ISSN : 0974 - 7427 Volume 5 Issue 3

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 5(3), 2011 [151-157]

Inhibition of urease immobilized in alginate beads by heavy metals

Neelam Mishra, Anita Bahadur*

Department of Zoology, Sir P.T.Sarvajanik College of Science, Surat - 395 001, Gujarat, (INDIA)

E-mail : anita26p@gmail.com; neelam1735@gmail.com

Received: 13th November, 2010 ; Accepted: 23rd November, 2010

ABSTRACT

Urease from jack bean meal (E.C. 3.5.1.5) was immobilized in calcium alginate beads. Effects of varying alginate and $CaCl_2$ concentration on immobilization yield were examined. The influence of pH, temperature and enzyme matrix ratio on the percent of the immobilization and activity was studied. An inhibiting effect of mercury (Hg^{2+}) and copper (Cu^{2+}) ion on immobilized urease has been investigated in order to elucidate the kinetics and mechanism of inhibition. Optimum sodium alginate and $CaCl_2$ concentration were found to be 2% (w/v). An increase in the concentration of alginate gives rise to a reduction in membrane thickness, while an increase in the concentration of calcium chloride leads to the formation of a thicker film. The straight lines intersecting at x-axis shows noncompetitive inhibition for both the Hg^{2+} and Cu^{2+}.

KEYWORDS

Immobilization;
Urease;
Alginate.

INTRODUCTION

The increasing use of immobilized enzymes in various industrial processes is mainly because of the advantages they confer over their soluble counterparts. These include increased enzymatic stability in extreme conditions of temperature, pH, storage and reuse of the enzymes. Several different methods have been employed for enzyme immobilization which includes adsorption, entrapment, encapsulation, and crosslinking[1]. The most common immobilization matrices used now a days are biocompatible polymers like alginate, chitosan, agarose gel, etc. Of this alginate gel beads have been widely used to entrap enzymes as low molecular weight substrates and products can diffuse freely into and out of the bead without disturbance by the pores in the gel[2]. The immobilization procedure on alginate beads is not only inexpensive but also easy to carry out and provides extremely mild conditions, so that the potential for industrial application is considerable[3].

Urease is amongst enzymes most extensively studied for immobilizations and practical applications[4,5]. This is because of many processes in which urease takes part and its possible exploitation in practical applications. The classic methods for the estimation of urease activity are based on the determination of NH_3 or CO_2 evolved from the conversion of urea by urease in solution[6].

In agricultural settings, rapid hydrolysis of fertilizer urea by soil bacterial ureases results in unproductive volatilization of ammonia and in ammonia toxicity or alkali-induced plant damage. Agricultural trials have

Regular Paper

shown that urease inhibitors can be combined with fertilizer to increase the overall efficiency of nitrogen utilization[7]. Control of the rate of urea hydrolysis with inhibitors would also lead to improved therapeutic strategies for treatment of infections involving ureolytic bacteria. Studies published so far on heavy metal ions inhibition of urease of both plant and bacterial origin have aimed either at listing the metals in order of their decreasing toxicity[8-12] or at detection of their trace amounts, e.g. Hg^{2+}, Cu^{2+} and Ag^{+} ions[13-16].

The purpose of this work was to investigate the potential use of alginates to improve the integrity of the matrix containing the enzyme urease. The influence of pH, temperature and enzyme matrix ratio on the percent of the immobilization and activity was studied. The work examines the influence of various parameters such as sodium alginate concentration, $CaCl_2$ concentration and hardening time of urease entrapped in crosslinked alginate beads for stability improvement. Also the work was carried out to find out the influence of inhibitors such as mercury (Hg^{2+}) and copper (Cu^{2+}) ion on immobilized urease.

EXPERIMENTAL

Materials

Crude urease from jack bean meal (E.C. 3.5.1.5) was purchased from Hi-Media Laboratories, India. Calcium chloride, urea, Nessler's reagent and Folin Ciocalteau reagent were from Qualigen's fine chemicals, India. Mercuric acetate and copper sulphate were obtained from E. Merck, India. All other chemicals were of AnalaR grade and were prepared in double distilled water.

