 55 85 3101 9822
Fax: 55 85 3299 2500
E-mail: rcbs@uece.br

**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

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*Corresponding author
The lysozyme enzyme was immobilized on vitreous surface (fragments with diameters of 0.3 and 1.0 mm) for remediation of the microorganism *Escherichia coli* JM 109 into fresh water and saline solutions with 0.9% NaCl (w/v). Characterization of enzymatic film was carried out by infrared spectroscopy and atomic force microscopy techniques. Bactericidal activity of the enzyme was evaluated by spectrophotometric analysis. It was verified that the enzymatic film was strongly coupled with the vitreous surface. The topographic analysis demonstrated that the deposited film was uniform and homogeneous. It was observed bactericidal activity of film deposited on vitreous surface with 0.3 mm in fresh and saline solutions. This fact was not verified to vitreous fragments with 1.0 mm of diameter.

There are different supports for enzymatic immobilization, for example, chitosan and alumina (Sobral et al. 2003). Indubitably, the largest advantage of the use of enzymatic film is that the method used has high capacity to process required chemical reactions.

The lysozyme film has called researchers’ attention in last years on account of the enzymatic activity being useful in the attack on many bacteria, with the breaking of mucopolysaccharides structure of the cell wall (Masschalck et al. 2001; Datta et al. 1973; Park et al. 2004). Moreover, the lysozyme has an important characteristic, when crystallized, it supports temperatures up to 120°C without perceiving the loss of its enzymatic activity (Kudou et al. 2003; Lavigne et al. 2004). The lysozyme film acts directly on gram positive bacteria and there is the possibility of interaction with phospholipids of external membrane of gram negative bacteria. It checks the enzyme, thus functions as a protection together with large amounts of biological fluids where the same is found (Masschalck et al. 2001; Min et al. 2005). The specific function of enzyme is represented by a deep opening inside the molecule. Inside this enzyme, the residues Glu-35 and Asp-52 are responsible for the hydrolysis of the glycosidic connection. It is believed that the Glu-35 has an acetyl link to the substrate, while the negative charge of the Asp-52 stabilizes the result of the ion cation (Datta et al. 1973; Dehong and Lu, 2004).

This work had the objective to evaluate the practical use of the immobilized lysozyme enzyme on vitreous surface for remediation process of pathogenic microorganism.
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*Escherichia coli* JM 109 found in fresh water and saline solution.

**MATERIALS AND METHODS**

**Preparation of the surface and film deposition**

The lysozyme film was prepared using the self-assembly technique, which is based on spontaneous irreversible adsorption of the protein. The deposition of the enzyme occurs though covalent chemical bonding between the substrate and the absorbent (Dickerson and Geis, 1993).

The substrate (fragments of glass of 0.3 and 1.0 mm) was submitted to chemical cleaning and hydrophilization, according to method proposed by Borato et al. (1997). H₂SO₄ (Merck) and H₂O₂ (Merck) solutions were prepared in proportions of (7:3, v/v). Afterwards, the fragments of glass were immerged in this solution at temperature of 80°C for 30 min, followed by exhaustive washes in Milli-Q distilled water.

These same surface was submitted to solution of H₂O/H₂O₂ (Merck)/NH₄OH (Merck) in proportions of (5:1:1, v/v) at temperature of 80°C. This procedure was carried out for 30 min and later were done various washes in distilled water.

This series of actions generated negative charges onto the glass surface.

The lysozyme aqueous solution from hen egg-white lysozyme (Sigma Chemical, 99%, 47,000 unit mg⁻¹, in the lyophilic form) was prepared in concentration of 10⁻⁴ mol L⁻¹ and pH 6.4 under moderate agitation for 4 hrs.

The enzymatic film was immobilized by immersion of the vitreous surface into the lysozyme aqueous solution for 2 hrs under gentle agitation. They were dried under vacummed at room temperature.

The atomic force microscope (TopoMetrix model TNX 2010) and the infrared spectrometer with Fourier analysis (BOMEM model DA8) coupled with MCT detector were utilized to confirm the presence of the film on the vitreous surface.

**Preparation of contaminated solution with *Escherichia coli* JM 109**

The *Escherichia coli* JM 109 stump was isolated from Eosin Methylene Blue Agar (Merck) and the growth of the colony was made in Trypticase Soy Agar (Merck), at 23°C, in incubator BOD (Fanem model 347).

The growth of the bacteria occurred in mixture EC Bouillon (Merck). The tubes remained in water bath at temperature of 44.5°C for 24 hrs. The development of the colony was detected visually although turbidness and the formation of gas in Duhran tube.

**Building of the filtration column**

The column, in which the filtration was carried out, was constituted of two separable bodies and united by thread.

Figure 2. Lysozyme film deposited on vitreous fragments with diameters of 0.3 mm.
(a) Topographic Micrograph of the vitreous substrate with diameter of 0.3 mm.
(b) Graphic representation of the covered surface with proteic film.
system. The superior part was formed by an acrylic tube of capacity of 50 mL whereas the inferior part was made of glass and had a tap.

**Monitoring of the bactericide activity of the enzyme**

The substrate of recycled glass with different sizes covered with lysozyme enzyme was packed inside filtration column. The *Escherichia coli* JM 109 solution was diluted in fresh water and salt water with solution of 0.9% NaCl (w/v) and added slowly into column.

