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Kinetics and diffusion studies in urease-alginate biocatalyst beads

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SUMMARY

Urease was immobilized with calcium alginate by entrapment method in the form of spherical beads and stored in Tris/acetate buffer (pH 7.3) at 4°C. Urease immobilized at different concentration of alginate beads (3%, 4% and 5%) showed higher apparent K_m values than the soluble urease. Furthermore, K_m has been shown to be corelated with effective diffusion coefficient (*De*) at different concentration of alginate gel. The present study showed that diffusion and reaction contribute to control the overall rate.

Key words: Urease; Calcium alginate; Biocatalyst; Kinetics; Effective diffusion coefficient

INTRODUCTION

Many papers have been published on the applications of the urease immobilization in kidney malfunctioning, urea detection in process fluids (especially biosensor) and in food industry (Trevan, 1980; Krajewaska *et al.*, 1990; Sungur *et al.*, 1992; Das *et al.*, 1997; Das and Kayastha, 1998; Laska *et al.*, 1999; Kayastha, *et al.*, 2003; Basu *et al.*, 2005; Reddy *et al.*, 2005; Reddy and Kayastha, 2006). As urea passes through the pores of biocatalyst beads, diffusion as well as reaction both takes place simultaneously. Therefore, studies of kinetics and diffusion in biocatalyst play very important role in biochemical as well as biocatalyst engineering. In this paper, we used the method (Peter *et al.*, 1997) for determination of effective diffusion coefficient (*De*). We have calculated effective diffusion coefficient and given relationship of K_m and *De* at different concentration of alginate beads (3%, 4% and 5%).

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One of the main disadvantages of enzyme immobilization is mass transfer resistance of support materials to the substrate. Due to immobilization, mass transfer resistance increases, which means that diffusion coefficient decreases. Thickness of the unstirred 'Nernst' layer around the beads in the case of (a) no external diffusion (b) slight internal diffusion (c) external and (d) strong internal diffusion limitation (Peter *et al.*, 1997). This 'Nernst layer' causing the external diffusion surrounds the bead. Its thickness 'd' depends on the relative velocity of the particle to the bulk solution. In the case of rapid stirring (above 300 rpm) this external diffusion can be neglected. A useful expression for the diffusion of molecules in a ball-shaped matrix with a radius 'R' (Crank, 1976):

$$\frac{C_t - C_\infty}{C_0 - C_\infty} = \frac{6}{\Pi^2}\sum_{n-1}^{\infty}\frac{1}{n^2}\exp\left(-\frac{n^2 \times \Pi^2 \times De \times t}{R^2}\right) \quad (1)$$

For sufficiently large values of time 't' this expression can be approximated by the first term of the series:

$$\frac{C_t - C_\infty}{C_0 - C_\infty} = \frac{6}{\Pi^2} \exp\left(-\frac{\Pi^2 \times De \times t}{R^2}\right) \quad (2)$$

$$\ln\left(\frac{C_t - C_\infty}{C_0 - C_\infty}\right) = -\frac{\Pi^2 \times De}{R^2} + \text{constant} \quad (3)$$

from this equation, the effective diffusion coefficient *De* is obtained by a plot of $\ln[(C_t-C_\infty)/(C_0-C_\infty)]$ versus 't', the slope of which is $\Pi^2 De/R^2$. The required data are obtained from effusion experiments.

The main aspect of this work is the simplification of the evaluation of Eq. (1), leading to a reduced number of parameters to be determined. The effusion process is divided here into the different stages; at the beginning of the experiment ($t = 0$) the whole substrate is within the beads, the concentration 'C' in the bulk solution is zero ($C = 0$). For a given time 't' ($C = C_t$) the substrate concentration within the catalyst beads is still higher than in the surrounding solution ($C = C_t$). Finally, as $t = \infty$, $C = C_\infty$ the substrate concentration is equally distributed in both phases.

Enzymes are typically immobilized on the internal surface of porous support or entrapped in matrices through which substrate can diffuse. In such a system, calculation of the observed rate of substrate disappearance requires evaluation of the concentration profile of substrate within the profile. Following assumptions were made: (a) the alginate beads have perfect spherical geometry (b) uniform distribution of enzymes within the beads (c) reaction occurs in the void volume of beads at pseudo steady state condition (d) the urea (substrate) molecules diffuse in r-direction only (e) neglected liquid film resistance (urea concentration on bead surface is equal to in bulk solution).

