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# Studies on Immobilization of Cutinases from *Thermobifida fusca* on Glutaraldehyde Activated Chitosan Beads

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***Authors' contributions***

*This work was carried out under the guidance of author VDV. Author KH designed the study, performed the experiments and wrote the first draft of the manuscript. Both the authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** To evaluate and optimize the activity and stability performance of two recombinant cutinases of *Thermobifida fusca*, Cut1 and Cut2 on glutaraldehyde activated chitosan beads.
**Place and Duration of Study:** Biochemical Engineering Laboratory, Department of Biotechnology, Indian Institute of Technology Guwahati, Assam India. Experiment conducted as a partial fulfillment to PhD degree from December, 2010 to January, 2014.
**Methodology:** Purified cutinase were immobilized on chitosan beads by covalently coupling with glutaraldehyde. The biophysical properties of immobilized cutinase was

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analysed by FTIR, FESEM and the operational stability and activity of the immobilized cutinase was studied at different pH and temperature.
**Results:** The optimal immobilization was achieved with 3% (v/v) glutaraldehyde activation and coupling pH of 8.5. Under this condition, 74% and 71% of immobilization was achieved for Cut1 and Cut2, respectively. Immobilized cutinase showed optimal activity at pH 8 with optimal functional range of pH 7.5 to 9 and 55ºC with operational stability in the range of 45ºC to 70ºC. The reusability and storage stability was found to be 80% after 10 reuse cycles and 50% after 13 days, respectively as compared to its initial activity. There was no loss in activity even after repeated freeze drying.
**Conclusion:** the cutinase immobilized on glutaraldehyde activated chitosan beads demonstrated better operational stability in comparison to free cutinase, showing chitosan as a potential support in the enzyme immobilization technology for industrial applications of *T. fusca* cutinases.

*Keywords: Chitosan; cutinase; glutaraldehyde; immobilization; Thermobifida fusca.*

## 1. INTRODUCTION

At present enzyme technology is considered as a powerful alternative to conventional process technology in various industrial and analytical fields. The benefits of biocatalyst over chemical catalysts are many, these include its capability to achieve multifarious chemical reactions under mild environmental conditions with high efficiency and specificity, remarkably efficient product conversion, reduced amount of undesirable by-products and increased product yield. Such features of biocatalyst have made them a prime target of exploitation by many emerging biotechnology and biopharmaceutical industries [1-4]. Regardless of all these advantages, the use of enzymes in industrial application has been hindered by various factors [5], for example, recovery of soluble enzyme from the reaction mixture and their recycle in subsequent reactions. To make use of enzyme economically feasible, recovery and reuse is vital [5]. A comprehensive research in the field of enzyme technology has provided various approaches to tackle such problems, among which enzyme immobilization technology is one, which offers promising economic exploitation of even expensive enzymes in various industries like bioprocessing, biopharmaceuticals, waste water treatment, development of biosensors, etc.

Chitosan is one of the abundant naturally occurring biopolymer composed of β-(1, 4)-linked D-glucosamine units, which is formed by the deacetylation of chitin extracted from shells of crabs, shrimps, and krills [6]. The ready availability, inexpensive method of preparation, its non-toxic and biocompatible nature has made it an excellent polymer in application in various fields [7-10]. Enzyme technology has exploited chitosan for enzyme immobilization by different methods including adsorption, entrapment and cross-linking. Chitosan have been reported as an ideal carrier for immobilization of many enzymes [11-14]. Chitosan also has been used along with alginate [15] and coated to magnetic nanoparticles [16] to immobilize enzyme efficiently. Amino and hydroxyl group present on the polymeric surface of chitosan greatly favor the immobilization process by covalent-linkage upon activation with glutaraldehyde or carbodiimide, respectively, followed by coupling of the enzyme [12,13,16,17].

Cutinase (EC 3.1.1.74) is a carboxylic esterase, which hydrolyzes plant cutin [18]. Both fungal and bacterial species have been reported to produce cutinase [18-23]. The

capabilities of cutinase to act on multi substrates like, synthetic short and long chain fatty acids, synthetic esters [24-25], water insoluble triglycerides, etc. has made it a versatile enzyme in industrial use. Besides substrate specificity, robustness to temperature, organic solvent and surfactant tolerance has made it a key enzyme in various industrial applications such as dairy, oleochemical industries, degradation of plastics, synthesis of polymers and surfactants, cotton scouring [26] and degradation of toxic substances [27]. Recently, cutinase from *Thermobifida fusca* has been shown its superiority over other cutinases in many aspects like thermostability, high activity in broad pH range, surfactant and organic solvent tolerance, which could have great biotechnological promise in many industrial applications [22,28]. In most of the applications of cutinase the use of organic solvents as reaction media can improve the productivity of the biotransformation of hydrophobic substrates like triglycerides, and short chain fatty acids. Nevertheless, when an organic solvent is present in the reaction medium containing the enzyme, the intermolecular interactions between the enzyme molecules may be changed, and the enzyme may become inactive. Another difficulty is poor solubility or poor dispersibility of enzymes in organic solvents, which may lead to apparent low or no enzyme activity. Enzyme immobilization seems to be a good approach to solve these problems, as often higher enzyme stability is obtained due to the interaction of the enzyme with the support, leading to a more rigid enzyme conformation [4].

