Enzymes are used in various industrial applications; such as pharmaceuticals, food, detergents and textile. However, the industrial applications of enzymes are restricted due to their instability under harsh industrial conditions, relatively low stability of the enzyme in liquid state and high production cost. Further, recovery of the active enzyme from the reaction mixture is quite difficult. One of the approaches to address these bottlenecks is the immobilization of the enzyme. The immobilization techniques have been used for making a number of biotechnological products with applications in biosensors, diagnostics and for therapeutic purpose (Wilson & Hu, 2000; Krajewska, 2004). Moreover, due to immobilization, catalysis and stability of the enzyme generally improve (Barbosa et al., 2013; Rodrigues et al., 2013). These changes are primarily due to the altered interaction between enzyme and substrate or changes in the three-dimensional conformation of the protein due to immobilization (Brena & Batista-Viera, 2006). Beside the intrinsic properties, the operational stability of the enzyme is also improved upon immobilization. For the successful immobilization, the nature of the enzyme or cells, the matrix or support and the methods of the immobilization are important factors. A successful immobilization improves the stability of the enzyme via multipoint or multi-subunit binding (Tavano et al., 2013). The glyoxyl and glutaraldehyde activated supports are reported to enhance stabilization via through covalent binding (Betancor et al., 2006; Mateo et al., 2006; Tavano et al., 2013). Various parameters to optimize the process of immobilization include the concentration of the enzyme, type of matrix, cross linkers and binders (Ertan et al., 2007; Kikani et al., 2013).
The immobilization of the enzymes from the extreme organisms has focused immense attention. The thermophilic enzymes, especially belonging to hydrolases, have extended applications in varied fields. For instance, amylases, account for approximately more than quarter of the global enzyme market (Haki & Rakshit, 2003; Kikani & Singh, 2012). The α-amylases have major application potential in starch liquefaction and saccharification, for removing starch sizer in textile industries, and the production of glucose, maltose, malto-oligosaccharides and ethanol. Since most of these applications occur at high temperatures, there is constant demand for the thermostable amylases. The thermostable enzymes have additional advantages such as reduction in cost of cooling, uniform mixing of the ingredients and reduced risk of the contamination (Haki & Rakshit, 2003). Majority of the thermostable enzymes have been obtained from the members of Bacillus; and only few are reported from the actinomycetes and fungi (Kikani et al., 2010).

In the present chapter, immobilization strategies of the partially purified amylase from a thermoactinomycete, Laceyella sacchari TSI-2 were explored. In the study, six matrices were employed using different methods of immobilization; entrapment, ionic binding and surface adsorption with covalent coupling. The immobilization efficiency and operational stability of the immobilized enzyme was further studied. The effect of temperature, pH, solvents and surfactants were assessed and compared for the immobilized and free enzymes. Further, various thermodynamic parameters were computed and the structural attributes of the enzymes probed by FT-IR. The starch hydrolytic and washing efficiency of the immobilized enzyme were evaluated to assess the potential of the enzyme in starch processing and detergent industries.
6.2. MATERIALS AND METHODS

6.2.1. Materials

Seralite SRA, Seralite SRC, hydroxyapatite and Silica (60-80 Mesh size) were purchased from Sisco Research Laboratories, Mumbai, India. DEAE cellulose and Folin’s ciocalteu were procured from Merck, Mumbai, India. All the microbial media components, agar, glutaraldehyde, Dinitro Salicylic acid and other chemicals were procured from Hi-Media, Mumbai, India.

6.2.2. The α-amylase production and partial purification

The α-amylase production in L. sacchari TSI-2 was carried out as described in the Chapter 4; section 4.2.3.; while, the partial purification was carried out by acetone precipitation method as described in the Chapter 5; section 5.2.2.2.

6.2.3. Enzyme assay

The α-amylase activity was measured as described earlier in the Chapter 4; section 4.2.2.2. Briefly, one unit of the α-amylase was defined as the amount of the enzyme that liberates 1 μg of the maltose per minute under the defined assay conditions, using maltose as the standard (100μg/ml – 1000μg/ml).

6.2.4. Immobilization of α-amylase

The study aimed at the immobilization of α-amylase on various matrix using different methods.

6.2.4.1. Immobilization by Entrapment method

Agar solutions of 4 and 5 % were prepared in 20 mM phosphate buffer, pH 7
by heating at 100 °C followed by cooling up to 60 °C. The 2 ml partially purified α-amylase (770 U/ml) was added in 10 ml of the agar solution, mixed gently and allowed to solidify in a sterile Petri dish. The agar entrapped immobilized enzyme was cut into 20 even size blocks. The blocks were then extensively washed with 20 mM Phosphate buffer, pH 7 to remove surface unbound enzyme. The enzyme entrapped in agar blocks were preserved at 4 °C for further studies (Prakash & Jaiswal, 2011; Kikani et al., 2013).