The substrate (urea) continuity equation with spherical bead in r-direction with Michaelis-Menten kinetics is given below (Bailley and Ollis, 1986):

$$\frac{d^2S}{dr^2} + \frac{2}{r}\left[\frac{dS}{dr}\right] = \left[\frac{V \times R^2}{De \times S_0}\right] = 9\phi^2 \frac{S}{1 + \beta S} \quad (4)$$

$$\phi = \frac{R}{3}\sqrt{\frac{V_{max}}{K_m \times De}} \quad (5)$$

$$\beta = \frac{S_0}{K_m} \quad (6)$$

Solution of the above equation is

$$\eta = \frac{\tanh\phi}{\phi} = \frac{V}{V_{sol}} \quad (7)$$

where, η: Effectiveness factor, ϕ: Thiele Modulus, V: activity of immobilized enzyme, V_{sol}: activity of soluble enzyme.

The magnitude of the saturation parameter β provides a measure of local rate deviations from first order kinetics, while very large values indicate an approach to zero order kinetics. When ϕ is sufficiently large ($\phi \geq 3$), diffusion of substrate is slow relative to consumption. In such a situation with diffusion limited rate, it may be assumed that all substrate is utilized in a thin region within the particle adjacent to its exterior surface. Criteria for assessing the magnitude of mass transfer effects on overall kinetics is as follows:

Criteria	η Value	Limiting rate process	Extent of mass transfer limitation
$\phi < 0.3$	≈ 1	Chemical reaction	Negligible
$\phi > 3$	$\propto \phi^{-1}$	Diffusion	Large

MATERIALS AND METHODS

Enzyme and chemicals

Urease (from jack beans), sodium alginate and urea (enzyme grade) were purchased from Sigma Chemical Co. St. Louis, MO, U.S.A. Trichloroacetic acid and Calcium chloride were purchased from Glaxo Smith Kline Pharmaceuticals Limited, Mumbai, India. Nessler's reagent was procured from HiMedia, Mumbai, India. All other chemicals were of analytical grade and Milli Q (Millipore, U.S.A.) water used all throughout.

Urease immobilization with calcium alginate

A 7% stock solution of sodium alginate was prepared by slowly adding alginate in 0.1 M Tris/

acetate buffers, pH 7.3 at 30°C. After the alginate had dissolved, bubbles are removed by cooling the solution. Enzyme (1 mg/ml) is then added and the volume made up in buffer so that the final concentration of the alginate gel is 4%. This was loaded in a 10 ml syringe and alginate-enzyme mixture was dropped into 500 ml of chilled 8% calcium chloride in 0.1 M Tris/acetate buffers, pH 7.0 with constant swirling on a magnetic stirrer. After stirring for 2 - 3 h at 30°C, beads of calcium alginate with entrapped enzyme were collected. Beads are washed with buffer to remove any calcium chloride solution and are stored in buffer at 4°C. Similarly, 3% and 5% alginate beads were prepared from different concentration of alginate solution (Das *et al.*, 1998; Kayastha and Das, 1999).

Activity measurement

1 ml of 0.1 M urea is added in 0.1 mg/ml of enzyme solution and total volume was made 3 ml by adding buffer solution (0.05 M Tris/acetate buffer, pH 7.3). After incubation with the substrate for 10 min, the reaction is stopped by the addition of 1 ml of 10% trichloroacetic acid. Nessler's reagent is added to the reaction mixture and the yellow color produced was measured at 405 nm. A blank is run without enzyme and suitable correction is applied. An enzyme unit is defined as the amount of enzyme required to liberate 1 μmol ammonia/min under the test conditions (0.1 M urea, 0.05 M Tris/acetate buffer, pH 7.3 at 30°C). Linearity in the activity was checked by varying the concentration of the urea and enzyme (Das *et al.*, 1998; Kayastha and Das, 1999; Ayhan, 2002). Similarly, activity was measured in case of immobilized enzyme by varying the number of beads to check the homogeneous distribution of the enzyme in beads.

Evaluation of K_m

K_m for soluble as well as immobilized urease (3%, 4% and 5% alginate) was evaluated at 30°C, Tris/acetate buffer, pH 7.3 using Lineweaver-Burk plot (Vasudevan *et al.*, 1990; Kayastha and Srivastava, 2001; Srivastava *et al.*, 2001).