Our previous studies on two homologous cutinases, Cut1 and Cut2 from *T. fusca* revealed that regardless of 93% amino acid similarities among them, they show different biochemical and biophysical properties and substrate specificity [22,29]. Hence, detailed study of both the cutinases needed in the case of *T. fusca* cutinase on optimization of immobilization parameters and evaluation of the catalytic performance under immobilized conditions. Thus, The present study focuses on immobilization of *T. fusca* cutinase, Cut1 (NCBI Acc. No. JN129499.1) and Cut2 (NCBI Acc. No. JN129500.1), on glutaraldehyde activated chitosan beads. For the first time, various immobilization parameters and catalytic parameters like, temperature and pH optima, pH stability, thermostability and the reusability of the immobilized cutinase from *T. fusca* has been evaluated.

## 2. MATERIALS AND METHODS

### 2.1 Materials

Chitosan (with a ≥75% degree of deacetylation and molecular mass of 310 kDa, ≥93 % purity), p-Nitrophenyl butyrate (≥98% purity) and Bradford reagent were purchased from Sigma Chemicals Co. USA. Glutaraldehyde was purchased from Merck India Co., India. All other chemicals used were of the highest purity and commercially available from Hi-Media, India or Merck India. *T. fusca* cutinase, Cut1 and Cut2 used in the present study were previously cloned, expressed in E. coli BL21 (DE3), purified to homogeneity using affinity purification and characterized in our laboratory [22].

### 2.2 Preparation of Chitosan Beads

3.0% (w/v) chitosan beads were prepared by dissolving powdered chitosan in 1.5% (v/v) glacial acetic acid at 50-60ºC. The mixture was then added drop wise into 150 ml 1 N KOH solution containing 25% (v/v) ethanol under stirring condition using a syringe needle (21 G). To get uniform sized beads, the syringe was fixed at an appropriate height (from larger height the beads became disk shaped). The solution was allowed to stand for 1-2 h for

hardening of beads. The beads of diameter nearly 0.4-0.5 mm and uniform shape obtained were immediately washed with sterile double distilled water and stored in 20% ethanol at 4ºC until activation with glutaraldehyde.

## 2.3 Bead Activation and Immobilization of Cutinase

Chitosan beads were activated at different concentration of glutaraldehyde in the range of 1.5% to 4% (v/v) at room temperature for 3 h. The activated beads were washed extensively with 50 mM potassium phosphate buffer (pH 8) to remove all traces of un-reacted glutaraldehyde. The activated beads were incubated with 0.5 mg/ml purified cutinase for 24 h at 4ºC for enzyme coupling. To determine optimal pH for cutinase coupling, the chitosan beads activated with 3% (v/v) glutaraldehyde were incubated for 24 h at 4ºC with 0.5 mg/ml cutinase in 50 mM potassium phosphate buffer of different pH. The beads were washed with 50 mM potassium phosphate buffer to remove un-bound cutinase. The activity of the chitosan coupled and unbound cutinase was measured by pNPB assay as mentioned in analytical methods. The protein content in the unbound fraction was estimated by Bradford method as mentioned in analytical methods.

The percentage immobilization (percentage activity retention) was calculated as follows:

$$\text{Immobilization } (\%) = \frac{\text{Total specific activity of immobilized cutinase}}{\text{Total specific activity of soluble cutinase}} \times 100 \quad \text{Eq. (1)}$$

The total specific activity of immobilized cutinase was determined by subtracting total specific activity of unbound cutinase from total specific activity of soluble enzyme.

## 2.4 Chitosan Bead Characterization

### 2.4.1 Fourier Transform Infrared (FTIR) Spectra of chitosan beads

The activation of chitosan beads by glutaraldehyde was confirmed using the FTIR (UNICAM Mattson 1000 FTIR spectrophotometer) spectra of normal chitosan beads and glutaraldehyde activated beads. For FTIR spectra, samples were crushed with potassium bromide (KBr) to form a very fine powder. This powder was then compressed into thin pellet for analysis.

