Chapter 3
Materials and Methods
Materials and Methods

Chitin is the most abundant polymer in the ocean and the second most abundant polymer on earth, after cellulose. But its insolubility in most of the solvents makes it an underutilized polymer. Thus to exploit the benefits of this most abundant polymer, it becomes necessary to convert it in to its soluble form i.e. chitosan. Chitin deacetylase is one such enzyme that catalyzes the bioconversion of chitin to chitosan. In the present study, an efficient microbial strain producing chitin deacetylase was isolated from natural source. To enhance the chitin deacetylase titer from the selected isolate, optimization of production conditions using submerged fermentation, solid state fermentation and statistical design methods was carried out. The purification of chitin deacetylase was carried out using gel filtration chromatography and potential application of enzyme was also studied.

3.1 Chemicals and raw material

The chemicals used during this investigation were of high purity, analytical grade and purchased from HiMedia Laboratories Pvt. Ltd., India. However Folin-Ciocalteau reagent was purchased from Loba Chemie, Mumbai India, Sephadex G-100 from Pharmacia and Ammonium sulfamate from Qualikems fine chem, Vadodara, India. Raw materials used as substrate for solid state fermentation were obtained from the local market and fields. P-Nitroacetanilide was prepared in lab (Annexure).

3.2 Composition of culture media and reagents

The composition of various media and reagents used in the present investigation is given in annexure I. All the media used for isolation, screening and production of chitin deacetylase were prepared in distilled water and autoclaved at 121°C at 15 lbs/inch for 20 min.

3.3 Isolation and screening of Chitin deacetylase producing microorganisms

Microorganisms were isolated from different natural materials and then tested for Chitin deacetylase production.

3.3.1 Collection of samples

Various soil samples were collected from rhizosphere of different plants such as rice, wheat, sugarcane, aloe vera, papaya, orange, potato, guava, money plant, tomato and guldavari with the help of a sterile scalpel in sterile polythene bags. Samples of rotten fruits and vegetables, manure soil (mixture of cow dung and straw), mushroom
soil, dried cow dung, goat fecal matter and dairy waste were also collected. The water samples from Jyotisar Lake and sewage were taken in sterile glass bottles.

3.3.2 Isolation of microorganisms

Both bacteria and fungi were isolated from the above samples on suitable agar medium plates by using spread plate method.

3.3.2.1 Isolation of Bacteria

Serial dilutions of the samples were prepared and spread on plates containing Nutrient agar (NA) medium supplemented with amphotericin-B (30µg/ml) as an antifungal. The plates were incubated at 37°C for 2 days and observed for bacterial growth. All the bacterial isolates thus obtained were streaked on fresh NA plates so as to get their pure isolated colonies. The purified isolates were then maintained on NA slants and stored in refrigerator for their use in primary screening for chitin deacetylase production.

3.3.2.2 Isolation of Fungi

Petri plates containing Czapek-Dox Agar medium supplemented with chloramphenicol (25µg/ml) were prepared and inoculated separately with different dilutions of each of the samples. The plates were incubated at 28±2°C for 6-7 days and observed for fungal growth. Fungal isolates thus obtained were transferred further on fresh Czapek-Dox Agar plates so as to get pure culture of isolates. The purified isolate was then maintained on Czapek-Dox Agar slants and stored in refrigerator for their use in primary screening for chitin deacetylase production.

3.3.3 Screening of isolates for Chitin deacetylase

All the fungal and bacterial isolates were screened for chitin deacetylase production on chitin agar plates.

3.3.3.1 Primary Screening by qualitative analysis

Screening of chitin deacetylase producing microorganisms was carried out on Chitin agar medium plates (Annexure). The plates of bacterial and fungal isolates were incubated at 37°C and 28±2°C respectively generally for 2-4 days but some take 6-10 days of incubation. The development of yellow color in the chitin agar plate indicated the presence of chitin deacetylase production. All the chitin deacetylase positive isolates were then maintained on Nutrient agar slants (bacteria) and Czapek Dextrose agar slants (fungi).
3.3.3.2 Secondary Screening by quantitative analysis

Each of the positive microbial isolate was grown in a 250ml flask containing 100ml of YPD medium (Annexure) supplemented with chitin (1%) and incubated at 37°C for 2 days. Crude enzyme was harvested by centrifugation at 10,000 x g for 15 min at 4°C and assayed for chitin deacetylase activity by MBTH method as explained in section 3.6.2.

3.4 Selection of isolate for Chitin deacetylase production

Among all the screened isolates, the isolate NS-1 showed maximum chitin deacetylase activity during secondary screening by quantitative analysis and was selected and maintained on Czapek Dox agar (CDA) medium and stored at 4°C for further study.

3.5 Identification and characterization of selected isolate

The selected isolate NS-1 was identified on the basis of its morphological, cultural, biochemical and molecular characteristics. The molecular identification of the selected isolate was done by the service provided by Xceliris Labs Ltd., Ahmedabad, India.

3.5.1 Morphological Characterization

The isolate NS-1 was stained with Lactophenol cotton blue and its morphological Characteristics were observed under compound light microscope.

3.5.2 Cultural Characterization

The isolate NS-1 was grown on Czapek Dox agar plates for colony morphology (color, shape, margin, elevation, consistency and opacity) and in Czapek Dox broth for the observation of pellicle formation.

3.5.3 Biochemical Characterization

The selected isolate NS-1 was biochemically characterized by performing various biochemical tests such as Urease, Nitrate reduction, Cycloheximide and Carbohydrate assimilation test. The carbohydrate assimilation ability of the selected isolate was tested for various sugars such as Glucose, Galactose, Sorbose, Sucrose, Maltose, Trehalose, Cellobiose, Lactose, Raffinose , Mannitol, D-ribose, D-xylose, L-arabinose, L-rhamnose and Soluble starch.

