Laccase Containing Sol-Gel Based Optical Biosensors

Article *in* Analytical Letters · August 1996

DOI: 10.1080/00032719608000797

CITATIONS
27

READS
80

7 authors, including:

Remigijus Simkus
Vilnius University
27 PUBLICATIONS **239** CITATIONS
SEE PROFILE

Terje Skotheim
Lightsense Technology
200 PUBLICATIONS **3,972** CITATIONS
SEE PROFILE

James Nakas
State University of New York College of Environmental Science and Forestry
40 PUBLICATIONS **1,220** CITATIONS
SEE PROFILE

David Slomczynski
Templeton Rye
8 PUBLICATIONS **370** CITATIONS
SEE PROFILE

ANALYTICAL LETTERS, 29(11), 1907–1919 (1996)

LACCASE CONTAINING SOL-GEL BASED OPTICAL BIOSENSORS

Key words: laccase, phenols, biosensor, sol-gel, fiber-optic

R.A. Simkus, V. Laurinavicius
Institute of Biochemistry, Vilnius 2021, Lithuania

L. Boguslavsky[1], T. Skotheim[1]
Moltech Corp., 25 East Loop Rd., Stony Brook NY 11790-3350 USA

S.W.Tanenbaum, J.P. Nakas, D.J. Slomczynski
SUNY Coll. Environ. Sci. and Forestry, Syracuse NY 13104 USA

ABSTRACT

Laccase-containing sol-gels were synthesized by hydrolysis and condensation - polymerization of tetramethylorthosilicates. Two types of laccase containing sol-gel based optical biosensors were designed and tested. The first type is based on 2-8 mm thick monolith sol-gel blocks. It was observed that laccase-containing sol-gel blocks accumulate high molecular weight products of

[1] Current address: Moltech Corporation, 9000 South Rita Road, Building 61, Tucson, Arizona 85747-9108

oxidation of the laccase substrate - 2,6-dimethoxyphenol. This leads to at least ten fold increase of optical bioassay sensitivity in comparison with the homogeneous phase. The response time of such biosensors is 3-24 hours. The second type of biosensor is based on laccase-containing 0.3 mm diameter sol-gel particles in a flow cell and includes an optical fiber measuring system. This sensor is characterized by a fast response time in comparison with the first type. The latter biosensor also possesses a homogeneous phase assay sensitivity and yields a linear calibration curve.

INTRODUCTION

Recently, novel sol-gel synthetic techniques were used to immobilize biologically active molecules in stable, optically transparent, porous silica glass matrixes under mild conditions such that biomolecules retained their characteristic reactivities[1,2]. The resulting glasses allowed transport of small molecules but nevertheless retained the protein molecules within their pores. It was stressed that spectroscopic changes occurring in the gel glasses could readily be quantified by optical measurement. Thus, the prospects are excellent for the use of these novel materials in optical biosensors[1,2]. However, the background signal of such biosensors depends mainly on the optical properties of the supporting material since absorbance and scattering of the matrix may lead to significant decrease in the sensitivity. Up to now there have been limited results concerning glassy sol-gel based optical biosensors or fiber optic biosensors[3-5].

In recent years a great deal of effort has been directed at developing enzyme sensors for the determination of phenolic compounds[6,7]. Although most of the enzyme based biosensors which have been developed are amperometric in nature[6-10], flow cell fiber optic sensors for phenols have also been reported [11].

Laccases are broad specified phenol oxidases which oxidatively polymerize phenols and/or aromatic amines with a concomitant electron transfer to oxygen [12]. The reaction products are often highly colored and have strong absorption in

the near ultraviolet [13] due mainly oxidized oligomers or to polymerization and polycondensation of intermediate products [14].

The aim of the present work was to investigate the possibilities to design laccase-containing sol-gels, including xerogels, for optical sensing of phenolic compounds. 2,6-Dimethoxyphenol was used as the model substrate for two types of biosensor constructs.

EXPERIMENTAL PROCEDURES

Laccase from *Botrytis cinerea*

The laccase enzymes were isolated by a facile purification scheme from a high - yield producer strain[15]. The activity was 1.2 mkatals/mg (substrate, 2,6-dimethoxyphenol; 99%, Aldrich). This specific activity is several thousand fold higher than the values reported for laccase enzymes resolved from other species. This laccase from *Botrytis cinerea* is active over a pH range of 1.5-7.0 with an optimum activity at 3.5. For activity measurements of the laccase containing sol-gel products, the pH of reaction mixtures was maintained at 3.5 by the addition of substrates in buffered solutions. The retention of activity over this wide range of pH allowed for the use of the enzyme without having to resort to additional pH controls. Therefore, unbuffered distilled water was used as a solvent in preparing enzyme containing sol-gels and 2,6-dimethoxyphenol solutions.