### 2.4.2 Field Emission Scanning Electron Microscopy (FESEM) of chitosan beads

The surface morphology of the chitosan beads, glutaraldehyde activated beads, and cutinase immobilized beads were analyzed by FESEM (Carl Zeiss, ΣIGMA). Samples were coated with a thin layer of gold, prior to examination using FESEM.

## 2.5 Characterization of Immobilized Cutinase

All the experiments were performed in duplicate for *T. fusca* cutinases, Cut1 and Cut2 separately using five chitosan beads per sample immobilized with Cut1 or Cut2 unless otherwise mentioned.

### 2.5.1 The pH and temperature optima

The activity of cutinase as a function of varying pH was measured to determine the pH optima under immobilized condition. Activity of cutinase was measured by p-nitrophenyl butyrate (pNPB) assay in a range of pH varying from 5 to 11. The effect of temperature on activity of immobilized cutinase was investigated by incubating chitosan beads immobilized with cutinase for 10 minutes followed by pNPB assay at different temperature ranging from 37ºC to 80ºC.

### 2.5.2 The pH and thermal stability

The ability of immobilized cutinase to retain its activity in different pH was studied by incubating immobilized cutinase in buffer of different pH, ranging from pH 5 to 11. Cutinase immobilized chitosan beads were incubated in corresponding buffer for 1 h at 37ºC. The buffer was removed and residual activity of the beads was measured by pNPB assay. Thermal stability of the chitosan bead immobilized cutinase was investigated at 55ºC. A series of tubes having cutinase immobilized chitosan beads in 50 mM potassium phosphate buffer (pH 8) was incubated at 55ºC. Tubes were removed at regular time intervals to check the residual activity by pNPB assay.

### 2.5.3 Freeze-drying and its effect

The effect of freeze drying on stability of cutinase immobilized chitosan beads and activity of immobilized enzyme was investigated as follows. Cutinase immobilized chitosan beads were subjected to freeze drying (Christ, ALPHA 1-4) at -50ºC under vacuum for 1 h. The freeze dried beads were reconstituted by soaking in 50 mM potassium phosphate buffer (pH 8) for 15 minutes. These reconstituted beads were then used to measure activity by pNPB assay. The used beads were washed 3 times with 50 mM potassium phosphate (pH 8) and subjected to repeated freeze drying and activity assay to assess the reusability.

### 2.5.4 Storage stability and enzyme leaching study

Storage stability of the immobilized cutinase at 4ºC was measured as a function of pNPB activity at different time intervals as follows. The cutinase immobilized beads were stored in 50 mM potassium phosphate buffer (pH 8) at 4ºC and cutinase immobilized beads were taken at regular time interval to measure the activity by pNPB assay. In order to study the leaching of coupled enzyme (if any) in a course of time, the immobilized bead storage buffer was used as a source of enzyme to check the activity by pNPB assay.

### 2.5.5 Reusability of immobilized cutinase

The reusability of the immobilized enzyme was assessed as follows. After each activity assay, the chitosan beads with immobilized cutinase were removed and washed with 50 mM potassium phosphate buffer (pH 8), to remove any residual substrate within the chitosan beads and stored in the same buffer at 4ºC until further use. They were then reintroduced into fresh assay buffer, and the enzyme activity was determined at optimum conditions as mentioned in analytical methods.

## 2.6 Analytical Methods

### 2.6.1 Cutinase assay

Cutinase activity against p-nitrophenyl butyrate (pNPB) was determined by measuring the amount of p-nitrophenol released by hydrolysis of pNPB, according to the method described earlier [22]. Cutin hydrolysis was measured using the cutinase specific substrate p-nitrophenyl (16-methyl sulphone ester) hexadecanoate (p-NMSH) [22], which was prepared in our laboratory using the method described by Degani et al. [30]. In brief, assay was performed using the method described in enzyme assay for pNPB, with the change in incubation period for 1 hour instead of 5 minutes at 55ºC. One unit of enzyme activity is defined as release of 1 µmol of p-nitrophenol per minute.

### 2.6.2 Protein estimation

Protein concentration was estimated by the Bradford protein assay method using bovine serum albumin as a standard.

## 3. RESULTS AND DISCUSSION

## 3.1 Chitosan Bead Preparation

The chitosan beads of ~ 0.42±0.035 mm size weighing ~ 5.68±0.66 mg/wet bead were prepared and then activated by glutaraldehyde. In addition to activation of chitosan beads, glutaraldehyde also cross-links the chitosan, thereby providing resistance against lower pH. The two terminal aldehyde groups of glutaraldehyde react with amino groups of D-glucosamine units of different chains, resulting in cross-linking of those chains through glutaraldehyde. The irreversible Schiff's base linking of aldehyde with amino group provides operational stability to beads. Chitosan beads of consistent size and shape were prepared and the immobilization of cutinase on glutaraldehyde activated chitosan beads was successfully optimized.