Measurement of effective diffusion coefficient

Diffusion coefficient of alginate beads (3%, 4% and 5% alginate) was determined experimentally at pH 7.3 and 30°C. For this, 30 beads (in which enzyme was not immobilized) were equilibrated 24 h in 10 ml of urea solution (0.1 M). These beads were then transferred to 10 ml of distilled water from

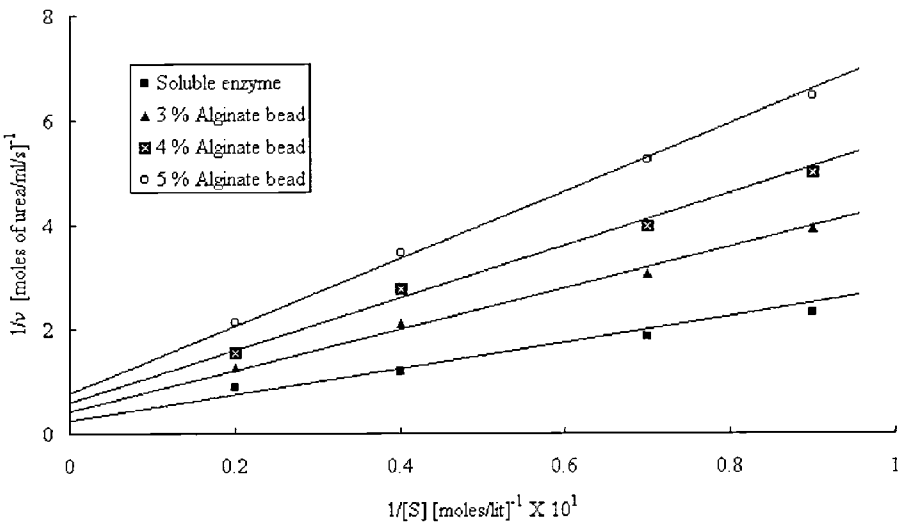

Fig. 1. Lineweaver-Burk plot for soluble and immobilized enzyme at different concentrations of alginate.

which 1 ml samples were taken at a given time in order to determine the amount of substrate effused from the Ca-alginate beads into the surrounding solution (Peter *et al.*, 1997).

RESULTS AND DISCUSSION

Effect of immobilization on K_m

Fig. 1 shows K_m values obtained from Lineweaver-Burk plot at 30°C, Tris/acetate buffer pH 7.3. It is clear that urease immobilized on different concentration of alginate beads showed higher apparent K_m values than the soluble urease (Table 1). It is postulated that an unstirred layer of solvent surrounds suspended water insoluble particles. This unstirred layer known as the 'Nernst layer' with water-insoluble enzyme, i.e. immobilized enzymes, a concentration gradient of substrate is established across the layer. Consequently, saturation of an enzyme attached to water-insoluble particle will occur at higher substrate concentration than normally required for the saturation of the soluble enzyme, thus leading to an increase in the K_m value. A similar change has also been observed by Reddy *et al.* (2005); values for soluble and immobilized pigeonpea urease were 3.0 mM and 4.75 mM, respectively.

Effective diffusion coefficient in biocatalysts

According to equation (3), there is a linear relationship between ln $[(C_t-C_\infty)/(C_0-C_\infty)]$ and effusion time 't', which allows the calculation of the effective diffusion coefficient *De* from the resulting slope (size of bead was 1.7 mm) as shown in Fig. 2. Value of *De* evaluated for different concentration of alginate beads (3%, 4% and 5%) is shown in Table 1. Values of *De* obtained were similar to a previous report (Peter *et al.*, 1997).

Table 1. Calculated result of kinetic parameter K_m and effective diffusion coefficient

Urease	K_m (mM)	Effective diffusion coefficient (cm^2/s) × 10^6
Soluble	3.13	-
Immobilized with 3% alginate	5.56	7.43
Immobilized with 4% alginate	11.11	5.77
Immobilized with 5% alginate	20.00	4.39

