



Immobilization of urease in alginate, paraffin and lac

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Abstract: The enzyme urease (EC.3.5.1.5) from jack bean meal was immobilized by various techniques, such as entrapment in calcium alginate gel spheres in aqueous suspension, lac impregnated muslin cloth as dry films and by embedding in paraffin wax impregnated muslin cloth. The activity of the free and immobilized enzymes as a function of pH, temperature, storage stability, kinetic parameters and periodic use were compared. The immobilized enzyme showed good storage stability. After repeated use, the alginate beads turned brown and deteriorated, hence the storage stability was not good. The paraffin films were preserved dry because during wet preservation, the film slightly softened and the protein leached out slightly. The alginate beads had moderate mechanical stability. The lac films were tougher than the paraffin wax films in terms of mechanical stability. The K_m and V_{max} values were altered after immobilization. The K_m values for calcium alginate and lac were low, while it was larger in paraffin film as compared to the free enzyme. This may be due to the fact that immobilization on calcium alginate and lac in presence of $CaCl_2$ and methanol exposed certain active sites of the urease. While immobilization on paraffin masks the active sites and may lead to reduced binding of the substrate.

Keywords: enzyme; immobilization; urease; calcium alginate; paraffin wax; lac.

INTRODUCTION

Immobilized enzymes are rooted deeply in the field of biotechnology due to their inherent properties, such as specificity, stability and reusability. The storage stability of free enzymes in solution is poor. Recovery from solution with their activity retained is not possible. Amongst supports used for immobilization, biological macromolecules (such as cellulose, chitin and chitosan) and their derivatives have been studied the most extensively through physical adsorption or chemical linkages.^{1–4} A few reports have appeared on the immobilization of enzymes on cellulosic supports, calcium alginate gel-spheres and paraffin wax.^{4–7} Studies are reported on the use of polymeric supports in the form of granules,

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powder, membrane, fibers and tubes.⁷⁻¹² Immobilization of chymotrypsin and trypsin on spherical particles of an anionic polymeric latex were also reported previously.^{13,14} Urease was immobilized earlier on many supports, such as PVC, cellulose, alginate¹⁵⁻¹⁸ and some natural seed coats.¹⁹ The present investigation was undertaken with the view of studying the retention of urease activity, reusability, storage stability, changes in kinetic parameters and also superiority of the matrices, *viz.*, calcium alginate beads, paraffin film and reinforced lac film for immobilization. Paraffin wax (m.p. 58–60 °C) is non-toxic and therefore finds diverse uses, including medication, *etc.*^{20,21}

Hitherto, there have been no reports of immobilization of enzymes on bio-materials such as natural lac (shellac). It is a naturally occurring resinous substance (chiefly derived from polysaccharides) and is a mixture of resin 60 %, coloring matter 10 %, wax 6 %, gum 5.5 %, sugar 4 % and remaining mud and dirt. Lac being non-toxic was tried for immobilizing enzymes. In this paper, the results of a systematic study of native and immobilized urease on different matrices and the effects of various factors, such as pH, temperature, storage stability and reusability on enzyme activity, are reported.

EXPERIMENTAL

Materials and methods

The enzyme urease from jackbean meal and its substrate urea (Loba Chem. Pvt. Ltd. India.) were used. Sodium alginate, Nessler's reagent, tris buffer (0.20 M) and all other employed chemicals were of Analar grade. Paraffin wax (m.p. 58–60 °C) was a Ranbaxy product and lac was obtained from a lac infected tree belonging to the genera *Albizia lebbek* and was used after purification by solvent extraction in methanol. Muslin cloth (Tata fabric pure cotton) was used for reinforcing.

Preparation of urease immobilized in calcium alginate gel spheres

Sodium alginate (200 mg) with 10 mg of urease in 10 ml aqueous medium was thoroughly mixed until a honey-like consistency was attained. The mixture was then filled in a syringe and allowed to drop into 50 ml of CaCl₂ solution (2 % w/v) from a constant height to form beads.⁷ The beads were then stirred slowly for 20 min and then removed from the solution and subsequently washed with buffer before use.