## 3.2 Effect of Glutaraldehyde Concentration and pH on Immobilization of Cutinase

Effect of glutaraldehyde concentration on activation of chitosan beads and immobilization of cutinase was investigated in the range of 1.5% to 4% at pH 8. Most efficient glutaraldehyde concentration was found to be 3% for both Cut1 and Cut2 with 67% and 65% immobilization, respectively (Table 1). Lower concentration of glutaraldehyde showed lesser immobilization efficiency, which could be due to the fact that enough aldehyde groups were not generated on surface of chitosan beads to efficiently couple cutinase. Nevertheless, at higher glutaraldehyde concentration there was no significant decrease in coupling efficiency but the chitosan beads become brittle. This could be due to the fact that, glutaraldehyde not only activates the chitosan beads but it also cross-links the chitosan molecules. Higher concentration of glutaraldehyde increases inter molecular crosslinking which leads to increase in stiffness of the chitosan beads thus, making them brittle.

The pH plays a vital role in coupling of enzyme to glutaraldehyde activated chitosan through the amino group. It is a well-known fact that the ionization state of various functional groups present on the enzyme predominantly depends on the pH of the surrounding environment.

The effect of pH on cutinase coupling with chitosan beads was investigated in the range of pH 6 to 9. As shown in Table 1, the best suited pH range for ideal cutinase immobilization was found to be pH 8 to pH 9, with more than 50% immobilization. Whereas the optimum pH was found to be pH 8.5, with 74% and 71% immobilization for Cut1 and Cut2, respectively. At lower pH, the immobilization was substantially decreased, which could be due to improper ionization state of surface amino groups of cutinase.

**Table 1. Immobilization condition optimization for cutinase1 (Cut1) and cutinase2 (Cut2) on glutaraldehyde activated chitosan beads**

| Variation of coupling pH | pH[a] | Immobilization (%)[c] | | Specific activity per bead(weighing ~ 5.68 ± 0.66 mg/wet bead) | |
|---|---|---|---|---|---|
| | | Cut1 | Cut2 | Cut1 | Cut2 |
| | 6 | 25±0.2 | 2±0.7 | 3±0.3 | 3±0.6 |
| | 6.5 | 30±0.6 | 32±0.3 | 4±0.3 | 4±0.1 |
| | 7 | 38±0.2 | 40±0.5 | 6±0.1 | 6±0.6 |
| | 7.5 | 48±0.8 | 51±0.8 | 6±0.2 | 6±0.1 |
| | 8 | 69±0.7 | 67±0.4 | 7±0.2 | 7±0.1 |
| | 8.5 | 74±0.5 | 71±0.4 | 7±0.4 | 7±0.9 |
| | 9 | 71±0.1 | 69±0.2 | 6±0.1 | 6±0.7 |
| **Variation of glutaraldehyde (GA) %** | | | **GA%[b]** | | |
| | 1.5 | 46±0.4 | 50±0.9 | 5±0.2 | 5±0.7 |
| | 2 | 61±0.3 | 59±0.0 | 6±0.4 | 7±0.9 |
| | 3 | 67±0.2 | 65±0.5 | 8±0.9 | 8±0.6 |
| | 4 | 64±0.6 | 62±0.1 | 4±1.0 | 5±0.6 |

*± correspond to the standard deviation of two determinations*

*[a] Coupling pH optimization was done using 3 % Glutaraldehyde (GA) activated chitosan beads. [b] Glutaraldehyde (GA) % optimization was done at coupling pH 8.*

*[c] Immobilization (%) = Total specific activity of immobilized cutinase/Total specific activity of soluble cutinase X 100*

## 3.3 Chitosan Bead Characterization

### 3.3.1 Fourier Transform Infrared (FTIR) Spectra of chitosan beads

The structural changes in chitosan after glutaraldehyde activation were analyzed by IR spectroscopy. As depicted in Fig. 1, the peak at 1495 $cm^{-1}$ represents the C-N bond resulting from a cross-linking reaction. A reaction occurs between aldehyde groups of glutaraldehyde molecules and the amino group of chitosan through Schiff base linkage. Linkage of both terminal aldehyde groups with separate chitosan molecules results in cross-linking, while the linkage of only one aldehyde group results in the activation of surface for immobilization [11,12]. Other characteristic absorption peaks of chitosan were observed at 3000-3500 $cm^{-1}$ (OH, $NH_2$), 1656 (CONH amide band II) and 1085 $cm^{-1}$ (may be attributed to polysaccharide structure).

