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Immobilization Imparts Stability to Watermelon Urease to Work in Water Miscible Organic Media

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Abstract The behaviour of alginate immobilized and soluble watermelon (*Citrullus vulgaris*) urease in water miscible organic solvents like, acetonitrile, dimethylformamide (DMF), ethanol, methanol, and propanol is described. The organic solvents exhibited a concentration dependent inhibitory effect on both the immobilized and the soluble urease in the presence of urea. Pretreatment of soluble enzyme preparations with organic solvents in the absence of substrate for 10 min at 30°C led to rapid loss in the activity, while similar pretreatment of immobilized urease with 50% (v/v) of ethanol, propanol, and acetonitrile was ineffective. Time-dependent inactivation of immobilized urease, both in the presence and in the absence of urea, revealed stability for longer duration of time even at very high concentration of organic solvents. The soluble enzyme, on the other hand, was rapidly inactivated even at fairly lower concentrations. The results suggest that the immobilization of watermelon urease in calcium alginate make it suitable for its application in organic media. The observations are discussed.

Keywords: urease, immobilized urease, water miscible solvents, watermelon, *Citrullus vulgaris*

INTRODUCTION

Over the past few decades non-aqueous enzymology has emerged as a major area of biotechnology research and development. Awareness of and interest in the use of enzyme in organic media started in 1980's [1] even though its root lies somewhere in 1930's with the attempts of Ernest Aleksander Sym, which was not very much successful. "Organic media results into denaturation of enzymes" was practically proved to be not true by many scientists working in the field of non-aqueous enzymology and shown that various crystalline enzymes retain their native structure in non-aqueous solvents [2-4]. Dramatic decrease in their catalytic efficiency and loss of substrate specificity, however, needs attention. Enzymes, as practical catalysts, are being exploited for synthetic transformation fueling growing demand of pharmaceutical, chemical and food industries [5]. Use of biocatalyst in organic media finds application in chemical processing, food related conversions and analyses. Several approaches such as biphasic liquid systems, reverse micelles and monophasic liquid systems utilizing various classes of enzymes such as oxidoreductases, isomerases, lyases, and hydrolases have been developed and are successfully being used in production process [6-9]. Not only enzymes but also abzyme and DNA in association with lipids have been studied for their activity and stability in organic media [10,11].

Most of the studies pertaining to the use of enzymes in organic media are restricted to either reverse micelle or lipid coated enzymes. Not much attention has been given on the study of physically immobilized enzymes in organic media. Lipase appears to be one enzyme which has gained maximum attention in the field of non-aqueous enzymology [1,7,12]. There still exists a need to study other enzymes too in non-aqueous media and explore their potentials.

Urease (urea amidohydrolase, EC 3.5.1.5) from watermelon (*Citrullus vulgaris*) seeds has been purified to apparent homogeneity and partially characterized [13]. We have also presented evidence for the presence of thiol groups on the active site of the enzyme. Its inactivation kinetics with thiol specific reagents showed biphasic kinetics in that half of the initial activity was destroyed more rapidly than the remaining half [14]. This unique phenomenon of molecular asymmetry or more specifically half-site reactivity in watermelon urease, reported for the first time, was further substantiated by thermal inactivation studies [15]. Earlier studies on jack bean urease have established that the thiols are inhibitors; β-mercaptoethanol (ME) being a competitive inhibitor in the hydrolysis of urea and dithiothreitol (DTT) being a poor inhibitor [16]. But we have recently demonstrated, for the first time, that not only ME but also DTT and L-cysteine at 30°C in 50 mM Tris-acetate buffer (pH 8.5) are excellent activators of the watermelon urease, and the order of effectiveness as activator was ME > DTT > L-cysteine [17]. Recently, we have immobilized watermelon

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urease in calcium alginate beads and detailed kinetic study has been performed [18]. In the present communication, we attempt to explore the potentials of alginate-immobilized urease in water miscible organic solvent system.