6.2.4.2. Immobilization by Ionic binding

Seralite SRA, Seralite SRC and Hydroxyapatite were used for the ionic binding. In order to activate the matrices, 2 gm of matrix was added to 50 ml of 20 mM Phosphate buffer, pH 7 and incubated for 3 hours with continuous slow stirring. The matrix was then filtered using Buchner funnel and incubated with 100 ml 0.1 N NaOH for 1 hour with continuous stirring followed by washing with distilled water thoroughly until pH 7. Further, the matrix was filtered on a Buchner funnel and treated with 100 ml 0.1 N HCl followed by the continuous slow stirring for 1 hour. It was then washed with distilled water until pH 7. The activated matrix was filtered and preserved in 25 ml 20 mM Phosphate buffer, pH 7 at 4 °C. For immobilization, 1 ml of free enzyme (770 U/ml) was mixed with 1 gm of each matrix. The mixture was stirred slowly at 4 °C for overnight. The unbound enzyme was washed out with the assay buffer and immobilized enzyme was preserved at 4 °C till further application.
6.2.4.3. Immobilization by Surface adsorption and cross linking

The Silica and DEAE Cellulose were used as matrix while Glutaraldehyde was used as cross linking agent. The Silica and DEAE cellulose were activated as mentioned above. For immobilization, 1 gm of the activated matrix was mixed with different concentrations of Glutaraldehyde (50 μg – 200 μg); stirred slowly at 4 °C for 5 hours. The glutaraldehyde charged matrix was added to 1 ml of free enzyme (770 U/ml) and 0.2 ml of 20 mM Phosphate buffer, pH 7. The mixture was stirred slowly at 4 °C for overnight. The unbound enzyme was washed with the assay buffer and immobilized enzymes preserved at 4 °C.

6.2.5. Enzyme assay of Immobilized enzyme

For the enzyme assay of the immobilized enzyme, 2 blocks of the agar entrapped enzyme or 0.1 gm of the immobilized enzyme by other methods was added into 1 ml of 1% starch solution prepared in 20mM Phosphate buffer, pH 7. The reaction mixture was incubated at 50 °C for 10 minutes under shaking condition and centrifuged at 5000 rpm for 2 minutes. The centrifugation step was not performed for agar entrapped enzyme. The supernatant was analyzed for maltose concentration using DNS method as mentioned in above section. The theoretical and practical immobilization efficiency was calculated using the following expression:

Theoretical immobilization efficiency (%) = \( \frac{(A - B) \times 100}{A} \) \hspace{1cm} (1)

Actual immobilization efficiency (%) = \( \frac{C \times 100}{A} \) \hspace{1cm} (2)

Where, \( A \) is the activity of the free enzyme (amount of the enzyme loaded for the immobilization); \( B \) is the activity of the unbound enzyme (wash out) and \( C \) is the activity of the immobilized enzyme.
Chapter 6

Immobilization and characterization of thermophilic amylase

6.2.6. Operational stability

The immobilized enzymes were incubated with 1 ml of 1 % (w/v) soluble starch prepared in 20mM phosphate buffer, pH 7 at 50 °C for 10 minutes. The immobilized enzyme was separated by centrifugation, washed thoroughly with the assay buffer and was used for the next reaction. The process was repeated till the measureable enzyme activity was detected.

6.2.7. Characterization of free and immobilized enzyme

The immobilized enzyme displaying good immobilization efficiency and high operational stability was selected for detailed characterization.

6.2.7.1. Temperature and pH optima

The temperature profile of the immobilized and free amylases was followed by incubating the reaction mixture at different temperatures ranging from 37 to 100 °C; wherein, the pH of the reaction mixture was maintained at 7. Similarly, the effect of pH on the immobilized and free amylases was studied over a pH range of 5 – 9. The enzyme assay was carried out with the substrate prepared in buffers with varying pH. The pH values were achieved with: 20 mM acetate buffer for pH 5, 20 mM Phosphate buffer for pH 6, 7 and 8 and 20 mM borax NaOH buffer for pH 9. The reaction mixtures were incubated at the optimum temperature for the enzyme assay.

6.2.7.2. Temperature and pH stability

The thermal stability of the free and immobilized enzyme was monitored at various temperatures in the range of 50 – 80 °C for 12 hours. Aliquots were
withdrawn at different time interval and amylase activity determined. In a similar manner, the pH stability of the enzyme was monitored at pH 6 – 8 for 12 hours and optimum stable temperature. The residual enzyme activity was the determined.