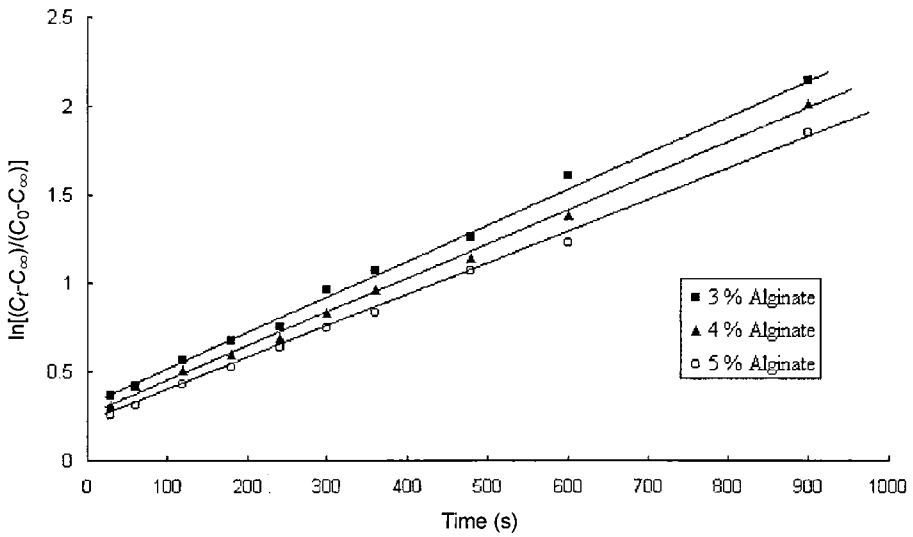

Fig. 2. Plot for determination of effective diffusion coefficient (*De*) for various concentrations of alginate beads.

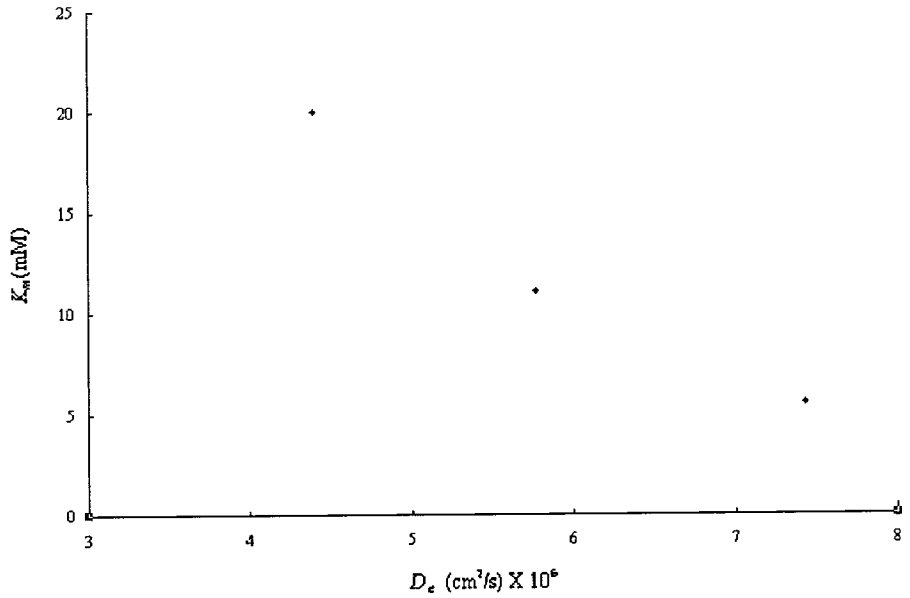

Fig. 3. Plot to show relationship of effective diffusion coefficient (*De*) with K_m.

Dependence of effective diffusion coefficient on K_m

Effective diffusion coefficient strongly depends on the kinetic parameters of the enzyme. In the present case, as the concentration of the alginate increased, value of K_m increased due to Nernst layer, as discussed earlier, which in turn decreased the effective diffusion constant. These relationships are clear from the Table 1 and Fig. 3. This is similar to the results by Miyama *et al.* (1982). By finding the value of 'Thiele Modulus', one can find out if the overall rate is controlled by diffusion or chemical reaction. In our case, mass transfer resistance was varied by changing concentration of alginate beads, which in turn varied their pore size. Results from Table 2, showed that either diffusion or reaction alone couldn't control the overall rate.

Table 2. Calculated result of Thiele Modulus

Urease	η Value	ϕ Value
Immobilized with 3% Alginate	0.903	0.56
Immobilized with 4% Alginate	0.82	0.825
Immobilized with 5% Alginate	0.699	1.19

CONCLUSION

Diffusion and reaction simultaneously take place inside the biocatalyst beads. A simple method for evaluation of effective diffusion coefficient (*De*) has been described in this paper. K_m has been shown to be corelated with *De* at different concentration of alginate gel. Present studies showed that overall rate was controlled by both diffusion as well as chemical reaction.