MATERIALS AND METHODS

Dehusked seeds of watermelon were purchased from the local market. Tris was obtained from Boehringer Mannheim Gmbh, Germany. Bovine serum albumin was obtained from Sigma Chemical Co., USA. Sephadex G-200 was from Pharmacia Fine Chemicals, Uppsala, Sweden. Ethanol was purchased from Shymlakhs International, London, UK. Acetonitrile and calcium chloride were obtained from E. Merck, India. Methanol, propanol, urea, Nessler's and Folin-Ciocalteau reagents were from Qualigens Fine Chemicals, Mumbai. *N,N*-Dimethyl formamide was obtained from Ranbaxy Laboratories Limited, New Delhi, India. All other reagents were analytical grade chemicals either from BDH or E. Merck, India. All the reagents were prepared in double distilled water from an all glass assembly.

Enzyme

Urease was isolated and purified from dehusked seeds of watermelon (*C. vulgaris*) as described earlier [17]. The enzyme preparation (sp. activity 3,000 ± 550 U/mg protein), showing a single enzyme and protein band on native 7.5% PAGE (at pH 8.3), was employed for the study.

Immobilization of Urease

Suitably diluted urease solution (0.7 mg protein/mL) was mixed in chilled 3.5% (w/v) sodium alginate solution and dropped into 100 mL of chilled and continuously stirring 400 mM calcium chloride solution with the help of a micropipette [18]. The beads formed were allowed to stir for 90 min for complete calcium alginate formation. Beads were collected, washed thoroughly with 25 mM Tris-acetate buffer (pH 7.5) to remove excess Ca^{2+} and stored in the same buffer at 4°C. During the course of immobilization, the left over calcium chloride solution was analysed for protein and enzyme activity to ascertain leaching, if any.

Urease Activity Assay

For routine measurement of soluble urease activity, the amount of ammonia liberated during a fixed time period at a saturating concentration of urea was determined as described earlier [13]. An aliquot (0.8 mL) of 50 mM Tris-acetate buffer (pH 8.5) and 1.0 mL of 250 mM urea in the same buffer were brought to 30°C. The reaction was started by adding 0.2 mL of suitably diluted enzyme. After 10 min, 1.0 mL of 10% trichloroacetic acid was added to stop the reaction. The total reaction mixture was transferred to a measuring flask (50 mL), and the volume was made to 50 mL with distilled water after adding 1.0 mL of Nessler's reagent as described earlier [17]. The yellow-orange colour produced was measured at 405 nm in a Spectronic 21 UVD spectrophotometer.

One enzyme unit was defined as the amount of enzyme required to liberate one μmol of ammonia in one min under the test conditions defined above (30°C, 50 mM Tris-acetate buffer, pH 8.5, 250 mM urea). Protein concentration was estimated by the method of Lowry [19] with Folin-Ciocalteau reagent calibrated with crystalline bovine serum albumin.

For assay of immobilized enzyme, the beads were incubated at 30°C for 10 min in standard assay medium comprised of 50 mM Tris-acetate buffer (pH 8.5) containing 250 mM urea. Following incubation, an aliquot of 1.0 mL was withdrawn from the reaction mixture and assayed as described above. The beads were recovered from the reaction mixture, washed thoroughly with the buffer and stored at 4°C.

Effect of Organic Solvents

Effect of various water miscible organic solvents on the activity of immobilized and soluble urease was studied in the presence of various concentrations of organic solvent added in the standard assay mixture. For the direct effect of organic solvents, enzyme preparation alone was incubated with the desired concentration of organic solvent for 10 min at 30°C and the treated enzyme preparation was assayed for activity. Time course of effect of organic solvents was studied for soluble and immobilized urease both in the presence and in the absence of urea. The enzyme preparation was incubated at 30°C with the desired concentration of organic solvent added in the standard assay mixture. The aliquots/beads were withdrawn at specific time intervals and assayed.