6.2.7.3. The deactivation constant

For the deactivation studies, the enzyme was deactivated at the temperatures between 50-80 °C. It is assumed that the active state of enzyme (E) is directly converted to inactive state (Ed) without providing any significant amount of intermediates.

\[ E \rightarrow Ed \]  \hspace{1cm} (3)

The first order deactivation constant (Kd) is a function of temperature and equation 3 is represented as:

\[ \frac{dE}{dt} = -Kd [E] \]  \hspace{1cm} (4)

Integration of equation 4 is represented as

\[ \frac{Ed}{E} = \exp(-Kd t) \]  \hspace{1cm} (5)

The slope of the plot of \( \ln(Ed/E) \) v/s t gives the value of deactivation rate constant (Kd). The half-life of an enzyme is defined as the time required by the enzyme to loses half of its initial activity, which can be represented as

\[ \frac{\ln 2}{Kd} = t1/2 \]  \hspace{1cm} (6)

6.2.7.4. Effect of surfactants on catalysis

The behavior of any potential laundry enzyme needs to be assessed in the presences of surfactants. The immobilized and free enzymes were incubated
Immobilization and characterization of thermophilic amylase
with the commercial surfactants, viz. Triton X 100, Tween 20 and Tween 80, dissolved in substrate at the concentration of 1 % and 5 % (v/v). The reaction mixture without surfactants was considered as control and the relative activities were calculated taking the activity of control as 100 %.

6.2.7.5. Effect of solvents on stability
The effect of solvents; acetone, ethanol, butanol, benzene and toluene were studied at two concentrations of 10 and 50 % (v/v). The solvents were selected based on the log Pow value which indicates the toxicity level of the solvents. The free and immobilized enzymes were incubated in the presence of solvents for 1 hour followed by the determination of the enzyme activity. The reaction mixture without solvents was considered as control and the residual activities calculated considering the activity of control as 100%.

6.2.8. Determination of Kinetic parameters
The free and immobilized enzymes were assayed at varying substrate (starch) concentrations, ranging 0.1-2 % (w/v). The Km and Vmax were estimated by Lineweaver-Burke double reciprocal plot of the 1/Vo v/s 1/[S]. The graph yielded straight line with a slope of Km/Vmax and an intercept of 1/Vmax on 1/Vo axis.

6.2.9. Structural elucidation of the enzyme by FT-IR
Conjugation of the free amylase with the activated DEAE cellulose was probed by analyzing free amylase, immobilized amylase and activated DEAE cellulose using Fourier Transform Infrared spectroscopy (FTIR Spectrometer,
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Immobilization and characterization of thermophilic amylase

Shimzdzzu). The spectra were recorded within a range of 4000 - 600 cm\(^{-1}\) with an average of three independent scans.

6.2.10. Applications of immobilized \(\alpha\)-amylase

6.2.10.1. Starch hydrolysis

In order to assess the efficiency of the immobilized amylase for the hydrolysis of the substrate, the immobilized enzyme was incubated with 1 % (w/v) soluble starch dissolved in 20mM phosphate buffer, pH 7 at 100 °C for 10 minutes for gelatinization. The gelatinized starch was further incubated at 90 °C for 1 hour to for liquefaction. Lastly, the liquefied starch was incubated at 70 °C for 3 hours for the saccharification of the starch. The aliquots were withdrawn after each step to measure the hydrolytic efficiency by estimating maltose using DNS method. The hydrolysis efficiency was expressed as:

\[
\text{% Starch hydrolysis efficiency} = 100 \times \frac{\text{number of glycosidic bonds broke}}{\text{number of glycosidic bonds present}}
\]  
(7)

In other words (Jana et al., 2013)

\[
\text{% Starch hydrolysis efficiency} = 100 \times \frac{\text{amount of reducing sugars produced}}{\text{total starch concentration}}
\]  
(8)

6.2.10.2. Detergent Additive

Beside starch processing industries, \(\alpha\)-amylases have major applications in detergent industry. The suitability of the immobilized \(\alpha\)-amylase in detergent was assessed by measuring the liberated glucose during the washing process. The experiment was performed as described by Dhingra et al. (Dhingra et al., 2006) with some modification. Clean cotton cloth piece (5 cm X 5 cm) was stained with 0.2 ml of 1 % starch solution followed by drying at 50°C for 30 minutes. The dried stained piece of cloth was placed in a beaker that contained
0.2 ml of the partially purified enzyme in 5 ml of tap water. The beaker was incubated at 45°C for 20 minutes under shaking conditions. After incubation, the cloth piece was removed and squeezed inside the beaker. The washout was used to estimate the reducing sugar using Anthrone’s method. For Anthrone’s test, 0.1 ml of the washout was mixed with 0.9 ml water followed by the addition of 4 ml freshly prepared chilled 0.2 % Anthrone’s reagent prepared in 95 % chilled sulphuric acid. The reaction mixture was allowed to cool to room temperature and O.D. was measured at 630 nm. The similar experiments were conducted replacing mixture of immobilized α-amylase and tap water with 5 ml - tap water, various detergents (viz. Tide, Ariel, Rin, Surf excel and Wheel) prepared in the concentration of 2 gm/liter and combination of enzyme with detergent. The glucose was estimated using the standard curve of glucose in the range of 10 -100 μg/ml. The starch concentration was estimated by multiplying the glucose concentration with 0.9 (Dhingra et al., 2006; Prakash & Jaiswal, 2011).

