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L-Arginase Immobilization on Chitosan Hydroxyapatite Complex: Effects of Immobilization Conditions

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Abstract: L-ornithine (L-Orn), is an intermediate metabolite in the urea cycle that plays an important role in the human beings and is used for the treatment of the liver diseases etc. Based on these versatile advantages, L-Orn is widely applied in the health care and pharmaceutical industries. In this study, *l-arginase* from *Idiomarina sediminium* was immobilized onto chitosan-hydroxyapatite. Effects of immobilization parameters including medium temperature and pH, enzyme concentration and coupling time was evaluated to obtain the best reaction conditions. The optimal immobilization pH was determined as pH 7.5 with the highest enzyme activity of 101.24 U chitosan-HAP beads and highest immobilization efficiency of 81.4%. Furthermore, the immobilized protein amount showed an increase with increasing enzyme concentration as expected. Characterization of immobilized enzyme was evaluated using l-arginine as a substrate. Immobilized enzyme exhibited better thermostability and higher stability at lower and higher pH values. Immobilization also resulted in a shift of 0.5 units in pH optimum for maximum enzyme activity. In contrast to optimum pH value, after immobilization no shift occurred in optimum temperature. Furthermore enzyme retained 81.8% of its initial activity during consecutive eight cycles. The results suggests that chitosan-HAP immobilized *l-arginase* can be employed on the large scale for industrial production of l-ornithine.
Keywords: Chitosan; Hydroxyapatite; Immobilization; *l-arginase*

INTRODUCTION

L-ornithine (L-Orn) is a non-protein amino acid that acts as an intermediate metabolite in the urea cycle [1]. It plays a significant role in humans, and is used for the treatment of the liver diseases, to strengthen the heart function, for weight loss and to boost immunity. Based on these versatile advantages, L-Orn is widely applied in the health care and pharmaceutical industries. Thus, this field has attracted considerable attention due to the enormous market [1, 2].

Production of l-ornithine can be carried out by one of these three methods - chemical synthesis, fermentation and enzymatic method. Chemical synthesis of l-ornithine is one of the oldest technique used, but it is chemically complicated process often associated with huge cost, hence not in use [3, 4]. Fermentation method though economical, is also associated with drawbacks such as back mutations of the strain used, difficulty in the product separation etc [5]. In contrast enzymatic synthesis offer wider advantages such as simple operation, ecofriendly biocatalyst, easy purification which can also be simplified by using immobilized technology. Enzyme immobilization technique is highly applicable for industrial production of the desired products. Hence the same method was employed for the production of l-ornithine [6, 7].

In this method we have immobilized the enzyme *l-arginase*, isolated from the marine strain *Idiomarina sediminium*, onto the chitosan using hydroxyapatite (HAP) and evaluated its characteristics. *L-Arginase* (EC 3.5.3.1; L-arginine amidinohydrolase) is a key enzyme in the urea cycle that catalyses the formation of urea in the mammalian liver [8, 9]. Currently *l-arginase* is considered as an important enzyme for its use in the cancer therapy for arresting the growth of l-arginine dependent cancer cells. It is also being developed as a biosensor for monitoring the

levels of the amino acid (l-arginine) in the blood samples [10].

L-Arginase is widely distributed in the plants, animals, human beings and the microorganisms. *Arginase* extracted from the livers of the animals exhibits low recovery, huge cost and contamination with the viruses [11]. Other bacterial sources showed the presence of huge number of inclusion bodies, hence difficult to purify .Some microorganisms gave low yield. Therefore, we employed the more efficient and facile marine bacterium *Idiomarina sediminium* in our work.

In our preliminary studies we have isolated potential enzyme producers from the marine source [12]. The free enzyme demonstrated a high level of enzyme activity, selectivity and specificity but lacked the long-term stability, recovery and recyclability, which restricted its application [13].

This problem was eradicated by immobilizing the enzyme with a suitable carrier. Out of the several carriers used for the enzyme immobilization, chitosan was of interest because it provided most of the characteristics that we needed. It possesses many qualities, such as high affinity for proteins, biodegradability, mechanical stability and diversity of geometrical configurations; moreover it was fairly suitable for the chosen biotransformation matrix [14]. Subsequently, we have immobilized enzyme on chitosan particles and investigated its properties under optimal conditions.

