# Lists of Tables

(Content wise)

<table>
<thead>
<tr>
<th>CONTENT</th>
<th>TABLE NUMBER</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>GENERAL</td>
<td>Table 1: Proteases producing fungi</td>
<td>11</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>Table 2: Characteristics of the extracellular proteolytic enzymes from basidiomycetes</td>
<td>25-27</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>Table 1: Kinetics of proteolytic enzyme production using Wheat Bran as complex C-source</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Table 2: Kinetics of proteolytic enzyme production in presence of protein supplements</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Table 3a: Effect of inhibitors on both proteases</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Table 3b: Effect of detergent, reducing agent, metal ions and commercial detergents</td>
<td>58</td>
</tr>
<tr>
<td>CHAPTER 2</td>
<td>Table 1a: Milk-clotting and proteolytic activities of AcP, ratio (R) between these activities</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>Table 1b: Extracellular AcP purification table</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Table 2a: Identification of the protein spot R1 from 2DE by PMF and Mascot search</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>Table 2b: Calculated and observed ions of trypsin digests of purified AcP (spot R1)</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>Table 3: Characteristics of some commercially known microbial proteinases</td>
<td>75</td>
</tr>
<tr>
<td>CHAPTER 3</td>
<td>Table 1a: Minimum and Maximum ranges (actual) for the parameters selected in Plackett-Burman design</td>
<td>92</td>
</tr>
</tbody>
</table>
Table 1b: Plackett-Burman experimental design variables along with the observed results

Table 2a: Box-Behnken experimental design of three independent variables with actual and predicted responses as protease activity

Table 2b: Analysis of variance (ANOVA) for the response surface quadratic model

Table 3: Purification of extracellular AkP

Table 4: NCBI protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search analysis

Table 5: Calculated and observed ions of trypsin digests of purified AkP (2DE protein spot)

Table 6: Susceptibility of protease towards different inhibitors

Table 7: Physico-chemical analysis of treated tannery effluent

CONCLUDING REMARKS

Table 1: Applications and properties of some microbial proteases
Bioremediation by alkaline protease (AkP) from edible mushroom *Termitomyces clypeatus*: optimization approach based on statistical design and characterization for diverse applications

Rajib Majumder, a Samudra Prosad Banik, b Lata Ramrakhiania and Suman Khowala a*

Abstract

BACKGROUND: Bioremediation using enzymes has become an attractive approach for removing hazardous chemicals from the environment. The present study investigated the production, optimization and applications of an extracellular alkaline protease (AkP) from the edible mushroom *Termitomyces clypeatus* focusing on bioremediation.

RESULTS: For optimized enzyme production, the variables significantly influencing the protease activity were screened through the 11 factorial Plackett–Burman design. The optimum values of the selected variables and their mutual interactions were determined through response surface methodology using the Box–Behnken experimental design. Overall a 10-fold increase in enzyme activity (1830 U mL⁻¹) was obtained which closely matched the yield predicted by the statistical model (1778.63 U mL⁻¹) with $R^2 = 0.9819$. AkP could efficiently dehair the goat skin, detached bird feather vanes from its shaft and reduce BOD, COD and pH of tannery waste effluent. The AkP also demonstrated bactericidal property against *Staphylococcus aureus* as detected by MIC and flow cytometry analyses.

CONCLUSION: As an ecofriendly alternative, the enzyme showed significant promise for bioremediation and industrial applications through time-saving bioprocesses. This is first report of alkaline protease from *T. clypeatus* or from a fungal source with wide-ranging application potential.

© 2014 Society of Chemical Industry

Keywords: alkaline protease; hide dehairing; feather detachment; bactericidal properties; Plackett–Burman; *Termitomyces clypeatus*

INTRODUCTION

The development and preparation of efficient enzymes for use in bioremediation process as well as in other industrial waste management remains a key challenge. The enzymatic processes have advantages over traditional physico-chemical methods as they are regarded as ‘clean and green’, safe and economic alternatives. Proteases and other hydrolytic enzymes have been used in bioremediation processes due to their involvement in the accelerated solubilization and hydrolytic stages of breakdown of polymeric organic compounds into smaller simple monomers.¹ The leather industry has been a source of significant environmental pollution. The consistent use of hazardous chemicals such as lime and sulfide for leather dehairing has also aggravated the environmental risk factors.²–⁴ Presence of these chemicals in tannery waste causes health hazards to the tannery workers. Lime produces a poisonous sludge while sodium sulfide is highly toxic and has an obnoxious odour.

Poultry farms are another concern that contributes to environment pollution. Keratinases play an important role in biotechnological applications such as improvement of feather meal in that respect. It is necessary to develop clean and eco-friendly technologies for leather processing and effluent treatment. Extracellular enzymes with keratinolytic activity have potential use as dehairing agent in leather and cosmetic industries.⁵,⁶ Some microorganisms producing extracellular enzyme with dehairing activity have been described.²–⁶,⁷–¹⁰ Many proteases are not suitable for dehairing, since they have associated collagen-degrading activity, which destroys the collagen structure in leather. Therefore, it is essential to find proteases with nil to low collagen-degrading activity while maintaining high dehairing activity.¹¹ In the past 60 years, antibiotics have been critical in the fight against infectious disease caused by bacteria and other microbes.

* Correspondence to: Suman Khowala, Drug Development Diagnostics and Biotechnology Division, CSIR-Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Kolkata-700032, West Bengal, India. E-mail: sumankhowala@iicb.res.in; sumankhowala@yahoo.com

a CSIR- Indian Institute of Chemical Biology, Drug Development Diagnostics and Biotechnology Division, 4, Raja S.C. Mullick Road, Kolkata, 700032, India

b Department of Microbiology, Maulana Azad College, 8, Rafi Ahmed Kidwai Road, Kolkata, 700013, India
Microbial development of resistance, as well as economic incentives, has resulted in research and development in the search for new types of antimicrobial agents or combined drugs in order to ensure continuous availability of a pool of effective drugs. Interest in this respect has grown and led to increased importance of proteases because of their antagonistic activity towards different human pathogens and reports of bactericidal activity. Proteases were assumed to play roles in the host defence mechanisms against various microorganisms by either killing them by increased permeability of both inner and outer membranes resulting in morphological changes of pathogen or by cleaving bacterial virulence factors.12

Microorganisms such as filamentous fungi are easily extracted and separated from mycelium and owing to their GRAS (generally regarded as safe) nature have now become popular for the production of enzymes. Fungal proteases are more versatile than bacterial enzymes in terms of substrate specificity and pH tolerance.13 For example, Aspergillus oryzae produces acid, neutral, and alkaline proteases.14 A 30kDa novel alkaline serine protease from wild edible mushroom Termitomyces alburninus was reported.15 Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases constitute two-thirds.16

Termitomyces clypeatus, an edible variety of basidiomycetes filamentous fungus, has gained considerable importance as a good producer of industrial enzymes with promising thermostabilities.17 Use of T. clypeatus biomass in bioremediation was reported for the first time through its chromium tolerance.18 Microorganisms such as filamentous fungi are easily extracted and separated from mycelium and owing to their GRAS (generally regarded as safe) nature have now become popular for the production of enzymes. Fungal proteases are more versatile than bacterial enzymes in terms of substrate specificity and pH tolerance.13 For example, Aspergillus oryzae produces acid, neutral, and alkaline proteases.14 A 30kDa novel alkaline serine protease from wild edible mushroom Termitomyces alburninus was reported.15 Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two-thirds.16

Termitomyces clypeatus, an edible variety of basidiomycetes filamentous fungus, has gained considerable importance as a good producer of industrial enzymes with promising thermostabilities.17,18 Use of T. clypeatus biomass in bioremediation was reported for the first time through its chromium tolerance.18 Microorganisms such as filamentous fungi are easily extracted and separated from mycelium and owing to their GRAS (generally regarded as safe) nature have now become popular for the production of enzymes. Fungal proteases are more versatile than bacterial enzymes in terms of substrate specificity and pH tolerance.13 For example, Aspergillus oryzae produces acid, neutral, and alkaline proteases.14 A 30kDa novel alkaline serine protease from wild edible mushroom Termitomyces alburninus was reported.15 Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two-thirds.16

Termitomyces clypeatus, an edible variety of basidiomycetes filamentous fungus, has gained considerable importance as a good producer of industrial enzymes with promising thermostabilities.17,18 Use of T. clypeatus biomass in bioremediation was reported for the first time through its chromium tolerance.18 Microorganisms such as filamentous fungi are easily extracted and separated from mycelium and owing to their GRAS (generally regarded as safe) nature have now become popular for the production of enzymes. Fungal proteases are more versatile than bacterial enzymes in terms of substrate specificity and pH tolerance.13 For example, Aspergillus oryzae produces acid, neutral, and alkaline proteases.14 A 30kDa novel alkaline serine protease from wild edible mushroom Termitomyces alburninus was reported.15 Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two-thirds.16

Termitomyces clypeatus, an edible variety of basidiomycetes filamentous fungus, has gained considerable importance as a good producer of industrial enzymes with promising thermostabilities.17,18 Use of T. clypeatus biomass in bioremediation was reported for the first time through its chromium tolerance.18 Microorganisms such as filamentous fungi are easily extracted and separated from mycelium and owing to their GRAS (generally regarded as safe) nature have now become popular for the production of enzymes. Fungal proteases are more versatile than bacterial enzymes in terms of substrate specificity and pH tolerance.13 For example, Aspergillus oryzae produces acid, neutral, and alkaline proteases.14 A 30kDa novel alkaline serine protease from wild edible mushroom Termitomyces alburninus was reported.15 Global requirements of thermostable biocatalysts are far greater than those of the mesophiles of which proteases contribute two-thirds.16

MATERIALS AND METHODS

Chemicals and reagents

All media components, Luria Bertani broth (LB) and agar were purchased from HiMedia, India. Azocasein, gelatine, collagen type I, propidium iodide (PI), sodium dodecyl sulphate (SDS), glycine, β-mercaptoethanol, coomassie brilliant blue R-250, acrylamide/bisacrylamide, tris, Brij-35 solution were purchased from Sigma-Aldrich, USA. Protease inhibitors were purchased from Roche Applied Science and Calbiochem-Merck, Germany. Eosin-Y was purchased from Merck-Millipore and Hematoxylin from Poly-sciences, Inc. All other chemicals and salts (analytical grade) were procured from Merck and Fisher Scientific International, Inc. Other materials were purchased locally.

Organism and inoculum preparation

T. clypeatus (MTCC-5091) was cultured under submerged condition in media containing (%; vv-1) glucose - 5.0, malt extract - 1.0, NH₄H₂PO₄ - 2.5 at pH 6.0 using 5% (vv-1) inoculum, and incubated for 40 h at 30°C to get the seed culture.

Protease production

Initial screening of the most significant carbon and nitrogen sources (simple and complex) allowing maximum protease production was performed by a one-variable-at-a-time approach. Cultures were inoculated at a level of 1% (vv-1) in 500 mL Erlenmeyer flasks (100 mL medium) and incubated on a rotator shaker (300 rpm) for 6 days.

Protease activity assay

Protease activity of cell extracts was assayed using azo-casein22 and keratin23 as substrates. Alkaline protease activities were assayed at pH 10, unless otherwise specified. In brief, azo-dye linked substrate azo-casein (1%, vv-1) was prepared in 0.1 mol L⁻¹ Tris-HCl buffer (pH 7.4). After 30 min incubation at 50 °C the reaction was stopped using 10% chilled trichloroacetic acid (TCA) and kept on ice for 15 min. Absorbance of the supernatant after centrifugation at 12 000 x g for 15 min was measured at 440 nm. One unit of activity is defined as the amount causing a change in optical density (OD) of 0.01 at 440 nm min⁻¹.