Washing efficiency was expressed as

\[
\% \text{ Washing Efficiency} = 100 \times \frac{A \times 0.9}{B} \tag{9}
\]

A = Concentration of glucose (μg/ml) in washout

B = Concentration of starch (μg/ml) applied on clean cotton cloth piece
6.3. RESULTS AND DISCUSSION

The study focused on the immobilization of a thermostable α-amylase and to assess changes in its inherent properties.

6.3.1. Immobilization of the enzyme by the entrapment method

The enzyme entrapped in 4 and 5 % agar showed 66 and 46.75 % immobilization yield, respectively. The results are depicted in Table 6.1. With increasing concentrations of agar, the immobilization efficiency of the enzyme was reduced. This may be due to low penetration of the starch in the agar blocks (Prakash & Jaiswal, 2011). The amylase was significantly entrapped in 4 % agar with high immobilization yield.

6.3.2. Immobilization by Ionic binding

The strong ionic resins viz. Seralite SRA, Seralite SRC and Hydroxyapatite were used for immobilization. The immobilization efficiency of Seralite SRA, Seralite SRC and Hydroxyapatite were 29.5, 35.3 and 41.3 %, respectively (Table 6.1). The α-amylase was stable at the neutral pH and thus Hydroxyapatite may have good ionic interaction with the amylase compared to Seralite SRA (strongly anionic) and Seralite SRC (strongly cationic).

6.3.3. Immobilization by Surface adsorption and cross linking

The enzyme was significantly adsorbed on the Silica and DEAE cellulose using gluteraldehyde as cross-linker. Silica and DEAE cellulose immobilized enzymes have only non-covalent forces between the support and enzyme molecules, hence it easily leads to desorption of the enzyme from the support.
However, the adsorbed enzyme cross-linked with glutaraldehyde prevent desorption of the enzyme (Evans C, 2011). The silica with 50, 100 and 200 μL glutaraldehyde resulted in 8, 20 and 10 % immobilization efficiency, respectively (Table 6.1). The enzyme was stable in 100 μL of 25% glutaraldehyde. An inhibitory effect on the enzyme catalysis was observed with the increasing concentrations of the glutaraldehyde. Further, in DEAE cellulose the immobilization yields, at 50 μL and 100 μL, were 44 and 68 %, respectively. The free amino groups of the enzyme couples with the free aldehyde groups of glutaraldehyde and form imines, arguably leading to improved binding of the enzyme on the matrix. Similar results using DEAE cellulose was observed by Kikani and Singh (Kikani et al., 2013), where an α-amylase was reported to be immobilized on DEAE cellulose with 84 % efficiency.

**Table 6.1:** Immobilization efficiency using different matrices

<table>
<thead>
<tr>
<th>Immobilization Strategies</th>
<th>Practical Efficiency</th>
<th>Theoretical efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Entrapment</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Agar 4%</td>
<td>66.88</td>
<td>66.88312</td>
</tr>
<tr>
<td>Agar 5%</td>
<td>46.75</td>
<td>63.76623</td>
</tr>
<tr>
<td><strong>Ionic binding</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>41.30</td>
<td>71.37681</td>
</tr>
<tr>
<td>Seralite SRA</td>
<td>29.50</td>
<td>70.01934</td>
</tr>
<tr>
<td>Seralite SRC</td>
<td>35.30</td>
<td>39.07157</td>
</tr>
<tr>
<td><strong>Surface adsorption with covalent cross linking</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE Cellulose + 50 μL Glutaraldehyde</td>
<td>44.48</td>
<td>74.64413</td>
</tr>
<tr>
<td>DEAE Cellulose + 100 μL Glutaraldehyde</td>
<td>68.15</td>
<td>68.77224</td>
</tr>
<tr>
<td>Silica + 50 μL Glutaraldehyde</td>
<td>7.97</td>
<td>72.94686</td>
</tr>
<tr>
<td>Silica + 100 μL Glutaraldehyde</td>
<td>20.13</td>
<td>23.76623</td>
</tr>
<tr>
<td>Silica + 200 μL Glutaraldehyde</td>
<td>10.39</td>
<td>19.22078</td>
</tr>
</tbody>
</table>
6.3.4. Operational stability