MATERIALS AND METHODS

Chemicals

Media used for culturing the microorganisms was prepared using readymade dehydrated media of Hi-Media laboratories. Remaining chemicals were purchased from Sigma Aldrich Bengaluru, India.

Preparation of Glutaraldehyde activated Chitosan Hydroxyapatite (chitosan-HAP) Microspheres

Chitosan-HAP beads of uniform dimensions were prepared using peristaltic pump. Equal amounts of powdered chitosan and HAP were dissolved in 1M of 100 ml acetic acid solution. The resultant solution was pumped through a sterile silicon tube into solution containing 1.5% w/v sodium tripolyphosphate crosslinking solution kept under constant stirring condition at room temperature. Pumping rate of the peristaltic pump was maintained at 5ml/minute. The beads thus formed were collected and washed with deionized water. For activation, chitosan-HAP beads were incubated in 0.1% glutaraldehyde solution for 24 hours with constant agitation. The excess glutaraldehyde was washed off and the beads were suspended in 0.025 M of phosphate buffer at pH 6.86.

Immobilization

1.5 g of activated chitosan beads were added to 5 ml enzyme solution in 0.025 mM of phosphate buffer at pH 6.86. The preparations were left at 15^0 C under continuous and gentle stirring (150 rpm) conditions for 24 hours. The preparations obtained were washed with the buffer until no enzyme remained in the washing solution.

Determination of the immobilized protein amount

The efficiency of enzyme immobilization was estimated on the basis of the difference between the amount of protein added to the beads and that recovered in the pooled supernatant and washing the fractions. Protein content of the enzyme solutions was determined according to the method of Lowry et al using Folin's reagent [15]. Bovine Serum Albumin (BSA) was used as a standard.

Determination of activity

Arginase activity was determined in terms of the rate of hydrolysis of L- arginine to l-ornithine and urea by measuring the amount of l-ornithine released in the reaction. L-ornithine was quantitated colorimetrically by ninhydrin assay [16] .One unit of enzyme activity is defined as the amount of enzyme that catalyzes the release of 1µmol of l-ornithine at 37^0C. The activity was tested at 40^0C with a reaction time of 10 minutes in a reaction mixture containing 500 µl of l-arginine in phosphate buffer, 0.1 ml $MnCl_2$ and 500 µl of the enzyme preparation. The reaction mixture was maintained at 40^0C for 5 minutes, then 100 µl of 10 % TCA was added, followed by 25 µ l of ninhydrin reagent. The mixture was heated for 5 min, centrifuged and the developed color was measured at 575 nm using spectrophotometer.

Same method was followed for assay of the immobilized enzyme, except that the immobilized enzyme suspension was used instead of free enzyme and that the reaction mixture was incubated with shaking.

Characteristics of the free and immobilized enzymes

Optimum temperature

The free enzyme and immobilized enzymes were incubated separately in 0.05 M bicarbonate buffer at temperatures ranging from 20-50 °C. Samples were collected and their activities were assessed as described above.

Optimum pH

Effect of the p H on the activity of both the derivatives were carried out at pH range of 4.0-9.0 using the following buffers: phosphate buffer (50 mM, pH 6.0–7.0), Tris-HCl buffer (50 mM, pH 7.0–8.0) and

bicarbonate buffer (50 mM, pH 9.0–11.0) at 40 °C. Their activities were assessed as described above.

Thermal stability

The two derivatives were incubated separately in 0.05 M bicarbonate buffer at pH 6.5 and between 25-55 °C. Periodically, samples were collected and their residual activities were assessed as described above.

Determination of kinetic parameters

Kinetic parameters, Km and Vmax values, for free and immobilized enzymes were carried out at the substrate concentration in the range of 0.5-20 m M. The rate of enzymatic hydrolysis of l-arginine can be expressed by Line weaver-Burk double reciprocal plot.

Operational stability of the immobilized enzyme

The operational stability was tested by repeated batch experiments using the method for activity determination. After each reaction run, immobilized beads were removed and washed with buffer to remove any residual substrate within the beads, then reintroduced to fresh reaction medium.