### 3.3.2 Field Emission Scanning Electron Microscopy (FESEM) of chitosan beads

FESEM images of the chitosan beads at different stages of immobilization are depicted in Fig. 2. The change in the roughness of the beads increased upon glutaraldehyde treatment and subsequent cutinase immobilization. The increase in coarseness may be due to

activation of the surface with glutaraldehyde and attachment of the enzyme on the activated surface.

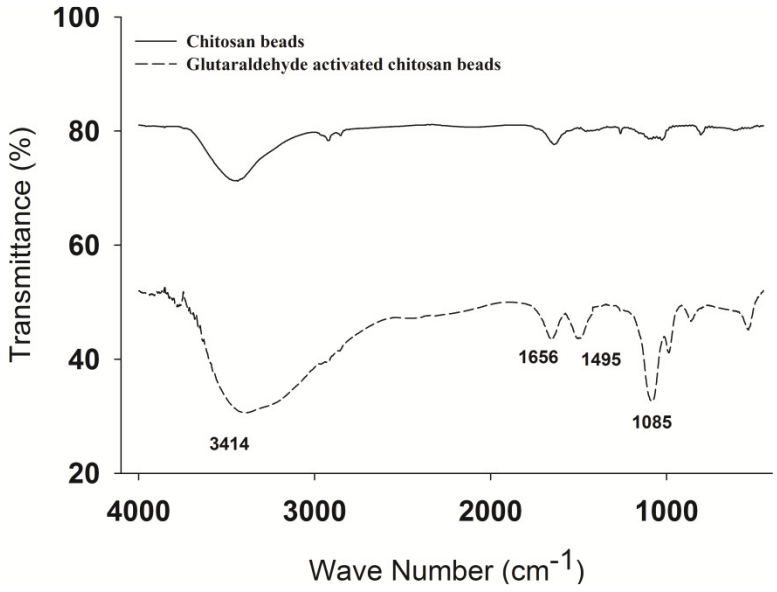


**Fig. 1. Comparison of FTIR spectra of normal chitosan beads (solid lines) and glutaraldehyde activated chitosan beads (dotted lines) for confirmation of glutaraldehyde activation**

## 3.4 Characterization of Immobilized Cutinase

Though there is 93% homology among the two variants of the cutinase, certain biochemical properties, substrate specificity of these enzymes are different. Moreover, there are very less reports available on these enzymes. Thus, evaluation of their behavior is essential to establish applications like esterification, transesterification [24], biodegradation [27], detoxification [27] and to determine what may be the best suited enzyme for a particular application. The efficiency of such catalysis might be greatly affected by varying pH and temperature. Thus, a prior knowledge of the immobilized enzyme performance at various pH and temperature would be a beneficial step in carrying out the experimental part of applications.

### 3.4.1 The pH and temperature optima

It was observed that free cutinase has broader pH optima as compare to immobilized cutinase (Fig. 3A). Though free as well as immobilized cutinase showed highest activity at pH 8, immobilized Cut1 as well as Cut2 showed considerably lesser activity at acidic pH (below pH 7) but were more active in alkaline pH (above pH 9) in comparison to free cutinase. One possible reason for the reduction in activity for immobilized cutinase at acidic pH could be due to the interaction of residual charges of the activated chitosan residues with coupled enzyme resulting in the unfavorable alteration of active site. Similar results were observed for some other enzymes in previous studies [31-33]. Thus, the immobilized cutinase is efficient at pH 8 as compare to free cutinase, which showed comparatively

broader pH curve. Similar results were observed for soluble cutinase from *T. fusca* and *F. solani* cutinase [21,22,34].

The temperature optima of the immobilized Cut1 and Cut2 were found to be 55ºC (Fig. 3B). The operational stability of the immobilized cutinase was considerably higher in the range of temperature studied. Increase in the operational stability at higher temperature is a reflection of conformational rigidity which makes immobilized enzyme resistant to denaturation.