The operational stability of an immobilized enzyme is one of the important parameters in the biotransformation processes. The amylase in the present report immobilized on the DEAE cellulose was significantly stable and retained almost 49% of its initial activity for 6 cycles. Following which, 4% agar and DEAE cellulose with 50 μL glutaraldehyde showed measurable residual activities up to 4th and 6th cycles, respectively. With the other supports in the study, the enzyme was easily lost leading to a steam drop in the residual activities even after 2nd cycles. The results are summarized in Table 6.2. The continuous loss in the enzyme activity during the successive cycles may be due to the leakage of the enzyme from the matrix, loss of the matrix during the washing, denaturation of the enzyme or low penetration of the substrate. A similar study by Kikani and Singh (Kikani et al., 2013) reported the operational stability for 20 cycles of a DEAE cellulose immobilized enzyme with a loss of 4% in the activity. In an another report, a α-amylase immobilized on the gum arabica and agar, retained 98 and 13% of the original activities, respectively, after 4 subsequent cycles (Wang et al., 2011).
### Table 6.2: Operational stability of different matrices

<table>
<thead>
<tr>
<th>Name of Matrix</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>4% Agar</td>
<td>100</td>
</tr>
<tr>
<td>5% Agar</td>
<td>100</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>100</td>
</tr>
<tr>
<td>Seralite SRA</td>
<td>100</td>
</tr>
<tr>
<td>Seralite SRC</td>
<td>100</td>
</tr>
<tr>
<td>DEAE Cellulose + 100 μL Glu.</td>
<td>100</td>
</tr>
<tr>
<td>DEAE Cellulose + 50 μL Glu.</td>
<td>100</td>
</tr>
<tr>
<td>Silica + 50 μL Gluteraldehyde</td>
<td>100</td>
</tr>
<tr>
<td>Silica + 100 μL Gluteraldehyde</td>
<td>100</td>
</tr>
<tr>
<td>Silica + 200 μL Gluteraldehyde</td>
<td>100</td>
</tr>
</tbody>
</table>

#### 6.3.5. Characterization of the free and immobilized enzymes

DEAE cellulose with 100 μl gluteraldehyde emerged as the best candidate for the immobilization of the amylase in this study. It displayed maximum immobilization efficiency and reusability. Hence, the enzyme immobilized on the DEAE cellulose was selected for the characterization and comparison with the free enzyme.

#### 6.3.5.1. Temperature and pH optima

The effect of temperature on the free and immobilized α-amylase was assessed at the temperatures between 37 and 100 °C. The optimal temperature for the catalysis of the enzyme was improved and shifted by 10 °C from 60 to 70 °C on immobilization (Figure 6.1a). A comparable effect of the temperature was observed by Wang et al. (Wang et al., 2011), where the optimum temperature of the free TAKA-amylase shifted from 40 to 50 and 60 °C due to the immobilization on polystyrene and pentaethylene hexamine resin.
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Immobilization and characterization of thermophilic amylase respectively. Similarly, the effect of temperature on another immobilized α-amylase was studied by Singh and Kumar (Singh et al., 2011), according to which the activity of the immobilized α-amylase significantly improved as compared to the free enzyme.

Further, the effect of pH on the free and immobilized enzyme catalysis was investigated in the range of pH 5.0–9.0 (Figure 6.1b). It was observed that the enzyme activity varied with pH. The maximum activity of the free and immobilized enzymes was observed at pH 7.0. Further, the impregnated enzyme exhibited improved catalysis in the alkaline pH when compared with the free enzyme. The improvement in the enzyme activity might be attributed to the altered configuration under immobilized environment (Abdel-Naby, 1993; Prakasham et al., 2007). Similar improvements in the enzyme activity under immobilized conditions have been reported with other microbial systems and enzymes (Prakasham et al., 2007; Singh et al., 2011).