Preparation of immobilized enzyme in reinforced paraffin film

To 4.0 g of molten paraffin wax in a thermostated water bath (at 65 °C), 1.0 g of enzyme powder was added under continuous stirring at a low speed. Sun dried muslin cloth previously washed in distilled water was cut into 1 cm² pieces which were added to the continuously stirred molten wax at 65 °C. After 3–5 s, these pieces were removed using pointed B. B. forceps, slightly shaken to remove dripping wax and allowed to dry at room temperature. In this way, 1 cm² reinforced films of paraffin wax with enzyme were made.

Procuring lac

Lac is produced by non-motile females of tiny insects belonging to the genera *Trachardia* or *Laccifer lacca* to protect themselves against adverse conditions of weather, especially winter. Lac insects are parasites on trees, *e.g.*, *Albizia lebbek*, *Schleicheia oleosa*, *S.*

trijuga, *Acacia catechu*, *A. arabica*, *Zizyphus jujuba*, *Z. mauritiana*, *Grewia sp.*, etc. *Albizia lebbeck*, *Schleicheia oleosa*, *S. trijuga*, *Accacia catechu*, *A. arabica*, *Zizyphus jujuba*, *Z. mauritiana*, *Grewia sp.*, *Ficus religiosa*, *Shorea talura*, *Butea monosperma*, *B. frondosa*, etc.

Natural lac, procured by scrapping infected twigs, was melted, squeezed through a cloth to obtain buttons. These were then dissolved in methanol and kept for 2 days. The thus obtained clear solution was centrifuged at high speed for 20 min to allow settling of impurities, if any. The clear supernatant was then poured into a porcelain evaporating dish and the methanol was allowed to evaporate in open air for 2 days, whereby a crust of pure crystalline lac was obtained on the evaporating dish. This was scrapped and used for the experiments.

Preparation of immobilized enzyme in reinforced lac

Purified lac (2.0 g) was dissolved in 5 ml of methanol. To this was added 1.0 g of urease powder and the mixture was stirred continuously on a magnetic stirrer. Cloth squares (as previously treated) were dipped into this mixture for 3–5 s and then allowed to dry and harden for a day at room temperature. These films were then weighed to find the amount of trapped enzyme. The enzyme in the washing was measured colorimetrically using the Lowry method²² to obtain the amount of bound enzyme using BSA as the standard.

Assay of native urease

For measurement of the urease activity, the ammonia liberated on incubating the enzyme with urea for a fixed time was determined using Nessler's reagent.¹⁹ One unit of urease activity liberates 1.0 mol of ammonia per min from 0.10 M urea under standard assay conditions.

Assay of immobilized urease

The alginate beads (17 beads) with urease entrapped were taken in 1 ml phosphate buffer (0.20 M, pH 7) solution and reacted with 1.0 ml of a 3 % urea solution for 15 min. The same procedure was used to follow the reaction and the measurements were performed in the same manner as for the native enzymes studies. Here the beads were removed before chilling the solution and adding H₂SO₄ to stop the reaction.

One film of paraffin wax and lac were each taken in 1 ml phosphate buffer (0.20 M, pH 7) and reacted with 1.0 ml of a 3 % urea solution for 30 min. The same procedure was used to follow the reaction and the measurements were performed as in the above-described experiment. The films were taken out from the reaction mixture to stop the reaction. The experiments were conducted in triplicate to confirm the reproducibility of the results.

Storage stability

For storage stability studies, the immobilized enzyme was kept at room temperature. The activity was measured for a month. Fresh preparations of immobilized enzymes were taken as controls for each assay. The enzyme immobilized on paraffin wax films was preserved in buffer, distilled water and under dry condition while the enzyme entrapped in beads was preserved in CaCl₂ solution because wet beads gave better result than dry beads.

Thermal stability

To determine the optimal temperature up to which the immobilized enzyme can withstand thermal stress, free and immobilized enzyme were suspended in phosphate buffer (0.20 M, pH 7) and incubated at different temperatures (30 to 80 °C) for 30 min before the activity was measured.