RESULTS AND DISCUSSION

Effect of process parameters on immobilization efficiency and activity

Effect of medium temperature & p H

Effect of various medium temperatures on immobilization efficiency, enzyme activity is shown in Figure 1. Results indicated that increasing temperature of the medium also resulted in an increase in immobilization efficiency. Highest amount of enzyme with 82.3% efficiency was obtained at 30 °C .It appeared that temperature above 20 °C had a negative effect on immobilization; causing some conformational changes due to thermal denaturation resulting in a decrease in enzyme activities. Hence the enzyme was immobilized at the optimal medium temperature of 20 °C. The results obtained in our studies are consistent with the studies conducted by Vasileva *et al*. [17]. They used modified polypropylene membrane as a support material for enzyme immobilization. Amount of bound protein and enzyme activity was recorded to decrease above 4 °C. Similarly, Jochems *et al*. [18] evaluated the effect of temperature on enzyme immobilization and observed an increase in immobilization yield with increasing temperature up to 60 °C, but above 25 °C, a sharp decrease in enzyme activity was seen.

From the results obtained in the present study (Figure 2) it is clear that pH of the immobilization medium had a significant effect on the enzyme activity and immobilization yield. Generally, it is expected to obtain the highest enzyme activity of immobilized enzyme at or around the optimum pH level. Results showed that immobilization efficiency followed the same trend with both activity values, yielding a peak of 81.4% at pH 7.5 with the maximum enzyme activity with chitosan-HAP beads. Moreover, enzyme activity was also had the highest value at pH 7.5 .Covalent binding between the activated support matrix and an enzyme is mainly depend on the reaction between the primary amino groups, usually lysine residues present on the surface of the enzyme and the aldehyde groups of glutaraldehyde introduced to the support previously. The loss in enzymatic activity at too high and too low pH values might be contributed to the changes in the enzyme conformation resulting in decrease in immobilization efficiency. Similarly, study of Liu et al. [19] demonstrated that fixing of *β-galactosidase* from *Klyveromyces fragilis* is primarily depended on the medium pH and between pH 6.0 to 9.0, with the optimum pH 7.0, higher enzyme loadings were achieved. Therefore, in our study, pH 7.5 was established as the optimal pH of the immobilization system.

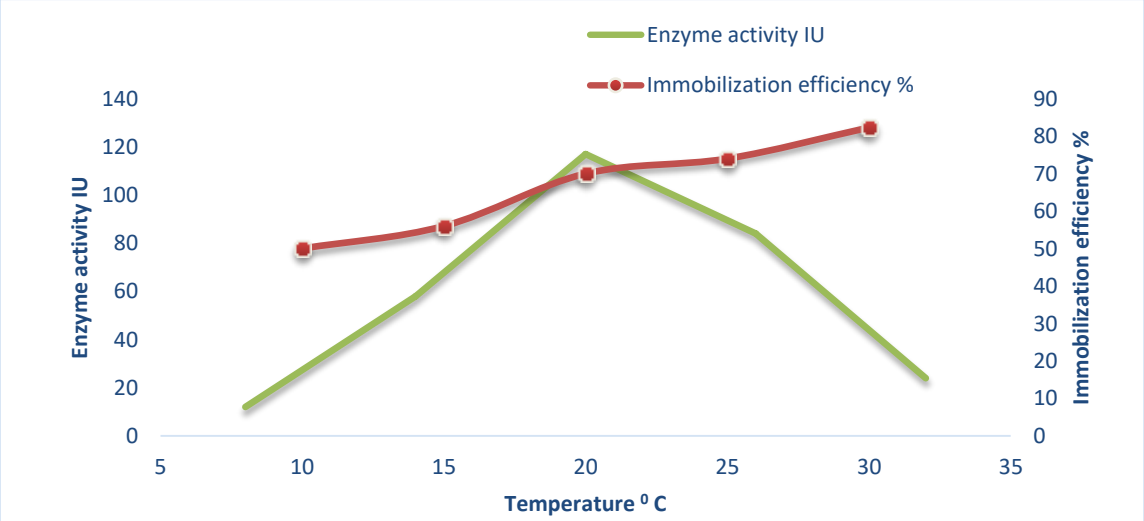

Fig-1 : Effect of medium temperature on immobilization efficiency activity (*l-arginase* was reacted with chitosan-HAP (1.5g) in phosphate buffer (5 ml, pH 6.86) at different temperatures with gentle shaking (150 rpm for 24 h)