3.5.4 Molecular characterization using ITS region based molecular technique

After the morphological, cultural and biochemical characterization, the isolate NS-1 was subjected to molecular characterization. The molecular characterization of the isolate was done by commercial service provided by Xceliris Labs Ltd.,
Ahmedabad, India. Internal transcribed spacer (ITS) sequences were employed for the molecular characterization of NS-1.

**Methodology**

DNA was isolated from the culture. Agarose gel (1.2%) electrophoresis was performed to check the purity of the DNA sample. PCR is performed for amplification of fragment of ITS region of this isolated plasmid DNA. A single discrete PCR amplicon band was observed on Agarose Gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with IF and IR primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of ITS region was generated from forward and reverse sequence data using aligner software. The ITS region sequence was used to carry out BLAST with the nrdatabase of NCBI genebank database. Based on maximum identity score, first ten sequences were selected and the phylogenetic tree was constructed using MEGA 4.

The selected isolate NS-1 was identified as *Trichosporon asahii*, a yeast strain on the basis of above mentioned identification procedures and used further for the whole study.

**3.6 Production of chitin deacetylase from *Trichosporon asahii***

Chitin deacetylase was produced from the *Trichosporon asahii* under submerged fermentation (SmF) and solid state fermentation (SSF). The conditions for both SmF and SSF were optimized using the conventional one variable at-a-time (OVAT) approach to obtain maximum production of the enzyme. The procedures employed for SmF and SSF are explained below in section 3.6.3 and 3.6.5 respectively.

**3.6.1 Preparation of inoculum**

The inoculum was prepared by growing the *Trichosporon asahii* on CDA plate at 37°C for 2 days. Then, a loopful of *Trichosporon asahii* culture was taken from the CDA plate and inoculated in 250 ml conical flask containing 100 ml of CDA broth and incubated overnight at 37°C to allow the growth of yeast cells. This overnight grown culture was used to calculate colony forming unit per ml by McFarland method (annexure1). The turbidity of the culture was adjusted to 1.0 McFarland standard (~3×10⁸ CFU/ml) by diluting the culture with sterile saline (0.85%) and used as inoculum. One ml of this inoculum was transferred to another 100 ml of autoclaved CDA broth in a conical flask, incubated overnight at 37°C and used for further study.
The inoculum for both SmF and SSF was prepared by this method. The enzyme was extracted from the fermented medium by centrifugation in SmF and by squeezing the fermented medium through a wet muslin cloth followed by centrifugation in SSF. The centrifugation for both SmF and SSF was done at 10,000 x g for 20 min at 4ºC. The clear supernatant thus obtained was used as crude enzyme for chitin deacetylase assay by MBTH method.

3.6.2 Assay of chitin deacetylase activity

Chitin deacetylase activity was estimated using glycol chitin as a substrate prepared from glycol chitosan by the procedure of Araki and Ito (1975) ( Annexure). The quantitative assay of chitin deacetylase was done according to MBTH method of Kauss and Bauch (1988). The reaction mixture containing 50µl of crude enzyme, 100µl of substrate and 100µl of 50mM sodium tetraborate/HCl buffer (pH 8.5) was incubated at 37ºc for 30 minutes. The reaction was terminated by heating in boiling water bath for 5 min followed by the addition of 250µl of 5% (w/v) potassium hydrogen sulphate (KHSO₄). Controls were prepared by adding substrate solution after the addition of KHSO₄. This was followed by addition of 250µl of 5% (w/v) sodium nitrite (NaNO₂). The tubes were incubated for 30 min at room temperature with occasional shaking. Then 250µl of 12.5% (w/v) ammonium sulfamate (NH₄OSO₂NH₂) was added and the tubes were kept for 5 min at room temperature. For color development, 250µl of 0.5% (w/v) freshly prepared MBTH was added. The tubes were heated for 3 min in boiling water bath. Allow the reaction mixture to cool at room temperature and then 250µl of 0.5% (w/v) ferric chloride (freshly prepared) was added. The tubes were allowed to stand at room temperature for 30 min. The absorbance of blue color developed was measured at 650nm. The concentration of enzyme was computed against a standard curve prepared by using Glucosamine-HCl as standard.

One unit of enzyme was defined as the amount of enzyme required to produce 1.0 µg of glucosamine (GlcN) per minute when incubated with glycol chitin as substrate under the specified reaction conditions.

Construction of standard curve

A standard curve was prepared with 0 to 40µg/ml of Standard D-glucosamine-HCl. Different concentrations of glucosamine were prepared from 0.1mg/ml stock solution of D-glucosamine-HCl in 50mM sodium tetraborate/HCl buffer (pH 8.5). The further procedure for construction of standard curve of D-glucosamine-HCl was same as explained above for MBTH assay.
3.6.3 Optimization of chitin deacetylase production under SmF

*Trichosporon asahii* was initially cultivated for chitin deacetylase production under submerged fermentation using unoptimized conditions. The SmF was carried out in conical flasks (250ml capacity) containing 100ml of YPD medium supplemented with 1% chitin (pH 7.0). The flasks were seeded with 1% inoculum containing $3 \times 10^8$ CFU/ml and incubated at 37°C. After 48h of incubation, fermented broth was centrifuged at 10,000 x g for 15 min at 4°C. The clear supernatant collected after centrifugation was referred as the crude enzyme and used for the assay of chitin deacetylase activity.