Sol-gel Syntheses

Enzyme solutions in water (2 mL, ~ 60:katals) were mixed at room temperature with tetra-methyl orthosilicate (TMOS, 98%, Aldrich, 2 ml). The reaction mixture was very gently shaken for 50-90 min until homogeneous. The solutions were poured into disposable polystyrene cuvettes (Sarstedt, 10 x 4 x 45 mm) and allowed to gel at room temperature. Gelation occurred within 2 hours. The process of aging starts after gelation and is accompanied by

shrinkage and partial drying of gel due to evaporation of methanol and water (products of hydrolysis and polycondensation).

After shrinkage of the gel (up to half of initial volume within 3 days), rectangular wet glassy bricks (8 x 3.2 x 20 mm) were obtained. These wet gels with entrapped laccase were washed in distilled water and sealed in polyethylene packs without further drying. Monolith glassy sol-gels were used for dipping sensors and were tested within 1 week.

The wet gels with entrapped laccase can form xerogels after drying. The characteristic opaque state[16] was observed within 30 days. The resulting xerogel monolith had 20% of its initial volume (6x2.4x12 mm). The density of dried xerogels was 1.1 g/cm^3. As the result of inner tension[16], the dried monolith transfers into 0.3 mm size laccase-containing xerogel particles. Xerogel particles obtained in this manner from the same xerogel monolith were dried and stored in the refrigerator and were employed as sensing material for the flow cell sensor.

Optical Measurements

Oxidation of 2,6-dimethoxyphenol in presence of laccase causes the appearance of a yellow product with an absorption maximum at 470 nm. The optical density of the monolith glassy sol-gels was recorded with a spectrophotometer (Specord UV VIS; Carl Zeiss, Jena, Germany).

A fiber optic biosensor was made, consisting of a stabilized light source; a monochromator (UM-2; LOMO, Russia) filtered at 470 nm; a pair of 70 cm long quartz optical fibers of 1 mm diameter (Livany Glass Factory, Latvia); photon counter; interface and PC computer and flow cell (Fig.1). A 2 mm thick xerogel particle layer was used. Due to the irreversible nature of the process, the biocatalyst (laccase containing xerogel particles) was changed after each measurement. Injection of substrate solution (5 ml) was provided by syringe. Time-resolved total scattering back from white Teflon cover and xerogel-water interfaces at 470 nm was measured.

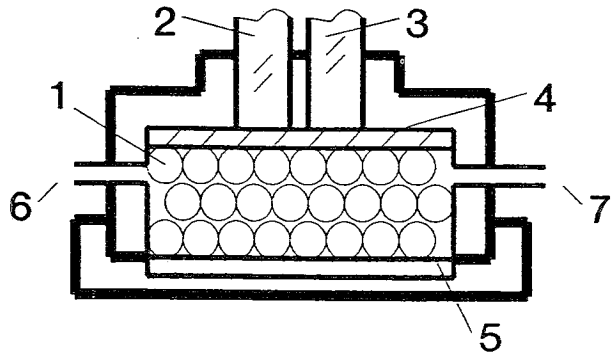

Fig. 1. A schematic of flow cell of the fiber optic biosensor. 1 - xerogel particles with immobilized laccase; 2 - optical fiber from monochromator; 3 - optical fiber to photon counter; 4 - quartz glass window; 5 - Teflon wall; 6 - inlet; 7 - outlet.

RESULTS AND DISCUSSION

2,6-Dimethoxyphenol in the presence of laccase is oxidized to quinone-like compounds, which are condensed to yield colored derivatives of high molecular weight. The process continues to ultimately yield polymeric insoluble wax[14]. Characteristic time dependent changes of the optical density of laccase-containing wet sol-gel monoliths dipped into micro molar 2,6-dimethoxyphenol solutions are shown in Fig. 2. Curves 1 and 2 correspond to the same wet monolith oriented differently in the spectrophotometer (different lengths of the optical path). The optical densities are equivalent within about 3 hours (see the picture inside). This result shows that oxidation starts at the boundary between water, containing 2,6-dimethoxyphenol, and the sol-gel monolith with entrapped laccase. The thickness of the layer of colored products of 2,6-dimethoxyphenol oxidation in the monolith exposed for a few hours is less than the thickness of the whole monolith. We conclude that there is no reason to form large path length monoliths of wet sol-gel for short time measurements.