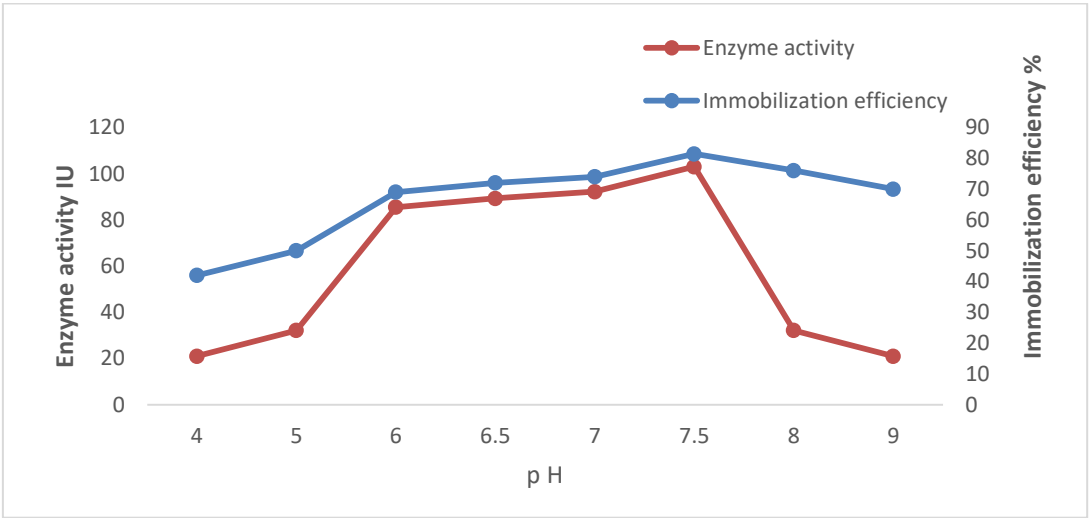

Fig-2: Effect medium pH on immobilization efficiency and enzyme activity (*l-arginase* was reacted with chitosan-HAP (1.5g) in phosphate buffer (5 ml) at different pH values with gentle shaking (150 rpm) for 24 h at 20 °C)

Effect of coupling time on immobilization and catalytic activity

As shown in the Figure 3, there is a sharp rise in immobilization efficiency as well as the enzyme activity within first hour there by reaching maximum activity within 9hrs of time. Optimum immobilization yield and enzyme activity were calculated as 91.4% and 113.2.7 U respectively at 9th hour. This is not an expected result since as the duration of coupling rises, increase in the extent of multipoint interactions between the support and enzyme resulting in a decrease in the enzymatic activity has been reported earlier in many studies [20- 22]. The results may be explained by the case that adsorption of the enzyme into porous support quickly occurred and covalent bonds acted as a barrier for substrate diffusion leading to a lower enzyme activity. Within time as the covalently bound enzyme amount increased, both activity values increased and at 9th hour, linkage reaction was almost completed.