For estimation of keratinase activity, keratin (0.5%, vv-1) was dissolved in Tris-HCl buffer of pH 7.4. Insoluble residues were removed by filtration through Whatman No. 1 filter paper and activity was assayed. 1 mL of substrate was incubated with 1 mL of appropriately diluted protease preparations at 50 °C for 30 min, and the reaction was stopped using 10% ice cold TCA. After 15 min, tyrosine released was estimated using folin-ciocalteau’s reagent and absorbance measured at 660 nm. One unit of protease was considered as the amount required for release of 1 pmol L⁻¹ of tyrosine per minute under optimal condition. Protein content was estimated by Bradford method using BSA as standard.

Plackett-Burman design

Plackett-Burman Design-Expert 7.1 statistical software package (Stat-Ease, Inc., Minneapolis, MN, USA) was used.23 Eleven culture

<table>
<thead>
<tr>
<th>Variables</th>
<th>Factors</th>
<th>Concentrations (A-H, (%; wv⁻¹))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sucrose</td>
<td>0.5</td>
</tr>
<tr>
<td>B</td>
<td>Casein</td>
<td>0.5</td>
</tr>
<tr>
<td>C</td>
<td>Mycological peptone</td>
<td>0.5</td>
</tr>
<tr>
<td>D</td>
<td>Proteose peptone</td>
<td>0.5</td>
</tr>
<tr>
<td>E</td>
<td>Soyabean meal</td>
<td>0.5</td>
</tr>
<tr>
<td>F</td>
<td>Wheat bran</td>
<td>0.5</td>
</tr>
<tr>
<td>G</td>
<td>Bispeptone</td>
<td>0.5</td>
</tr>
<tr>
<td>H</td>
<td>Yeast extract</td>
<td>0.5</td>
</tr>
<tr>
<td>I</td>
<td>Inoculum volume (ml)</td>
<td>1.0</td>
</tr>
<tr>
<td>J</td>
<td>Agitation (rpm)</td>
<td>200</td>
</tr>
<tr>
<td>K</td>
<td>Dummy (D1)</td>
<td>–1</td>
</tr>
</tbody>
</table>

11 culture parameters including one dummy (D1) were evaluated at two levels (high (+1) and low (−1)) based on the experimental design developed by Plackett and Burman. All media components were taken into media in percentage (%; wv⁻¹)
### Table 1(b). Plackett-Burman experimental design variables (actual level) along with the observed results

<table>
<thead>
<tr>
<th>Variables (actual)</th>
<th>Trial</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
<th>K</th>
<th>Protease activity (U mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>300</td>
<td>–1</td>
<td>231</td>
<td>200</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>200</td>
<td>1</td>
<td>176</td>
<td>1</td>
<td>300</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>3</td>
<td>300</td>
<td>–1</td>
<td>539</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>3</td>
<td>200</td>
<td>–1</td>
<td>234</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>200</td>
<td>–1</td>
<td>234</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>300</td>
<td>–1</td>
<td>1101</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>3</td>
<td>–1</td>
<td>944</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>3</td>
<td>200</td>
<td>–1</td>
<td>176</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>200</td>
<td>1</td>
<td>539</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>0.5</td>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>3</td>
<td>300</td>
<td>–1</td>
<td>234</td>
<td>1</td>
</tr>
<tr>
<td>11</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>201</td>
<td>300</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>3</td>
<td>300</td>
<td>1</td>
<td>284</td>
<td>300</td>
</tr>
</tbody>
</table>

The significant variables were screened in 12 combinations in accordance with the design matrix. The responses were measured in terms of protease activity on day 5. The principal effect of each variable on response (AkP activity in U mL⁻¹) was calculated using the Design-Expert 7.1 statistical software package (Stat-Ease, Inc., Minneapolis, MN, USA).

### Table 2(a). Box-Behnken experimental design of three independent variables with actual and predicted responses as protease activity

<table>
<thead>
<tr>
<th>Actual variable level (% w/v⁻¹)</th>
<th>Protease activity (U mL⁻¹)</th>
<th>Standard order</th>
<th>X1</th>
<th>X2</th>
<th>X3</th>
<th>Actual value</th>
<th>Predicted value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1220</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.38</td>
<td>1248.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1830</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>0.38</td>
<td>1778.63</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1044</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0.38</td>
<td>1095.5</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1638</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>0.38</td>
<td>1609.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>950</td>
<td>5</td>
<td>1</td>
<td>2</td>
<td>0.25</td>
<td>928.0</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>980</td>
<td>6</td>
<td>3</td>
<td>2</td>
<td>0.25</td>
<td>1060.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>637</td>
<td>7</td>
<td>1</td>
<td>2</td>
<td>0.5</td>
<td>556.5</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1400</td>
<td>8</td>
<td>2</td>
<td>3</td>
<td>0.5</td>
<td>1489.75</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1420</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td>1390.88</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1401</td>
<td>10</td>
<td>2</td>
<td>3</td>
<td>0.25</td>
<td>1339.38</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1477</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>0.5</td>
<td>1528.63</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>1218</td>
<td>12</td>
<td>2</td>
<td>3</td>
<td>0.5</td>
<td>1247.13</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>1582</td>
<td>13</td>
<td>2</td>
<td>2</td>
<td>0.38</td>
<td>1567.00</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1548</td>
<td>14</td>
<td>2</td>
<td>2</td>
<td>0.38</td>
<td>1567.00</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>1560</td>
<td>15</td>
<td>2</td>
<td>2</td>
<td>0.38</td>
<td>1567.00</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>1570</td>
<td>16</td>
<td>2</td>
<td>2</td>
<td>0.38</td>
<td>1567.00</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>1575</td>
<td>17</td>
<td>2</td>
<td>2</td>
<td>0.38</td>
<td>1567.00</td>
<td></td>
</tr>
</tbody>
</table>

X₁ = Mycological peptone; X₂ = Soyabean meal; X₃ = Sucrose.

### Parameters were evaluated at two levels (high (+1) and low (-1)) (Table 1(a)). The significant variables were screened in 12 combinations in accordance with the design matrix (Table 1(b)). The responses were measured in terms of protease activity (U mL⁻¹) on day 5.

### Response surface methodology based on Box-Behnken design

The Box-Behnken design for three independent variables, each at three levels, was used to develop a second-order polynomial model for determining further optimum levels. A total of 17 experimental runs with various combinations of mycological peptone (X₁), soyabean meal (X₂) and sucrose (X₃) were conducted (Table 2(a)) for 5 days. The behavior of each variable, their interactions, and statistical analysis to obtain predicted responses was explained by the following second-order polynomial equation:

\[ Y = B_o + \sum B_i X_i + \sum B_{ij} X_i X_j + \sum B_{ii} X_i^2 \]

where Y is the predicted response, B₀ - offset term, Bᵢ - linear effect, Bᵢᵢ - squared effect, Bᵢᵢ - interaction effect, and Xᵢ is the ith independent variable. Xᵢ Xᵢ and Xᵢ² represent the interaction and quadratic terms, respectively, followed by the quadratic model verification.

### Effect of additives, inhibitors, reducing agent and commercial detergents on AkP activity

Residual AkP activity was determined in the presence of reagents: SDS (0.5%, w/v⁻¹), Triton X-100 (1%, w/v⁻¹), E-64 (10 μmol L⁻¹), ethylenediaminetetraacetic acid (EDTA) (5 mmol L⁻¹), iodoacetamide (5 mmol L⁻¹), phenylmethanesulfonyl fluoride (PMSF) (0.1 mmol L⁻¹), 1,10-Phenanthroline (10 mmol L⁻¹), Antipain (50 μmol L⁻¹), β-mercaptoethanol (β-ME) (0.5%, w/v⁻¹), Pepstatin A (1 mmol L⁻¹), and different concentrations of salts. 24

### Zymography of AkP

100 μg of protein was run on 7.5% SDS-polyacrylamide gels for zymography in the presence of gelatin and collagen. 25

### Bioremediation/applications

Optimized AkP activity obtained by Box-Behnken method in culture filtrate of T. clypeatus was taken for all downstream applications while uninoculated media was used as control. AkP enzyme preparation (specific activity of 788.21 U mg⁻¹) was used for feather detachment and goat skin dehauling studies. (i) Feather detachment studies. This was done according to Bockle et al.27 Feathers having a central shaft length of 13.7 ± 0.5 cm and total vane-width on either side of 2.55 ± 0.1 cm with average dry weight of 0.19 ± 0.027 g were used. Approximately 900 U (1.17 mg) of appropriately diluted AkP were

---

The image contains a table and text discussing the production, optimization, and application of alkaline protease of *T. clypeatus*. The text includes a Plackett-Burman experimental design variables (actual level) along with the observed results, as well as a Box-Behnken experimental design of three independent variables with actual and predicted responses as protease activity. The tables are used to analyze the effects of various factors on protease activity, including protease activity in culture filtrate of *T. clypeatus* for all downstream applications while uninoculated media was used as control.
Table 2(b). Analysis of variance (ANOVA) for the response surface quadratic model

<table>
<thead>
<tr>
<th>Sum of squares</th>
<th>Degree of freedom</th>
<th>Mean square</th>
<th>F-value</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression (model)</td>
<td>1.496E + 006</td>
<td>9</td>
<td>1.662E + 005</td>
<td>42.10</td>
</tr>
<tr>
<td>Residual</td>
<td>27641.75</td>
<td>7</td>
<td>3948.82</td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>26933.75</td>
<td>3</td>
<td>8977.92</td>
<td>50.72</td>
</tr>
<tr>
<td>Pure error</td>
<td>708.00</td>
<td>4</td>
<td>177.00</td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>1.524E + 006</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

R² = 0.9819; adjusted R² = 0.9585; CV = 4.62%.

RESULTS AND DISCUSSION

Various simple and complex carbon and nitrogen sources along with other supplements were used to study the effects on AKP production. Maximum AKP production was calculated in myco-

logical peptone supplemented media (149.4 U mL⁻¹) followed by activities 138.7, 99.50, 68, 25.07, and 18.52 U mL⁻¹ respectively for soyabean meal, proteose peptone, yeast extract, casein and biopeptone containing media preparation with sucrose as sole carbon source. Specific nitrogen supplement on protease produc-

tion differ from organism to organism although complex nitrogen sources are usually used for protease production. In this study, it appeared that nonutilization of inorganic nitrogen in the form of KNO₃, led to reduced protease production. A similar observation was reported for fungus R. solani where significant decrease in proteo-

telic activity was found when KNO₃, was added to medium for growth.31 The strain T. clypeatus is an edible variety of fungus, thus secreted enzymes can be used safely for different industrial pur-

poses. T. clypeatus AKP could hydrolyze the large globular protein azo-casein, showed good keratinase activity and was characterized for its susceptibility towards different inhibitors and additives.

Plackett-Burman design

The main effects of variables (Table 1(a)) on AKP production are shown in Table 1(b). Variables with confidence levels of P < 0.01 were considered to be significant. On the basis of statistical
analyses, the variables evidencing the most significant effects were as follows: mycological peptone \((P = 0.0007)\), soyabean meal \((P = 0.006)\), sucrose \((P = 0.002)\) and agitation in rpm \((P = 0.004)\). For AkP production, sucrose was one of the significant variables with increasing sucrose concentration causing lower AkP production. Mycological peptone and soyabean meal also exhibited high levels of significance, both having positive effects. Thus, three variables (sucrose, mycological peptone and soyabean meal) exerting significant impacts on AkP production were selected for further optimization by Box-Behnken design and response surface (RSM) analysis.