![Effect of Temperature on the catalysis of free and immobilized enzyme](image1.png)

![Effect of pH on the catalysis of free and immobilized enzyme](image2.png)

**Figure 6.1.** a) Effect of Temperature on the catalysis of free and immobilized enzyme b) Effect of pH on the catalysis of free and immobilized enzyme
6.3.5.2. Temperature and pH stability

The effects of temperature on the stability of the free and immobilized enzymes were studied at the temperatures between 50 and 80 °C. The free enzyme had maximum stability at 60 °C; however, on immobilization, the stability of the enzyme improved and shifted to 70 °C (Figure 6.2). The half-life of the free enzyme shifted from 26.67 hours at 60 °C to 38.38 hours at 70 °C after immobilization. At temperatures above 60 °C, the $t_{1/2}$ value decreased and Kd increased for the free enzyme; while, for the immobilized enzyme, at the temperatures above 70 °C, $t_{1/2}$ decreased and Kd increased. The $t_{1/2}$ and Kd values of the free and immobilized enzymes at different temperatures are given in Table 6.3. The improved thermal stability appears to be due to the formation of imines formed during the binding of the enzyme with the matrix. The matrix generally has a protecting effect on the enzyme at the elevated temperatures. The conformational flexibility of the enzyme may get altered on immobilization and thus lead to increased rigidity (Abdel-Naby, 1993). The higher thermal stability of the immobilized enzyme might be due to the reduction in the conformational flexibility in immobilized enzyme compared to the free enzyme (Çetinus & Öztop, 2003). Chang and Juang reported an improved temperature stability of a catalase in comparison to the free enzyme in the entire range of the temperature (Chang & Juang, 2005). A similar effect of the temperature on the stability of a $\alpha$-amylase secreted from Bacillus amyloliquifaciens TSWK1-1 has been recently reported (Kikani et al., 2013), where the half-life of the enzyme was enhanced from 31.5 to 86.5 hours at 60 °C after immobilization.
Table 6.3: Thermodynamic parameters: Deactivation constant (Kd) and Half-life ($t_{1/2}$) of the free and immobilized enzyme at different temperatures

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Half-life ($t_{1/2}$) in hours</th>
<th>Deactivation constant (Kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Free enzyme</td>
<td>Immobilized enzyme</td>
</tr>
<tr>
<td>50</td>
<td>22.991</td>
<td>27.656</td>
</tr>
<tr>
<td>60</td>
<td>26.678</td>
<td>30.748</td>
</tr>
<tr>
<td>70</td>
<td>23.615</td>
<td>38.389</td>
</tr>
<tr>
<td>80</td>
<td>20.235</td>
<td>24.12</td>
</tr>
</tbody>
</table>

**Figure 6.2** Effect of temperature on the stability: a) free and b) immobilized enzyme

The effect of pH on the enzyme due to immobilization is depicted in Figure 6.3. The free enzyme showed maximum stability at pH 6 with the residual activity at 68 % after 12 hours, while the immobilized enzyme at pH 7 retained 80 % of the activity after 12 hours. Overall, a 10 % enhancement in the residual activities was evident for the immobilized $\alpha$-amylase compared to the free enzyme. The methods of the enzyme immobilization on insoluble
carriers have a variety of effects on the protein conformation and state of the ionization and dissociation.

**Figure 6.3:** Effect of pH on the stability: a) free and b) immobilized enzyme

It is not uncommon about the resultant changes in the relationship between pH and enzyme. In few cases, when the enzyme is coupled with a polyanionic carrier, the pH optimum usually shifts in the alkaline direction whereas if the carrier is polycationic, the shift is in the acidic range (Costa et al., 2001). A similar study on a fungal amylase at different pH suggested a 30% reduction in the residual activity of the free fungal amylase as compared to the immobilized enzyme (Pascoal et al., 2011).

**6.3.5.3. Effect of surfactants on the enzyme catalysis**

The amylases are widely used in the detergent industry. However, in order to have potential applications in detergent, the amylase must be alkali tolerant and stable in various detergent ingredients, such as surfactants (Kikani & Singh, 2012). The immobilized α-amylase in the present study distinctly showed improved catalysis in the presence of surfactants with an exception of Tween 80. The Tween 80 adversely affected the α-amylase in the immobilized
Immobilization and characterization of thermophilic amylase state as compared to the free enzyme (Figure 6.4). The surfactants associate with the binding sites of the active protein through electrostatic and/or hydrophobic interactions. Further, type and size of the carbon chains may also influence the protein stability (Costa et al., 2001). Similar effect of surfactant has also been observed for a DEAE cellulose immobilized α-amylase (Kikani et al., 2013). The immobilized enzyme showed significant activity in presence of all the studied surfactants compared to its free enzyme. Thus, the inherent properties of the immobilized enzyme make it a promising candidate for detergent supplement.

Figure 6.4: Effect of surfactant on the catalysis of free and immobilized enzyme

6.3.5.4. Effect of solvents on the enzyme catalysis

The effect of solvents of varying log Pow values were studied on the enzyme stability. On immobilization, the stability of the enzyme improved in the presence of solvents (Figure 6.5). The maximum stability of immobilized enzyme was observed at 10 % ethanol and 50 % acetone; while the free
enzyme possessed significant stability at the varying concentrations of ethanol. With increasing concentrations of the solvents, the stability of the free enzyme was reduced except in case of benzene; wherein, marginal increase in the relative activity was observed. The stability of the immobilized enzyme was decreased with increasing concentrations of the solvent, the exceptions being butanol and acetone. Overall, the residual activity of the immobilized enzyme was higher compared to the free enzyme.