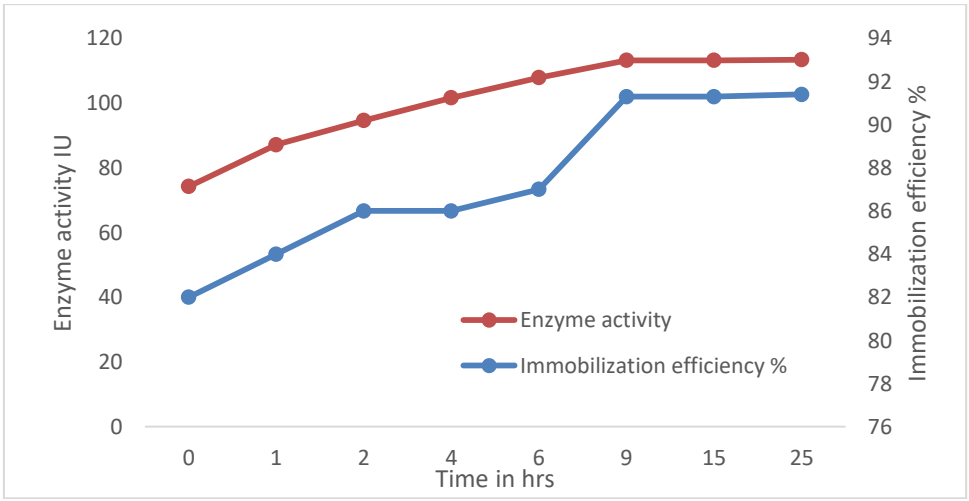

Fig-3: Effect of duration of coupling on immobilization efficiency, activity and specific activity (*l-arginase* was reacted with chitosan-HAP (1.5g) in phosphate buffer (5 mL, pH 7.5) with gentle shaking (150 rpm) for 24 h under 20 ºC temperature)

Effect of enzyme concentration on immobilization and catalytic activity

In order to optimize immobilization process, effects of varying enzyme concentrations on the immobilization and catalytic activity of the enzyme were evaluated. It seems that the increase in the enzyme concentration resulted in an increase in the activity of immobilized *l-arginase* (Figure 4).

Whereas, in contrast the immobilization efficiency was decreased with the increase in the enzyme concentration. Thus, it is also seen that

maximum specific enzyme activity was achieved at lowest enzyme concentration. It was assumed that the diffusion of the substrate got restricted as the concentration of the enzyme was increased on the support. Moreover, it might be due to the inactivation of the active sites of the enzyme due to protein-protein interactions [23-25]. The results obtained in our studies correlates with those of Rhimi *et al*. [26] and Alonso *et al* [27] .They reported decreased enzyme activity and the immobilization yield values of *Streptococcus thermophilus β-galactosidase* and *Pseudomonas glutaryl acylase* above a certain concentration value due to steric hindrance. In contrast, Jochems *et al.* [18] reported an increasing enzyme activity with the increasing in the enzyme concentration in contact medium when the enzyme was immobilized on a mixed-matrix membrane containing zirconium dioxide.

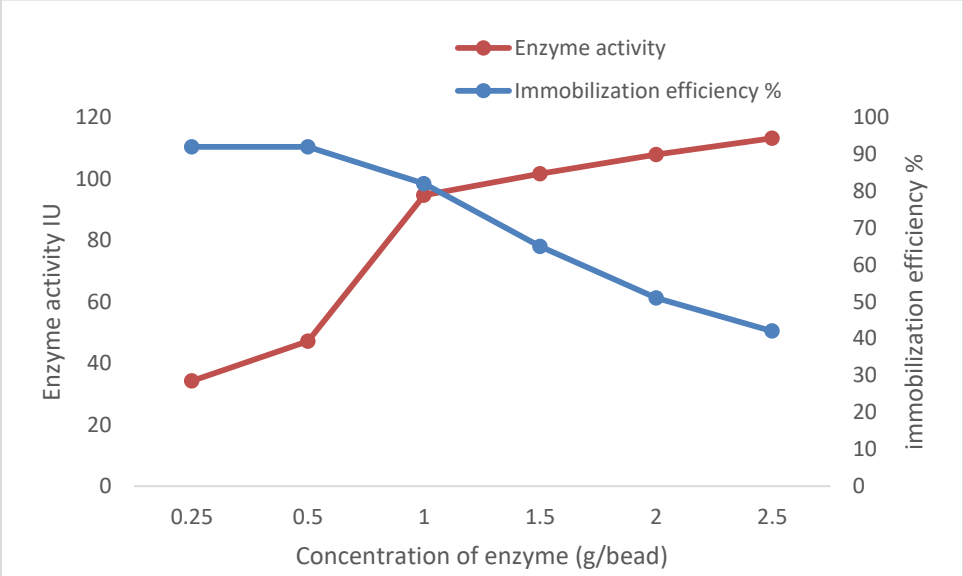

Fig-4: Effect of enzyme concentration on immobilization efficiency and enzyme activity (*l-arginase* was reacted with chitosan HAP (1.5g) in phosphate buffer (5 mL, pH 7.5) with gentle shaking (150 rpm) for 24 h at 20 °C)