Preparation of immobilized calcium alginate beads

Urease powder (40 mg) and sodium alginate was dissolved in water (10 ml), gently stirred and dropped into 50 ml of $CaCl_2$ solution by using syringe befitted with needle (18G). The filtered $CaCl_2$ solution and the two washings were collected for protein determination and beads were stored at 6°C.

Preparation of immobilized calcium alginate beads with inhibitor

Urease (40mg) and sodium alginate solution 2% (w/v) was dissolved in water, thorough mixing with 0.2 ml of mercury acetate or copper sulphate (inhibitor solution, 0.2µM) and dropped into 50 ml of chilled calcium chloride solution (2%) with the help of a syringe as done before. The beads formed were allowed to stir for 10 min.

Protein estimation and urease activity

The free and immobilized urease protein content was evaluated using Lowry's method[17]. The activity of urease was estimated by the known method[18]. The absorbance was measured at 405 nm using UV-Spectrophotometer (Elico, India).

Optimization of immobilization parameters

Two sets of immobilized beads were prepared and used for comparative study.

1) To study the effect of sodium alginate concentration on bead permeability for urease activity was conducted at various concentrations ranging from 0.5%, 1.0%, 1.5%, 2.0%, and 2.5%. The formation of beads was carried out in a 2% $CaCl_2$ solution.
2) In order to investigate the effect of $CaCl_2$ concentration on the hardness of beads, the 2% sodium alginate solution was extruded drop-wise into different $CaCl_2$ concentrations from 1 to 3.5%. They were washed with (0.1 M) *Tris* HCl buffer (0.1 M). The immobilization efficiency and retained activity was calculated.

pH profile for free and immobilized urease

Free or immobilized urease was added into 1 ml buffers having different pH values in the range of 4-9. Three different buffers were used for this study: 0.1 M citrate phosphate buffer (pH 4.0-6.6), 0.2 M phosphate buffer (pH 6.6-7.6) and 0.1 M *Tris*-acetate buffer (pH 7.2-9.0). The solutions were placed into a shaking water bath for 30 min at 55°C. The activity of the enzyme was determined as described earlier.

Repeated use of urease immobilized in the alginate beads

In order to test the reuse of entrapped urease, the activity in the beads was assayed several times. After each urease activity assay, the beads were removed, washed thoroughly with distilled water and stored at room temperature. Then, the beads were assayed again

Regular Paper

TABLE 1a : Effect of sodium alginate and $CaCl_2$ concentration on beads diameter and membrane thickness

	Sodium alginate – 0.5 % w/v				
	$CaCl_2$ Concentration %				
	1%	1.5%	2%	2.5%	3%
Diameter (mm)	4.1	4.5	4.7	5.0	5.2
Membrane Thickness (mm)	0.12	0.17	0.25	0.30	0.32
	Sodium alginate – 1 % w/v				
	$CaCl_2$ Concentration %				
	1%	1.5%	2%	2.5%	3%
Diameter (mm)	3.9	4.2	4.4	4.9	5.1
Membrane Thickness (mm)	0.11	0.16	0.23	0.29	0.30
	Sodium alginate –1.5 % w/v				
	$CaCl_2$ Concentration %				
	1%	1.5%	2%	2.5%	3%
Diameter (mm)	3.1	3.4	4.0	4.2	4.7
Membrane Thickness (mm)	0.10	0.14	0.18	0.24	0.21
	Sodium alginate – 2 % w/v				
	$CaCl_2$ Concentration %				
	1%	1.5%	2%	2.5%	3%
Diameter (mm)	3.1	3.2	3.8	4.2	4.5
Membrane Thickness (mm)	0.08	0.12	0.16	0.22	0.21

TABLE 1b : Effect of $CaCl_2$ and sodium alginate concentration on beads diameter and membrane thickness