**Box-Behnken design and RSM analysis**
Sucrose, mycological peptone and soyabean meal were utilized to determine their optimal levels. As shown in Table 2(a), a total of 17 runs were conducted to optimize these three independent variables in the Box-Behnken design. The data were analyzed via multiple regression analysis, and the following second-order polynomial equation was obtained to express AkP production as

\[
	ext{AkP} = f(\text{sucrose}, \text{mycological peptone}, \text{soyabean meal})
\]

\[
	ext{AkP} = \beta_0 + \beta_1 \text{sucrose} + \beta_2 \text{mycological peptone} + \beta_3 \text{soyabean meal} + \beta_4 \text{sucrose}^2 + \beta_5 \text{mycological peptone}^2 + \beta_6 \text{soyabean meal}^2 + \beta_7 \text{sucrose} \times \text{mycological peptone} + \beta_8 \text{sucrose} \times \text{soyabean meal} + \beta_9 \text{mycological peptone} \times \text{soyabean meal}
\]

The coefficients \(\beta_0, \beta_1, \beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7, \beta_8, \beta_9\) were estimated using the least squares method. The model was found to be significant with a high coefficient of determination \(R^2 = 0.98\). The predicted AkP production was found to be in close agreement with the experimental values, indicating the reliability of the model.

**Table 3(a).** Susceptibility of proteases towards the effects of inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor class</th>
<th>Inhibitor name</th>
<th>Concentration</th>
<th>Residual activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Cysteine protease</td>
<td>E-64</td>
<td>10 μmol L(^{-1})</td>
<td>3.18</td>
</tr>
<tr>
<td>Iodoacetamide</td>
<td>5 mmol L(^{-1})</td>
<td>15.0</td>
<td></td>
</tr>
<tr>
<td>Serine protease</td>
<td>PMSF</td>
<td>0.1 mmol L(^{-1})</td>
<td>40.9</td>
</tr>
<tr>
<td>Antipain</td>
<td>50 μmol L(^{-1})</td>
<td>26.4</td>
<td></td>
</tr>
<tr>
<td>Metalloprotease</td>
<td>EDTA</td>
<td>5 mmol L(^{-1})</td>
<td>97.20</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>10 mmol L(^{-1})</td>
<td>80.10</td>
<td></td>
</tr>
<tr>
<td>Aspartic acid protease</td>
<td>Pepstatin A</td>
<td>1 mmol L(^{-1})</td>
<td>77.0</td>
</tr>
<tr>
<td>All four classes above</td>
<td>Inhibitor cocktail</td>
<td>1:100 dilution</td>
<td>34.90</td>
</tr>
</tbody>
</table>

*Four independent sets of experiments were performed with triplicate for calculating the residual activities. Activities of the samples incubated without the additives were treated as controls.

**Table 3(b).** Susceptibility of proteases towards the effect of detergent, reducing agent, metal ions and commercial detergents

<table>
<thead>
<tr>
<th>Applications</th>
<th>Concentration</th>
<th>Residual Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>1%</td>
<td>95.27</td>
</tr>
<tr>
<td>SDS</td>
<td>0.5%</td>
<td>62.48</td>
</tr>
<tr>
<td>β-ME</td>
<td>0.5%</td>
<td>90.8</td>
</tr>
<tr>
<td>ZnSO(_4) (\times) 7 H(_2)O</td>
<td>10 mmol L(^{-1})</td>
<td>58.1</td>
</tr>
<tr>
<td>FeSO(_4) (\times) 7 H(_2)O</td>
<td>10 mmol L(^{-1})</td>
<td>67.59</td>
</tr>
<tr>
<td>CaCl(_2)</td>
<td>10 mmol L(^{-1})</td>
<td>91.33</td>
</tr>
<tr>
<td>MnCl(_2)</td>
<td>10 mmol L(^{-1})</td>
<td>67.1</td>
</tr>
<tr>
<td>MgSO(_4)</td>
<td>10 mmol L(^{-1})</td>
<td>71.5</td>
</tr>
<tr>
<td>CuSO(_4) (\times) 5 H(_2)O</td>
<td>20 mmol L(^{-1})</td>
<td>23.7</td>
</tr>
<tr>
<td>HgCl(_2)</td>
<td>20 mmol L(^{-1})</td>
<td>10.4</td>
</tr>
<tr>
<td>Commercial detergents</td>
<td>Surf Excel</td>
<td>1%</td>
</tr>
<tr>
<td>Tide</td>
<td>1%</td>
<td>88.94</td>
</tr>
<tr>
<td>Sunlight</td>
<td>1%</td>
<td>93.4</td>
</tr>
</tbody>
</table>

*Values are the means of three independent determinations \((n = 3)\) and expressed as a percentage of control (set as 100%). Activities of the samples incubated without the additives were treated as controls.
Partial characterization of AkP

Susceptibility towards different inhibitors and additives on enzyme activity

Proteases are classified based on their sensitivity to various inhibitors. Maximum inhibition of enzyme activities was obtained in the presence of cysteine protease inhibitors E-64 (95–97.2%) and with iodoacetamide (85%) (Table 3(a)). Significant inhibition of enzyme activities up to 60% with serine protease inhibitor PMSF and 74% in the presence of antipain were also observed. In the presence of metallo-protease inhibitors EDTA (5 mmol L⁻¹) and phenanthrolin (10 mmol L⁻¹), 97.2% and 80% protease activity was observed, respectively. In presence of pepstatin-A (aspartic protease inhibitor), AkP retained 77% of its activities. The results indicated that aspartic acid residue might not be present at the catalytic centre. The AkP appeared to be cysteine-serine cross group proteinases on the basis of their susceptibility towards cysteine and serine protease inhibitors. In cysteine protease the inhibition by E-64 is due to nucleophillic attack from the thiol group of the cysteine on C-2 of the epoxide. PMSF can bind specifically only to the serine residue in the active site in a serine protease and does not bind to any other serine residues in the protein. Active site serine residue sulfonylation was obvious in the presence of PMSF as reflected in activity inhibition of more than 60%. Reports on cysteine–serine dual-type proteases were rare and reported in fungus Metarhizium anisopliae and in Aspergillus flavus. No significant inhibition by EDTA and phenanthrolin confirmed the absence of metalloprotease the fungal preparations.

With β-ME (0.5%, w/v⁻¹), approximately 90.8% of residual activities were found confirming that the reducing agent had minimal effect on AkP (Table 3(b)). Iodoacetamide irreversibly inhibited cysteine peptidases by covalently binding to the thiol group thus preventing the formation of disulfide bond. As a result, β-ME exhibited negligible effects on protease due to virtual absence of disulfide bonds at the catalytic site.

Residual activities of AkP were of a similar range (58–72%) as observed in presence of ZnSO₄, MnCl₂, and MgSO₄ while Ca²⁺ apparently had very mild or no effect on activity (Table 3(b)). In the presence of Cu²⁺ approximately 77% inhibition of enzyme activities was observed. The effect of enzyme inhibition (90–95%) by Hg²⁺ also suggested that cysteine is present either in or near the active site of the enzyme. It is known that ions like mercury
react with the protein thiol groups (converting them to mercaptides), as well as with histidine and tryptophan residues. Moreover, by action of mercury, the disulphide bonds were found to be hydrolytically degraded. In the presence of Triton X-100 and commercial detergents, the AkP retained up to 95.27% and (88–100%) enzyme activity respectively (Table 3(b)). However, on addition of 0.5% SDS, loss of 40% of the total activity was observed. The use of alkaline protease-based detergents is preferred over the conventional synthetic ones, partly because of their better cleaning properties and higher performance efficiency at elevated washing temperatures.\(^{1,3}\) Proteases are present in different brands of detergents for use in home and commercial establishments.\(^{24,35}\) Therefore, the search and screening of microorganisms that produce proteases with laundry detergent compatibility and high dehairing ability would be of paramount importance.

**Zymography of protease:** AkP was capable of hydrolysing gelatin detected in gelatine zymogram in terms of clear zones (Fig. 3(a)). In comparison, no collagenolytic activity was observed in collagen zymography (Fig. 3(b)). Absence of glycosylation in both protease preparations was confirmed by periodic acid Schiff (PAS) staining and through affinity binding with ConA-Sepharose 4B where more than 95% protein was eluted (data not given here).

**Bioremediation studies by AkP**

*Hide dehairing*

On a scale of 1 to 10, AkP showed dehairing efficiency on goatskin of 9.9 (experiments conducted in CSIR-CLRI, India), which confirmed high potency of the enzyme for dehairing applications. Optimal conditions for the production of dehaired pelts from goatskins for 20 h were established as 5% enzyme, 0.1% sodium
carbonate and 100% distilled water at pH 10.0. Efficacy of this process was corroborated with normal and magnified photographs of dehaired pelts (Fig. 4). No removal of hair was observed in the control specimen after 24 h (Fig. 4(a), (b)). The presence of depleted areas was noted in the skins treated with the AkP. The hairs were very easily released in enzyme-treated pelts with forceps. After incubation intact hairs could be taken out of the skins simply by scraping. In controls, hair loosening was not observed, even with forceps. Visual observation studies on the negative control (using lime-sulfide) showed incomplete removal of hair (Fig. 4(c), (d)) while enzyme treated dehaired pelts (Fig. 4(e), (f)) from goat skins apparently revealed complete absence of fine hairs, and surfaces of pelts were whiter than the negative control. Dehairing by enzyme was slightly slower than traditional lime-sulfide process (16 h) and reached completion in ∼20 h in the absence of any dehairing aids and the hair released was apparently intact leaving much whiter dehaired pelt than traditional treatments. This observation also indicated the ability of the alkaline protease to selectively break down the keratin tissues in the follicle, capable of releasing the intact hair. Microscopic observations showed the presence of stubble in the lime-sulfide treated skin against the smoothen enzymatically treated hide (Fig. 4(e), (f)).

Efficiency of dehairing process was supported with histological studies on sections of dehaired pelts. Staining by hematoxylin and eosin (H and E) differentiated the features in terms of the quality of dehaired pelts by removal of hair shaft, granular structures and follicles. Incomplete removal of these structures was observed in conventional lime-sulfide treated skin (Fig. 4(g)), whereas complete absence was evident for enzyme treated skin (Fig. 4(h)). Use of enzymes in the dehairing process yielded better quality leather with simultaneous reduction of the pollution load of the leather manufacturing process. Similar results were found in dehairing goat hide by keratinolytic serine protease from a Bacillus subtilis isolate. Most of the proteases used in the leather industry for dehairing often met the problem of collagen degradation and were not suitable for intact hair removal.

Feather detachment study
After periodic monitoring of detachment patterns in culture tubes, no feather detachment was observed up to 120 h in the control (Fig. 5(a)). Periodic disintegration pattern of feather vanes from shafts treated by AkP was visible (Fig. 5(b)). Almost complete disintegration of the feather shaft was observed after 100 h as evidenced by the periodic loss in dry weight of disintegrated feather. Dry weight of AkP treated bird feather at 0 h was noted as 0.19 ± 0.027 g, which was reduced periodically to 0.16 ± 0.07, 0.1 ± 0.03 and 0.08 ± 0.004 g, respectively, after 24, 60, and 100 h. The weight did not change after 100 h (checked up to 120 h). The results showed that the efficacy of feather-biodegradation can be employed for bioremediation in the poultry industry in management of keratin-rich waste as a time saving bioprocess.