Determination of the kinetic parameters

For the determination of the kinetic parameters, the substrate concentration was varied and the optimum pH for native and immobilized enzyme was determined by varying the pH of the assay buffer. The relative enzyme activity was determined for each pH by the method described above for the assay of the enzyme. The absorbance was read at 500 nm. The rate of the reaction was measured as mmoles of ammonia produced / min / mg enzyme. The values of K_m and V_{max} were determined using Lineweaver–Burke plots.

RESULTS AND DISCUSSION

The structural integrity and activity of the urease were retained due to mild and precise physical condition during the immobilization procedure. The calcium alginate beads were chemically inert to the entrapped enzyme. The porosity of the gel was such that it allowed easy movement of the substrate molecules through the beads. The beads were quite stable in tris buffer (pH 7) and in phosphate buffer with pH > 7.8. However at higher pH values, the beads showed softening and stickiness of the surface. The entrapment of urease in alginate gel was approximately 60 %. The immobilized enzyme retained 41 % of its activity. Urease is a thermostable enzyme that is denatured at/above 80 °C²³ and, therefore, it could be easily immobilized on paraffin wax. Alginate beads are hydrophilic in nature while paraffin and lac are hydrophobic so only the surface molecules react. The reinforced films of paraffin were durable; the enzyme embedded could catalyze and retained 45–50 % of its activity.^{24,25}

As the above immobilization procedures were not applicable for thermolabile enzymes, immobilization on lac films was a step refinement as it involved extremely mild physical conditions. The lac films were tougher, thermostable and the enzyme could easily catalyze and retained 28–30 % of its activity.

The results of the studies performed with these preparations were compared with those of native enzymes to determine the utility of immobilized urease in the hydrolysis of urea.

Storage stability and reusability

The native enzyme retained the same activity for about seven days, after which it slowly decreased and was lost completely after a month. The enzyme immobilized on calcium alginate retained the same activity for a fortnight, then decreased and was lost in 30 days. The beads darkened (slightly brown) and deteriorated (beads were stored at room temperature in CaCl₂ solution).

The enzyme immobilized on paraffin wax films preserved in buffer retained the same activity for a week and had retained about 73 % of its activity after a month. When the films were preserved in distilled water, 70 % activity of the immobilized enzyme was observed even after a month. When preserved under dry condition, the enzyme retained 88 % of its activity. During wet preservation, the film softened somewhat and the protein leached out slightly (Fig. 1).

The storage stability of the enzyme immobilized on lac films when preserved in dry condition was the greatest and the immobilized enzyme had retained 98 % of its activity even after a month.

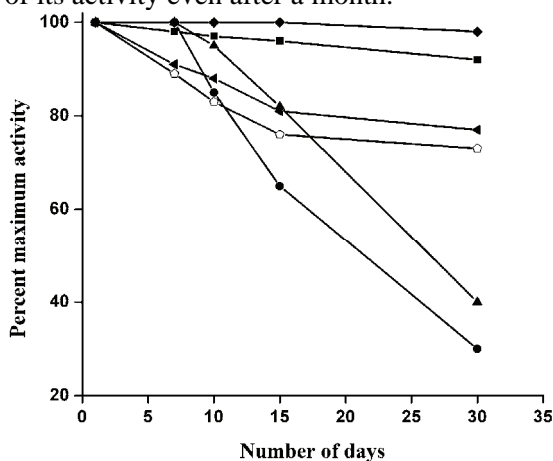


Fig. 1. Plot of the percentage of the maximum activity vs. time (days) for estimating the storage stability of urease on paraffin wax as dry films (■), films kept in buffer (◄) and films kept in distilled water; (△), native enzyme (●), Ca-alginate (▲), lac as a dry film (◆).

The immobilized enzyme lost activity after every use. The urease entrapped in alginate could be reused at the most five times, while the enzyme immobilized on paraffin and lac could be reused more than eight or nine times with less than 40 % activity. The native enzyme, being water soluble, could not be recovered and reused (Fig. 2).