	$CaCl_2$ Concentration – 1 % w/v				
	Sodium alginate %				
	0.5%	0.5%	0.5%	0.5%	0.5%
Diameter (mm)	4.1	4.1	4.1	4.1	4.1
Membrane Thickness (mm)	0.12	0.12	0.12	0.12	0.12
	$CaCl_2$ Concentration – 1.5 % w/v				
	Sodium alginate %				
	0.5%	0.5%	0.5%	0.5%	0.5%
Diameter (mm)	4.5	4.5	4.5	4.5	4.5
Membrane Thickness (mm)	0.17	0.17	0.17	0.17	0.17
	$CaCl_2$ Concentration – 2 % w/v				
	Sodium alginate %				
	0.5%	0.5%	0.5%	0.5%	0.5%
Diameter (mm)	4.7	4.7	4.7	4.7	4.7
Membrane Thickness (mm)	0.25	0.25	0.25	0.25	0.25
	$CaCl_2$ Concentration – 2.5 % w/v				
	Sodium alginate %				
	0.5%	0.5%	0.5%	0.5%	0.5%
Diameter (mm)	5.0	5.0	5.0	5.0	5.0
Membrane Thickness (mm)	0.30	0.30	0.30	0.30	0.30

for urease activity and the same process was repeated.

Optimization of temperature for free and immobilized urease

Free or immobilized urease was added into 1 ml phosphate buffer (pH 7.2), and the solutions were placed into a shaker for 30 min in water bath which was adjusted to temperature in the range of 10-80°C. Enzyme activities were evaluated as described above.

Determination of storage stability for free and immobilized urease

The free and immobilized enzymes were kept at 6°C in order to examine the storage stabilities, the activities of the enzymes were measured upto 35 days after an interval of five days.

Determination of K_m and V_{max} values

K_m and V_{max} values were determined from Lineweaver–Burk plots by measuring the initial rates of the reactions by using various concentrations (0.2 to 2.0 M) of urea solutions at 55°C and pH 7.2. For this purpose, urease solutions containing 1 ml free enzyme (in phosphate buffer) or immobilized alginate beads were used. Both form of enzyme were incubated with 0.2 ml of inhibitor solution.

RESULTS AND DISCUSSION

Alginate and $CaCl_2$ concentration are major parameters for enzyme entrapment because the cross-linking between alginate and Ca^{2+} ions leads to gelation and the instantaneous interfacial crosslinking leading to entrapment of enzyme. Therefore, effects of alginate and $CaCl_2$ concentration on urease activity were first investigated.

The enzyme amount and the needle size were kept constant. The percentage of immobilization and the bead size were found to be directly proportional to alginate concentration upto a certain limit (2%) and inversely proportional to calcium chloride concentration. When alginate concentration was increased from 0.5% to 2.5 % at a fixed $CaCl_2$ concentration (2% w/v), the highest urease activity & immobilization yield was found to be at 2% (w/v) and decreases with increases in the amount of alginate used (Figure 1). Similarly standardization of $CaCl_2$ solutions (1% - 3.5%)