Characterization of immobilized *l-arginase*

Effect of temperature and temperature stability

Considering that free enzyme demonstrates lack of long-term stability, recovery and recyclability that restricts its further use, a crosslinking method for the immobilization of *l-arginase* on chitosan particles was employed here to improve its stability. Upon immobilization, characteristics of enzymes may alter as a result of changes in the microenvironment conditions, in molecular conformation, and in the molecule charge of the adsorbed solid support. The results of the activity of both free and immobilized enzymes at various temperature and pH conditions are shown figure 5 and 6 respectively. The activities increased and then decreased with increasing temperature and pH values; the maximum values were achieved at 35 °C and pH 7.5. Additionally, the residual activity of the immobilized enzyme was considerably higher than the free enzyme at temperatures greater than 45 °C, which suggests that immobilization of *arginase* contributed to its temperature stability.

Moreover, the soluble enzyme exhibited more than 60 % activity from pH 7.5 to 10, whereas the immobilized enzyme displayed the most activity from pH 7.5 to 11. This result indicates that the immobilized enzyme had a higher adaptability to pH compared to the free enzyme.

Both the thermal and long-term stability, which are the most essential parameters for the measurement of the practicability of enzymes. The immobilized enzyme retained more than 80 % of its activity after incubation for 240 min at 40 °C, whereas the free enzyme lost approximately 30 % of its activity. There is no effect on the optimal temperature of the enzymes. The optimal temperature was found to be 35^0C for both the enzymes.

Thermostability is the capability of an enzyme to resist against thermal unfolding in the absence of substrates. Higher thermostability due to immobilization was shown in Figure 7. At 40 °C, immobilized enzyme on chitosan-HAP beads retained an activity of about 41.1%, however the activity retained by the free *l-arginase* was decreased at the same temperature to 30.7%. In view of these results, the immobilized *l-arginase* presented remarkable enhancement in terms of thermal and long term stability compared with the free enzyme, which is meaningful for future industrial applications.

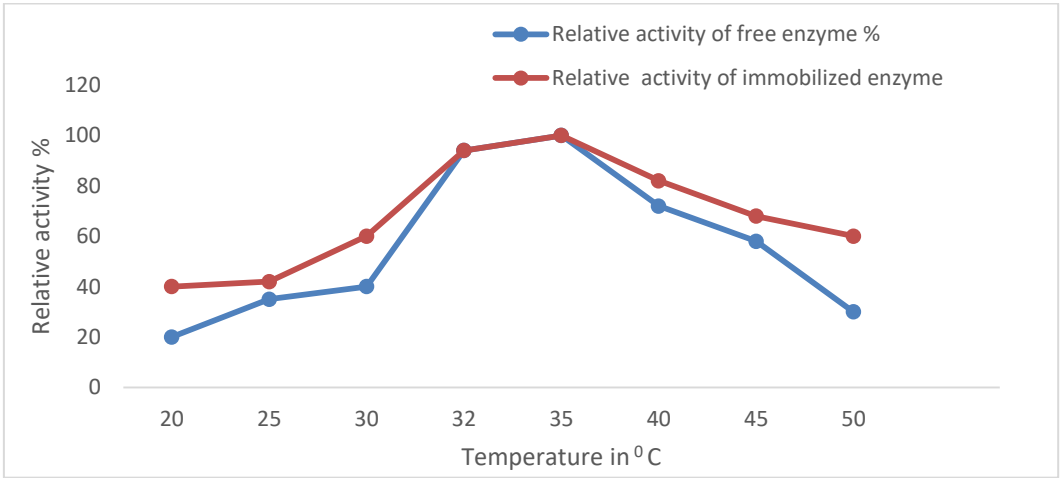

Fig-5:The effect of temperature on activity of free and immobilized *l-arginase* (Reactions were carried out at 37 °C using 500µl of l-arginine in phosphate buffer at pH 6.5)