**Bactericidal property**
(i) By MIC: AkP showed antibacterial activity against *S. aureus* NCTC 3750 (MTCC 3160) strain by measurement of diameter of zone of inhibition in petri plate (Fig. 6). The enzyme showed a clear...
zone of bacterial growth inhibition with MIC value 14.3 U mg⁻¹ equivalents AkP, compared with the control (Fig. 6(a), (b)). The zone of inhibition diameter for AkP was calculated as 14mm for 19.24 U mg⁻¹ (supra-MIC) (Fig. 6(c)) and 11mm at MIC (Fig. 6(d)). No bactericidal activity was detected against S. aureus was observed in serine protease of Sarcophaga peregrina pupae. In coccoïd cells like S. aureus, it is known that new peptidoglycan is inserted at the division septum.77 It may be speculated that exposure to MICs of protease led to accumulation of peptidoglycan and teichoic acid precursors, which were synthesized in the cytoplasm under conditions where the translocation of lipid-linked precursors from the cytoplasmic side to the outer side of the membrane is disturbed. Gram negative bacteria contain high lipoprotein in their outer membrane. However AkP could not disrupt E. coli cells, and was not capable of substituting divalent cations like Mg²⁺ ions in the thick lipopolysaccharide (LPS) layer on the outer membrane and thereby could not destabilize the outer surface. Such destabilization of the outer membrane would promote the penetration of anti-microbial proteins or peptides which may lead to local disruption of the inner membrane.28 The antibacterial activity of AkP might be applied in combination with broad-spectrum antibiotics in the treatment of S. aureus borne diseases. In future, methods allowing direct monitoring of protease activity against different pathogenic microorganisms will improve our understanding of the antibacterial function of protease.

Bioremediation of tannery waste effluent
In tanneries proteinaceous effluents are present as pollutants in different inlet/outlet areas. The untreated BOD and COD values of direct CETP inlet tannery wastes were, respectively, 600 mg L⁻¹ and 1850 mg L⁻¹. After AkP treatment to the waste, BOD and COD values were calculated at 93 mg L⁻¹ (84% reduction) and 216 mg L⁻¹ (88% reduction) respectively which is within the permissible limits (BOD: 100 mg L⁻¹; COD: 300 mg L⁻¹) of West Bengal Pollution Control Board standard and therefore safe for disposal into mainstream. AkP was capable of lowering the high alkaline pH of raw
effluents. The alkaline pH of CETP inlet raw effluent could affect biological properties of the receiving water body. Alkalinity is taken as an indication of carbonate, bicarbonate and hydroxide contents of water. Increasing alkalinity suggests an increase in ion concentration with increased conductivity. The higher conductivity alters the chelating properties of water bodies and creates an imbalance of free metal availability for flora and fauna. The high BOD and COD values of raw effluent indicated an increase in the organic matter content. As the number of organisms increases, the demand for oxygen increases proportionately. The reduction in protein load in these effluents is one of the major aspects of control of tannery wastes. Accordingly, the potential of AkP in ‘clean up’ the effluents with zero emission held significant promise for bioremediation. The T. clypeatus AkP can also be valuable for the reduction of energy costs in the application of enzymes in industrial processes as an alternative to mesophilic and thermophilic microorganisms.

The present study is the first report towards the production of alkaline protease from mushroom fungus *Termitomyces clypeatus* and showed the potency of AkP on multifunctional bioremediation processes. So far, no other proteases from any microorganisms have been reported to exhibit simultaneous applications in respect of hide dehairing, feather disintegration and bactericidal properties (Table 4). Considering the unprecedented increase in global demand for industrially useful proteases, the present studies are believed to be of substantial interest with reference to the quest for cheap multifunctional ecofriendly sources of the enzyme which can be used directly in the form of crude culture medium without any interference. The fact that filamentous fungi are natural hosts of both homologous and heterologous eukaryotic and prokaryotic protein production also eliminates the need for cloning of the protease gene in other expression hosts. Studies on complete characterization of AkP for its molecular size, N-terminal sequence and MALDI-TOF/MS MS analyses are ongoing.

**ACKNOWLEDGEMENTS**

Funding to R. M. by Council of Scientific and Industrial Research (NWP-0044) is acknowledged. Validation of the leather dehairing properties of the enzyme preparations were done at CSIR-Central Leather Research Institute, Chennai, India. We thank Ms. Anushila Gangopadhyay (flow cytometry technologist, BD Biosciences) for flow cytometry analyses.

**REFERENCES**


Production, optimization and applications of alkaline protease of *T. clypeatus* www.soci.org


**In situ Reversible Aggregation of Extracellular Cellobiase in the Filamentous Fungus *Termitomyces clypeatus***

Samudra Prosad Banik, Swagata Pal, Shakuntala Ghorai, Sudeshna Chowdhury, Rajib Majumder, Soumya Mukherjee, and Suman Khowala

Received: 2 January 2012 / Revised: 10 April 2012 / Accepted: 14 April 2012
© The Korean Society for Biotechnology and Bioengineering and Springer 2012

**Abstract** Cellobiase (E.C. 3.2.1.21), is a widely exploited industrial glycosidase with a major role in biofuel industry. Its stability and shelf life are major bottlenecks in achieving a superior formulation for industry. In the filamentous fungus *Termitomyces clypeatus*, the enzyme is secreted in a co-aggregated form with sucrase; the separation of this co-aggregation results in substantial loss of the enzyme’s activity. The aim of the present study was to examine the mode of aggregation of the secreted cellobiase-sucrase coaggregate and its role in the stabilization of cellobiase. Transmission electron microscopy and dynamic light scattering of purified co-aggregates revealed reversible, concentration driven self-aggregation of the extracellular enzymes to form larger entities. However, the intracellular enzyme aggregates were rigid, non-interacting, and possessed a higher percentage of disulphide bonds. Circular dichroic spectra of the two co-aggregates indicated no significant difference in secondary structures. Self-association increased the stability of extracellular aggregates towards heat by 1.5 fold, SDS by 4 ~ 7 fold, and chaotropic agents, by 1.5 ~ 2 fold, than the intracellular counterpart. The $K_m$ of extracellular aggregate varied between 0.29 and 0.45 mM as a result of spontaneous aggregation and disaggregation, whereas that of intracellular aggregate was 0.22 mM irrespective of its concentration status. In *in situ* detection of cellobiase in native PAGE revealed two activity bands of the extracellular enzyme, which indicated a minimum of two active dissociated aggregate species, as compared to a single band for the intracellular enzyme. These studies are believed to improve the understanding of aggregation of the fungal glycosidases, which remains to be a blackbox, to increase the efficacy of these enzymes.

**Keywords:** *Termitomyces clypeatus*, cellobiase-sucrase coaggregate, natural aggregation, reversible and irreversible aggregation

**1. Introduction**

The rise in the demand for industrially important enzymes over the last decade has necessitated the search for enzyme forms that show greater stability, higher catalytic efficiency, and enhanced shelf life. Several approaches, such as immobilization [1], site-directed mutagenesis, and directed evolution [2] have been tried. Two methods that have met with maximal success lately are the Cross Linked Enzyme Crystals (CLEC) [3] and the Cross Linked Enzyme Aggregates (CLEA) [4]. However, insolubility of the synthetic enzyme aggregates generally renders them unsuitable for use in aqueous phase systems without prior immobilization to an appropriate matrix. A more recent challenge has been encountered with the realization that enzymes tend to show lower catalytic efficiency in nonaqueous environments than in conventional aqueous medium. Therefore, the naturally aggregated enzyme complexes provide a valuable platform in understanding the mechanistic attributes of aggregation for creating better industrial recipe. It has been proven over...
years of studies that aggregation enhances the stability and catalytic efficiency of enzymes [5-8].

Over recent years, the use of cellulolytic enzyme systems has vastly increased in industries due to their large scale involvement in the upstream processes of biohydrogen & biofuel production [9]. Cellobiase (E.C. 3.2.1.21) has attracted a lot of commercial ventures in the last few decades, because of its indispensable role in utilization of cellulosic biomass and other useful industrial applications. Cellobiase is co-produced along with other cellulolytic enzymes like endo-glucanase, cellobiohydrolase etc. [10,11]. However, for issues pertaining to titer, efficiency, and stability, people had often resorted to either mixed cultivation [12], use of recombinant strains [13], or even immunization [14] for optimized product accumulation. Again, these methods have been confronted with challenges of cost-efficiency and source heterogeneity, which are constraints in developing an industrial recipe. Recently, a few efficient natural producer organisms have been screened [15]. Cellobiase extracted from *Termitomyces clypeatus* may be another potential option for the biotechnological industry. Therefore, elucidation of the attributes of its aggregation with sucrase and the subsequent impacts are of utmost significance, since it would serve the dual purpose of invading the black box of protein aggregation, as well as provide useful alternatives to the synthetic cross-linked enzymes aggregates in terms of better, industrial enzyme formulation.

The cellobiase-sucrase coaggregate from the filamentous fungus *Termitomyces clypeatus* is one of such unique enzyme co-assembly, in which the hetero-aggregated state represented the native functionally optimal state of both the enzymes [8,16]. The fungus secreted the enzyme complex, constitutively, in the culture medium where sucrase played a pivotal role in governing the secretion [17], activity and stability [8] of the cellobiase. Aggregation of sucrase led to both stabilization and improvement of catalytic efficiency of cellobiase [8]. Along with the secreted high titer of cellobiase [18], the enzyme can be recovered from mycelial extracts, as a useful industrial candidate with novel properties apart from cellobiase hydrolysis [19]. Previous studies showed considerable differences between the extracellular and intracellular enzyme forms during stages of purification; both stability and catalytic efficiency of cellobiase were differentially affected as a consequence of removal of sucrase from the co-aggregates [8]. Sucrase-free cellobiase, purified from the two different cellular domains, were found to possess different CD spectra [8]. This led to the proposition that differential aggregation of sucrase-cellobiase, co-aggregate in extracellular and intracellular domains were responsible for their altered kinetic and physicochemical profile. Present studies have been conducted to understand the aggregational behaviour of cellobiase in co-aggregation with sucrase. Accordingly, the co-aggregates have been purified from culture medium and mycelial extract for subsequent analyses.

### 2. Materials and Methods

#### 2.1. Materials

DNS reagent, Sephacryl S-200, cellobiose, p-nitrophenyl-β-D-glucopyranoside, Bradford reagent, phenyl methyl sulfonyl fluoride (PMSF), pepstatin, diithiothreitol, iodoacetamide, 2,3,5 triphenyltetrazolium chloride monohydrate, and uranyl acetate (EM grade) were purchased from Sigma (USA). Dialysis tubing (SnakeSkin Dialysis tubing, MWCO 3.5 kDa) was purchased from Pierce (USA). Electrophoretic reagents except the pre-stained MW marker (SM 0441, Fermentas) were obtained from Bio-Rad (USA). Filtration products were obtained from Millipore (USA). All other chemicals and salts (AR grade) were procured locally.