Regular Paper

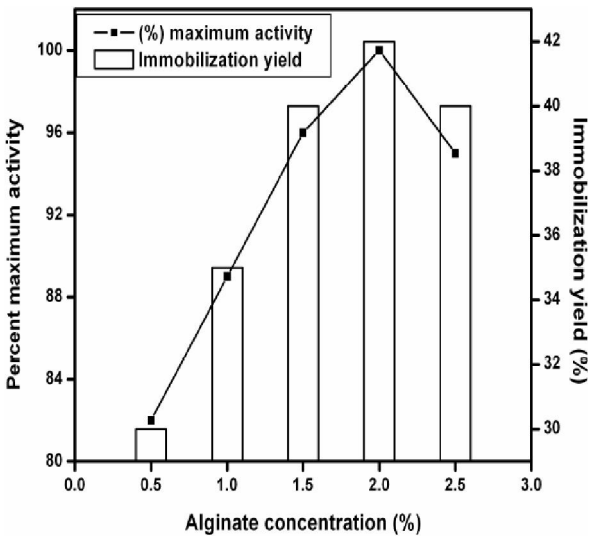

Figure 1 : Effect of sodium alginate concentration on urease activity

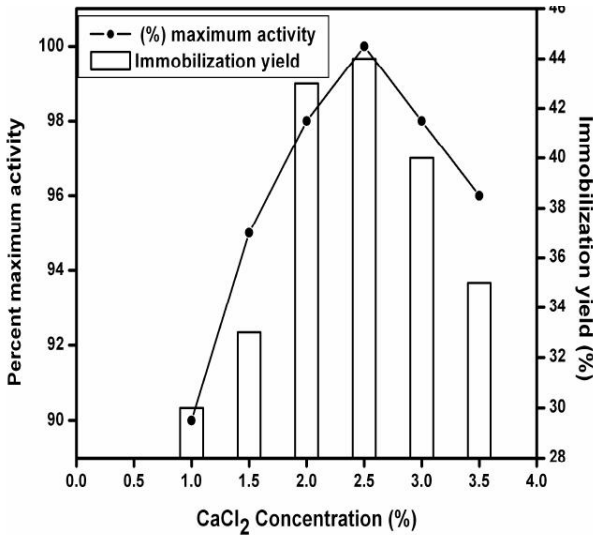

Figure 2 : Effect of Calcium chloride concentration on urease activity

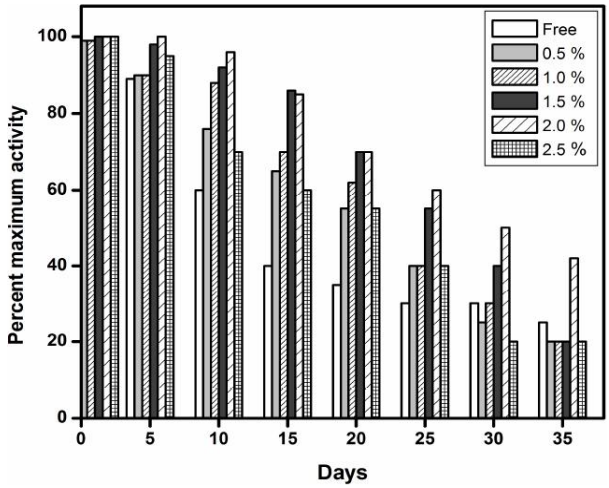

Figure 3a : Plot of percent maximum activity vs. time (days) for storage stability and immobilized urease in calcium alginate beads by changing sodium alginate concentration at constant amount of calcium chloride (2%)

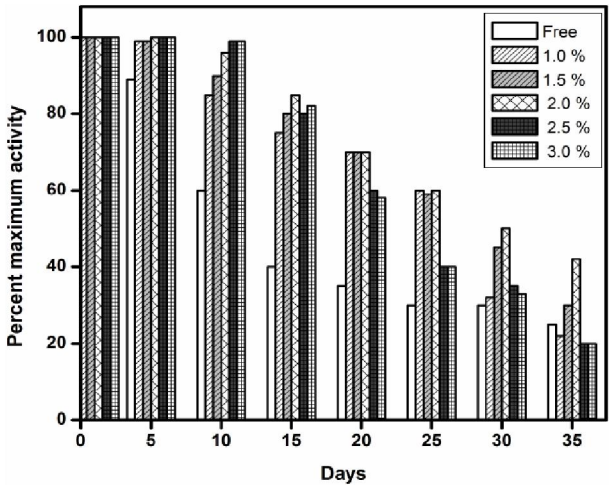

Figure 3b : Plot of percent maximum activity vs. time (days) for storage stability and immobilized urease in calcium alginate beads by changing calcium chloride concentration at constant amount of sodium alginate (2%)

was done against 2% sodium alginate with 1mg/ml urease solution for both free and immobilized. The best urease activity was observed at 2.5% $CaCl_2$ solution (Figure 2) but beads were disrupted after 24 hour so it was presumed that 2% $CaCl_2$ solution is stable for immobilization of urease.

It has been reported that alginate and $CaCl_2$ concentration in the enzyme and cell entrapment changed in the range of 2-4% (w/v) and 2–5% (w/v), respectively[19-21]. Devi and Sridhar[22] found that $CaCl_2$ concentration had a profound effect on cephamycin C production, using *Streptomyces clavuligerus* immobilized on alginate beads. The range of alginate and $CaCl_2$ concentration used in our study were enough to hold urease inside the gel during immobilization. The highest immobilized urease activities were obtained at 2% alginate (42%) and 2.5% $CaCl_2$ concentration (61%). However, beads were not stable at 2.5% $CaCl_2$ solution and therefore, 2% sodium alginate and 2 % $CaCl_2$ solution were used for further study.