For the enhancement of chitin deacetylase production from *Trichosporon asahii*, the enzyme production under SmF was optimized with respect to various physical and nutritional parameters using OVAT approach. For optimization, the enzyme production was carried out in YPD medium and the different parameters were varied one at a time keeping all other parameters constant. In each case, enzyme was produced at different values of the respective parameters followed by extraction of chitin deacetylase from the culture filtrate by centrifugation at 10,000 x g for 15 min at 4°C and assayed for enzyme activity. Condition optimized in each experiment was used in all the next experiments.

3.6.3.1 Effect of pH

The effect of pH on enzyme production was determined by using the autoclaved production medium of various pH ranging from 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0

![Fig 3.1 Standard curve of Glucosamine-HCl](image)
and 12.0. Each flask (250ml) containing 100ml of autoclaved production medium was inoculated with 1% of inoculum and incubated at 37\(^{0}\)C for 48 hours. The enzyme was extracted and assayed for chitin deacetylase activity.

### 3.6.3.2 Effect of temperature

Each flask (250ml) containing 100ml of autoclaved production medium was adjusted to optimized pH and inoculated with 1% of inoculum. The flasks were incubated for 48 hours at different temperature such as 25, 30, 37, 40, 45, 50, 55, and 60\(^{0}\)c. The enzyme was extracted and assayed for chitin deacetylase activity.

### 3.6.3.3 Effect of Incubation period

Each flask (250ml) containing 100ml of autoclaved production medium was adjusted to optimized pH and inoculated with 1% of inoculum. The flasks were incubated at optimized temperature for different time intervals viz. 24h, 48h, 72h, 96h, 120h, 144h and 168h. The enzyme was extracted and assayed for chitin deacetylase activity.

### 3.6.3.4 Effect of Agitation speed

Each flask (250ml) containing 100ml of autoclaved production medium was adjusted to optimized pH, inoculated and incubated at optimized temperature in a rotator shaker incubator with different rates of speeds viz. 50, 100, 150, 200, 250, 300, 350 and 400 rpm for optimized incubation period. The enzyme was extracted and assayed for chitin deacetylase activity.

### 3.6.3.5 Effect of Inoculum size

Using all the optimized physical conditions including agitation speed as described in section 3.7.5.4, the inoculum size was optimized by inoculating the production medium with the different size of inoculum ranging from 1.0 to 8.0%. The enzyme was extracted and assayed for chitin deacetylase activity.

### 3.6.3.6 Effect of carbon source

To study the effect of carbon source on chitin deacetylase production, various c-sources such as glucosamine, glucose, fructose, lactose, sucrose, maltose, starch, cellulose, mannitol and galactose were added separately at the concentration of 2% (w/v) in the minimal medium containing chitin as an inducer. Then, all the flasks containing medium were inoculated with optimized inoculum size and incubated at optimized conditions. In control, carbon source was not added. The enzyme was extracted and assayed for chitin deacetylase activity.
3. 6.3.7 Effect of Nitrogen source

To study the effect of nitrogen source on chitin deacetylase production, various nitrogen sources namely peptone, yeast extract, potassium nitrate, beef extract, tryptone, sodium nitrate, ammonium chloride, ammonium sulphate, ammonium phosphate, ammonium nitrate and urea were added separately in the minimal medium at the concentration of 1% (w/v) each. Then all the flasks containing medium were inoculated and incubated at optimized temperature and period. The control was devoid of nitrogen source. The enzyme was extracted and assayed for chitin deacetylase activity.

3. 6.3.8 Effect of Inorganic salts

Different inorganic salts namely MgSO\(_4\), FeSO\(_4\), ZnCl\(_2\), CuSO\(_4\), K\(_2\)HPO\(_4\), CoCl\(_2\) and MnSO\(_4\) were added separately to the minimal medium at the concentration of 0.08% (w/v) to observe their effect on chitin deacetylase production. Then all the flasks containing medium were inoculated and incubated at optimized temperature and period. The control was devoid of inorganic salt. The enzyme was extracted and assayed for chitin deacetylase activity.

3.6.4 Response Surface Methodology (RSM) and Central Composite Design (CCD) of experiments

The four most influential factors for chitin deacetylase production were found by “one-variable-at-a-time” (OVAT) approach. These factors were further optimized by Response Surface Methodology (RSM) using the Central Composite Design (CCD) of experiments. The RSM was used to develop a mathematical correlation between four independent variables on production of chitin deacetylase by the *Trichosporon asahii*. The four independent variables, chitin (A), yeast extract (B), sucrose (C) and magnesium sulfate (D) were chosen to study their effect on Chitin deacetylase production by *Trichosporon asahii* (Table 3.1).

Table 3.1 Range and levels of four selected variables in terms of actual and coded factors for central composite design for the production of chitin deacetylase

<table>
<thead>
<tr>
<th>Variables</th>
<th>Name</th>
<th>Levels</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-(\alpha)</td>
<td>-1</td>
</tr>
<tr>
<td>A</td>
<td>Chitin (%)</td>
<td>0.50</td>
</tr>
<tr>
<td>B</td>
<td>Yeast extract (%)</td>
<td>0.01</td>
</tr>
<tr>
<td>C</td>
<td>Sucrose (%)</td>
<td>0.50</td>
</tr>
<tr>
<td>D</td>
<td>Magnesium sulphate (%)</td>
<td>0.07</td>
</tr>
</tbody>
</table>
Table 3.2 Experimental plan for optimization of chitin deacetylase production using RSM

