

## **Protease production from marine microorganism by immobilized cells**

Usama Beshay<sup>1\*</sup> and Antonio Moreira<sup>2</sup>

1&\* Bioprocess Development Dept., Genetic Engineering and Biotechnology Research Institute (GEBRI), Mubarak City for Scientific Research and Technology Applications, New Bourg El-Arab City, Post code 21934, Research Area, Alexandria, Egypt, E-mail:

[ugabra10@hotmail.com](mailto:ugabra10@hotmail.com), Fax: +2-03-45 93 423

2 Dept. of Chemical & Biochemical Engineering, University of Maryland Baltimore County (UMBC), Baltimore, Maryland, U.S.A.

\* Author for correspondence

### **Abstract**

Proteases represent the most important kind of enzymes from an industrial point of view. Alkaline proteases have applications in leather processing, laundry detergents, production of protein hydrolysates, and food processing. In this study, cell immobilization has been used for enhancing enzyme titer. Our aim was to choose carriers, methods, and optimal conditions for immobilization of growing cells of *T. turnirae* and evaluate the immobilized biocatalysts in repeated batch fermentations for production of alkaline protease. *Teredinobacter turnirae* cells were adsorbed onto different matrices namely, ceramic support - CeramTec<sup>®</sup> F1/porous PST 5, broken pumice stone (Particle size: 1.5-2.5 mm, 2.5-3.5 mm, 5-15 mm) and Silicone foam ImmobaSil D. These matrices effectively retained biomass, and increased volumetric productivity by over 207%, when compared to free cells. Enzyme productivity by immobilized cells in ceramic support (CeramTec<sup>®</sup>) and Silicone foam ImmobaSil D, was about 2.1 fold higher than the corresponding free cells. Repeated batch production of alkaline protease by immobilized cells in Silicone foam ImmobaSil D matrix was achieved for 5 repeated batches without a significant decrease in the production of protease enzyme. Moreover, electron micrograph images indicate how *T. turnirae* colonizes porous support matrices.

### **Introduction**

Proteases are a complex group of enzymes collectively known as peptidyl peptide hydrolases (EC 3.4.21.14) and constitute one of the most important groups of industrial hydrolytic enzymes. This enzyme occupies about 60% of the global enzyme market (Sachidanandham et al., 1999). These may be extracellular or intracellular in nature and degrade proteins to peptides and amino acids. These enzymes possess a wide variety of physiochemical and catalytic properties. Alkaline proteases have applications in leather processing, laundry detergents, production of protein hydrolysates, and food processing (Godfrey and Reichet 1985; Zukowski 1992).

A major trend in the detergent industry is a shift to non-phosphate detergent formulations. Because of concerns about their ecological effect the phosphate builders in phosphate detergents have been replaced with builders that tend to decrease the cleaning power of the detergent formulations and increase the pH of their suds to the range 10-12 rather than the typical value of about 9 for phosphate detergents. To replace the lost cleaning power detergent makers have turned to proteases. However, the availability of proteases that possess high activity and stability in the required pH range is limited. The shipworm protease is highly active and stable in this pH range and increases the cleaning power of a standard non-phosphate detergent independent of pH over the range 10-11.9 better than the common commercial protease additive subtilisin.

In this study, a novel protease was produced by *Teredinobacter turnirae*, a new isolate symbiotic bacterium found in the gland of Deshays of the marine shipworm *Psiloteredo healdi* (Waterbury et al., 1983). The secreted protease is somewhat unusual because it has an alkaline iso-electric point. It exhibits complete thermal stability up to 40 °C, but retains a high level of activity above 50 °C for at least 60 min. The enzyme remains active over a broad pH range and exhibits salt tolerance up to saturated sodium chloride (Griffin et al., 1992). Additionally, the produced protease is resistant to the trypsin inhibitor PMSF, which reacts with active site serine residues. These properties render the produced protease useful in detergents and other low-moderate temperature industrial applications.