Figure 6.5: Effect of solvents on the stability of free and immobilized enzyme

In a study reported in literature, an α-amylase immobilized on DEAE cellulose showed fairly good stability against various solvents up to 50 % (v/v) concentrations when compared with the free enzyme and other immobilized forms (Kikani et al., 2013). The improved enzyme characteristics, due to immobilization on DEAE cellulose, have also been reported earlier (Musthapa M et al., 2004; Reddy K et al., 2004). Enzymes immobilized on DEAE cellulose have only non-covalent forces between the support and enzyme.
Immobilization and characterization of thermophilic amylase
molecules, and therefore, it may lead to desorption of the enzyme from the
support. However, the adsorbed enzyme on cross-linking with bi-functional or
multifunctional reagents prevents desorption of the enzyme from the matrix
(Musthapa M et al., 2004). Thus, the improved stability of the immobilized
enzyme appears to be due to glutaraldehyde which arguably prevent desorption
of the enzyme by creating multipoint attachment of the enzyme with the
matrix.

6.3.6. Determination of Kinetic parameters

The kinetic parameters for the starch hydrolysis using free and immobilized
enzyme were determined. The Km values of an enzyme suggest the affinity of
the enzyme for the substrate; whereas, Vmax provide information about the
maximum rate of the enzyme reaction at the saturated concentration of the
substrate. The Km of the free and immobilized amylase was 3 mg/ml and
0.45mg/ml, respectively (Figure 6.6). The substantial decrease in Km
suggests a positive distortion of the enzyme structure during the
immobilization. The matrix probably provides an environment which prevents
large conformational changes in the enzyme structure which usually cause the
enzyme deactivation. Unstrained non-covalent interactions can be assumed
between the functionalities available at the matrix and enzyme surface. The
reduction in Km value of the enzyme under immobilization conditions has
been observed in few other studies reported in literature (Heitmann et al.,
1997; Sanjay & Sugunan, 2005). A similar trend was observed by Singh and
Kumar (Singh et al., 2011), where a drop in Km value from 6.2 mg/ml to 4.2
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mg/ml on immobilizing of a amylase on the silica nano-hybrids was evidently reported.

![Graph](image)

a) Lineweaver-Burke Double reciprocal plot of free enzyme

b) Lineweaver-Burke Double reciprocal plot of immobilized enzyme

Further, the Vmax of the free and immobilized enzyme in the present study was 2500 and 909 mg/ml/min, respectively. A similar trends of the reduction in the Vmax was reported, wherein Vmax decreased from 2632 mol/ml/min for free amylase to 2186, 1771, 1563 and 1600 mol/ml/minas for the immobilized amylase on DEAE cellulose, gelatin, polyacrylamide and agar,
respectively (Kikani et al., 2013). The decrease in Vmax of the immobilized enzyme may be due to the increased stearic hindrance on immobilization.

6.3.7. Structural elucidation by FT-IR

The covalent binding between the α-amylase and various functional groups on the DEAE cellulose and glutaraldehyde was revealed by FT-IR spectroscopy (Figure 6.7). The FT-IR scanning was carried out between 4000 and 600 cm\(^{-1}\) to ascertain characteristic functional groups and bonding patterns. The variations in the peaks between 3200 – 3500 cm\(^{-1}\) appeared due to the changes in the stretches of alcohol (O-H) and amines (N-H) of the enzyme (Sinha & Khare, 2014). The characteristic peak of the cellulose was obtained at 1041.56 cm\(^{-1}\), which on immobilization altered, both in terms of the position and intensity, suggesting increase in the number of the stretches of the aliphatic amines (N-H) and/or ester (C=O). The maximum alteration in the peak positions and intensity of the DEAE cellulose and free enzyme was observed between 1800 – 1600 cm\(^{-1}\) when compared with the immobilized enzyme. The peak of the ester stretch (C=O) of DEAE cellulose obtained at 1743.65 cm\(^{-1}\) shifted to 1735.93 cm\(^{-1}\) with reduced intensity on immobilization. Whereas, the peak of alkenes (-C=C-) obtained at 1651.07 cm\(^{-1}\) of the free enzyme shifted to 1643.35 cm\(^{-1}\) with the reduction in the intensity on immobilization. Thus, alteration of peaks position and intensity in amide I region suggests its significance in binding of the free enzyme with matrix on immobilization in this region. Further, changes observed between 1700 – 1600 cm\(^{-1}\) indicated major alteration in the amide I and II region. As reported earlier, the two characteristic IR peaks for amylase enzyme observed in amide I band exists at
1650 cm$^{-1}$ due to the $\nu$ (C=O) in plane stretching vibration of the protein backbone and the amide II band exists at 1550 cm$^{-1}$ due to the $\delta$ (N–H) in plane bending mode (Fitter & Heberle, 2000). The peak at 1650 cm$^{-1}$ is a combination of peaks due to $\alpha$ helix, $\beta$ sheet, $\beta$ turn and random coil of the enzyme (Claverie et al., 2003), known to be sensitive to the environment. Conformational changes in protein can also be inferred from the amide I and II bands that appear between 1500 – 1700 cm$^{-1}$ region (Sinha & Khare, 2014). Thus, on immobilization the intensity of the aliphatic amine and the ester of the DEAE cellulose increase; while the alkenes of the enzyme are reduced, this may be responsible for the binding of the enzyme on the matrix. Sharma and Chattopadhyay (Sarma & Chattopadhyay, 2004) reported the peak of amylase at 1650 cm$^{-1}$. 