The results reported are mean of 5~7 replicate experiments carried out with fresh batch of purified and immobilized enzyme.

RESULTS AND DISCUSSION

Effect of various water miscible organic solvents like acetonitrile, ethanol, methanol, propanol, and DMF on alginate immobilized watermelon urease was investigated by adding desired concentrations (0~50%, v/v) of organic solvent in the standard reaction mixture comprising of 50 mM Tris-acetate buffer (pH 8.5) and 250 mM urea. The results (Fig. 1) revealed a concentration-dependent effect of organic solvents on alginate-immobilized urease. There was almost complete loss of the activity at 50% concentration of DMF, methanol, and propanol. On the other hand, the enzyme still retained about 40% activity in acetonitrile and ethanol.

In another set of experiments, the beads of the immobilized urease were first exposed to the various concentrations of organic solvents, in the absence of urea, for 10 min at 30°C and were then transferred to the standard assay system and assayed for the residual activity. It was

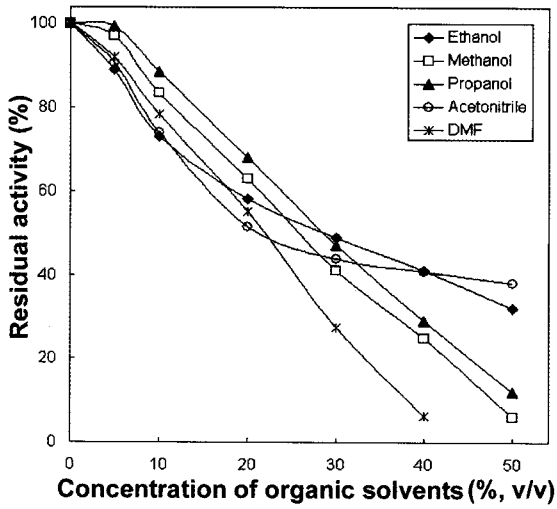

Fig. 1. Effect of organic solvents on immobilized watermelon urease. One bead (3 μg protein/bead) was incubated at 30°C in the presence of varying concentrations of organic solvents added in the standard assay mixture comprising of 50 mM Tris-acetate buffer (pH 8.5) and 250 mM urea.

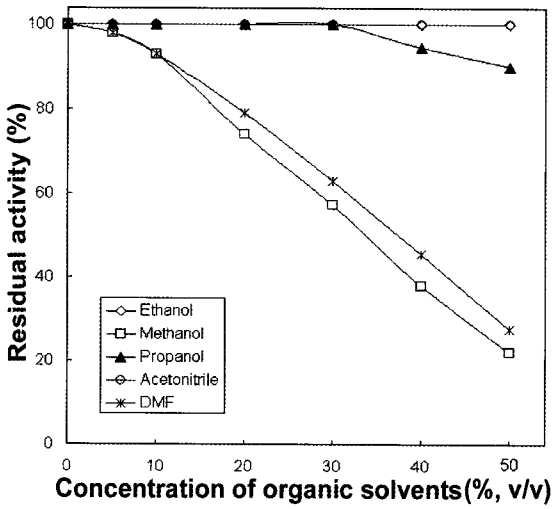

Fig. 2. Effect of organic solvents on immobilized watermelon urease. Desired number of beads (3 μg protein/bead) was treated with varying concentrations of organic solvents for 10 min at 30°C. One bead was immediately transferred into the standard assay mixture and assayed for the residual activity.