One of the main problems concerning the batch process of *T. turnirae* cells is the low yield of protease enzyme. Therefore, the recent research is focused on new approaches for increasing the cell concentration and protease production, respectively. One of the methods applied for maintaining high cell concentration and higher productivities is immobilization (Beshay et al., 2003a; Beshay et al., 2003b). Cell immobilization offers several potential advantages to fermentation systems from the standpoint of process engineering (Navarro and Durand 1977; Helmo et al. 1985; Karel et al., 1985; Brodelius and Vandamme 1987). Like enzyme immobilization, the immobilization of cells, has the same advantages, when comparing the immobilized cells with the free cells. Thus the immobilization process makes possible the reuse or the continuous use of this type of biocatalyst (Zhang et al., 1989; Galazzo and Bailey 1990; Ban et al., 2002). Immobilized cells are often more stable than the equivalent free cells; immobilized cell processes are easier to automate and enable exploitation of the advantages of various reactor configuration. Immobilized cells are convenient to handle, appear to be less susceptible to microbial contamination, and permit easy separation of products from the biocatalyst. The use of immobilized

cells enables greater control throughout the reaction. Immobilization also facilitates the use of dense cell populations by altering the rheological properties of the suspending medium. The fluid viscosity is lower than when comparable numbers of cells are freely suspended in solution. Lower viscosities contribute to better mixing and mass transfer properties in the reactor.

Our previous work (Beshay & Moreira 2003c) has indicated that porous sintered glass SIRAN<sup>®</sup> is a suitable matrix for the immobilization of *T. turnirae* cells by means of adsorption. In addition, immobilized cells are capable of producing a high protease activity. Therefore, our aim in this study was to use cheaper immobilization supports and choose suitable carriers, methods and optimal conditions for immobilization of growing cells of *Teredinobacter turnirae* and to evaluate the immobilized biocatalysts in repeated batch fermentations for production of alkaline protease.

## Materials and methods

### *Microorganism and cultivation conditions*

The bacterium *Teredinobacter turnirae* was generously supplied by Dr. Richard Greene (USDA, Peoria, IL, USA). A basal medium (Plackett-Burman media, PB) was used throughout all experiments (Beshay & Moreira 2003b). Growth was carried out with 50-ml cultures in 250-ml Erlenmeyer flasks shaken at 120 rpm and 30 °C.

### *Supporting matrices for cell immobilization*

Three different inorganic porous supports, ceramic support CeramTec® F1/porous PST 5, broken pumice stone and Silicone foam ImmoBaSil D were used to immobilize *T. turnirae* cells. Ceramic support matrix was obtained from CeramTec® AG Innovative Ceramic Engineering, Wunsiedel, Germany. It had a diameter of 1.5-2.5 mm, a particle density of 1430 g/l, a pore volume of 0.25 ml/g and a specific surface area of 20 cm<sup>2</sup>/cm<sup>3</sup>. The second carrier (broken pumice stone) was provided by Joseph Raab GmbH & Cie.KG, Neuwied, Germany. Three different particle sizes were used. The chemical composition of broken pumice stone is as follows: Silicic acid SiO<sub>2</sub> 55%, Aluminium oxide Al<sub>2</sub>O<sub>3</sub> 22%, Alkalis K<sub>2</sub>O+Na<sub>2</sub>O 12%, Iron oxide Fe<sub>2</sub>O<sub>3</sub> 3%, Calcium oxide CaO 2%, Magnesium oxide MgO 1%, Titanium dioxide TiO<sub>2</sub> 0.5%, Ignition loss 4%. The physical properties of broken pumice stone are summarized in Table 1.

**Table 1:** Characteristics of porous broken pumice stones

Carrier	Diameter (mm)	Pore volume (g/ml)	Particle density (g/l)	Specific surface area (cm <sup>2</sup> /cm <sup>3</sup> )
Pumice stone	1.5 - 2.5	0.93	670	27
	2.5 - 3.5	0.93	670	27
	5 - 15	0.93	670	27

The third carrier, Silicone foam ImmoBaSil D was supplied from Ashby Scientific Ltd, England. The material consists of silicone rubber formed into a sponge-like structure with randomly interconnecting pores. It is resistant to both biological and chemical attack. Its mechanical strength, coupled with its intrinsic elasticity, means that it can withstand unusually high impact and

shear stresses. In addition, it can withstand wet heat up to 200 °C and this makes it eminently suitable for all standard steam sterilization processes.