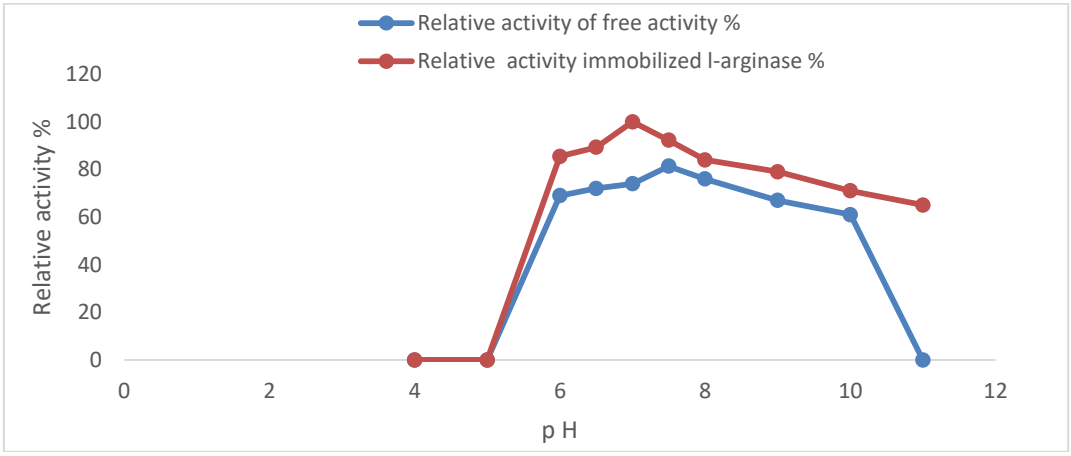

Fig-6: The effect of pH on the activity of free and immobilized *l-arginase* (Reactions were carried out at 37 °C using 500µl of l-arginine in phosphate buffer at pH 6.5)

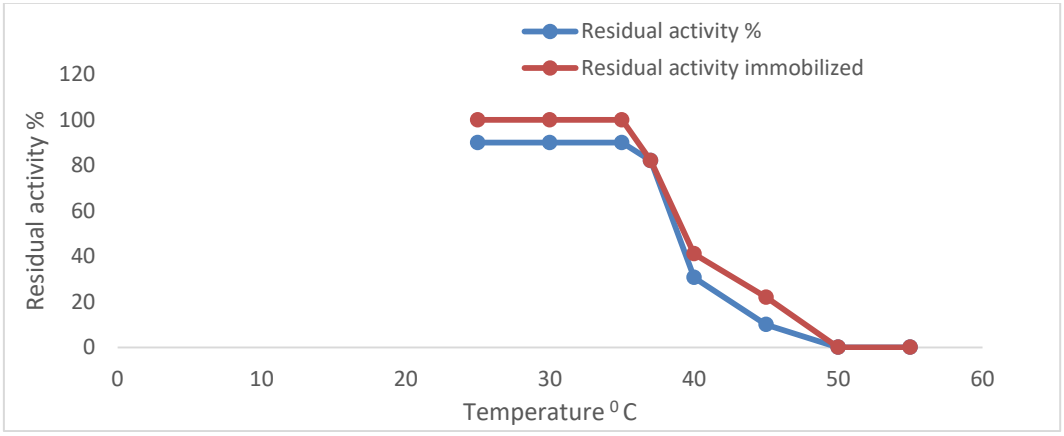

Fig-7: The thermal stability of free and immobilized *l-arginase* (Reactions were carried out at 37 °C using 500µl of l-arginine in phosphate buffer at pH 6.5 after incubation at indicated temperatures)

Optimal conditions for bioconversion

Next, we carefully evaluated the usage of immobilized *l-arginase* to catalyse L-Arg into L-Orn. Based on the series of experiments explained above, the immobilized *l-arginase* was utilized to synthesize l-ornithine under the optimal conditions of 35 °C and pH 7.5. To further increase the conversion rate, the concentrations of glutaraldehyde and Mn^{2+} and the

reaction time were systematically studied. According to the results obtained in the present studies, there was no significant change in the activity of the immobilized *arginase* when the change in the glutaraldehyde concentration [28]. The influence of the Mn^{2+} concentration on the conversion efficiency showed that the best result was obtained with the use of 1 mM Mn^{2+}.