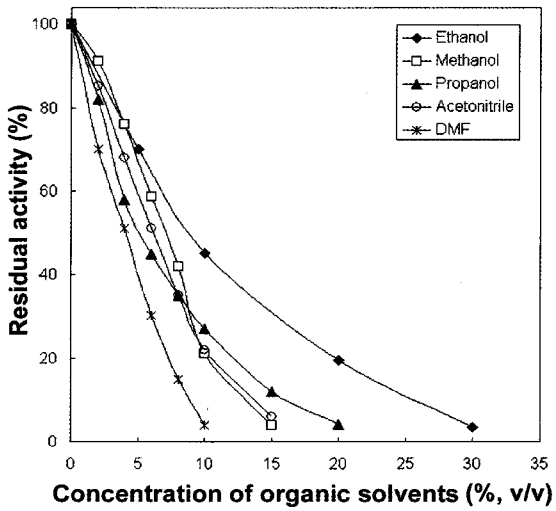

Fig. 3. Effect of organic solvents on soluble watermelon urease. Suitably diluted enzyme (9~10 U/mL, 6~7 μg protein/mL) was incubated at 30°C in presence of varying concentrations of organic solvents added in the standard assay mixture comprising of 50 mM Tris-acetate buffer (pH 8.5) and 250 mM urea.

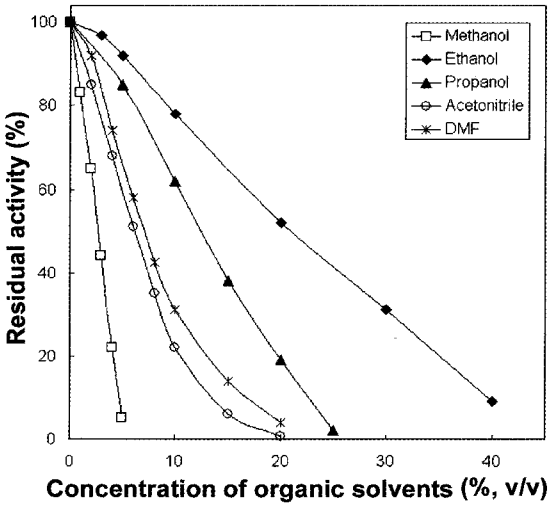

Fig. 4. Effect of organic solvents on soluble watermelon urease. Suitably diluted enzyme (9~10 U/mL, 6~7 μg protein/mL) was treated with varying concentrations of organic solvents for 10 min at 30°C. 200 μL of enzyme was immediately transferred into the standard assay mixture and assayed for the residual activity.

found that the immobilized enzyme was fairly stable to direct exposure to the organic solvent in absence of the substrate. Almost 100% activity was retained when the beads were exposed directly to the 50% concentration of ethanol, propanol, and acetonitrile for 10 min (Fig. 2).

On the other hand, only 30% of the initial activity was retained on direct interaction of the immobilized enzyme with 50% methanol and DMF. These observations suggest that the organic solvents affect the activity of the immobilized urease severely in the presence of the substrate. It is probable that urea facilitates percolation of organic solvents in the alginate beads and results in the replacement of the aqueous layer around the enzyme with organic solvent thereby suppressing the activity.

Similarly, the probable effect of various organic solvents on the soluble urease was studied in the presence of organic solvents added in the standard assay mixture (Fig. 3) as well as by pretreating the enzyme for 10 min at 30°C with desired concentrations of organic solvents in

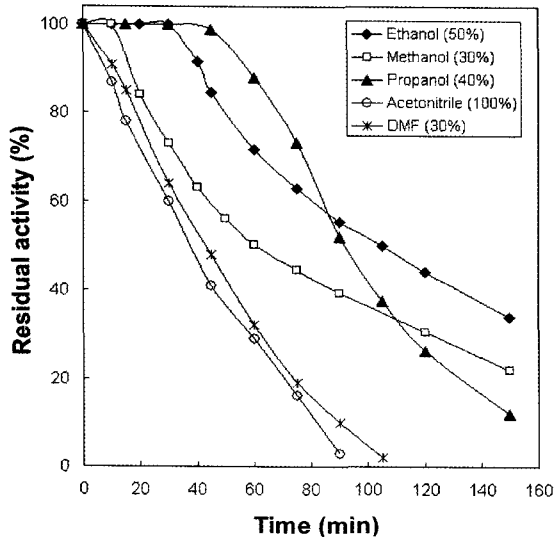

Fig. 5. Time-dependent effect of organic solvent on immobilized urease. Desired number of beads (3 μg protein/bead) was incubated with desired concentrations of organic solvents (in absence of urea) at 30°C for varying time intervals. One bead was withdrawn at specified time interval and assayed for the residual activity by immediately transferring into the standard assay mixture.