The analysis of affluent (solution that drained off from the column) was performed by spectrophotometric measurements (Shimadzu model 1601PC) and the efficiency evaluation followed the Kawabata et al. (1996) relation:

\[
\text{Efficiency (\%)} = \frac{\text{PFU}_{\text{inf}} - \text{PFU}_{\text{eff}}}{\text{PFU}_{\text{inf}}} \times 100
\]

Where PFU$_{\text{inf}}$ and PFU$_{\text{eff}}$ are the concentration of the specimens in the influent (solution added into column) and affluent suspension, respectively. The tests of bactericide activity were done in triplicate in order to have reliable results.

**RESULTS AND DISCUSSION**

**Presence of immobilized lysozyme on vitreous substrate**

After depositing the lysozyme film on vitreous surface (fragments with diameters for values 0.3 and 1.0 mm), measurements of infrared spectrometry were carried out to confirm the deposit formation (Pérez and Griebenow, 2000) and the topographic analysis were done to verify its distribution.

The Figure 1 illustrates the infrared spectrum ranging between 400 to 2000 cm$^{-1}$ for lysozyme film on vitreous fragments with diameters of 0.3 mm. The bands ranging between 1237 and 1401 cm$^{-1}$ are related to amino acid tryptophane; another characteristic of the lysozyme is also observed in the region between 1530 and 1700 cm$^{-1}$ (bands in 1542 and 1659 cm$^{-1}$), which corresponds to the amino band, as verified in the literature (Alix et al. 1985). The bands were similar for the two granulometries confirming the presence of lysozyme on them.

Figure 2a is a topographic image of small part of the vitreous substrate with diameter of 0.3 mm covered with the enzymatic film, made up of approximately 30 overlapping layers (Borato et al. 1997).

Figure 2b is a graphic representation of a part of the covered surface with proteic film. It is noted that the vitreous surface is covered homogeneously by enzymatic film. The topographic analysis in micrometric scale not revealed difference between the two granulometries.

**Bactericide activity of protein film**

Previous experiments showed promising results for bactericide activity of immobilized lysozyme on vitreous substrates with diameters equal or inferior to 0.3 mm. However, size of fragments smaller than 0.3 mm have as disadvantage to provide slow speed of liquid by column. In this work, we showed glass fragments with granulometries of 1.0 mm to compare size bigger than 0.3 mm.

We also tested high concentration of microorganisms and dissolved salt in water to evaluate if in extreme conditions the enzymatic activity was remained. In comparative experiments prepared with fresh water and salt-water solutions, it was noticed that the enzyme maintains its bactericide activity for concentrated solutions with approximately 6,000 bacteria/cm$^3$.

We used the spectrophotometric technique because this is one of the simplest ways to determine the density of a bacterial culture. In dense culture, the light is scattered, and less light reaches the phototube of the spectrophotometer. In fact, turbidity is more closely related to cell biomass (cell dry weight) than to the number of cells present. In dilute samples, absorbance is directly proportional to biomass.

In the presence of the immobilized lysozyme occurs the rupture cell of the bacteria. This fact is evidenced with a significant decrease in the optical density of wavelength 600 nm (visible spectrum). We verified that in the presence of lysozyme the optical density showed a progressive decrease of the turbidity, resultant from effect of the bactericide activity. Moreover, it was observed that this activity has shown reduction of the turbidity on the first 30 mL, confirming the process of lysis cell as presented in Figure 3. After sieving 30 mL of contaminated solution in column, it was noted decreases in remediation process, probably owing to the fact that the film is covered with...
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Figure 3 (curve a) indicates that the bactericide efficiency of lysozyme in fresh water was on average 60%, this results are near to 50% found for Assis and Claro (2003). On the other hand, in curve b, for the saline solution, the efficiency was on average 30%. It was observed that the activity of the immobilized enzyme is reduced because of the presence of ions (electrolytic solution of NaCl). According to Campbell and Dwek (1984), when ions are present, the lysozyme in solution undergoes a modification in its conformation. In this way, there is a great loss of the enzymatic activity.

The influence of the glass particles with immobilized lysozyme in the remediation efficiency, it was also tested on vitreous surface with diameter of 1.0 mm. In Figure 4, it is no observed variation in the absorbance for solutions of bacteria, indicating that there is no bactericide activity of the lysozyme. The glass fragments with diameter of 1.0 mm leave big spaces for bacterial solution to drain off by column. This fact becomes ineffective the percolation process because the time of contact between immobilized protein and *Escherichia coli* is very short, thus the solution of microorganism passes by enzymatic film without undergoing bactericide action of the lysozyme.

In this work, we demonstrated that the lysozyme efficiency in the remediation process depend on size substrate and other factors that can affect the enzyme and consequently the specific action sites, as was seen in saline medium.

**CONCLUDING REMARKS**

Lysozyme enzymatic film grew on recycled glass fragments of 0.3 mm has potential for applications in decontamination systems, especially in no saline medium, in function of the good bactericide activity and the low cost. Contra wise, the enzymatic film deposited on fragments of 1.0 mm had not bactericide action in solutions with approximately 6,000 bacteria/cm³. One of the biggest problems for the practice application of methodology developed (decontamination system) is in the fact of fragments of bacteria’s cell wall to accumulate on the enzymatic film obstructing the lysis of other bacteria.

**REFERENCES**


KNIGHT, Kirsty; BARROS PIMENTEL, Maria do Carmo; CAMARGO DE MORAIS, Marcia Maria; LEDINGHAM, William M.; DE LIMA FILHO, José Luiz and DINIZ MAIA, Maria de Mascena. Immobilization of lipase from Fusarium solani FS1. Brazilian Journal of Microbiology, July-September 2000, vol. 31, no. 3, p. 220-222.