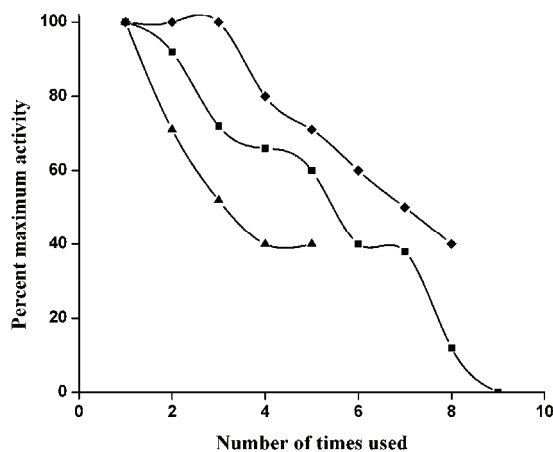


Fig. 2. Plot of the percentage of the maximum activity vs. the number of times used for reusability studies of urease immobilized on calcium alginate (▲), paraffin wax (■) and lac (◆).

Thermal stability

The temperature dependence of the relative activity of the enzyme is shown in Fig. 3. When kept for 30 min, the optimum temperature was found to be 55 °C for both the immobilized and native enzymes at pH 7, while the enzyme immo-

bilized on lac showed a comparatively higher relative activity up to 60 °C. Both the native and the immobilized enzyme were stable up to 70 °C, although a remarkable drop in percent maximum activity from 97 to 50 % was observed for the immobilized and native enzyme. Thermostability studies could not be realized for the enzyme immobilized on paraffin films (melting point 58–60 °C).

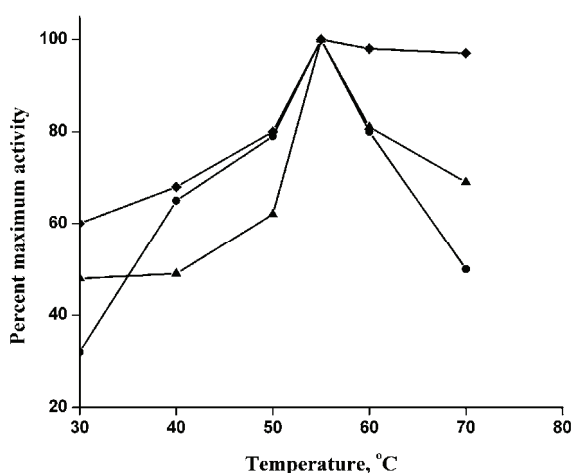


Fig. 3. Plot of the percentage of the maximum activity vs. temperature for temperature stability of free (●) and urease immobilized on calcium alginate (▲) and lac (◆).

Mechanical stability

Five to six beads of calcium alginate were centrifuged at 1200 rpm for 2–5 min. All the beads remained intact and none were broke or adhered. In addition, beads subjected to stirring for 5–10 min at slow speeds showed no breakage; similarly five to six films of paraffin wax were resistant to breakage when centrifuged but broke when subjected to stirring using a magnetic stirrer. The lac films were very tough, durable and highly resistant to mechanical wear when centrifuged as well as when stirred vigorously.

pH activity profile

The pH dependence of the relative activity of the enzyme was compared with that of the enzyme immobilized on the different supports. The reaction was performed in the pH range 5.6–7.8. The optimum pH for the native and the enzyme immobilized on calcium alginate, paraffin and lac were 6.8, 7.0, 7.0 and 7.2, respectively. This showed that immobilization shifted the optimum pH range in the basic direction by 0.2 units for calcium alginate and paraffin and 0.4 units for lac. Thus, there was an increased possibility of using urease over a broader range of pH (6.8–7.2) compared to native urease (Fig. 4).

Kinetic parameters

The kinetic behavior of native and the immobilized enzyme is shown in (Fig. 5). The values of K_m and V_{max} are given in Table I. They are in accordance with the Michaelis–Menten rate equation.