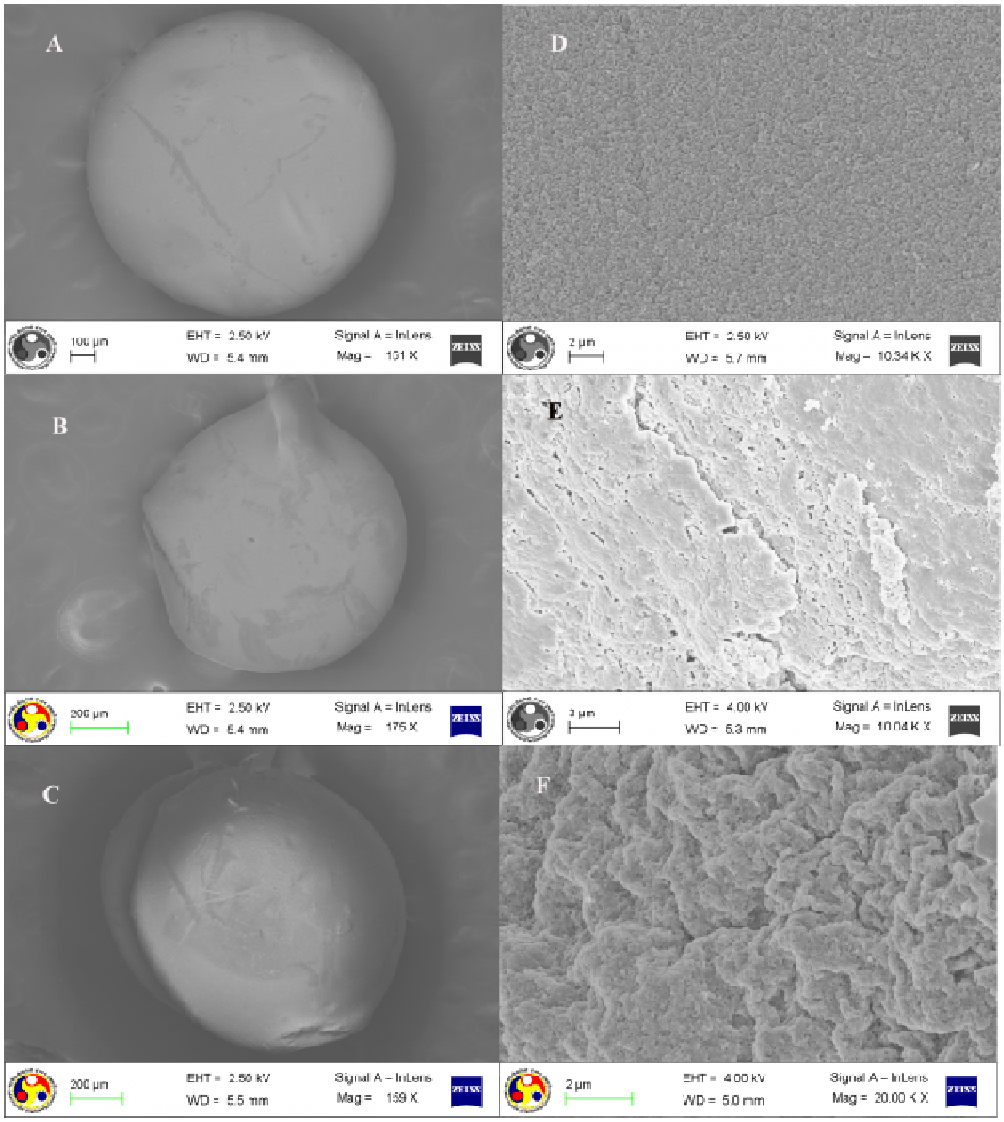


**Fig. 2. FESEM images of chitosan bead and detailed surface view of normal chitosan bead (A and D), glutaraldehyde-activated chitosan bead (B and E), and immobilized chitosan bead (C and F)**

### 3.4.2 The pH and thermal stability

Both the immobilized Cut1 and Cut2 were found to be highly stable at pH 7.5 to 9, with more than 80 % activity retention. However, there was noticeable activity loss at pH 9 to 11. The result is further summarized in Fig. 4A. Thermal stability of the immobilized cutinase was carried out at 55℃, which was found to be the optimal operational temperature. Moreover most of the industrial processes are employed in the range of 40-60℃ for carrying out any catalytic reactions. As depicted in Fig. 4B, the deactivation process was faster after 12 h for free cutinase in comparison to immobilized cutinase. There was almost 60% activity retention even after 50 h for immobilized cutinase, whereas the free cutinase activity was dropped to 40% at 40 h which indicates that the immobilized cutinases are thermally more stable in comparison to free cutinase.