#### 2.2. Organism & growth conditions

*Termitomyces clypeatus* (strain no. MTCC 5091) was a CSIR depository and was cultured in the lab. The strain was grown in shake-flasks at 30°C in synthetic medium (400/2,000 mL) containing (% w/v), cellobiose, 1; NH₄H₂PO₄, 2.5; sodium succinate, 0.5; CaCl₂·2H₂O, 0.037; KH₂PO₄, 0.087; MgSO₄·7H₂O, 0.05; boric acid, 0.057; FeSO₄·7H₂O, 0.025; MnCl·4H₂O, 0.0036; NaMoO₄·4H₂O, 0.0032; ZnSO₄·7H₂O, 0.03; at pH 5.0, as described earlier [8]. The cultures were harvested on the third day of growth, and the mycelia were separated from the culture filtrate by filtration. Washed mycelia collected by filtration through a pre-weighted filter paper were pressed to soak the water. Subsequently, they were macerated in 0.1 M acetate buffer at pH 5, which contained 1 mM PMSF and 15 μg/mL Pepstatin A in order to obtain the crude intracellular extract. The culture filtrate was centrifuged at 8,000 rpm for 10 min and passed subsequently through a 0.22 μ filter.

#### 2.3. Purification of cellobiase-sucrase coaggregates

The culture filtrate was concentrated 10 times by ultrafiltration through a PM-5 membrane (Millipore, USA). The retentate was loaded on a Sephacryl S-200 gel permeation column (200 × 1.8 cm) in batches of 3.0 mL. The intracellular extract was loaded directly on the same column. Both the samples were subsequently eluted with 0.1 M Acetate buffer, pH 5. Protein fractions, eluted at the rate of 8.0 mL/h, were monitored for protein (A₂₈₀) and cellobiase activities, the peak pools (fractions 10 ~ 12) were desalted against 0.01 M of the same buffer by dialysis. The dialyzed preparations were concentrated by lyophilization, and
In situ Reversible Aggregation of Extracellular Cellobiase in the Filamentous Fungus Termitomyces clypeatus... each case from the slope of the plots [24-26]. The thermodynamic data were calculated by rearrangement of the Eyring

2.4. Enzyme and protein assays
Cellobiase activities were measured as β-D-glucosidase by measuring the OD_{500} of p-nitrophenol liberated from p-nitrophenyl-β-D-glucopyranoside in 0.1 M acetate buffer (pH 5.0) for 10 min at 45°C. The enzyme activity (U/mL) corresponded to the amount of enzyme that could produce 1 μmole p-nitrophenol per minute under the assay conditions [8]. Sucrase activities were measured as librated releasing groups employing DNS reagent [20]. Briefly, enzyme samples were incubated with 2% sucrose in 0.1 M acetate at 40°C for 15 min. Then, the incubation mixtures (0.5 mL) were mixed with 1.5 mL DNS reagent and were kept in a boiling water bath for 10 min. The solutions were cooled to RT, diluted to 10 mL with water and subsequently, color intensity was measured at 540 nm. Protein was estimated by Bradford reagent according to manufacturer’s technical bulletin.

2.5. Dynamic light scattering and circular dichroic studies
Dynamic light scattering measurements were performed with different concentrations of the purified enzyme aggregates ranging between 6 and 60 µg/mL. The concentration range was selected according to the response and sensitivity of the instrument and the selected range was used in further concentration dependent characterizations. Data were acquired in a Nano zeta-sizer (Malvern Instruments) DLS instrument at a fixed 90° scattering angle using a Nd-doped solid state laser of 632.5 nm with 100 sec of integration time; the intensity correlation function was obtained by acquiring data between 5 and 1,000 ms, in 200 channels. Circular dichroic (CD) spectra of the proteins (30 μg) in the far-UV wavelength range (190 ~ 250 nm) were recorded at room temperature (22°C) on a JASCO J-720 spectropolarimeter (calibrated with d-10-camphorsulfonic acid) using a cylindrical quartz cuvette of path length 1 mm. The following scan parameters were used: 1 nm bandwidth, 2 sec. response time, 0.1 nm step resolution and 20 nm/min scan speed. Each spectrum had an average of four continuous scans. The acquired spectra were corrected by subtracting blank runs on appropriate protein-free buffer solutions (10 mM-acetate buffer, pH 5.0) and subjected to a moderate degree of noise- reduction analysis.

2.6. TEM analyses of purified enzyme aggregates
10 µL aliquots of HPLC preparations of the enzyme aggregates at different concentrations were applied over 400 mesh carbon coated grids and stained with 2% uranyl acetate. Then they were observed under a TECNAI SPIRIT instrument (FEI, The Netherlands) with an accelerating voltage of 60 kV and a magnification of 60,000X. The sizes of the different aggregated species were measured with the associated software (TECNAI G² Version 2.1.5).

2.7. Measurement of protein thiol groups
Sulhydryl groups of proteins were measured by employing DTNB reagent. Colorimetric reactions were conducted under the conditions described by Ellman [21]. Unless otherwise indicated measured amounts of purified protein samples were suspended in 1.0 mL of reaction buffer consisting of 8 M urea, 10 mM DTNB, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.2 M Tris-HCl, pH 8.0, and 1% SDS (1%). Samples were incubated under N₂ for 30 min. Determination of total thiols (after reduction of disulfides) was carried out according to Thannhauser [22]. Protein samples were suspended in 1.0 mL of reaction buffer consisting of 8 M urea, 0.1 M sodium sulfite, 3 mM EDTA, 0.2 M Tris-HCl, pH 9.5, 1% SDS, and 10 mM NTSB²⁻, synthesized from DTNB in the presence of sodium sulfite and O₂, as described by Thannhauser [22].

2.8. Assessment of thermal stability
Thermal inactivations of the cellobiase preparations from intracellular and extracellular domains were determined by incubating the enzyme solutions in 100 mM sodium acetate, pH 5 buffer at a particular temperature. Aliquots were withdrawn at different times, cooled on ice for 3 h [23] and assayed for cellobiase activity at 45°C, as described before. This procedure was repeated at four different temperatures ranging from 45 to 52°C. The values of kₐ were obtained as the slopes of thermal inactivation at the respective temperatures, as indicated in Fig. 3. The values of ΔG* were calculated from the relationship ΔG* = - RT ln (kₐ/kₐT) h being the Planck constant, R the gas constant and Kₐ the Boltzmann constant. The data were subsequently used for calculation of activation energies of denaturation. Arrhenius plots were drawn with the first-order rate constants for denaturation (kₐ) of cellobiase in the two cellobiase-sucrase aggregates and activation energy of denaturation (Eₐ) was calculated in each case from the slope of the plots [24-26]. The thermodynamic data were calculated by rearrangement of the Eyring
absolute rate equation [27]:

\[ k_d = \left( K_d T / h \right) e^{-\Delta H^*/RT} = e^{\Delta S^*/R} \]

where \( h \) (Planck constant) = \( 6.63 \times 10^{-34} \) Js and \( K_B \) (Boltzmann constant)

\[ [R/N] = 1.38 \times 10^{-23} / JK \]

\[ E_a RT = 8.314 JK/mol \]

\[ RT k_d / \left( K_B T \right) \]

\[ H^* + G^* \Delta \left( T \right) \]

with

\[ X \], 12 ng (2X), 30 ng (5X), and 60 ng (10X) of proteins

Burk plot at four concentrations of the aggregates: 6 ng

Kinetic parameters were determined using Lineweaver-

2.10. Elucidation of kinetic constants

Kinetic parameters were determined using Lineweaver-Burk plot at four concentrations of the aggregates: 6 ng (1X), 12 ng (2X), 30 ng (5X), and 60 ng (10X) of proteins with \( pNPG \) concentrations ranging between 20 and 400 \( \mu \)M. The 1X preparation was obtained by tenfold dilution of 10X in 0.1 M acetate buffer (pH 5.0) using same volumes of enzyme samples for assay in all cases. The same 1X aliquot was re-concentrated in both the intracellular and extracellular aggregates to 10X by lyophilization.

2.9. Stability in presence of urea, guanidium hydrochloride and SDS

Stability in presence of the denaturants were measured by incubating the enzyme with 0.25 ~ 4 M urea and 0.15 ~ 1.5 M guanidium hydrochloride solution respectively for 1 h at room temperature and residual cellobiase activities were determined subsequently. Stability in presence of SDS was determined by incubation of enzyme preparations (50 ng) in presence of varying concentrations of SDS ranging between 0.01 and 0.4% at room temperature for 1 h and subsequent elucidation of enzyme activity.

3. Results and Discussions

3.1. Purification of cellobiase-sucrase coaggregates

Cellobiase-sucrase coaggregates were purified from culture filtrate (extracellular) and mycelial extract (intracellular) as described in materials and methods section. Around 39% of sucrase activity was filtered out with 3.6% of cellobiase in ultrafiltration step (step 2, Table 1A). As a result, the specific activity of cellobiase in the retentate was increased by 1.36 times in comparison to that of the culture filtrate. The retentate was then subjected to gel filtration chromatography (Sephacryl S-200), and was separated in two cellobiase peaks, pool E1 and pool E2. Both peaks contained associated sucrase in activity ratio (C/S) of 1.2 and 14.3 respectively (step 3, Table 1A). The specific activities of both the enzymes in pool E1 were higher (4 and 47.5 times for cellobiase and sucrase respectively) than pool E2. Previous studies had indicated that removal of sucrose affected cellobiase activity, stability and the conformation of the native coaggregate substantially [8]. Since, in the present studies, our main objective was to characterize the cellobiase in the co-aggregated form, Pool E1, which contained better sucrase activity was concentrated by lyophilization and subjected to HPGPLC, which revealed a higher MW OD280 aggregate peak at 11.25 min (step 4, Table 1A), followed by a low molecular weight species at 15.2 min (not shown in a purification table). Cellobiase with a specific activity 28.83 U/mg was observed in the fraction corresponding to the first peak. Sucrase was detected in this peak; however its specific activity declined drastically to 0.167. This was pooled and used as the purified extracellular cellobiase-sucrase coaggregate in further analyses. No sucrase or cellobiase activity was detected in the fraction corresponding to the second peak. The second peak was also devoid of any other glycosidase activity normally present in the culture filtrate of filamentous fungi.

Activity ratio (C/S) and specific activity of cellobiase in mycelial fraction was different from those of the culture filtrate protein (Table 1B). On gel chromatography in Sephacryl S-200, the intra-cellular cellobiase did not separate from sucrase, as observed in case of the extracellular co-aggregate. However, unlike the extracellular enzyme, only one cellobiase activity peak was obtained also containing sucrase with specific activities of cellobiase and sucrase being 24.69 and 10.1 U/mg, respectively with C/S ratio at 2.44. This was pooled and subjected to HPGPLC under the same conditions, as the extracellular enzyme preparation. The cellobiase activity peak of the intracellular fraction gave two OD280 peaks at identical positions as that observed in case of the extracellular aggregate. The first peak containing cellobiase (12.16 U/mg)
In situ Reversible Aggregation of Extracellular Cellobiase in the Filamentous Fungus Termitomyces clypeatus

and sucrase (0.33 U/mg) was pooled and used as purified intracellular cellobiase-sucrase coaggregate for further analyses. (The first peak contained cellobiase (specific activity, 12.16 U/mg) with sucrase (specific activity, 0.33 U/mg).