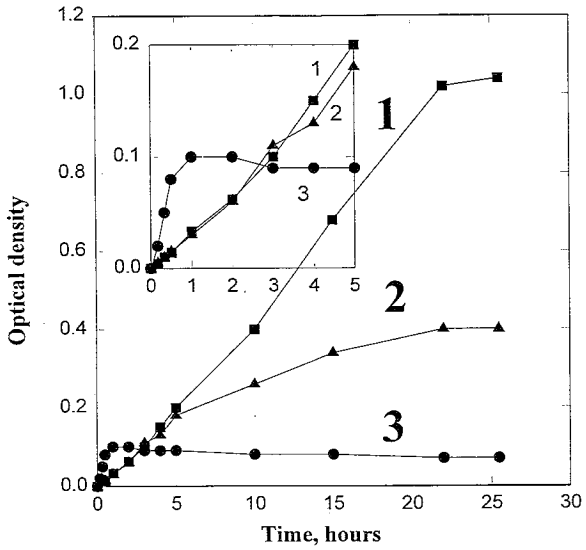

Fig. 2. Time-dependence of optical density of sol-gel monoliths dipped into 10:M 2,6-dimethoxyphenol solution: 1 - wet monolith (optical path 8 mm); 2 - wet monolith (optical path 3.6 mm); 3 - homogeneous phase assay of same solution (optical path 10 mm).

Curve 3 in Figure 2 shows the rate of accumulation of colored products in the homogeneous conditions, with laccase present in the aqueous solution of 2,6-dimethoxyphenol. Increasing concentration of soluble colored products within 2 hours was observed and the optical density did not increase by more than 0.1. Later the solution became more transparent as the result of the sedimentation of poorly soluble, high molecular weight products. Homogeneous media are therefore suitable for optical sensors based on the measurement of optical density.

The accumulation of colored products in the sol-gel matrix at the same range of concentrations keeps the sol gel monolith transparent, possibly as a result of the 3-dimensional restriction to grow molecular chains of the colored products.

It can be expected that the accumulation of colored products in other porous matrixes with entrapped laccase will also occur. However in contrast to the sol-gel monoliths most of other matrixes are not transparent and this process would not be detectable spectrophotometrically.

The phenomenon of product accumulation characteristic of sol-gel monoliths with entrapped laccase was checked by additional experiments with aniline solutions as an alternate substrate. Characteristic time dependent changes of the absorption indexes (the ratio between optical density and optical path) of laccase containing wet sol-gel monolith dipped into 20 mM solution of aniline (curve 1) and the same solution with laccase added (curve 2) were noted (Fig.3). Absorption index of monolith colored by products of oxidation at absorption maximum (448 nm) is 31-fold higher than the index achieved by homogeneous phase assay.

Dipping Sensor

In stationary conditions (24 hours), the optical densities of gels are proportional to the length of the optical path. Close to equilibrium, the absorbance of the gel (Fig. 2, curve 1) is more than 10 times greater than for the correspondent homogeneous phase assay (Fig. 2, curve 3). This is caused by accumulation of high molecular weight colored products into the silicate network. This feature of sol-gel monoliths could be useful for long term in situ determination of phenolic compounds.

Slow diffusion of substrate into the gel causes a slow accumulation of colored products and an increase in optical density. Calibration curves for sol-gel based dipping biosensor are shown in Fig. 4 for relatively short (curve 1) and long (curve 2) time exposure. Curve 1 corresponds to optical density measurements after 3 hours. In this case, slow diffusion of substrate caused detectable accumulation of product near front and back walls of the sensing dipping monolith. Although the process of accumulation of products is not complete, close to linear dependence was observed. After a 24 hours exposure