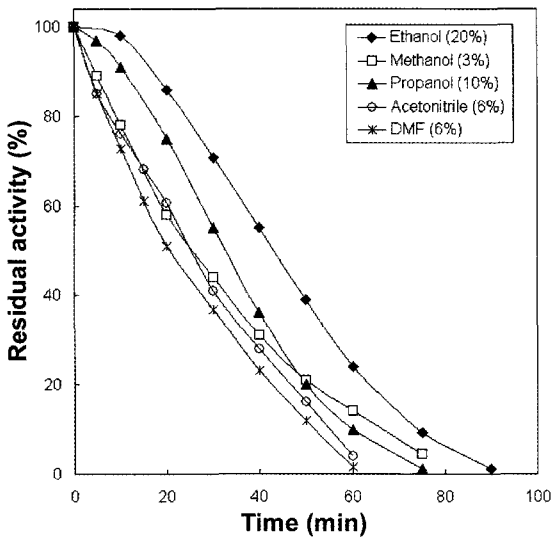

Fig. 6. Time-dependent effect of organic solvent on soluble urease. Suitably diluted enzyme (9~11 U/mL, 6~7 μg protein/mL) was incubated with desired concentrations of organic solvents (in absence of urea) at 30°C for varying time intervals. 200 μL of enzyme was withdrawn at specified time interval and assayed for the residual activity by immediately transferring into the standard assay mixture.

the absence of the substrate and then assaying the residual activity (Fig. 4). The results revealed a concentration dependent decline in the activity of the soluble urease in the presence as well as in the absence of urea, respectively. Moreover, the stability of the soluble urease in the presence of urea was also reduced as compared with the enzyme in the absence of urea. Soluble urease became almost inactive at much lower concentrations of the hydrophilic solvents than the corresponding alginate-immobilized enzyme. However, soluble enzyme appeared to be slightly more stable in ethanol than the other solvents. DMF and methanol appeared to be fairly toxic to the soluble enzyme. Hence, this may be one of the probable reasons for employing ethanol and acetone in enzyme purification process.

Upon comparison of the alginate-immobilized enzyme with that of the soluble one (Figs. 1 and 3), immobilized enzyme exhibited about 50 and 75% residual activity in the presence of 30% ethanol and 10% acetonitrile, respectively. But for the same concentration of the solvents, soluble enzyme exhibited only 3.4 and 22% residual activity, respectively. Moreover, ethanol, propanol, and acetonitrile at a concentration of 50% could not affect the immobilized urease (even after direct exposure for 10 min). On the other hand, these solvents completely suppressed the activity of the soluble enzyme under similar experimental conditions (Figs. 2 and 4). Comparative analyses of soluble and immobilized enzyme reveal that immobilization imparts added stability to the enzyme in organic media. Such enhanced stability has also been reported for catalytic monoclonal (lipase-like) antibody immobilized on an inorganic support (porous glass beads) [20], papain immobilized on CM-Cellulose and chymotrypsin immobilized on alginate [21]. This stability could be an outcome of interplay of water activity and support. The correlation between support and water activity was investigated by Adlercreutz [22] using immobilized enzymes in organic solvents. Horse liver alcohol dehyrogenase and chymotrypsin exhibited greatly different activities in organic solvents when immobilized to different supports with the same water activity [22]. The significance of support to immobilized enzyme activity has also been demonstrated by Khmelnitsky *et al.*, who studied the effect of non-buffer salts on the transesterification activity of subtilisin Carlsberg in anhydrous hexane [23].