It has been reported earlier that extracellular cellobiase can assume characteristic oligomeric forms by virtue of spontaneous protein-protein interaction [31]. The higher molar mass cellobiase aggregates exhibit concentration-dependent dissociation as under HPGPLC the enzyme dissociates into multiple lower molar mass peaks with subsequent decrease in specific activities [7]. The higher molar mass and lower mass aggregates observed in gel filtration chromatography of the extracellular retentate were the results of this spontaneous in vitro association of the two aggregate species. In case of the intracellular enzyme, single cellobiase-sucrase coaggregate peak was observed which was different from the extracellular coaggregate [8]. The lower molar mass second OD280 peak which was observed on HPGPLC of the sephacryl preparation of both of the extracellular and intracellular aggregates were also part of the higher molar mass cellobiase-sucrase coaggregate rendered inactive, as a result of dissociation below the minimal active conformation, as confirmed by western blot analyses, for the intracellular [19] and extracellular aggregates [32].

3.2. Light scattering studies
In order to understand the aggregational behaviour of the extracellular and intracellular aggregates, dynamic light scattering studies were performed with different concentrations of the purified enzyme aggregates (Fig. 1). The intracellular aggregate at a concentration of 60 µg/mL (Fig. 1C) showed a polydisperse mixture of essentially three species of hydrodynamic radii 11.6, 162.5, and 559.6 nm, respectively with scattering intensities of 6.1, 21.3, and 69.3%, respectively. When the same sample was analyzed at a 10-fold diluted state (Fig. 1D), the 11.6 nm species persisted with the same scattering intensity, but the two bigger species were replaced by a single 181 nm species with an approximately additive scattering intensity of the two earlier species (94%). This was in all probability attributable to the averaging out of the diffusion coefficients of the two species, due to low population density especially of the 559.6 nm species. The polydispersity of the sample also decreased by 24% in the diluted state indicating that no new species was formed due to dilution and subsequent disintegration of the aggregate. However, the results were of different nature in case of the extracellular aggregate. At

<table>
<thead>
<tr>
<th>Table 1. A. Purification of extracellular cellobiase-sucrase coaggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>(1) Culture filtrate</td>
</tr>
<tr>
<td>Filtrate (40 mL)</td>
</tr>
<tr>
<td>Retentate (10 mL)</td>
</tr>
<tr>
<td>Pool E1 (10 ~ 12) 96 mL</td>
</tr>
<tr>
<td>Pool E2 (13 ~ 15) 96 mL</td>
</tr>
<tr>
<td>Pool 1 (10 ~ 12) 6 mL</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 1. B. Purification of intracellular cellobiase-sucrase coaggregate</th>
</tr>
</thead>
<tbody>
<tr>
<td>STAGE</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>(1) Mycelial extract</td>
</tr>
<tr>
<td>Pool In1 (10 ~ 12) 96 mL</td>
</tr>
<tr>
<td>Pool 1 (10 ~ 12) 6 mL</td>
</tr>
</tbody>
</table>
a 60 µg/mL concentration, only a single species of 188 nm was detected (Fig. 1A), but after dilution by 10 times, at 6 µg/mL concentration, this was replaced by two new species of hydrodynamic radii 5.6 and 58.7 nm (Fig. 1B). This was also accompanied by a substantial increase in polydispersity of the sample by 66%. On concentrating the aggregate samples again to 60 µg/mL, their original size distribution pattern was restored (data not shown). In case of the extracellular aggregate, the smaller units again reassociated to form the larger species; for the intracellular aggregate, it was a mere regain of concentration of the two species within the detection level so that they were discernible individually. In order to substantiate the studies DLS was performed with two additional intermediate concentrations of the aggregates (12 and 30 µg/mL).

However, 12 µg/mL gave results similar to 6 and 30 µg/mL gave results similar to 60 µg/mL). These data indicated that the extracellular aggregate underwent further concentration-dependent aggregation to develop into larger entities, unlike the intracellular aggregates that were individually rigid and non-interacting.

### 3.3. TEM studies

TEM images of the two purified aggregate preparations confirmed the associative tendency of the extracellular enzymes, as well as the size distribution of the two aggregates. At a concentration of 6 µg/mL, both the co-aggregate forms showed heterogeneous populations with respect to sizes ranging in multiples of around 10 ~ 15 nm. The extracellular preparation (Fig. 2A) showed a predominant species of around 60 nm, which was approximately the same as that calculated through DLS (58.7 nm) and a distinct aggregational tendency as marked by the arrowheads in the Figure. This was not observed in the intracellular assemblies (Fig. 2C) which maintained their distinct individual entities. At a 10-fold higher concentration of 60 µg/mL, the extracellular aggregates were visibly enlarged in sizes ranging between 110 and 150 nm by virtue of aggregation (Fig. 2B). The corresponding

---

**Fig. 1.** Determination of hydrodynamic radii of purified extracellular and intracellular cellobiase-sucrase co-aggregates by dynamic light scattering. Figs. 1A and 1B represent the extracellular aggregate at 60 and 6 µg/mL concentrations respectively; Figs. 2C and 2D represent the intracellular aggregate at same concentrations as above respectively.

**Fig. 2.** TEM images of purified extracellular (Figs. 2A and 2B) and intracellular (Figs. 2C and 2D) cellobiase-sucrase coaggregates; 10 µL aliquots of each of the protein aggregates at two concentrations; (6 µg/mL: Figs. 2A and 2C; 60 µg/mL; Figs. 2B and 2D) were visualized under 60,000X magnification. Measurement of aggregate size was done with the associated software (TECNAI G2 Version 2.1.5). Aggregates along with their point of association (extracellular only) are shown by arrowheads.
intracellular enzymes did not show any such associative tendency; seemingly scattered aggregation at places were a consequence of sheer increase in density but mostly the smaller sized species of 15 nm where retained (Fig. 2D). The data obtained from DLS and TEM studies cumulatively demonstrated the concentration dependent aggregational tendency of the extracellular enzyme.

3.4. Secondary structure analyses
In order to negate the possibility that the differences in aggregational behaviors and resultant physicochemical attributes were due to differences in the secondary structure of the two enzyme forms, their CD spectra were elucidated. Both the aggregates showed essentially the same spectrum and no clear interpretable secondary structural elements were discernible in either case.

3.5. Determination of protein thiols
Rigidity of aggregation as observed in case of the intracellular cellobiase often results due to disulphide bond formation between previously unpaired free thiols. For some proteins, a covalent interaction between monomers is required to form a stable protein structure. Many of the growth factors, including Vascular Endothelial Growth Factor (VEGF), Transforming Growth Factor- b1, and Nerve Growth Factor, have extremely stable structures owing to the presence of several disulfide bonds, including one that exists between monomers to lead to a native covalent dimer [33]. To determine the role of disulphide bonds in modulating the aggregation of sucrose-cellobiase coaggregates, free thiol and total thiol content of the two coaggregates were determined. Total thiol content of the extracellular aggregates was higher, as compared to the intracellular aggregates, however, most of the thiol groups of the intracellular enzyme were disulphide bonded (Table 2). As a result the ratios of free thiol to total thiol groups were 5.5 times higher for the extracellular enzyme in comparison to the intracellular preparation. It may be proposed that disulphide bonded subunits of the intracellular preparation could not be dissociated by dilution as observed for extracellular aggregates.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>k_d (1/sec)</th>
<th>∆H* (kJ/mol)</th>
<th>∆G* (kJ/mol)</th>
<th>∆S* (J/mol/K)</th>
</tr>
</thead>
<tbody>
<tr>
<td>318</td>
<td>0.66</td>
<td>254.68</td>
<td>79.15</td>
<td>551.98</td>
</tr>
<tr>
<td>321</td>
<td>1.2</td>
<td>254.65</td>
<td>78.32</td>
<td>549.31</td>
</tr>
<tr>
<td>323</td>
<td>2.28</td>
<td>254.63</td>
<td>77.10</td>
<td>549.63</td>
</tr>
<tr>
<td>325</td>
<td>4.14</td>
<td>254.62</td>
<td>75.98</td>
<td>549.63</td>
</tr>
</tbody>
</table>

\[ E_a = 257.32 \text{ kJ/mol.} \]


3.6. Assessment of thermostability
Thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with a parallel increase in the enthalpy of activation (\( \Delta H^* \)) [34]. The opening up of the enzyme structure is accompanied by an increase in the disorder or entropy of activation (\( \Delta S^* \)) [35]. Thermodynamic parameters of cellobiase aggregate from extracellular and intracellular milieu were elucidated according to standard procedures as described in materials and methods section. The inactivation processes for both the aggregates showed first-order kinetics (Figs. 3A and 3B for extracellular and intracellular aggregates respectively) resembling monomolecular denaturation mechanism, which ensured the homogenous nature of the aggregates with respect to their activities. Both \( \Delta H^* \) and \( \Delta S^* \) were found to decrease for the extracellular (Table 3A) and intracellular forms (Table 3B) with increasing temperatures, which was testimonial to the gradual conformational changes of both the enzyme forms. Arrhenius plots of the enzymes revealed that the extracellular form (Fig. 3C) was more thermostable (\( E_a = 257.32 \text{ kJ/mol} \)) than the intracellular counterpart (Fig. 3D) (\( E_a = 396.16 \text{ kJ/mol} \)). High molar mass enzymes generally tend to have larger values for \( \Delta H^* \) and \( \Delta S^* \) compared to the ones having smaller molecular weights [26,36]. The extracellular aggregate had \( \Delta H^* \) and \( \Delta S^* \) values of 254.68 kJ/mol & 551.98 J/mol, whereas the corresponding values for the intracellular aggregates were 393.52 kJ/mol & 982.77 J/mol.

### Table 3. Thermal inactivation rate constant and thermodynamic parameters for irreversible thermal inactivation of cellobiase aggregates

- **A: Extracellular cellobiase (AS2)**
- **B: Intracellular cellobiase (AS2)**

Free thiols groups in the aggregates were ascertained by Ellman’s reagent (Pierce) as described in materials and methods. Sodium borohydride and urea were employed for determination of total thiol groups in the proteins. Reducing conditions were maintained by purging liquid nitrogen through the solutions before addition of DTNB.
985.94 J/mol/K, respectively. These parameters demonstrated that the intracellular aggregates were larger in size. DLS and TEM studies had indicated that extracellular aggregates assumed higher average diameters than their intracellular counterparts by virtue of reversible self association; the $\Delta H^*$ and $\Delta S^*$ values were therefore the means of different aggregated forms of the extracellular enzyme. The temperature stability of the associated sucrase was also found to be about 20% higher than the intracellular form.

In both thermophilic bacteria and fungi, cellobiase is the thermostability-limiting enzyme of the cellulolytic system [37,38]. Therefore, the prospect of extracellular cellobiase, which acquired thermostability via self-association, is of substantial industrial importance.

3.7. Effect of chaotropes and SDS on enzyme aggregates
The effects of differential aggregation on the chaotrope induced unfolding of the enzyme forms were investigated. The impact of destabilization imparted by both urea and GuHCl was found to be much greater on the intracellular aggregate as the residual activities diminished to 49% in presence of 2 mM urea (Fig. 4A) and to 31% (Fig. 4B) in presence of 1 mM GuHCl. The extracellular enzyme showed a markedly better stability and retained about 76% (Fig. 4A).

![Fig. 3. First order plot thermal denaturation plots and Arrhenius plots of extracellular and intracellular cellobiase in the cellobiase-sucrase coaggregate.](image1)

![Fig. 4. Effect of chaotropes on cellobiase activity of extracellular and intracellular aggregate.](image2)
In situ Reversible Aggregation of Extracellular Cellobiase in the Filamentous Fungus *Termitomyces clypeatus* and 59% (Fig. 4B) activities respectively in presence of similar concentrations of urea and GuHCl. Stability of the associated sucrase was more or less similar as both the extracellular and intracellular enzyme retained about 75% activity at 0.2 M GuHCl concentration. The extracellular enzyme also showed significantly higher tolerance to 0.02% SDS by retaining 56% activity (Fig. 4C) as compared to the intracellular enzyme which showed only 8% residual activity.