<table>
<thead>
<tr>
<th>Run</th>
<th>Variables</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chitin (% w/v)</td>
<td>Yeast extract (% w/v)</td>
<td>Sucrose (% w/v)</td>
<td>Magnesium sulfate (% w/v)</td>
</tr>
<tr>
<td>1</td>
<td>2.00</td>
<td>1.30</td>
<td>2.00</td>
<td>0.08</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>5.0</td>
<td>2.00</td>
<td>0.08</td>
</tr>
<tr>
<td>3</td>
<td>1.50</td>
<td>3.15</td>
<td>6.50</td>
<td>0.09</td>
</tr>
<tr>
<td>4</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.11</td>
</tr>
<tr>
<td>5</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>6</td>
<td>2.00</td>
<td>5.00</td>
<td>2.00</td>
<td>0.10</td>
</tr>
<tr>
<td>7</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.07</td>
</tr>
<tr>
<td>8</td>
<td>1.00</td>
<td>1.30</td>
<td>2.00</td>
<td>0.08</td>
</tr>
<tr>
<td>9</td>
<td>1.50</td>
<td>6.85</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>10</td>
<td>0.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>11</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>12</td>
<td>2.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>13</td>
<td>1.00</td>
<td>5.00</td>
<td>5.00</td>
<td>0.08</td>
</tr>
<tr>
<td>14</td>
<td>1.00</td>
<td>1.30</td>
<td>5.00</td>
<td>0.10</td>
</tr>
<tr>
<td>15</td>
<td>1.50</td>
<td>0.01</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>16</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>17</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>18</td>
<td>1.00</td>
<td>1.30</td>
<td>2.00</td>
<td>0.10</td>
</tr>
<tr>
<td>19</td>
<td>1.00</td>
<td>5.00</td>
<td>2.00</td>
<td>0.10</td>
</tr>
<tr>
<td>20</td>
<td>2.00</td>
<td>1.30</td>
<td>5.00</td>
<td>0.08</td>
</tr>
<tr>
<td>21</td>
<td>2.00</td>
<td>1.30</td>
<td>5.00</td>
<td>0.10</td>
</tr>
<tr>
<td>22</td>
<td>2.00</td>
<td>5.00</td>
<td>5.00</td>
<td>0.08</td>
</tr>
<tr>
<td>23</td>
<td>1.50</td>
<td>3.15</td>
<td>0.50</td>
<td>0.09</td>
</tr>
<tr>
<td>24</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>25</td>
<td>2.00</td>
<td>1.30</td>
<td>2.00</td>
<td>0.10</td>
</tr>
<tr>
<td>26</td>
<td>1.50</td>
<td>3.15</td>
<td>3.50</td>
<td>0.09</td>
</tr>
<tr>
<td>27</td>
<td>1.00</td>
<td>1.30</td>
<td>5.00</td>
<td>0.08</td>
</tr>
<tr>
<td>28</td>
<td>2.00</td>
<td>5.00</td>
<td>5.00</td>
<td>0.10</td>
</tr>
<tr>
<td>29</td>
<td>1.00</td>
<td>5.00</td>
<td>5.00</td>
<td>0.10</td>
</tr>
<tr>
<td>30</td>
<td>2.00</td>
<td>5.00</td>
<td>2.00</td>
<td>0.08</td>
</tr>
</tbody>
</table>
Experimental design was generated by using the statistical software package ‘Design Expert® 6.0’ Stat-Ease Inc., Minneapolis, MN, USA. Statistical analysis of experimental data was also performed using this software. A multiple regression analysis of the data was carried out for obtaining an empirical model that relates the response measured to the independent variables. A second-order polynomial equation for a four factor system was also calculated. A total of 30 experiments were necessary to study the coefficients of model (Table 3.2). All the experiments were done in triplicate and the average of chitin deacetylase yield obtained was taken as the dependent variable or response.

The three dimensional response surfaces were plotted to study the interaction among the various selected factors. The plots were generated by plotting the response using z-axis against two independent variables, while keeping the other independent variable at the O-level.

3.6.5 Chitin deacetylase production under SSF

To carry out chitin deacetylase production under solid state fermentation, the substrate is selected as explained below.

Selection of solid substrate for production of chitin deacetylase

Prior to optimization, chitin deacetylase was produced by *Trichosporon asahii* under SSF using various natural agricultural residual materials viz. wheat bran, wheat straw, rice straw, ground nut shells and sugarcane bagasse as substrate. These substrates were washed with water and oven dried at 60°C for 48h. The dried substrates were then pulverized and utilized as the substrates for SSF. Various agricultural residues each weighing 5g were taken separately in 500 ml Erlenmeyer flasks and moistened with 5ml of tap water so as to make the final moisture ratio of 1:1 (w/v) and 1% of chitosan was added as an inducer. The flasks were autoclaved at 121°C for 20 min. After cooling, the solid medium was inoculated with 20% (v/w) inoculum and kept in an incubator (which was humidified with water) at 37°C for 72h. The flasks were tapped intermittently in order to mix their contents. Chitin deacetylase was extracted from the fermented medium by adding 50ml of sodium tetraborate buffer (pH 8.5) to each flask along with shaking in an orbital shaker at 100 rpm for 30 minutes. The enzyme was collected by squeezing the fermented medium through a wet muslin cloth followed by its centrifugation at 10,000 x g for 20 min at 4°C. The clear supernatant thus obtained was used as crude enzyme for chitin deacetylase assay by
MBTH method. The rice straw was selected as substrate for SSF on the basis of maximum chitin deacetylase production tested among various substrates.