Figure 6.7: FT-IR spectra of a) free enzyme b) Immobilized enzyme and c) DEAE cellulose

6.3.8. Applications of the immobilized α-amylase

6.3.8.1. Starch processing industry

The α-amylase hydrolyzes the glycosidic bond of the starch molecules liberating maltoligosaccharides which on further hydrolysis release maltose and glucose molecules. The hydrolytic efficiency of α-amylase is based on the
number of maltose or glucose molecules liberated during reaction with soluble starch as the substrate under defined conditions. The efficiency values of the pure starch, maltose and glucose are 0, 50 and 100 %, respectively. The value indicates complete conversion of the starch to maltose and glucose; and is based on the theoretical concept; where in the total number of the glycosidic bonds hydrolyzed due to the enzyme action is equal to the number of maltose or glucose liberated. Thus, the ratio of maltose or glucose concentration at the end of the reaction to the initial concentration of the starch would give the hydrolytic efficiency of α-amylase. In the present study, the hydrolytic efficiency of α-amylase was calculated by taking ratio of the maltose concentration to the initial starch concentration. The hydrolysis efficiency values, for the studied α-amylase, were 4.75, 6.65 and 15.55 % for gelatinization, liquefaction and scarification of the starch, respectively. The result indicates that the α-amylase can also be useful for gelatinization process besides being helpful in scarification. Thus, the studied α-amylase can be favorable choice for the starch processing industries especially in the maltose syrups preparation. Kikani and Singh (Kikani et al., 2013) have earlier reported on the hydrolytic efficiencies of an amylase from Bacillus amyloliquifacines TSWK-1 as 1.5, 4.0 and 27 % for the gelatinization, liquefaction and scarification of starch, respectively.

6.3.8.2. Detergent industry

The starch stained cotton cloth pieces were washed with water, detergents, immobilized enzyme and combination of detergent and immobilized enzyme. The removal of the starch content from the cloth as washout was determined,
where higher amount of the starch content in the washout indicates the better washing efficiency. The washing efficiency was quite low in the tap water, which, however, improved in the presence of detergent, immobilized enzyme and the combination of the immobilized enzyme and detergent. The washing efficiency significantly enhanced when detergent in combination with the immobilized enzyme was used. The findings of the washing efficiency are summarized in Table 6.4. Similar studies are reported for the immobilization of α-amylases from Aspergillus niger and soyabean seeds with evidently improved washing efficiency (Dhingra et al., 2006; Prakash & Jaiswal, 2011).

**Table 6.4:** Determination of starch content in the washout after washing with various detergents and combinations of detergents and enzyme

<table>
<thead>
<tr>
<th>Washing agent</th>
<th>% Starch content in washout</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
</tr>
<tr>
<td>Tap water</td>
<td>13.378</td>
</tr>
<tr>
<td>Immobilized enzyme</td>
<td>27.81</td>
</tr>
<tr>
<td><strong>Detergents (2 gm/L)</strong></td>
<td></td>
</tr>
<tr>
<td>Detergent alone</td>
<td>Detergent + Immobilized enzyme</td>
</tr>
<tr>
<td>Ariel</td>
<td>31.239</td>
</tr>
<tr>
<td>Surf excel</td>
<td>31.176</td>
</tr>
<tr>
<td>Rin</td>
<td>28.656</td>
</tr>
<tr>
<td>Wheel</td>
<td>25.425</td>
</tr>
<tr>
<td>Tide</td>
<td>28.197</td>
</tr>
</tbody>
</table>