Time-dependent inactivation of immobilized urease upon direct exposure to the organic solvent (in the absence of substrate) was studied next by incubating the beads of the alginate immobilized urease in the desired organic solvent at a concentration at which 50% activity was retained (at 30°C). Beads were withdrawn at specified time intervals, transferred immediately to the standard assay medium and assayed for the residual activity. The results (Fig. 5) revealed that immobilized enzyme retained its complete ureolytic activity even though the beads were exposed for 45 and 30 min in 40% propanol and 50% ethanol respectively, and then gradually declined (12 and 34% activity was still retained even after the end of 150 min of incubation, respectively). Interestingly, immobilized enzyme was fairly stable in 50% (v/v) acetonitrile for 3 h with no measurable loss in the activity. Therefore, neat acetonitrile was used (100% solvent only). As is evident in the Fig. 5, it took 90 min for complete

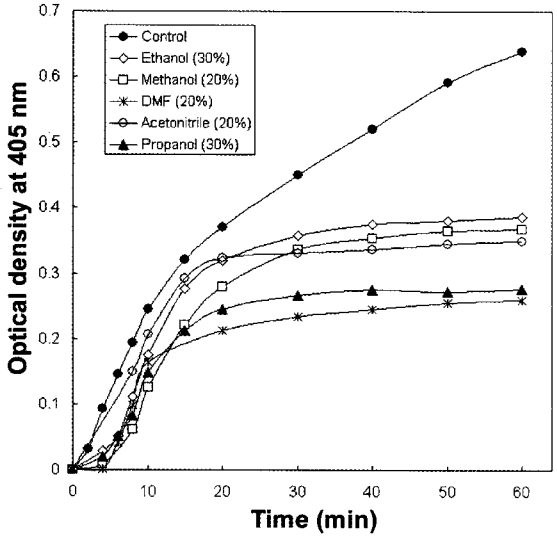

Fig. 7. Time-dependent hydrolysis of urea by immobilized urease in presence of organic solvent. Desired concentrations of organic solvent were added into the standard assay mixture containing urease beads (3 μg protein/bead). 100 μL of the reaction mixture was withdrawn at specified time interval and employed for colour development by treating with Nessler's reagent.

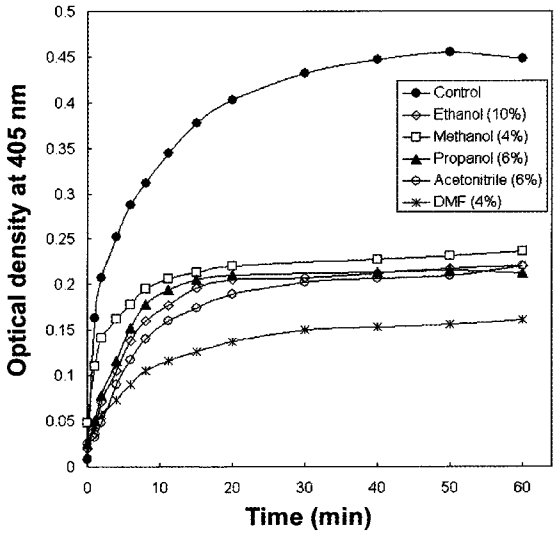

Fig. 8. Time-dependent hydrolysis of urea by soluble urease in presence of organic solvent. Desired concentrations of organic solvent were added into the standard assay mixture containing suitably diluted urease (9~10 U/mL, 6~7 μg protein/mL). 100 μL of the reaction mixture was withdrawn at specified time interval and employed for colour development by treating with Nessler's reagent.

inactivation of the activity in 100% acetonitrile too. However, DMF and methanol (both at 30%) were not found to be suitable organic media for immobilized urease.