3.6.5.1 Optimization of chitin deacetylase production under SSF

Chitin deacetylase production in SSF was optimized by the conventional one variable at-a-time approach. The fermentation was carried out in Erlenmeyer flasks (500 ml) each of which contained 5 g of rice straw which was selected on the basis of above mentioned experiment. Each flask containing rice straw was moistened with 5 ml of tap water. The flasks were autoclaved, cooled and inoculated with 20% (v/w) inoculum. After incubation for 72 h at 37°C in an incubator, chitin deacetylase was extracted from the fermented rice straw as described above. The conditions for production of enzyme were optimized with respect to various factors such as moistening agents, pH, temperature, inoculum size, incubation time, moisture level and nitrogen source etc.

To evaluate the effects of various factors on the production of chitin deacetylase, the fermentation conditions were varied according to the experimental requirement. Condition optimized in each experiment was used in all the next experiments.

3.6.5.1.1 Effect of moistening agents

In 500 ml Erlenmeyer flasks containing 5gm of rice straw & 1% (w/w) chitosan, 5ml of different moistening agent (MAI- MA VII) was added. The flasks were autoclaved, inoculated and incubated at 37°C for 72 h. The enzyme was extracted and assayed for chitin deacetylase activity. The composition of different moistening agents is given in annexure1.

3.6.5.1.2 Effect of pH

5gm of rice straw and 1% chitosan was taken in 500 ml Erlenmeyer flasks. The initial pH of the contents of the flasks was adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0 and 12.0 by using the optimized moistening agent. The flasks were autoclaved, inoculated and incubated at 37°C for 72 h. The enzyme was extracted and assayed for chitin deacetylase activity.
3.6.5.1.3 Effect of temperature
5gm of rice straw and 1% chitosan moistened with 5ml of moistening agent adjusted to optimized pH were taken in 500 ml Erlenmeyer flasks. The flasks were autoclaved, inoculated and incubated at 25°C, 30°C, 37°C, 40°C, 45°C, 50°C, 55°C and 60°C temperature for 72 h. The enzyme was extracted and assayed for chitin deacetylase activity.

3.6.5.1.4 Inoculum size
Rice straw (5g) and chitosan 1%, after addition of 5 ml of moistening agent in 500-ml Erlenmeyer flasks were autoclaved and inoculated with different amounts (10%, 20%, 30%, 40%, 50%, 60%, 70% and 80%) of inoculum and incubated at optimized temperature for 72 h. The enzyme was extracted and assayed for chitin deacetylase activity.

3.6.5.1.5 Incubation period
5g of moistened rice straw along with 1% chitosan was inoculated with optimized inoculum size and incubated at optimized temperature for different time periods. Enzyme was extracted from fermented rice straw at 24h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h of incubation and tested for chitin deacetylase activity.

3.6.5.1.6 Effect of moisture level
5g of rice straw along with 1% chitosan was taken in Erlenmeyer conical flasks of 500 ml capacity and moistened with moistening agent at optimized pH to adjust the solid-liquid ratio as 1:1.0, 1:1.5, 1:2.0, 1:2.5, 1:3.0, 1:3.5 and 1:4.0. All the flasks were inoculated and incubated at optimized temperature. Extracts of fermented medium were assayed for chitin deacetylase activity.

3.6.5.1.7 Effect of nitrogen source
5g of moistened rice straw along with 1% chitosan was taken in 500 ml Erlenmeyer flasks and supplemented with 1% (w/w) of the following nitrogen sources viz. beef extract, yeast Extract, peptone, ammonium sulphate, potassium nitrate and sodium nitrate. A control devoid of nitrogen source was also prepared. All the flasks were inoculated and incubated at optimized temperature and period. The extracts of fermented medium were assayed for chitin deacetylase activity.

3.7 Purification of the enzyme
For purification, chitin deacetylase was produced from Trichosporon asahii under SmF and cell free extract was used as crude enzyme.
3.7.1 Preparation of crude enzyme extract

The *Trichosporon asahii* was grown in optimized medium under SmF. The fermented medium containing enzyme was centrifuged at 10000×g for 20 minute at 4°C to obtain the supernatant (crude enzyme extract). The supernatant was then dialyzed against sucrose to concentrate the enzyme. After dialysis, ammonium sulfate precipitation (0-80%) was performed. Total enzyme activity and protein in the crude extract was estimated at each step. The protein content was measured by the method of Lowry et al. (1951). The crude enzyme was purified using sequential conventional techniques. All steps of purification were carried out at 4°C.

**ESTIMATION OF PROTEIN**

Quantitative estimation of protein at various stages of purification was done by the method of Lowry et al. (1951) using Bovine serum albumin (BSA) as standard.

**Reagents**

A. \( 2\% \text{Na}_2\text{CO}_3 \text{in 0.1 N NaOH} \)

B. \( 0.5\% \text{CuSO}_4\cdot5\text{H}_2\text{O in H}_2\text{O} \)

C. \( 1\% \text{ Sodium potassium tartrate in H}_2\text{O} \)

D. \( 48 \text{ml of reagent A, 1 ml of reagent B and 1 ml of reagent C were mixed just before use.} \)

E. \( 1 \text{ part Folin-Ciocalteau reagent [2 N]: 1 part distilled water (Freshly prepared 1N Folin-Ciocalteau reagent)} \)

F. \( \text{BSA Standard protein Stock solution- 10 mg bovine serum albumin (BSA) was dissolved in 10ml of distilled water.} \)

Appropriately diluted 0.5 ml of chitin deacetylase enzyme was mixed with 4.5 ml of Reagent D and incubated for 10 minutes at room temperature. After incubation 0.5 ml of reagent E was added and incubated for 30 minutes at room temperature. A blank was also prepared in which 0.5 ml of distilled water instead of culture filtrate was added. The color developed was read at 660 nm on a spectrophotometer (Systronics) against reagent blank containing 0.5 ml distilled water. The concentration of soluble protein was computed against a standard curve prepared by using Bovine serum albumin (BSA) as standard.