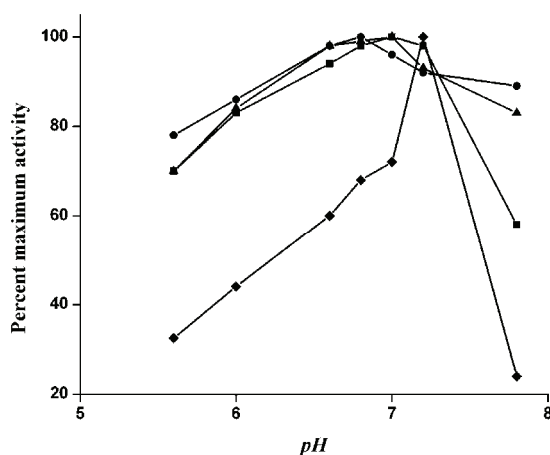


Fig. 4. Plot of the percentage of the maximum activity vs. pH, for the pH profile of free (●) and urease immobilized on calcium alginate (▲), paraffin wax (■) and lac (◆).

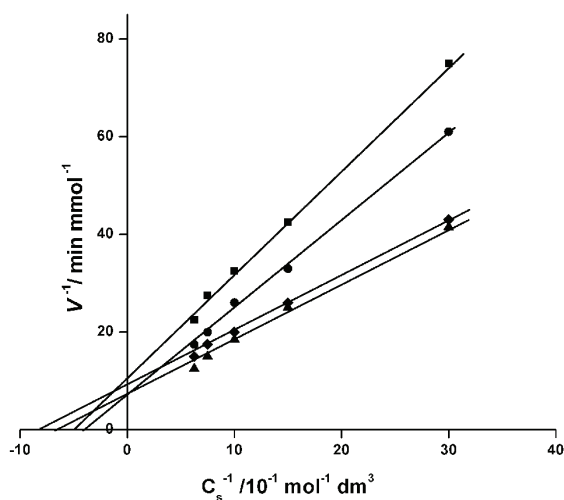


Fig. 5. Double reciprocal plot of rate V (mmol of NH_3 produced per min and mg of enzyme) vs. the concentration of urea for free (●) and urease immobilized on calcium alginate (▲), paraffin wax (■) and lac (◆).

TABLE I. Kinetic parameters of free and enzymes immobilized on calcium alginate, paraffin wax and lac

Support material	$K_m / \text{mol dm}^{-3}$	$V_{max} / \text{mmol min}^{-1} \text{mg}^{-1}$
Native enzyme	0.25	0.154
Ca alginate	0.18	0.154
Paraffin wax	0.20	0.100
Lac	0.13	0.125

It can be seen that the K_m values for the immobilized enzyme decreased, which shows that immobilization led to a masking of certain active sites of enzymes in all cases. The K_m values in the case of calcium alginate and lac as support materials were low compared when paraffin wax was used as the support. This might be attributed to the use of chemicals, viz. CaCl_2 and methanol, respectively, during the immobilization procedure.

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ИЗВОД

ИМОБИЛИЗАЦИЈА УРЕАЗЕ У АЛГИНАТУ, ПАРАФИНУ И ЛАКУ

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Ензим уреазе (EC.3.5.1.5) из пасуља је имобилизован различитим техникама: у сферном гелу калцијум-алгината у воденој суспензији, у лаку којим је у виду танког филма импрегнирано платно и у парафинском воску за импрегнирање платна. Упоредивана је активност слободног и имобилизованог ензима у функцији рН, температуре, стабилности при чувању, кинетичких параметара и учесталости употребе. Имобилизован ензим је био стабилан током чувања. После вишеструке употребе, зрна алгината су променила боју у браон и распала су се, што се одразило на стабилност овог матрикса. Парафински филм треба да се чува сув, пошто чување у влажним условима омекшава филм и доводи до губитка протеина. Зрна алгината су имала задовољавајућу механичку стабилност. Лак се показао механички чвршћим од парафинског воска. K_m и V_{max} вредности за ензим су се промениле после имобилизације. K_m вредност у калцијум-алгинату и лаку је била ниска, док је у парафинском филму била слична као код слободног ензима. Ово се може објаснити присуством CaCl_2 и метанола, који олакшавају излагање активних места уреазе. Имобилизација у парафину маскира активна места, што може довести до смањеног везивања супстрата.

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