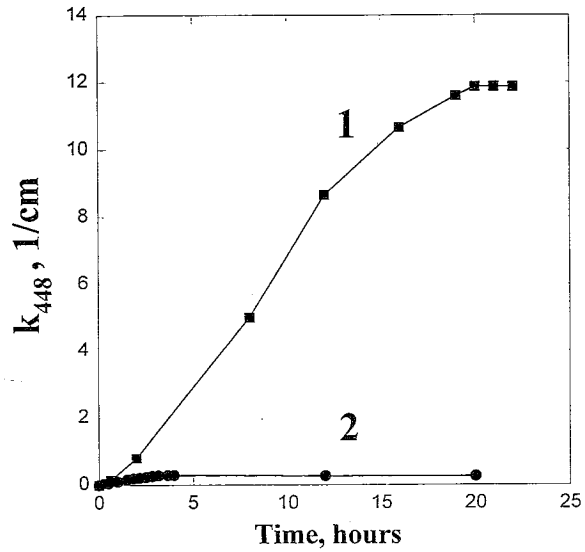

Fig. 3. Time dependence of absorption indexes of wet sol-gel monolith (optical path is 3.6 mm) dipped into 20 :M aniline solution (1) and homogeneous phase assay (optical path is 10 mm) of the same solution (2).

(curve 2) maximum optical densities are obtained corresponding to a homogeneous distribution of colored products.

Flow Cell Sensor

Flow cell sensors based on xerogel particles (Fig. 1) have much more faster response times than monolith-based dipping sensors. Figure 5 demonstrates the time dependence for the responses of this type of sensor. The decrease of the back scattered light intensity $\mathbf{I}_{10\ mim}$ (the measurement after 10 minutes of contact with analyte) was chosen to find the calibration dependence of the sensor expressed as $\log\mathbf{I}_{10\ min}/\mathbf{I}_{0\ min}$. The linear calibration curve in concentration region 0-60 :M as shown in Fig. 6 does not cross the origin. This

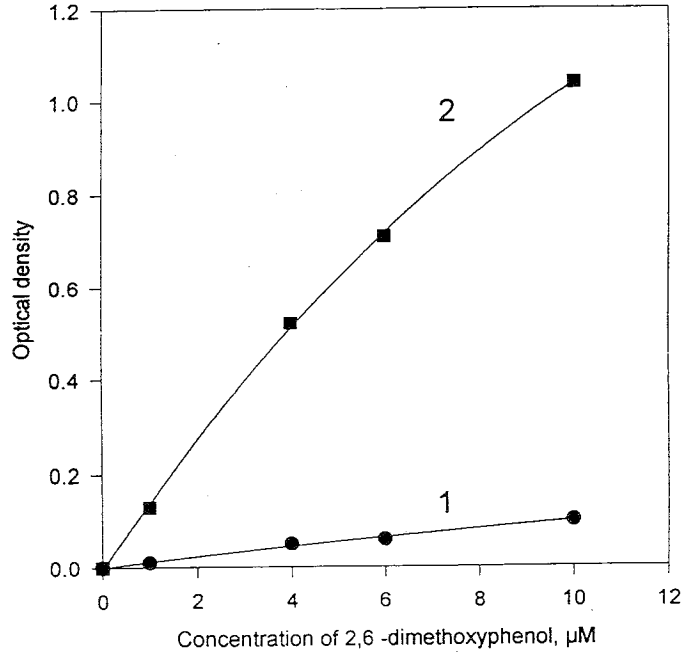

Fig.4. Dependence of optical density of 8 mm thick wet sol-gel on concentration of substrate (2,6-dimethoxyphenol). Time of exposure in substrate solution: (1) - 3 hours; (2) - 24 hours.

measurable response in the absence of substrate is caused by different scattering features of dry and wet xerogel particles and can be regarded as a background signal.

Xerogel particle based biosensors are as fast as homogeneous phase assay (Fig. 2, curve 3). The time required for internal diffusion t, may be related to the thickness of the gel L, and diffusion coefficient D, by the equation [17]:

$$t = L^2/D.$$

Thus, the smaller the xerogel particles, the shorter the response time.