### ***Alkaline protease production by free and immobilized *T. turnirae* in batch culture fermentation***

Batch culture fermentation was carried out in Erlenmeyer flasks (250 ml), each containing 50 ml of PB-medium. Flasks were inoculated with 5 ml of bacterial cell suspension and 5 gm of each supporting materials. Flasks were incubated in a shaker incubator (120 rpm and 30 °C) for the required time.

### ***Repeated batch fermentation***

Here, fermented broth was decanted, the beads were washed with sterile water and fresh medium was introduced at 48 h intervals over a period of 10 days. Similar flasks containing free *T. turnirae* cells, permitted comparisons of alkaline protease yields with those of immobilized bacteria fermentations. In this case, centrifuged and washed bacteria from a previous batch were introduced into the fresh medium. Cultivation conditions were as previously described for each set.

### ***Electron Microscopy***

Different supporting matrices containing *T. turnirae* cells were observed by electron microscopy. The matrices were fixed using a 2% glutaraldehyde solution in 0.1 M phosphate buffer pH 7.3, for 2 hours. After fixation, the samples were washed twice in ice-cold phosphate (0.1 M, pH 7.3) for 15 minutes. The material was dehydrated in an ethanol gradient (30-100%), being left for 20 minutes. The air dried particles were coated with 120-130 µm gold in argon medium. Finally the specimens were observed with a Philips XL30 scanning electron microscope attached to an EDX unit.

### ***Analyses***

Cell growth was determined spectrophotometrically at 600 nm and converted to cell dry weight (CDW) by using a conversion factor. On the other hand, immobilized cells were measured indirectly via determination of total protein content of the immobilized cells according to BCA-assay (Smith *et al.*, 1985; Beshay & Moreira 2003d). The method was calibrated against cells from suspension culture.

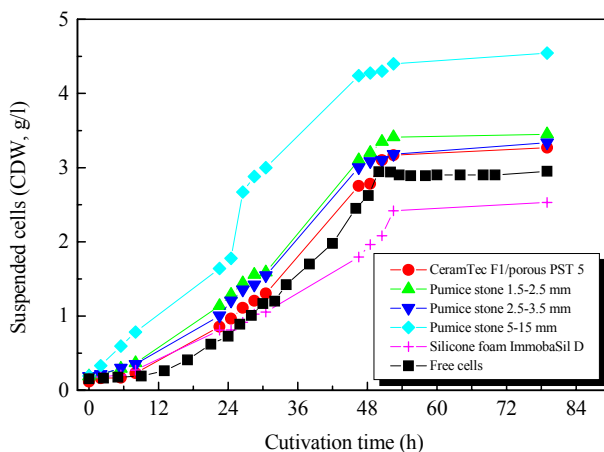
Proteolytic activity was determined using azocasein as substrate, as described elsewhere (Greene *et al.*, 1989). The Units of

activity represent the micrograms of azocasein digested per hour at pH 7.0 and 25 °C.

## Results and discussion

### *Cell growth and protease production by immobilized *T. turnirae* cells in different inorganic matrices:*

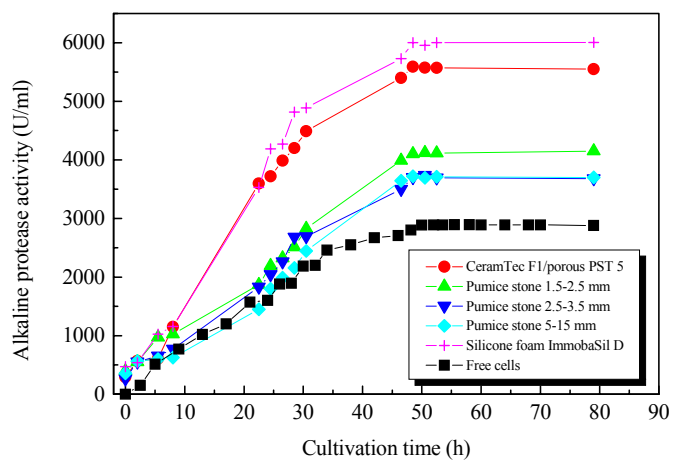
Firstly, different inorganic matrices have been used to select the most suitable one, which supports both cell growth and protease production. In the previous study (Beshay and Moreira 2003a), we have found that cell immobilization in porous sintered glass beads SIRAN® appears to be quite promising and very attractive for the production of alkaline protease from *T. turnirae* cells. Therefore, and according to the promising results obtained elsewhere, we have tried in the present study to find out more cheap supporting matrices to be adapted for cell immobilization. Figure 1 shows cell growth profiles of both free and immobilized *T. turnirae* cells in different matrices, namely, ceramic supports (CeramTec®) F1/porous PST, broken pumice stone and Silicone foam ImmobaSil D, respectively. Cells grew exponentially after a lag phase of about 6 h, and reached a maximum cell density after 48 h cultivation time. The obtained results showed that all supporting matrices used, were suitable for cell immobilization. Pumice stones of 5-15 mm size supported the best cell growth in suspension culture.