Chaotropes, such as urea and GuHCl, disrupt the hydrogen bonding network within water molecules. Proteins, in turn, maximize the presentation of their hydrophilic surface to stabilize their solvent exposed interfaces [39]. This leads to their gradual unfolding and subsequent departure from the native state. The extracellular co-aggregated cellobiase was affected to a lesser extent by the chaotropes probably because the active dissociated smaller units were still able to present a considerable amount of hydrophilic surface and therefore underwent little unfolding in comparison to the intracellular aggregate. Recent observations have indicated that GuHCl at moderately low concentrations (0.1 ~ 0.7 mM, similar to that used in this study) assisted the refolding of molten globule intermediates of many proteins [40,41]. At these concentrations, GuHCl probably shielded the positively charged groups on the protein surface, which in turn stabilized the interfaces undergoing unfolding processes and consequently decreased the extent of aggregation [42]. Unlike most other proteins, the aggregated state in this fungus represents the native state of these unique fungal glycosidase assemblies. It would be interesting to perform molecular simulation studies to elucidate the gradual shift in charge distribution patterns over the interacting subunits in presence of GuHCl; these could provide valuable insights to distinguish between the two enzyme assemblies in terms of chaotrope induced unfolding or refolding.

### 3.8. Effect of aggregation on catalytic efficiency

Kinetic parameters of cellobiase were evaluated with 6 ng (1X), 12 ng (2X), 30 ng (5X), and 60 ng (10X) of proteins of each of the extracellular and intracellular aggregates. The $K_m$ of the extracellular enzyme was found to increase steadily from 0.291 to 0.454 mM, as it was diluted from 10 to 1X and improved to 0.321 mM again as the enzyme was reconcentrated to 10X. The intracellular enzyme was catalytically superior to extracellular enzyme. Its substrate affinity was found to be 0.22 mM for all the enzyme concentrations (Table 4). Earlier studies had also confirmed that the substrate affinity of the intracellular cellobiase was better than the extracellular enzyme [8]. The corresponding $V_{max}$ values decreased for both the enzymes as their specific activities were reduced 10-fold by decrease in concentration. Further decrease in $V_{max}$ values for both the extracellular and intracellular samples after reconcentration was probably due to loss of activity caused by lyophilization. The present study showed that unlike extracellular cellobiase, aggregation did not affect the substrate affinity of the intracellular enzyme. Change in substrate affinity of the extracellular cellobiase by change in concentration also showed that spontaneous reversible self-association played a dominant role in modulating its catalytic efficiency.

### 3.9. Migration on native and SDS-PAGE

The enzymes in their native state were tracked in 5% native PAGE by cellobiase zymography (Fig. 5A). The extracellular enzyme revealed two distinct activity bands whereas the intracellular one showed a single aggregated band. It was reported that cellobiase develops into a high molar mass aggregate of around 450 kDa by spontaneous protein-protein interaction in *Termitomyces clypeatus* [31].

### Table 4. Effect of aggregation and disulphide reduction on catalytic efficiency of cellobiase

<table>
<thead>
<tr>
<th>Sample</th>
<th>$K_m$ (mM)</th>
<th>$V_{max}$ (U/mL)</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 X</td>
<td>0.291 ± 0.023</td>
<td>0.052 ± 0.004</td>
<td>0.179</td>
</tr>
<tr>
<td>5X</td>
<td>0.334 ± 0.029</td>
<td>0.045 ± 0.003</td>
<td>0.135</td>
</tr>
<tr>
<td>2X</td>
<td>0.381 ± 0.033</td>
<td>0.041 ± 0.004</td>
<td>0.108</td>
</tr>
<tr>
<td>1 X</td>
<td>0.454 ± 0.043</td>
<td>0.038 ± 0.003</td>
<td>0.084</td>
</tr>
<tr>
<td>10 X Reconstituted</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 X</td>
<td>0.221 ± 0.021</td>
<td>0.231 ± 0.021</td>
<td>1.045</td>
</tr>
<tr>
<td>5X</td>
<td>0.223 ± 0.023</td>
<td>0.142 ± 0.011</td>
<td>0.637</td>
</tr>
<tr>
<td>2X</td>
<td>0.223 ± 0.023</td>
<td>0.073 ± 0.006</td>
<td>0.327</td>
</tr>
<tr>
<td>1 X</td>
<td>0.221 ± 0.019</td>
<td>0.043 ± 0.003</td>
<td>0.195</td>
</tr>
<tr>
<td>10 X Reconstituted</td>
<td>0.222 ± 0.022</td>
<td>0.022 ± 0.001</td>
<td>0.099</td>
</tr>
</tbody>
</table>

Change in catalytic activities as an effect of dilution. Enzyme aliquots (50 ng protein) for each of the preparations were incubated with different substrate (pNPG) concentrations. Kinetic parameters of the partially purified enzyme samples from different media were then determined from the intercept values of the respective Lineweaver - Burk plots.
In case of the extracellular enzyme this aggregation is largely driven by concentration. As such, when migrating through the gel, the large aggregate disintegrated into two smaller active units of the enzyme, whereas the intracellular aggregate showed only a single activity band of intermediate molecular weight. The corresponding SDS-PAGE (Fig. 5B) showed protein bands of 124, 82, 70, 66, 62, and 52 kDa for the intracellular preparation (lane 1). The lower molecular weight bands were of low intensity for the extracellular enzyme and the 52 kDa band was not detectable (lane 2).

Reversible protein aggregation typically occurs due to relatively weak non-covalent protein interactions [43]. In some cases, it is indicative of the presence of equilibrium between the monomer and the aggregated higher order assemblies. A shift in this equilibrium may be affected by changes in solution conditions such as pH or protein concentration [44]. A weak, reversible self-association of this type has been reported in a monoclonal antibody to VEGF [45]. The extracellular aggregate was seen to disintegrate by decrease in concentration and had clear tendencies of associations as revealed in DLS and TEM studies. Evaluation of the effects of altered charge distributions in the local environment induced by solvent pH and/or presence of other charged co-solutes will immensely help in deciphering the subtle mechanistic attributes of aggregation.

It may be also possible that the intracellular cellobiase stays in a pro-enzyme form, and processing before secretion alters the aggregational tendency of its constituent units. However, processing of pro-enzymes known for mammalian cells [46] has not been reported yet in filamentous fungi. In filamentous fungi, cellular regulation over secretion of glycosidases was reported for the first time in T. clypeatus [47,48] and it was deciphered through later studies that sucrose plays a pertinent role in modulating the traffic of cellobiase in this regulated secretory pathway [17]. A final possibility is that co-aggregation of cellobiase with sucrase at a co-translational/post-translational level have a linkage in rendering the intracellular aggregates non-associative, whereas after translocation through vacuoles and subsequent secretion into the culture medium, there may be changes in molecular make-up of the enzyme so that it acquires the property of spontaneous aggregation [49].

4. Conclusion

Over recent years, the demand for the perfect industrial recipe in terms of a strong and stable cellobiase titer for use in biofuel production has increased greatly. However, we are yet to achieve the optimal formulation to meet the demanding conditions in conversion processes. The current study on these uniquely adapted enzyme assemblies presented a two-fold significance on a commercial perspective. Primarily, it described a stable extracellular cellobiase that retained 90% activity until 1 year at 4°C, until 1 month at room temperature, and can be produced in high titers for an efficient industrial formulation. In addition, the studies also provided useful insights into the process of aggregate formation together with the practical impacts involved therein. These are of the utmost importance in designing therapeutics for pharmaceutical industries and bioprocesses.
Acknowledgements

The authors are grateful to Dr. Anjan K. Dasgupta, Dept. of Biochemistry, Calcutta University and Dr. G. Suresh Kumar, Scientist, Biophysical Laboratory, IICB for kindly providing facilities respectively for the Dynamic Light Scattering and Circular Dichroism studies and also to Mr. S. N. Dey, IICB for providing technical assistance in acquiring the TEM images. The research fellowship support to SPB by University Grants Commission, Govt. of India is acknowledged.

Nomenclature

C : Cellobiase
S : Sucrase
C/S : Activity ratio of cellobiase to sucrase
SDS : Sodium dodecyl sulphate
PAGE : Polyacrylamide gel electrophoresis
HPGPLC : High performance gel permeation liquid chromatography
TEM : Transmission electron microscopy
DLS : Dynamic light scattering
CD : Circular dichroism spectroscopy
BSA : Bovine serum albumin
pNPG : Paranitrophenyl β-D-glucopyranoside
DNS : Dinitrosalicylic acid reagent
PMSF : Phenyl methyl sulfonyl fluoride
RT : Room temperature

References

1. Introduction

Increased industrialization and human activities have created impact on the environment through the disposal of waste containing heavy metals. The existence of heavy metals in the environment represents a significant and long-term environmental hazard. Even at low concentrations these metals can be toxic to humans, including humans. Chromium is a contaminant that is a known mutagen, teratogen and carcinogen [1,2]. Hexavalent chromium, Cr(VI), is contained in wastewaters produced by industrial processes, such as those employed in the electroplating, metal finishing, metallurgical, leather tanning, dye, wood preservation and battery manufacturing industries. Among the several oxidation states (di, tri, penta and hexa), the hexavalent state, together with trivalent chromium, can be the main forms present in aquatic environments [3]. Chromate (CrO$_4^{2-}$) and dichromate (Cr$_2$O$_7^{2-}$), are the prevalent species of Cr(VI) in natural aqueous environments, as major pollutant from chromium-related industries which create significantly higher levels of toxicity than the other valency states of the metal [4,5]. The maximum permissible limit of Cr(VI) in natural water is only 0.05 mg/L by the U.S. Environmental Protection Agency [4].

The removal of Cr(VI) from aqueous solution has received considerable attention in recent years. Traditionally, these removals are made by electrochemical treatment, chemical precipitation, membrane process, reverse osmosis, ion exchange, liquid extraction, electro dialysis, evaporation and sorption [6]. However, the application of these treatment processes has been found to be sometimes restricted, because of expensive investment, operational costs, potential generation of secondary pollution, and its disposal is not eco-friendly [7]. Furthermore such processes may be ineffective or extremely expensive when the initial heavy metal concentrations are in the range of 10–100 mg/L. At present emphasis is given to the utilization of biological adsorbents for the removal and recovery of heavy metal contaminants. Biomass involving pure microbial strains has shown high capacities for the selective uptake of metals from dilute metal bearing solutions [8]. Several investigations have been reported on metal binding efficiencies of various strains of bacteria, algae, fungi and seaweed [8–11]. Among them fungi are fast growing; low cost (less nutrient requirement) has adaptability to natural environments, available...
as industrial/laboratory byproduct [12–16]. Fungi can tolerate and detoxify metals by several mechanisms including passive accumulation processes which may include ion exchange, complexation, adsorption, extra and intracellular precipitation, valence transformation and also active uptake [15,17]. The cell wall of fungi is composed typically of chitin (a long linear homopolymer of beta-1,4-linked N-acetylglucosamine), glucan, mannan, proteins and other polymers [18] that possess carboxyl, phosphoryl, hydroxyl, amino, amine and imidazole functional groups at the surface.