**Construction of standard curve of Bovine serum albumin**

A standard curve was prepared with 0 to 50µg/ml of Bovine serum albumin (BSA). Different concentrations of BSA were prepared from 1mg/ml stock solution of
BSA in distilled water. The Lowry procedure for construction of standard curve of BSA was same as explained above for protein estimation.

**Fig 3.2 Standard curve of Bovine serum albumin**

### 3.7.1.2 Ammonium sulfate [(NH₄)₂SO₄] precipitation

The crude enzyme was subjected to (NH₄)₂SO₄ precipitation (0-80% saturation). Precipitation was carried out by addition of small amounts of ammonium sulfate with constant stirring at 4°C. It was ensured that all lumps of ammonium sulfate are broken. When all the ammonium sulfate get dissolved homogenate was kept overnight at 4°C. To recover the precipitates, it was centrifuged at 10,000×g for 20 min. Decant off the supernatant and the protein precipitates collected was dissolved in the minimum amount of 10 mM sodium phosphate buffer (pH 8.0). The solution was dialyzed for 24 hours in the dialysis bags (using cellulose dialysis tubing- Size 43×27mm Hi-media, India) against the same buffer at 4°C to remove other heat-unstable proteins. During dialysis, buffer was changed repetitively after every 6h. The dialyzed protein sample was analyzed for enzyme activity and protein content. The sample was used for further purification.

### 3.7.1.3 Sephadex G-100 column chromatography

15g Sephadex G-100 was slowly added to 10mM sodium phosphate buffer (pH 8.0) and allowed to swell at room temperature for 72h. The fine particles of the slurry were removed by decantation. A fresh batch of the elution buffer was added to the slurry. After this, the slurry was gently poured down with the help of glass rod into a column and was allowed to settle down under gravity for 3-4h. The Sephadex gel finally settled down to a height of 23 cm after several volumes of elution buffer were
passed through. Therefore, the size of the gel bed in the column was 23 x 3 cm. The column was equilibrated with 10mM sodium phosphate buffer (pH 8.0). The crude enzyme obtained after 0-80% ammonium sulfate precipitation was loaded on the top of column and eluted with same buffer. The unbound proteins were eluted by passing about one bed volume of 10mM sodium phosphate buffer (pH 8.0) through the column and then a continuous gradient of 0-1.5M NaCl (prepared in 10mM sodium phosphate buffer (pH 8.0) was applied to elute the proteins bound to Sephadex G-100. The column was run at a flow rate of 20 ml/h. Fractions of 5ml each were collected and analyzed for protein and enzyme activity.

The protein content was analyzed by measuring their absorbance at 280 nm. The protein containing fractions were tested for chitin deacetylase activity using MBTH method. The fractions containing chitin deacetylase activity were pooled. The enzyme activity and protein content were determined in the pooled enzyme as well as in crude extract so as to calculate specific activity, recovery and purification fold of the purified fraction. The purity of purified enzyme fraction was checked by both Native-PAGE and SDS-PAGE using a 12% polyacrylamide gel.

3.7.1.4 Native Polyacrylamide Gel Electrophoresis (Native-PAGE)

Native polyacrylamide gel electrophoresis was performed using the method of Davis (1964) using a 12% polyacrylamide gel.

Gel polymerization

The Glass plates, plastic combs and spacers were cleaned with detergent and then with 70% (v/v) ethanol. Properly cleaned and dried glass plates (8x7 cm) were assembled on gel caster of vertical slab gel assembly using 1mm spacers. The bottom of assembled glass plates was sealed by using resolving gel mixture and tested for leakage. The resolving gel mixture was prepared just before use as described in Table 3.3. Resolving gel of 12% Acrylamide was prepared by mixing all the components except TEMED. Then TEMED was added in to resolving gel mixture and immediately poured down into the space between the gel plates. Few drops of distilled water were layered over the resolving gel with the help of a micropipette to ensure the production of a flat gel surface and to exclude oxygen. The gel was allowed to polymerize completely. Polymerization was indicated by a sharp interface between water and gel. After polymerization of the resolving gel, the water layer was removed and soaked off with a filter paper. The stacking gel solution was then prepared according to its composition given in Table 1 and poured over the polymerized separating gel. A comb
was placed immediately at the top of the stacking gel solution so as to make wells for sample loading. The stacking gel was allowed to polymerize. After the stacking gel was polymerized, the comb was removed and the wells were cleaned thoroughly with reservoir buffer using a syringe so that no unpolymerized acrylamide was left in the wells. The gel plates were transferred to the gel electrophoresis unit and the reservoir buffer was poured in the lower and upper chambers of the apparatus in such a manner that no air bubble was trapped between the gel and the buffer.