**Fig. 1:** Cell growth of free and immobilized *T. turnirae* cells in different carriers

Figure 2 shows protease activities in the broth of flask cultures initiated either with free or with immobilized cells. The profile of protease activity curves for cells immobilized on ceramic supports (CeramTec®), broken pumice stone and Silicone foam ImmobaSil

D as well as free cells was similar and maximal protease activity was obtained after 48 h of cultivation. Maximum enzyme titers for immobilized cell cultures were about 207% higher than those for free cell cultures. This was probably due to the large amount of cells immobilized in matrices during this period. Enzyme productivity by immobilized cells in ceramic supports (CeramTec®) and ImmoBaSil D, was about 2.1 fold higher than the corresponding free cells (6000 vs. 2890 U/ml).



**Fig. 2:** Alkaline protease production profiles by free and immobilized *T. turnirae* cells in different carriers.

### **Comparison of batch fermentations with free and immobilized cells**

The comparison on the production of alkaline protease in different supports and by free cells is given in Table 2. The results show that the enzyme activity was the maximum for immobilized cells on Silicone foam ImmobaSil D followed by cells immobilized in ceramic supports (CeramTec®), broken pumice stone and free cells. The above results reveal that the quantity of the immobilized cells may have a direct correlation to the extracellular enzyme yield in the medium. Determination of immobilized cells revealed that there was no hindrance to the diffusivity of *T. turnirae* cells in the matrices used for immobilization. In comparison to all other supports, Silicone foam ImmobaSil D have offered better microenvironmental conditions for cell immobilization (Immobilized cells CDW= 25.4 g/l) and protease production. In the case of free cells, the activity in the culture broth was maximum (2890 U/ml) at a period of 48 h, while immobilized cells in Silicone foam ImmobaSil D showed maximum activity (6000 U/ml) at 48 h.

**Table 2:** Numerical comparison between free and immobilized *T. turnirae* cells in different carriers (Results obtained after 48 h cultivation).

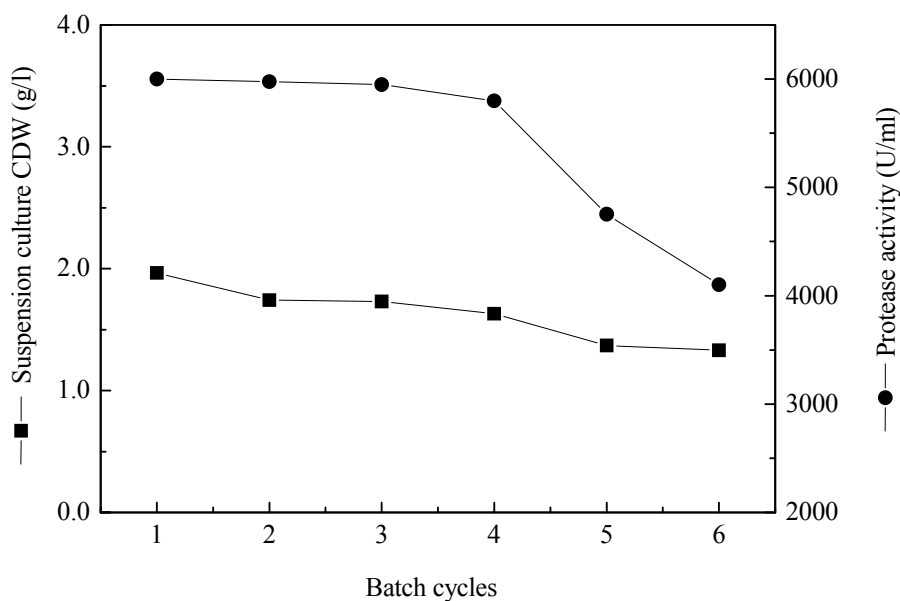
	<b>Suspended cells CDW (g/l)</b>	<b>Immobilized cells CDW (g/l)</b>	<b>Alkaline protease activity (U/ml)</b>
Free cells	3.0	-----	2890
Ceramic supports (CeramTec®)	3.1	18.9	5590
Pumice stone (1.5-2.5 mm)	3.4	16.1	4100
Pumice stone (2.5-3.5 mm)	3.3	11.3	3700
Pumice stone			