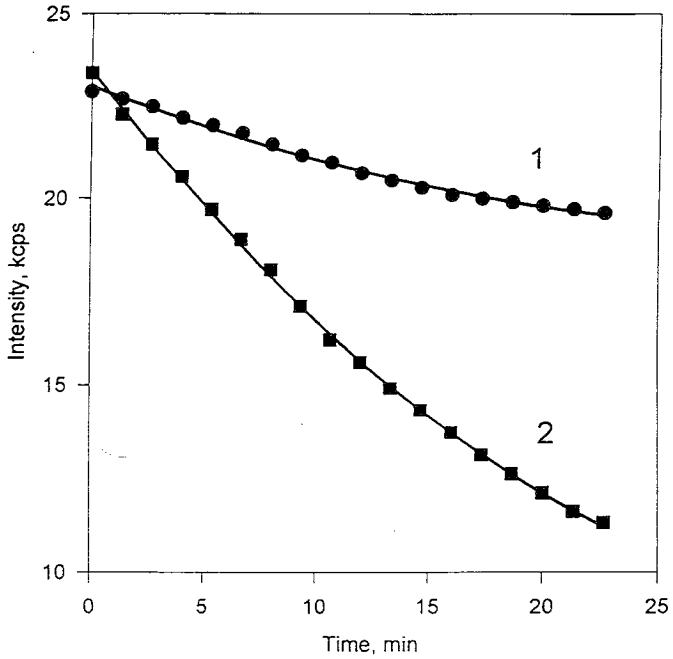

Fig. 5. Time-dependence of back-scattered light intensity detected by fiber optic biosensor: (1) - in presence of 1.5 :M of 2,6-dimethoxyphenol; (2) - 60:M substrate.

The experimental error of routine optical density measurements does not exceed 0.01. Concentrations of substrates required to achieve such optical density can be regarded as a detection limit of sensors. By comparing corresponding detection limit of homogeneous phase assays (10 mm optical path) and sensors based on xerogel particles (4 mm optical path) one can extrapolate 1 :M and 5:M . Almost the entire difference in detection limits can be explained by differences in optical paths and by the presence of unused volumes, e.g. interparticle volume and the volume of the glassy silicate network. An advantage of the flow cell sensor is the possibility of fast precalibration by the use of the same batch of xerogel particles.

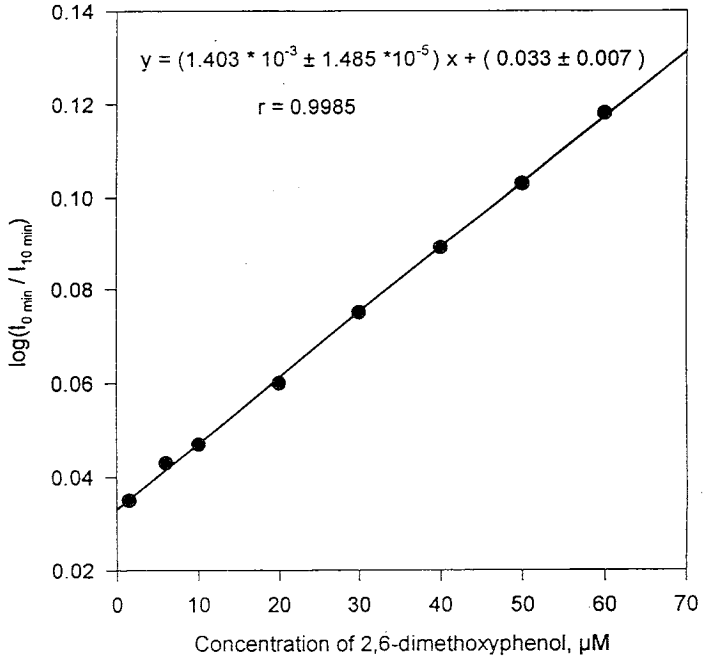

Fig.6. Calibration curve of fiber optic biosensor for 2,6-dimethoxyphenol based on laccase containing xerogel.

Enzyme activity in the dried xerogel vs. storage time at 4 C^0 was tested with fiber optic sensors as described above. It can be seen from Fig. 7 that the half-life of laccase activity is 4 months. This result is in accordance with the proposed high stability of other biological molecules in TMOS xerogel networks [1,2].