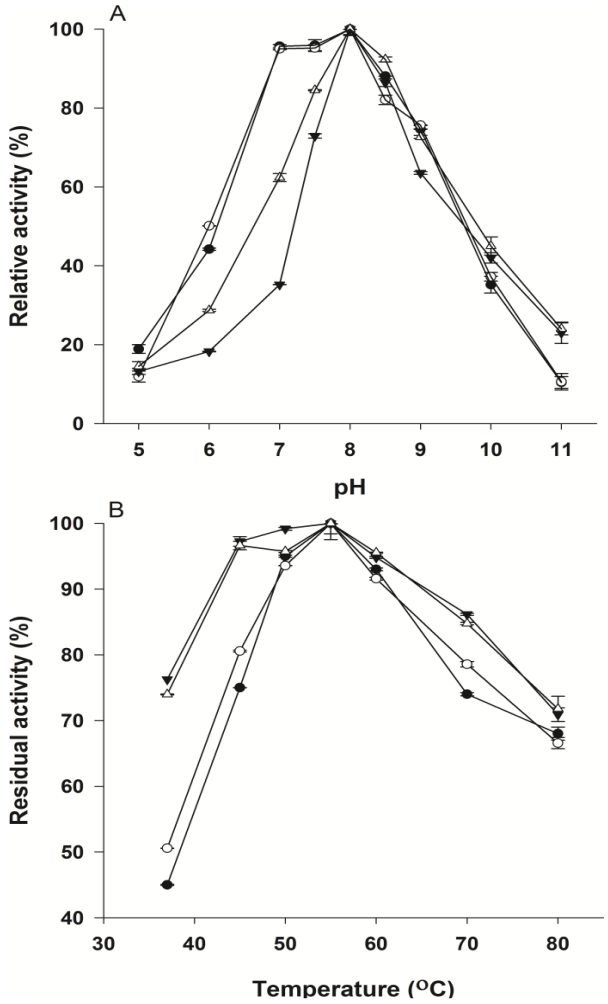


**Fig. 3A. Effects of pH on activity of Cut1 (▼) and Cut2 (∆) immobilized on chitosan beads, in comparison to free Cut1 (●) and Cut2 (○). (B) Effects of temperature on activity of Cut1 (▼) and Cut2 (∆) immobilized on chitosan beads, in comparison to free Cut1 (●) and Cut2 (○). Error bars correspond to the standard deviation of two replicates**

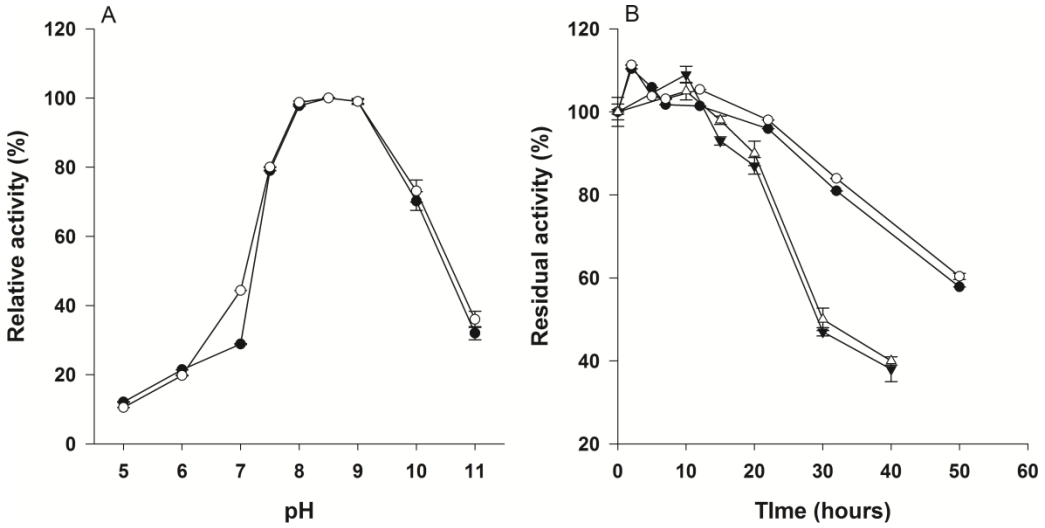


**Fig. 4A. Stability of Cut1 (●) and Cut2 (○) immobilized on chitosan beads, in different pH (Incubated in corresponding buffer for 1 h at 37℃). (B) Thermostability of Cut1 (●) and Cut2 (○) immobilized on chitosan beads, at 55℃ (pH 8) in comparison to free Cut1 (▼) and Cut2 (∆). Error bars correspond to the standard deviation of two replicates**