(5-15 mm)	4.3	5.2	3720
Silicone foam			
ImmobaSil D	2.5	25.4	6000

### **Repeated-batch of immobilized cells (Operational stability of biocatalysts)**

The activity of immobilized *T. turnirae* cells for the continuous production of alkaline protease was explored by using cells immobilized in Silicone foam Immobasil D carrier repeatedly for several batches. The medium was replaced every 48 h and the carrier was washed thoroughly with sterile water at the end of each cycle before reuse. Fig. 3 shows suspended cell growth and protease activity results of 5 repeated cycles. The activity of immobilized cells remained in the range of 5500 - 6000 U/ml throughout the first two cycles. Further replacing of the production medium every 48 h was accompanied by a decrease in protease activity and reached 4103 U/ml at the end of the fifth cycle, which is still higher than that obtained by free cells (2890 U/ml). On the other hand, washed free cells showed lower productivities than the immobilized ones and their activity decreased markedly with increasing reaction cycles (data not shown).



**Fig. 3:** Repeated-batch production of alkaline protease by *T. turnirae* cells immobilized in Silicone foam ImmobaSil D.

### **Scanning electron microscope (SEM)**

*T. turnirae* cells immobilized in Silicone foam ImmobaSil D and broken pumice stone were studied by scanning electron microscopy. The SEM photographs plates clearly showed that cells were randomly distributed in the pores of the carrier matrix as shown in Figure 4. The photographs show the inner pores of carriers used, which were

densely populated by the immobilized cells as compared to the outer surface of the matrix.

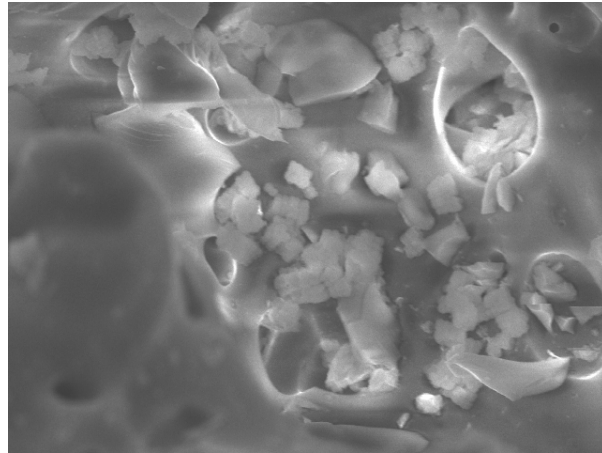


Plate B: *T. turnirae* cells immobilized in broken pumice stone

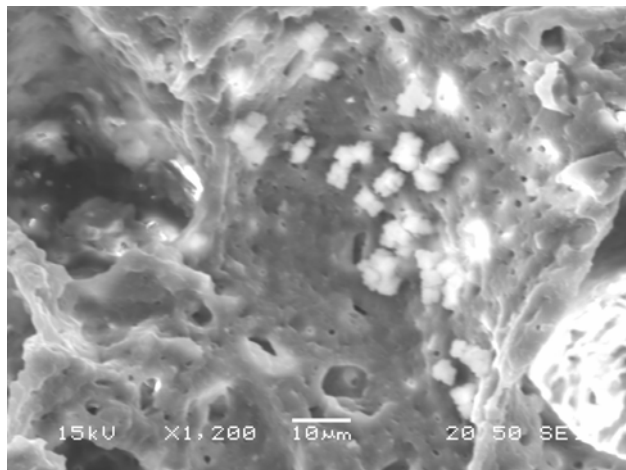


Plate C: *T. turnirae* cells immobilized in Silicone foam ImmobaSil D

**Fig. 4:** Scanning electron microscope SEM plates of *T. turnirae* cells immobilized in pumice stone and Silicone foam ImmobaSil D.