CONCLUSION

The characteristic features of these biosensors are slow (several hours) response times and detection limit around 0.1:M. This particular type of biosensor could be used for in situ determination of phenolic compounds. The second type (flow

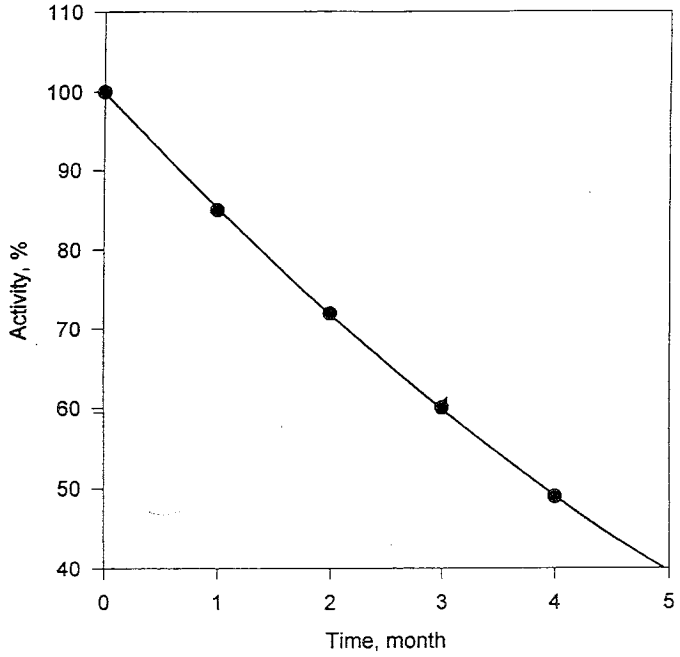

Fig.7. Dependence of xerogel-immobilized laccase activity on storage time.

cell sensor) is based on laccase-containing xerogel particles. It is a fiber optic sensor and characterized by fast (minutes) response times and around 5:M detection limit. A high stability of laccase activity in dried xerogels was observed.

ACKNOWLEDGEMENTS

This work was partially supported by the Soros International Science Foundation (contract LAB000) (R.A.S. and V.L.), and an award from the REMCO Program of the SUNY College of Environmental Science and Forestry (S.W.T., J. P. N., and D.J.S.).

REFERENCES

1. L.M. Ellerby, C.R. Nishida, F. Nishida, S.A. Yamanaka, B. Dunn, J.S. Valentine, and J.I. Zink, *Science* **255**, 1113 (1992).

2. S. Braun, S. Shtelzer, S. Rappoport, D. Avnir, and M.J. Ottolenghi, *Non-Cryst. Solids,* **147/148**, 739 (1992).

3. S. Shtelzer, S. Braun, *Biotechnol. Appl. Biochem.*, **19**, 293 (1994).

4. U. Narang, P.N. Prasad, F.V. Bright, A. Kumar, N.D. Kumar, B.D. Malhorta, M.N. Kamalasalan and S. Chandra, *Chem. Mater.* **6** , 1596 (1994).

5. U. Narang, P.N. Prasad, F.V. Bright, A. Kumar, N.D. Kumar, B.D. Malhorta, M.N. Kamalasalan, and S. Chandra, *Anal. Chem.* , **66** (19), 3139 (1994).

6. J. Wang, Y. Lin, and Q. Chen, *Electroanalysis* (N.Y.) , **5** (1), 23 (1993).

7. S. Cosnier, and C. Innocent, *Bioelectrochem. Bioeng.*, **31** (2), 1993 (1993).

8. P. Skalada, Coll. Czech. *Chem. Commun.*, **56** (7), 1427 (1991).

9. A.L. Ghindilis, V.P. Gavrilova, and Y. Yaropolov, Biosens. Bioelectron., **7** (2), 127 (1992).

10. L. Campanella, T. Beone, M.P. Sammartino, and M. Tomasseti, *Analyst,* **118**, 979 (1993).

11. D.B. Papkovsky, A.L. Ghindilis, and I.N. Kuroshkin, *Analytical Letts.*, **26** (7), 1505 (1993).

12. F.S. Sariaslani, in *Critical Reviews in Biotechnol.*, CRC Press Boca Raton, Florida, **9** (3), 171 (1989).

13. R. Sterjiades, J.F.D. Dean, G. Gamble, D.L. Himmelsbach, and K.E. Eriksson, *Planta*, 190, 75 (1993).

14. B.R. Brown, in *Oxidative coupling of phenols* (Eds. W.I.Taylor and A.R. Battersby), Marcel Dekker, Inc., New York, 167 (1967).

15. D.J. Slomczynski, J.P. Nakas, and S.W. Tanenbaum, *Appl. Environ. Microbiol.*, **61** (3), 907 (1995).

16. L.L. Hench, and J.K. West, *Chem. Rev.*, **90**, 33 (1990).

17. M.A. Icasa and U. Bilitewski, *Analytical Chem.*, 65(11), 522 (1993).

Received: July 12, 1995
Accepted: May 2, 1996