### 3.4.3 Freeze-drying and its effect

Most of the catalytic applications like esterification and transesterification using cutinase are carried out in different organic media such as isooctane, hexane, and other organic solvents where the high water contents are not the desirable criteria [35]. Thus, the method of choice in the case of free enzyme is lyophilization whereas in case of immobilized enzymes, they are freeze dried or desiccated to remove water contents. The ability of the chitosan immobilized cutinase to retain its activity after freeze drying was investigated. As shown in Fig. 5A, both the immobilized Cut1 and Cut2 showed good stability after freeze drying, with no loss of activity even after 3 freeze cycles. However the chitosan beads become brittle and powdery after 3 freeze drying cycles. After first freeze drying cycle, there was slight increase in the relative activity of both Cut1 and Cut2. The possible reason would be that during coupling by glutaraldehyde the protein molecules (enzyme) not only couples to chitosan but some protein molecules couple among themselves and some protein molecules might be coupled in wrong orientation which might not favor the active site for proper hydrolysis. As the number of freeze drying increases, the chitosan beads become powdery; this might be releasing some of the enzyme molecules to free solution, which include wrongly oriented and self-coupled, thus favoring the proper hydrolysis to enhance the activity after 1st freeze dry cycle. In summary, cutinase immobilized with chitosan beads showed good resistance with freeze drying which could be a promising application in esterification and transesterification reactions. There was less than 5% decrease in the activity after one freeze drying for free enzyme (data not shown). Nevertheless, the free enzyme can be freeze dried only once and can't be recovered after pNPB assay. Thus, there can only be one freeze drying cycle for free enzyme.

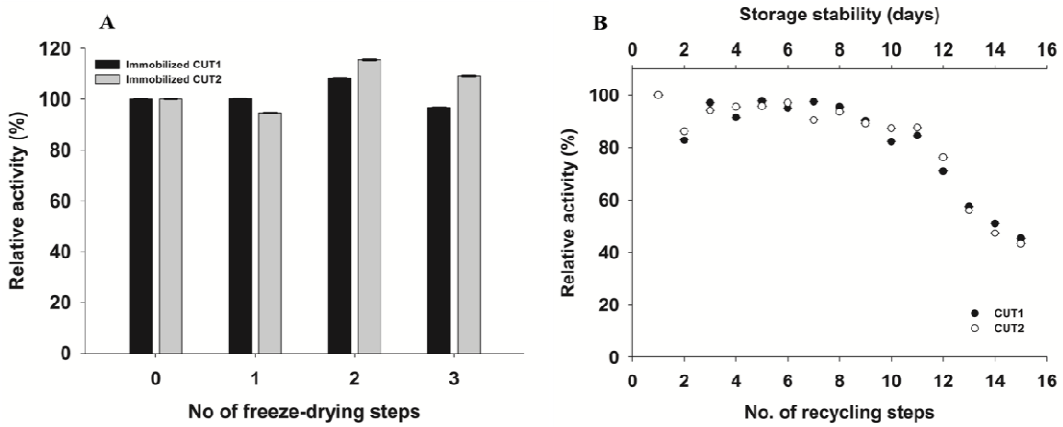


**Fig. 5A. Effect of freeze drying on activity of chitosan immobilized Cut1 (■) and Cut2 (■). (B) Reusability and storage stability of chitosan immobilized Cut1 (●) and Cut2 (○). Error bars correspond to the standard deviation of two replicates**

### 3.4.4 Reusability, storage stability and enzyme leaching study for immobilized cutinase

Reusability and storage stability are the most important criteria in enzyme immobilization for economic and cost effective application of enzyme in various industrial processes. Both the immobilized Cut1 and Cut2 showed good reusability and storage stability at 4ºC (Fig. 5B). More than 90% of the activity was retained even after reusing for 9 times. Both the immobilized cutinase showed more than 50% activity even after 13 days storage at 4ºC. However, after 11 uses, there was a gradual decrease in activity which could be explained by inactivation of activity by denaturation and stripping of the enzyme upon repeated use. The possible enzyme leaching during storage was studied by pNPB assay using immobilized enzyme storage buffer as a source of enzyme. There was no noticeable activity found even after 15 days in immobilized enzyme storage buffer, which confirms that the loss in activity after repeated reuse (after 11 cycles) is due to either enzyme deactivation or leakage of enzyme from support upon use.

## 4. CONCLUSION

In the present study, immobilization of *T. fusca* cutinases on glutaraldehyde activated chitosan beads has been successfully developed for the first time resulting in more than 70% immobilization. The operational stability of the immobilized cutinase (pH, temperature, reusability) was evaluated, which showed good pH stability at alkaline pH and thermostability. An outstanding reusability and storage stability have observed even after 10 uses and 11 days of storage, with retention of more than 90% activity, which make it ideal for the economic production of immobilized biocatalysts for the industrial applications. A good resistance to activity loss after repeated freeze drying could be a promising parameter in various applications like esterification and transesterification reactions. In summary, the cutinase immobilized on glutaraldehyde activated chitosan beads demonstrated better operational stability in comparison to free cutinase, showing chitosan was a potential support in the enzyme immobilization technology for industrial applications of *T. fusca* cutinases.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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