### Conclusions

Immobilization has been shown to be an adequate means for obtaining high enzyme titer. With immobilized cells, alkaline protease activity obtained was higher than with freely suspended cells. In repeated batch cultivations, Silicone foam ImmobaSil D biocatalyst could be successfully used for 5 subsequent batches.

## References

Ban K, Hama S, Nishizuka K, Kaieda M, Matsumoto T, Kondo A, Noda H, and Fukuda H (2002): Repeated use of whole-cell biocatalysts immobilized within biomass support particles for biodiesel fuel production. *J. Molecular catalysis B: Enzymatic*. **17**, 3-5, 157-165.

Beshay U, Friehs K, Azzam AE and Flaschel E (2003a): Analysis of the behaviour of *Dictyostelium discoideum* in immobilized state by means of continuous cultivation. *Bioprocess Biosyst. Eng.* **26**: 117-122.

Beshay U, Friehs K, Azzam AE and Flaschel E (2003b): Cultivation of *Dictyostelium discoideum* in immobilized form by colonization of porous supports. *Process Biochemistry*. **38**: 1521-1529.

Beshay U and Moreira A (2003c): Repeated batch production of alkaline protease by using porous sintered glass as carriers. *Process Biochemistry*. **38** (10): 1463-1469

Beshay U and Moreira A (2003d): Effect of Medium composition on the production of alkaline protease by *T. turnirae*. *Deutsche Lebensmittel-Rundschau*, (8): 319-323.

Brodelius P, Vandamme EJ (1987): Immobilized cell systems. In: Rehm HJ, Reed G, editors. *Biotechnology*. Weinheim: VCH Verlagsgesellschaft. 405-64.

Galazzo JL and Bailey JE (1990) Growing *Saccharomyces cerevisiae* in calcium alginate beads induces cell alterations which accelerate glucose conversion to ethanol. *Biotechnol. Bioeng.* **36**, 417-426.

Godfrey T and Reicht P (1985): *Industrial Enzymology: The applications of enzymes on industry*. The Nature Press. London.

Greene RV, Cotta MA and Griffin HL (1989) A novel symbiotic bacterium isolated from marine shipworm secretes proteolytic activity. *Current Microbiology*. **19**, 353-356.

Griffin HL, Greene RV and Cotta MA (1992): Isolation and characterization of an alkaline protease from the marine shipworm bacterium. *Current Microbiol.* **24**, 111-117.

Helmo K, Winther NM and Emborg C (1985) Protease productivity in chemostat fermentations with retention of biomass on suspended particles. *Enzyme Microb. Technol.* **7**, 443-444.

Karel SF, Libicki SB and Robertson CR (1985) The immobilization of whole cells: Engineering principles. *Chemical Eng. Science* **40**, 1321-1354.

Navarro JM, Durand G (1977): Modification of yeast metabolism by immobilization onto porous glass. *Eur Appl Microbiol.* **4** : 243.

Sachidanandham R, Ranjit IA, Krishnan MRV, Jayaraman K (1999): Mathematical model based estimation of volumetric oxygen transfer coefficients in the production of proteolytic enzymes in *Bacillus amyloliquefaciens*. *Bioprocess Engineering.* **21**: 319-322.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC (1985): Measurement of protein using Bicinchoninic acid. *Analytical Biochemistry.* **150**: 76-85.

Waterbury JB, Calloway CB and Tutner RD (1983): A cellulolytic nitrogen-fixing bacterium cultured from the gland of Deshayes in shipworms (*Bivalvia: Teredinidae*). *Science.* **221**: 1401-1403.

Zhang X, Bury S, DiBiasio D and Miller JE (1989): Effects of immobilization on growth, substrate consumption in *Escherichia coli*. *J. of Industrial Microbiology.* **4**: 239-246.

Zukowski MM (1992): Production of commercially valuable products. In: *Biology of Bacilli: Application to industry* (RH Doi and M Mc Gloughlin, eds), Butterworth-Heinemann. London. 311-337.