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REFERENCES

Ayhan F, Ayhan H, Piskin E, Tanyolac A. (2002) Optimization of urease immobilized onto non-porous

HEMA incorporated poly (EGDMA) microbeads and estimation of kinetic parameters. *Biores. Technol.* **81**, 131-140.

Bailley JE, Ollis DF. (1986) Biochemical Engineering Fundamentals, pp. 78-214, McGraw Hill publication, Singapore.

Basu I, Subramanian RV, Mathew A, Kayastha AM, Chadha A, Bhattacharya E. (2005) Solid state potentiometric sensor for the estimation of tributyrin and urea. *Sens. Actua. B Chem.* **107**, 418-423.

Crank, J. (1976) The Mathematics of Diffusion, pp. 89-103, 2nd ed, Clarendon Press, Bristol.

Das N, Kayastha AM. (1998) Immobilization of urease from pigeonpea (*Cajanus cajan* L.) on flannel cloth using polyethyleneimine. *World J. Microbiol. Biotechnol.* **14**, 927-929.

Das N, Kayastha AM, Malhotra OP. (1998) Immobilization of urease from pigeonpea (*Cajanus cajan* L.) in polyacrylamide gels and calcium alginate beads. *Biotechnol. Appl. Biochem.* **27**, 25-29.

Das N, Prabhakar P, Kayastha AM, Srivastava RC. (1997) Enzyme entrapped inside the reversed micelle in the fabrication of a new urea sensor. *Biotechnol. Bioeng.* **20**, 329-332.

Kayastha AM, Das N. (1999) A simple laboratory experiment for teaching enzyme immobilization with urease and its application in blood urea estimation. *Biochem. Edu.* **27**, 114-117.

Kayastha AM, Srivastava PK. (2001) Pigeonpea (*Cajanus cajan* L.) urease immobilized on glutaraldehyde-activated chitosan beads and its analytical applications. *Appl. Biochem. Biotechnol.* **96**, 41-53.

Kayastha AM, Srivastava PK, Miska B, Slomkowski S. (2003) Unique activity of ureases immobilized on poly(styrene-co-acrolein) microspheres. *J. Bioact. Compat. Polym.* **18**, 113-124.

Krajewaska B, Leszko M, Zabroska W. (1990) Urease immobilized on chitosan membrane: Preparation and properties. *J. Chem. Tech. Biotechnol.* **48**, 337-350.

Laska J, Wodarczyk J, Zaborska W. (1999) Polyaniline as a support for urease immobilization. *J. Mol. Catal. B Enzym.* **6**, 549-553.

Miyama H, Kobayashi T, Nosaka, Y. (1982) Immobilization of urease on synthetic polymers. *Biotechnol. Bioeng.* **24**, 2757-2763.

Peter G, Kristin H, Walter G. (1997) Determination of effective diffusion coefficients in a polysaccharide matrix used for the immobilization of biocatalysts. *Solid State Ionics* **101**, 863-867.

Reddy KRC, Kayastha AM. (2006) Improved stability of urease upon coupling to alkylamine and arylamine glass. *J. Mol. Catal. B Enzym.* **38**, 104-112.

Reddy KRC, Srivastava PK, Kayastha AM, Dey PM. (2004) Immobilization of pigeonpea (*Cajanus cajan*) urease on DEAE-Cellulose paper strips for urea estimation. *Biotechnol. Appl. Biochem.* **39**, 323-327.

Reddy KRC, Turcu F, Schulte A, Kayastha AM, Schuhmann W. (2005) Fabrication of a potentiometric/amperometric bifunctional enzyme microbiosensor. *Anal. Chem.* **77**, 5063-5067.

Srivastava PK, Kayastha AM, Srinivasan. (2001) Characterization of gelatin-immobilized pigeonpea urease and preparation of a new urea biosensor. *Biotechnol. Appl. Biochem.* **34**, 55-62.

Sungur S, Elicin E, Akbulut U. (1992) Studies on immobilization of urease in gelatin by cross-linking. *Biomaterials* **13**, 795-800.

Trevan MD. (1980) Immobilized Enzymes, p.11, John Wiley & Sons, New York.

Vasudevan PT, Ruggiano L, Weiland RH. (1990) Studies on the deactivation of immobilized urease. *Biotechnol. Bioeng.* **35**, 1145-1149.