Table 3.3 Composition of gel solution for Native and SDS-PAGE

<table>
<thead>
<tr>
<th>Stock Solutions</th>
<th>Native PAGE</th>
<th>SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stacking gel (5%)</td>
<td>Resolving gel (12%)</td>
</tr>
<tr>
<td>Acrylamide-bisacrylamide</td>
<td>2.00 ml</td>
<td>12.00 ml</td>
</tr>
<tr>
<td>Stacking gel buffer (0.5M, pH-6.8)</td>
<td>3.0 ml</td>
<td>-</td>
</tr>
<tr>
<td>Resolving gel buffer (1.5M, pH-8.8)</td>
<td>-</td>
<td>7.50 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>6.87 ml</td>
<td>8.58 ml</td>
</tr>
<tr>
<td>APS (1.5 % w/v)</td>
<td>0.12 ml</td>
<td>1.90 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.01 ml</td>
<td>0.015 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>12 ml</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

Electrophoresis, staining and de-staining of gels

The sample to be analyzed by Native PAGE was prepared by mixing an equal volume of protein sample and sample buffer (annexure1) in the ratio of 1:1. Sample was loaded into the wells of the polyacrylamide gel using a Hamilton syringe. The electrodes were connected to an electrophoretic power supply unit and the electrophoresis was carried out initially at 10mA to allow the sample to enter the gel and then the current was increased to 20mA till the tracking dye reached close to the bottom of the resolving gel. After electrophoresis, the polyacrylamide gel was taken out from the electrophoresis unit and stained with Coomassie brilliant blue R-250 staining
solution for 10h while shaking to visualize the protein bands. The staining solution was then removed and the gel was transferred to destaining solution and kept on a gel rocker with gentle shaking. The destaining solution was changed several times till the gel background was clear. The gel was then photographed and preserved in distilled water containing 10% glycerol in a dark and cool place.

3.7.1.5 SDS-PAGE

SDS-PAGE was performed to check the purity and molecular weight of enzyme by using the method of laemmli (1970).

Gel polymerization

SDS-PAGE was performed using 12% resolving and 5% stacking gel. The compositions of which are given in Table1. The procedure for gel polymerization was similar to Native-PAGE except the composition of resolving and stacking gels.

Molecular weight markers

A pre-stained mixture of SDS-PAGE molecular weight markers viz. phosphorylase b (97.4 kDa), bovine albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.3 kDa) was run simultaneously with the protein sample.

Electrophoresis, staining and destaining of gels

The sample to be analyzed by SDS-PAGE was prepared by mixing protein sample and sample buffer (5X) in the ratio of 4:1. The prepared sample was then boiled for 5 min in a water bath followed by cooling. The procedure for electrophoresis and processing of the gel for visualization of protein bands was same as described for native-PAGE.

Determination of molecular weight

The molecular weight of purified enzyme was determined by using SDS-PAGE. Purified chitin deacetylase was co-electrophoresed with pre-stained mixture of SDS-PAGE molecular weight markers in the lanes labelled as (A) protein molecular weight markers, (B) crude extract, (C) purified CDA and (D) fraction obtained after ammonium sulfate precipitation (0-80%). The molecular weight of purified protein was calculated with respect to molecular weight of markers which were run in the adjoining lane.

3.8 Characterization of purified chitin deacetylase

The purified chitin deacetylase was characterized with respect to their pH and temperature optima, stability of purified enzymes at different pH and temperature
values, effect of different metal ions, additives and substrate concentration on enzymes activity.

3.8.1 Effect of pH on chitin deacetylase activity

The pH optimum of purified chitin deacetylase was determined by carrying out the enzyme assay at different pH values using sodium acetate (pH 4.0-6.0), sodium phosphate (pH 7.0-8.0), Tris-HCl (pH 9.0) and glycine-NaOH (pH 10.0-12.0) buffers of 50mM concentration.

For this, the substrate (glycol chitin) solution was prepared in the above mentioned buffers and used in the reaction mixture, which led to different assay pH values. The observed enzyme activity at each pH value was calculated as relative activity (%) by considering the maximum activity as 100%. A pH profile was drawn between pH on x-axis and relative chitin deacetylase activity (%) on y-axis. The pH showing maximum chitin deacetylase activity was taken as the pH optimum.

3.8.2 pH stability profile of chitin deacetylase

The effect of pH on stability of purified chitin deacetylase was examined by pre-incubating 50 µl of enzyme and 50 µl of the above mentioned buffers in the pH range 4.0-12.0 in the absence of substrate for 2 h at room temperature followed by measurement of chitin deacetylase activity at 30 min intervals under optimal assay conditions. A control was run simultaneously in which the enzyme was incubated with distilled water instead of a buffer. The residual enzyme activity (%) at each pH value was calculated as a percent of the control. A profile for pH stability of the enzyme was drawn between time on x-axis and residual chitin deacetylase activity (%) on y-axis.

3.8.3 Effect of temperature on chitin deacetylase activity

The optimum temperature of purified chitin deacetylase was determined by carrying out the enzyme assay at different temperatures ranging from 25-60ºC. The observed enzyme activity at each temperature was calculated as relative activity (%) by considering the maximum activity as 100%.

A temperature profile was drawn between temperature on x-axis and relative chitin deacetylase activity (%) on y-axis. The temperature showing maximum chitin deacetylase activity was taken as the optimum temperature.

3.8.4 Thermal stability of chitin deacetylase

Thermal stability of purified chitin deacetylase was examined by pre-incubating the enzyme at different temperatures ranging from 25-60ºC for 2h in the absence of the substrate. Samples were taken at 30 min intervals for measurement of chitin
deacetylase activity under optimal assay conditions. The residual enzyme activity (%) at each temperature was calculated as a percent of the control in which the enzyme was not pre-incubated. A profile for enzyme thermostability was drawn between time on x-axis and residual chitin deacetylase activity (%) on y-axis.