Time course of inactivation of soluble urease (in the absence of substrate) following direct exposure to the organic solvents was similarly explored by incubating suitably diluted urease solution in the desired organic solvent at 30°C. Two hundred μL of enzyme samples were withdrawn at each specific time interval, transferred immediately to the standard assay medium and assayed. As is apparent (Fig. 6), the soluble urease was inactivated by fairly low concentrations of organic solvents. Thus, methanol (3%), acetonitrile, and DMF (both at 6%) completely inactivated the enzyme within 60 min of study. Maximum stability was observed only in ethanol (20%) where the enzyme retained its full activity for an initial 10 min and then declined gradually up to 90 min. Upon further increase in the concentration of organic solvents, the rate of inactivation increased drastically. The results (Figs. 5 and 6) thus indicate that immobilization not only imparts added stability in the organic media but also allows continuous ureolysis for a longer duration of time.

It was of interest to explore the persistence of the catalytic activity of the enzyme in the presence of organic solvents. This was done by quantitating the ammonia produced through time-dependent urea hydrolysis by immobilized urease in presence of miscible solvents. The desired concentration of organic solvents (at which 50% activity was retained in Fig. 1) was added to the standard assay mixture comprising of 50 mM Tris-acetate buffer (pH 8.5) containing 250 mM urea and immobilized urease beads (at 30°C). One hundred μL reaction mixture was withdrawn at specific time interval and treated with Nessler's reagent to develop color. The result (Fig. 7) revealed that the rate of ureolysis was maximum in ethanol (30%) and minimum in DMF (20%). The rate of urea hydrolysis in ethanol, methanol, acetonitrile, and propanol progressed for 20 min and then attained a steady state for rest of the period of study. However, in DMF, the activity progressed only for 10 min and then a plateau was obtained. The soluble enzyme under the similar conditions, on the other hand (Fig. 8), hydrolysed urea for 10 min (at a much slower rate) and then attained a steady state. Unlike immobilized enzyme, soluble enzyme was active only in the lower concentration range of organic solvents.

In the present communication we report for the first time that immobilization of watermelon urease in alginate beads imparts stability making it suitable to work in water miscible organic solvents. Such enhanced stability has also been demonstrated for catalytic antibody immobilized on an inorganic support [20], papain immobilized on CM-Cellulose and chymotrypsin immobilized on alginate [21]. In addition, immobilization also leads to some conformational change in the enzyme [24]. Chymotrypsin immobilized to cyanogen bromide-Sepharose exists in distinct form that has a different active site conformation and very different intrinsic specific activity [25]. Moreover, choice of matrix also controls the properties of immobilized enzyme, *e.g.*, horse liver alcohol dehydrogenase immobilized to hydrophobic and hydrophilic support exhibited significantly different substrate specificities and also expressed different activities in different organic solvent systems [26]. With due consideration of the above,

the difference in the behaviour of watermelon urease immobilized in alginate beads in water miscible organic media, therefore, is expected.

REFERENCES

[1] Zaks, A. and A. M. Klibanov (1984) Enzymatic catalysis in organic media at 100°C. *Science* 224: 1249-1251.

[2] Griebenow, K. and A. M. Klibanov (1996) On protein denaturation in aqueous-organic mixtures but not in pure organic solvents. *J. Am. Chem. Soc.* 118: 11695-11700.

[3] Schmitke, J. L., L. J. Stern, and A. M. Klibanov (1997) The crystal structure of subtilisin Carlsberg in anhydrous dioxane and its comparison with those in water and acetonitrile. *Proc. Natl. Acad. Sci. USA* 94: 4250-4255.

[4] Zhu, G., Q. Huang, Z. Wang, M. Qian, Y. Jia, and Y. Tang (1998) X-ray studies on two forms of bovine β-trypsin crystals in neat cyclohexane. *Biochim. Biophys. Acta* 1429: 142-150.

[5] Zaks, A. and D. R. Dodds (1997) Application of biocatalysis and biotransformations to the synthesis of pharmaceuticals. *Drug Discov. Today* 2: 513-531.