Calculation of kinetic parameters of immobilized enzyme

Kinetic parameters of the free and immobilized enzymes towards l-arginine were investigated at different concentrations using standard assay. A Line weaver Burk analysis gave K_m and V_{max} value of the free *l-arginase* as 1.26± 0.41 mM and 138.88 ± 0.24 U/ml/min, respectively. Whereas for immobilized enzyme as 1.21± 0.41 mM and 133.33 ± 0.24 U/ml/min. There was no major change in the Km values. Free and the immobilized enzyme showed the same affinity towards the substrate.

Figure 9 shows the reusability of immobilized enzyme. The activity of the first batch was taken as 100%. As seen, the immobilized *l-arginase* retained 81.8% of its initial activity during consecutive eight cycles emphasizing strong linkages between enzyme and support matrix that enzyme did not leached out with repeated use. The loss of enzymatic activity is an important aspect to investigate the industrial feasibility.

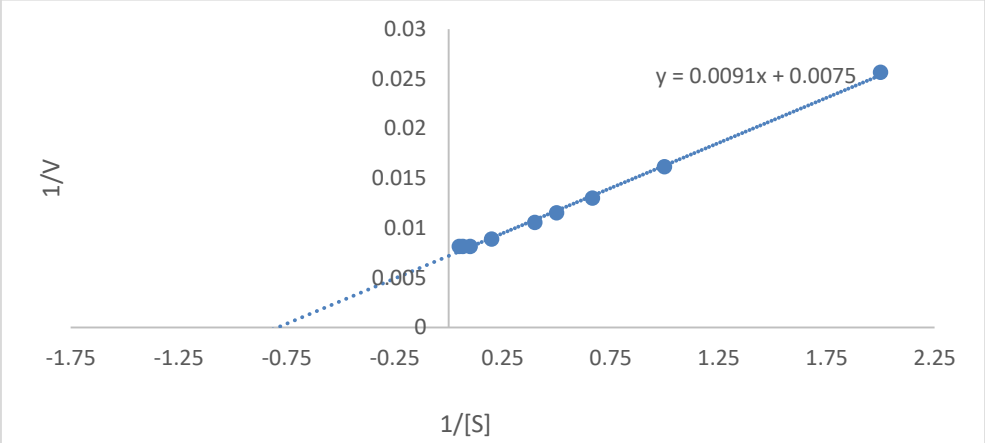

Fig-8: Double reciprocal plots to determine constants for l-arginine by free and immobilized *l-arginase*

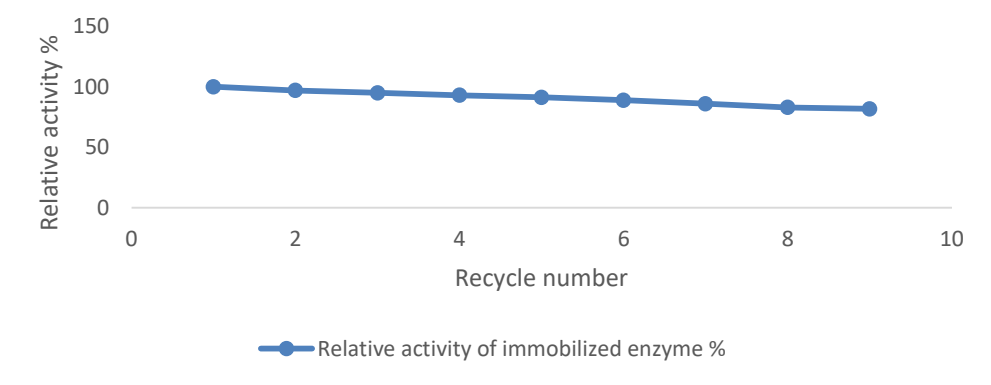

Fig-9: Batch wise reuse of chitosan-HAP immobilized l-arginase with 500µl of l-arginine (Reactions were carried out at 37 °C using 500µl of l-arginine in phosphate buffer at pH 6.5)

CONCLUSION

In this work, chitosan-HAP complex was efficiently used as an immobilization matrix for *l-arginase* from *Idiomarina sediminium.* The highest enzyme activity was recorded at the operation conditions of 20°C at pH 7.5.More over the *l-arginase* in immobilized form showed thermal and long-term stability, which are the most essential parameters for the measurement of the practicability of enzymes. The results suggests that chitosan-HAP immobilized *l-arginase* can be employed on the large scale for industrial production of l-ornithine.

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