3.8.5 Effect of additives on chitin deacetylase activity

Solution of metal ions and additives were prepared in 50 mM sodium tetraborate buffer (pH 8.5) at 1mM concentration. The effect of various metal ions viz. NaCl, KCl, MgCl₂, CaCl₂, MnCl₂, HgCl₂, CoCl₂, ZnSO₄, FeSO₄, FeCl₃, MgSO₄, CuSO₄, EDTA and urea on purified chitin deacetylase was studied by pre-incubating the purified enzyme with equal volume of 1 mM metal salts solution for 30 min at room temperature followed by measurement of chitin deacetylase activity under standard assay conditions. The residual activity (%) was calculated with reference to the control in which enzyme was not incubated with any metal ion. The activity assayed in the absence of metal ions was recorded as 100%.

3.8.2.4 Determination of Michaelis-Menton constant (Kₘ) and Vₘₐₓ

The activity of purified extracellular chitin deacetylase was measured at different concentrations (0.003-0.01%) of the substrate (glycol chitin). A Lineweaver-Burk plot also called double reciprocal plot was then drawn using the reciprocal of the substrate concentrations on x-axis and reciprocal of the corresponding chitin deacetylase activity values on y-axis. The Michaelis-Menton constant (Kₘ) and Vₘₐₓ of chitin deacetylase were calculated from this Lineweaver-Burk plot.

3.9 Application of chitin deacetylase

The chitin deacetylase produced from *Trichosporon asahii* was tested for its potential in bioconversion of chitin to chitosan.

3.9.1 Bioconversion of chitin to chitosan

The bioconversion of chitin to chitosan was carried out by the method of Kaur et al. (2012). The flasks containing production medium (Annexure) for bioconversion of chitin to chitosan were inoculated with 3% of inoculum of *Trichosporon asahii*. The un-inoculated flask was used as control. All the flasks were incubated on rotary shaker (300rpm) at 37°C for three days. After incubation, each flask was taken for chitosan recovery. The fermented broth from each flask was centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatant was discarded. 10mL of 0.1N NaOH was added to the pellet which contains a mixture of bacteria, chitin, and chitosan. The contents were mixed thoroughly and taken in separate clean flasks and autoclaved for 20 minutes at
The flasks were then allowed to cool at R.T. The alkali treatment was given to dissolve all the cells. The contents from the flasks were again centrifuged at 12000 rpm for 15 minutes at 4°C. The supernatants were carefully removed. The pellets containing chitin, chitosan, and small amount of cell debris were mixed with 10mL of 2% acetic acid and left on a shaker overnight at room temperature to solubilize chitosan in acetic acid. Then, the mixtures were taken in clean flasks. The contents of the above flasks were again centrifuged at 12000 rpm for 15 minutes. Pellet was discarded and supernatant was collected. The presence of chitosan in it was checked by neutralization with 1N NaOH.

3.9.1.1 Estimation of Chitosan

The precipitate obtained above in section 3.9.1 was centrifuged at 5000 rpm for 15 minutes and then washed twice with distilled water (pH-7). After this, the precipitate was resuspended in 0.5mL of distilled water and this suspension was taken in watch glass. It was allowed to dry at 55°C for 2–4 hours. The dried precipitate was used for the confirmatory test. The dried precipitate was mixed with a few drops of iodine solution and the mixture was then acidified with 2-3 drops of 1% H₂SO₄.

For quantitative estimation, 1ml of chitosan suspension in distilled water as obtained in section 3.9.1 was taken in the clean beaker and kept at 55°C for 2–6 hours for drying. After drying, loss on drying of the prepared chitosan will be determined by the gravimetric method. The water mass loss will be determined by drying the sample to constant weight and measuring the sample after and before drying. The water mass (or weight) will be the difference between the weights of the wet and oven dried samples (Hu et al., 2002; Puvvada et al., 2012).

The weight of chitosan produced was also determined by the gravimetric method. The weight of a clean & dried empty beaker was taken. In that beaker, the dried sample of chitosan was taken and plate was again weighed.

**Percentage yield of chitosan**

Weight of empty beaker = x gm
Weight of beaker with dried chitosan = y gm
Weight of chitosan produced = y-x = z gm
Initial amount of chitin taken= a gm
Amount of chitosan produced= z gm

Yield % = Amount of chitosan produced x 100/ Initial amount of chitin

\[ z \times 100/a = b\% \]
Loss on drying
The percent loss on drying was calculated as follows:
Weight of empty beaker = x gm
Weight of beaker with wet sample of chitosan = n gm
Weight of wet sample of chitosan= n-x = p gm
Weight of dried sample of chitosan = z gm
% loss on drying = (Wet weight- Dry weight)/ Dry weight × 100
% loss on drying = (p - z)/ z × 100 = q %

3.9.2 Characterization of chitosan
To utilize the chitosan samples for various industrial and biomedical applications, it is essential to determine the degree of deacetylation (DD). Because this parameter affects the property of chitosan. Different methods have been reported for determination of degree of deacetylation of chitosan but proton nuclear magnetic resonance (\(^1\)H-NMR) and FTIR are frequently used. In the present study the chitosan produced was analyzed for its quality using analytical methods viz. FTIR and NMR. The degree of deacetylation was calculated from \(^1\)H-NMR.

3.9.2.1 FTIR Spectroscopy
The spectra of chitosan samples were obtained by using Perkin Elmer-Spectrum RX-IFTIR instrument with in a frequency range of 4000-400 cm\(^{-1}\) (SAIF PU, Chandigarh). Measurements have been performed in the transmission mode.

3.9.2.2 NMR Spectroscopy
Proton nuclear magnetic resonance (\(^1\)H-NMR) spectra of samples were recorded in D\(_2\)O solution using BRUKER Avance 11 400 NMR spectrophotometer (SAIF PU, Chandigarh). NMR spectroscopy is one of the most accurate methods for determining the degree of deacetylation. Interpretation of results is clear and the values obtained are reproducible.