[6] Luisi, P. L. and C. Laane (1986) Solubilization of enzymes in apolar solvents via reverse micelles. *Trends Biotechnol.* 4: 153-161.

[7] Klibanov, A. M. (1989) Enzymatic catalysis in anhydrous organic solvents. *Trends Biochem. Sci.* 14: 141-144.

[8] Zaks, A. and A. M. Klibanov (1988) Enzymatic catalysis in nonaqueous solvents. *J. Biol. Chem.* 263: 3194-3201.

[9] Carrea, G., G. Ottolina, and S. Riva (1995) Role of solvents in the control of enzyme selectivity in organic media. *Trends Biotechnol.* 13: 63-70.

[10] Okahata, Y., M. Yamaguchi, F. Tanaka, and I. Fujii (1995) A lipid-coated catalytic antibody in water-miscible organic solvents. *Tetrahedron* 51: 7673-7680.

[11] Tanaka, K. and Y. Okahata (1996) A DNA-lipid complex in organic media and formation of an aligned cast film. *J. Am. Chem. Soc.* 118: 10679-10683.

[12] Carrea, G. and S. Riva (2000) Properties and synthetic applications of enzymes in organic solvents. *Angew. Chem. Int. Ed. Engl.* 39: 2226-2254.

[13] Prakash, O. and G. Bhushan (1997) Isolation, purification, and partial characterization of urease from seeds of water melon (*Citrullus vulgaris*). *J. Plant Biochem. Biotechnol.* 6: 45-47.

[14] Prakash, O. and G. Bhushan (1998) A study of inhibition of urease from seeds of the water melon (*Citrullus vulgaris*). *J. Enzym. Inhib.* 13: 69-77.

[15] Prakash, O. and G. Bhushan (1998) Molecular asymmetry in urease of water melon (*Citrullus vulgaris*). *J. Plant Biochem. Biotechnol.* **7**: 53-55.

[16] Blakeley, R. L. and B. Zerner (1984) Jack bean urease: the first nickel enzyme. *J. Mol. Catal.* 23: 263-292.

[17] Prakash, O. and L. S. B. Upadhyay (2003) Effect of thiols on the activity of urease from dehusked seeds of watermelon (*Citrullus vulgaris*). *Plant Sci.* 164: 189-194.

[18] Prakash, O. and L. S. B. Upadhyay (2005) Physicochemical characterization of watermelon urease upon immobilization in alginate beads. *J. Plant Biochem. Biotechnol.* 14: 209-213.

[19] Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.

[20] Janda, K. D., J. A. Ashley, T. M. Jones, D. A. McLeod, D. M. Schloeder, and M. I. Weinhouse (1990) Immobilized catalytic antibodies in aqueous and organic solvents. *J. Am. Chem. Soc.* 112: 8886-8888.

[21] Anupma (1997) *Immobilization and Chemical Modification of Proteases.* Ph.D. Thesis. Banaras Hindu University, Varanasi, India.

[22] Adlercreutz, P. (1991) On the importance of the support material for enzymatic synthesis in organic media. Support effects at controlled water activity. *Eur. J. Biochem.* 199: 609-614.

[23] Khmelnitsky, Y. L., S. H. Welch, D. S. Clark, and J. S. Dordick (1994) Salts dramatically enhance activity of enzymes suspended in organic solvents. *J. Am. Chem. Soc.* 116: 2647-2648.

[24] Clark, D. S. and J. E. Bailey (1983) Structure-function relationships in immobilized chymotrypsin catalysis. *Biotechnol. Bioeng.* 25: 1027-1047.

[25] Clark, D. S. and J. E. Bailey (1984) Characterization of heterogeneous immobilized enzyme subpopulations using EPR spectroscopy. *Biotechnol. Bioeng.* 26: 231-238.

[26] Skerker, P. S. and D. S. Clark (1988) Catalytic properties and active-site structural features of immobilized horse liver alcohol dehydrogenase. *Biotechnol. Bioeng.* 32: 148-158.

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