Alginate and $CaCl_2$ concentration also affect the membrane thickness and diameter of beads (TABLE 1a & b). It is seen from the data that on increasing the sodium alginate concentration (0.5% to 2% w/v) for

Regular Paper

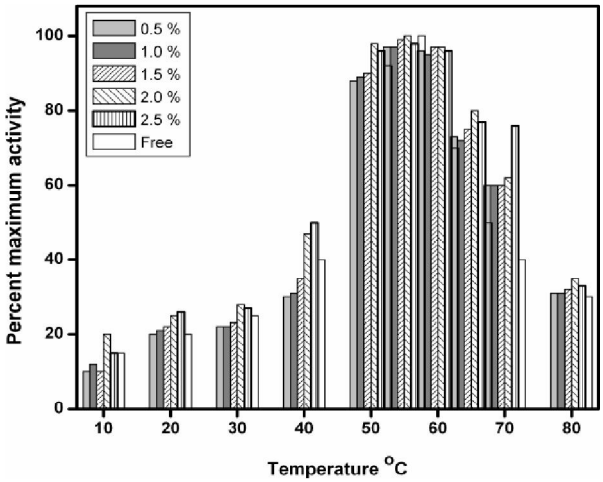

Figure 4a : Plot of percent maximum activity vs. temperature stability for immobilized urease in calcium alginate beads by changing sodium alginate concentration at constant amount of calcium chloride (2%)

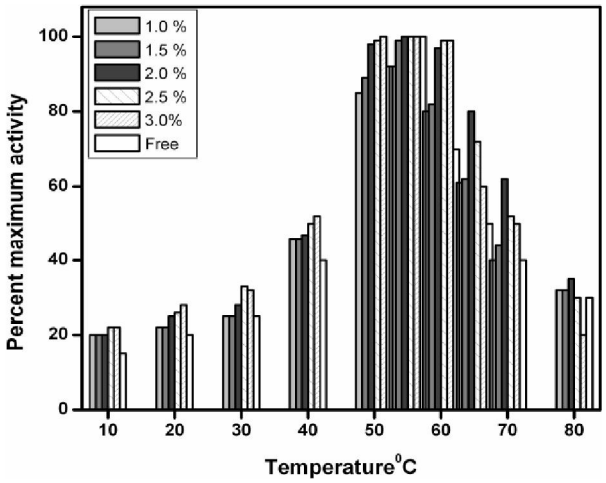

Figure 4b : Plot of percent maximum activity vs. temperature stability for immobilized urease in calcium alginate beads by changing calcium chloride concentration at constant amount of sodium alginate (2%)

bead formation, the thickness of the membrane and the diameter decrease for a given calcium chloride concentration. This effect is presumably due to the fact that on increasing the number of biopolymer molecules per unit solution volume, the number of binding sites for Ca^{2+} ions also increases. As a result, a more densely cross-linked gel structure will probably form and, consequently, it will have a lower thickness. On the other hand, on increasing the calcium chloride concentration (1% to 3% w/v) the thickness and diameter of the bead increases for a given sodium alginate concentration. This behaviour also confirms that the membrane thickness increases continuously until complete consumption of the calcium ions. Similar results were observed by Blandino et al.[23] by immobilizing glucose oxidase in alginate.

In general all these results can be explained by taking into consideration the gel formation process, which is assumed to be an almost instantaneous and irreversible process that is governed by the diffusion of the two components involved in it: sodium alginate and Ca^{2+} ions.

Storage stability

Enzyme stability is one of the factors affecting productivity. Enzymes are very delicate, and lose their activity during storage. From storage studies, increased rate of urease leaching was observed at lower concentrations of alginate (Figure 3a). Therefore, the concentration of sodium alginate was fixed at 2% for subsequent experiments. Similar results were obtained for calcium chloride concentration (Figure 3b). As can be seen, the immobilization increases storage stability of the enzyme and 2% of sodium alginate/calcium chloride concentration proved to be ideal in both cases. The activity of enzyme remains about 45% of the initial activity after 35 days whereas in case of free it drops down to 40% of its initial value after 15 days.

pH stability

The pH optimum of the free and immobilized urease was determined by using three buffer systems, i.e., citrate acetate (0.1M, pH 3.5–5.5), phosphate (0.2 M, pH 6.0-8.0), and *Tris*-HCl (0.1 M, pH 7.0–9.0) buffers. The immobilized and free urease was incubated in 1ml of phosphate buffer with 1 ml of urea solution (0.5 M) in a test tube at 55 °C. It was found that the calcium alginate beads formed by sodium alginate and $CaCl_2$ (2% concentration of each) were stable only in the range of 6-8 pH, in rest pH ranges beads were disrupted.

Thermal stability

The effect of temperature on the urease activity is shown in figure 4a & b. The optimum activity for free urease was obtained at 55°C and where as for immobilized urease 55-60°C. At 65°C the free and immobilized urease in 2% of each concentration of sodium alginate/calcium chloride retained their activity to a level of 50% and 80% during a 30-min incubation period. Increasing temperature beyond 60°C and below 50°C

Regular Paper

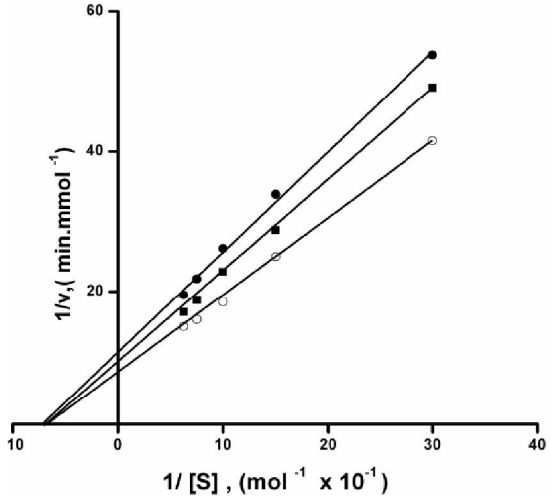

Figure 5 : Lineweaver–Burk plot for inhibition of urease immobilized in calcium alginate beads by Cu^{+2} and Hg^{+2}:(○) no inhibitor,(■)Cu^{+2}, (•) Hg^{+2}

shows reduced activity, at temperature beyond 60°C probably because they became more rigid at high temperature. If the thermal stability of an enzyme were enhanced by immobilization, the potential utilization of such enzymes would be extensive. In principle, the thermal stability of an immobilized enzyme revealed the affinity of urease to urea can be enhanced, diminished, or unchanged relative to free counterparts, and several examples for each kind have been previously reported[24].

Effects of inhibitors on kinetic parameters

The effect of Hg^{2+} and Cu^{2+} on the inhibition activity of free and alginate entrapped urease was studied in the concentration range that had measurable inhibition. In figure 5 the straight lines intersecting at x-axis, shows noncompetitive inhibition for both the Hg^{2+} and Cu^{2+}. In this kind of inhibition V_{max} modified by the inhibitor and the effect was more pronounced for Hg^{2+} compared to Cu^{2+}. The values are reported in TABLE 2. The maximum activity, V_{max}, decreased significantly upon inhibition of urease. The V_{max} value of the free urease was found to be 1.17×10^{-1} mmol/min/mg enzyme, whereas the V_{max} values of the enzyme inhibited with Cu^{+2} and Hg^{+2} were estimated from the data as 1.00×10^{-1} and 0.87×10^{-1} mmol/min/mg respectively. There was no effect observed on the value of K_m (1.48mM) by both the heavy metal ions. The literature data clearly show that heavy metal cations strongly interfere by the inhibition of urease as they strongly inhibit enzymatic reaction of urea hydrolysis[25-28].

TABLE 2 : Kinetic parameters for inhibition of urease immobilized in calcium alginate beads by Cu^{+2} and Hg^{+2}

Inhibitor	K_m , mol	V_{max} , mmol/min/mg enzyme$\times10^{-1}$
Free enzyme	1.48	1.17
Cu^{+2}	1.48	1.00
Hg^{+2}	1.48	0.87

ACKNOWLEDGEMENT

Anita Bahadur thanks U.G.C. India for Major Research Project[F.No.33-347/2007 (SR)] for financial assistance.

REFERENCES

[1] A.M.Klibanov; Science, **219**, 722 **(1983)**.
[2] H.Tanaka, M.Matsumura, I.A.Veliky; Biotechnol. Bioeng., **26**, 53 **(1984)**.
[3] C.Le-Tien, M.Millette, M.Lacroix, M.A.Mateescu; Biotechnol.Appl.Biochem., **39**, 189 **(2004)**.
[4] B.Krajewska; Journal of Molecular Catalysis B: Enzymatic, **59**, 9 **(2009)**.
[5] B.Krajewska; Journal of Molecular Catalysis B: Enzymatic, **59**, 22 **(2009)**.
[6] J.B.Nielands, P.K.Stumpf; 'Outline of Enzyme Chemistry', Wiley, New York, 411 **(1958)**.
[7] Y.Chun-lu, S.Tie-hang, H.E.Wen-xiang, Z.Q.Xing, C.Su; Journal of Environmental Sciences, **19**, 210 **(2007)**.
[8] W.H.R.Shaw, D.N.Raval; J.Am.Chem.Soc., **83**, 3184 **(1961)**.
[9] E.C.Toren, F.J.Burger; Mikrochim.Acta, (Wien), **5**, 1049 **(1968)**.
[10] B.Krajewska; J.Chem.Technol.Biotechnol., **52**, 157 **(1991)**.
[11] C.Preininger, O.S.Wolfbeis; Biosens.Bioelectron., **11**, 981 **(1996)**.
[12] K.Oehlschläger, R.Huttl, G.Wolf; Thermochim.Acta, **310**, 185 **(1998)**.
[13] E.Wittekind, M.Werner, A.Reinicke, A.Herbert, P.Hansen; Environ.Technol., **17**, 597 **(1996)**.
[14] F.Winquist, I.Lundström, B.Danielsson; Anal.Lett., **21**, 1801 **(1988)**.
[15] G.Schwedt, D.O.Waldheim, K.D.Neumann, K.Stein; Fresenius J.Anal.Chem., **346**, 659 **(1993)**.
[16] D.Narinesingh, R.Mungal, T.T.Ngo; Anal.Chim. Acta, **292**, 185 **(1994)**.
[17] O.H.Lowry, N.J.Rosebrough, A.L.Farr, R.J.Rasdall;

J.Biol.Chem., **193**, 265 **(1951)**.
[18] J.Jayaraman; 'Laboratory Manual in Biochemistry', Wiley Eastern Limited, New Delhi, 130 **(1981)**.
[19] A.Idris, W.Suzana; Proc.Biochem., **41**, 1117 **(2006)**.
[20] G.Dey, B.Singh, R.Banerjce; Braz.Arch.Biol. Techn., **46**, 167 **(2003)**.
[21] Z.Konsoula, M.Liakopoulu-Kyriakides; Proc.Biochem., **41**, 343 **(2005)**.
[22] S.Devi, P.Sridhar; World J.Microbiol.Biotechnol., **15**, 185 **(1999)**.
[23] A.Blandino, M.Macýas, D.Cantero; Enzyme Micro.Technol., **27**, 319 **(2000)**.
[24] M.Y.Arýca, V.Hasýrc, N.G.Alaeddinoglu; Biomaterials, **14**, 806 **(1993)**.
[25] T.K.Krawczyk, M.Moszczynska, M.Trojanowicz; Biosen.Bioelec., **15**, 681 **(2000)**.
[26] G.Schwedt, D.O.Waldheim, K.D.Neumann, K.Stein; Fresenius J.Anal.Chem., **346**, 659 **(1993)**.
[27] C.Preininger, B.Danielsson; Analyst, **121**, 1717 **(1996)**.
[28] A.Prell-Swaid, G.Schwedt; Acta Hydrochim.Hydrobiol., **22**, 70 **(1994)**.