Studies on biosorption using different fungal biomasses were focused on the removal of metal ions from aqueous solutions. However, some studies interpreted and established the mechanism involved in Cr(VI) binding [25,19–25]. Still the binding sites for chromium were not specifically identified. The fungal species, Termitomyces clypeatus, used in this study is an edible variety of mushroom, commonly found in near-surface system. The inactivated/dead fungal biomass is of little use and is preferred as a source of biomaterial for biosorption processes with no risk of contamination during biosorption process as well as better easy handling [12,25]. To study the biosorption of Cr(VI), an investigation of surface characteristics of T. clypeatus was required to understand the mechanisms of the metal biosorption. The objective of the present work was to study the mechanism of biosorption of heat inactivated biomass of T. clypeatus for the biosorption of Cr(VI) using different experimental approaches involving biochemical, FTIR and SEM–EDX surface analysis. The biomass was chemically pretreated by acid, alkali and salts for the assessment of change in biosorption efficiency. The functional groups involved in Cr(VI) biosorption were identified by potentiometric titration, pH of zero charge, modification of functional groups by chemical treatment and FTIR analysis. The results would contribute to a better understanding of biosorption and aid in the development of potential biosorption that possess almost complete removal efficiency for Cr(VI) from aqueous environment.

2. Materials and methods

2.1. Reagents

All the reagents used in this study were of analytical grade and purchased from Across, India, Merck, Germany and Sigma, USA. Media ingredients were procured from Himedia, India.

2.2. Culture conditions and biomass preparation

The fungal strain of T. clypeatus (MTCC-5091) was routinely cultured in complex medium under submerged condition at 30 °C for 5 days and mycelia were taken as byproduct. The complex medium (% w/v) comprised of the following ingredients: sucrose 5.0, malt extract 1.0, boric acid 0.057, KH2PO4 0.15, CaCl2 2H2O 0.037, MgSO4 0.05, MnCl2 4H2O 0.0036, ZnSO4 ·7H2O 0.031, FeSO4 ·7H2O 0.025, (NH4)2PO4 2.5, at pH 3.5. Inoculum (2%) was added and growth was continued under submerged condition (150 rpm) at 30 °C for 4 days.

Heat inactivated biomass of T. clypeatus was obtained by filtering the culture medium and the mycelia was washed thoroughly with double distilled water to attain neutral pH (6.8–7.2). Finally the biomass was dried at 50 ± 5 °C for 24 h and kept in air tight containers.

2.4. Metal solution and analysis

1 g/L K2Cr2O7 stock solution was prepared and diluted to desired concentrations. Cr(VI) was measured by spectrophotometric method using 1,5-diphenyl carbazide [26]. Total chromium concentration was estimated through AAS.

2.5. Batch biosorption studies

The biosorption experiments were carried out in 250 mL Erlenmeyer flasks containing 25 mL K2Cr2O7 solution and 0.2 g the biomass by shaking (150 rpm) at 30 °C for period of contact time up to 48 h. At the end of each experiment solutions were separated from the biomass by filtration through filter paper (Whatman no. 42), for the analysis of Cr(VI) ions left in solution after biosorption. Care was taken to wash all glassware used for experimental purpose with (phosphate free) 50% (v/v) followed by 60% (v/v) nitric acid and subsequent rinsing with DDW to remove any possible interference of T. clypeatus by other metals.

The differences in Cr(VI) concentration before and after biosorption were found to use out the percentage of hexavalent chromium adsorbed by the biomass as Eq. (1).

\[ \text{Biosorption (\%)} = \frac{C_i - C_f}{C_i} \times 100 \]  

where \(C_i\), metal uptake (mg/g); \(C_f\), initial metal concentration (mg/L); \(C_e\), concentration after biosorption; \(m\), biomass weight (g); \(V\), volume of metal solution (mL).

2.6. Surface chemistry characterization of the biomass

Surface chemistry of the biomass was characterized by Boehm titration, pH of zero charge, modification of functional groups by chemical treatment and FTIR analysis of all treated biomass.

2.6.1. Potentiometric titration of the biomass

Acidic and basic sites on heat inactivated biomass of T. clypeatus were determined by the acid–base titration (potentiometric titration) method proposed by Boehm [27]. The total acidic sites were neutralized using NaOH (0.1 mol/L) while the basic sites were neutralized with HCl (0.1 mol/L) using the pH meter (Eutech pH 510, India). The titration was performed in Erlenmeyer flasks over the magnetic stirrer. Protonation was carried out by soaking the biomass (1 g) in 0.1 N HCl (50 mL) and agitating for 2 h on a rotary shaker at room temperature. The titration procedure was executed slowly, by the stepwise addition of the titrant (0.1 M NaOH) to the biosorbent slurry. After each addition of titrant (0.25 mL), the system was allowed to equilibrate until a stable reading was obtained. The total volume of NaOH utilized for neutralization was recorded for total basic sites calculations. Likewise, the total acidic sites were determined by keeping biomass (1 g) in 0.1 mol/L NaOH solution (50 mL) and preceded for titration similar to as described above. The potentiometric titration curve was obtained by plotting the volume of titrant against the recorded pH.
2.6.2. Determination of pH of zero charge

The pH or point of zero charge (pZC) for the biomass (0.2 g) was determined by boiling 100 mL deionized water for 20 min to eliminate dissolved CO₂ and quickly cooling and capping the solution. The biomass was placed in the CO₂-free water (15 mL), then sealed and continuously agitated for 48 h at room temperature before measuring the pH of the solution – taken as the point of zero charge [28,29].

2.6.3. Modification of functional groups by chemical treatment of the biomass

Different chemical treatments were given (i) methylation of amines [16], (ii) esterification of carboxylic acids [30], (iii) esterification of phosphates [31], (iv) modification of sulfhydryl group [32] and (v) extraction of lipids [16]. During treatment with the chemicals the biomass was shaken (150 rpm) for 5 h. The solution was filtered using Whatman no. 42 filter paper and the biomass was dried at 50 ± 5 °C for 24 h, unless otherwise specified. Weight of the biomass group was carried out by mass assessed after each treatment [2].

2.6.3.1. Chemical modification of amino groups. Methylation of amines was carried out by treating the biomass (1 g) with formaldehyde (20 mL) and formic acid (40 mL) [16]. The general reaction takes place as followed:

\[
\text{RCH}_2\text{NH}_2 + \text{HCHO} \rightarrow \text{RCH}_2\text{N(CH}_3\text{)}_2 + \text{CO}_2 + \text{H}_2\text{O}
\]

2.6.3.2. Chemical modification of carboxyl groups. Esterification of carboxyl group was carried out by suspending the biomass (1 g) in benzene (75 mL) under reflux conditions for 6 h [16]. Esterification of carboxylic acids present on the cell wall occurs according to following reaction:

\[
\text{RCOOH} + \text{CH}_3\text{CH}_2\text{OH} \rightarrow \text{RCOOCH}_3\text{CH}_2\text{H} + \text{H}_2\text{O}
\]

2.6.3.3. Esterification of phosphates. Esterification of phosphates was carried out by suspending the biomass (1 g) in a mixture of triethyl phosphate (40 mL) and nitromethane (30 mL) under reflux conditions for 6 h [31].

2.6.3.4. Modification of sulfhydryl group. Modification of sulfhydryl group was carried out by dithiopyridine treatment [32]. The biomass (1 g) was washed in HCl (0.1 mol/L) followed by sodium acetate (0.1 mol/L) at pH 5. The biomass was then suspended in 0.001 mol/L dithiopyridine prepared by dissolving 0.22 g of 2,2-dithiopyridine in 2 mL of concentrated HCl and diluting to 1 L with 0.1 mol/L sodium acetate at pH 5. The reactions occur with available sulfhydryl groups and are chemically blocked and converted to thiol groups as followed.

2.6.3.5. Extraction of lipids. Extraction of lipids was carried out by suspending the biomass (1 g) in benzene (75 mL) under reflux conditions for 6 h [16].

2.7. FTIR spectroscopy

FTIR spectra of powdered heat inactivated and chemically modified functional groups of biomass were obtained using JASCO FTIR instrument-410. The samples were pressed into spectroscopic quality KBr pellet with a sample/KBr ratio about 1/100. The FTIR spectra were recorded in the region 4000–400 cm⁻¹.

2.8. SEM–EDX analysis

The surface structure of biomass was analyzed by scanning electron microscopy (SEM) coupled with energy dispersive X-ray analysis (EDX) using JEOL 560 LV SEM at 20 kV with background subtraction and a summation of 60 scans. Unloaded and chromium-laden heat inactivated T. clypeatus biomass samples were mounted on a stainless steel stab with a double stick tape followed by coating with a thin layer of gold under vacuum to increase the electron conduction and to improve the quality of the micrographs.

3. Result and discussion

3.1. Enhancement of Cr(VI) removal efficiency by chemical pretreatment

Various chemical pretreatments of the biomass were applied to improve the Cr(VI) adsorption capacity and removal efficiency. A series of biosorption batch experiments were carried out with heat inactivated (control) and with acid/alkali/salts pretreated biomass at pH 5.0 and 7.0. The obtained results (Table 1) indicated that HCl and CaCl₂ pretreatments enhanced the biosorption efficiency 20–25% than the control biomass. Acid-treatment has been used for washing the cell wall to enhance uptake capacity of biomass.
by increasing the surface area and porosity of original sample [2,13,33–35]. Enhancement in biosorption may be due to the protonation of functional groups (e.g. carboxyl and amino groups) at low pH condition giving an overall positive charge to the biomass, which adsorbed negatively anionic metal ions like CrO$_4^{2-}$. Calcium stabilized the biomass, causing the precipitation of inorganic substances present in the mycelia. Due to this precipitation vacant sites were created on the mycelial surface resulting in the enhancement of Cr(VI) biosorption by pretreatment using CaCl$_2$ [2,33].

Biosorption capacity of the alkali pretreated biomass exhibited approximately 65.4% and 61.7% reduction in Cr uptake at pH 5.0 and 7.0, respectively. Furthermore, alkali treatment resulted in desorption of biomass and caused 41.9% mass loss (Table 2). The alkali treatment caused the hydrolysis of protein constituents and deacetylation of chitin in biomass, due to polymer chain breakage and thereby hindered the overall operational stability. The reduction in Cr uptake was reported for fungal biomass of *Rhizopus nigricans* [24] on alkali treatment though improved Cr uptake in *Lentinus sajor-caju* biomass was observed, when compared to untreated form [22].

Pretreatment of biomass by NaCl decreased the biosorption of Cr(VI) to 40.02% at pH 5.0 and 37.43% at pH 7.0 as compared to the control biomass, indicating that sodium ions inhibited the biosorption. Biosorption of Pb$^{2+}$, Cd$^{2+}$, Ni$^{2+}$, and Zn$^{2+}$ by biomass of *Mucor rouxii* was reduced on treatment with CaCl$_2$ [20].

### Table 2

<table>
<thead>
<tr>
<th>Chemical-treatment</th>
<th>Mass loss (%)</th>
<th>PZC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat treated (control)</td>
<td>0.0</td>
<td>ND</td>
</tr>
<tr>
<td>Lipid extraction</td>
<td>8.3</td>
<td>ND</td>
</tr>
<tr>
<td>Methylation of amine</td>
<td>3.1</td>
<td>ND</td>
</tr>
<tr>
<td>Esterification of carboxyl group</td>
<td>11.1</td>
<td>ND</td>
</tr>
<tr>
<td>Modification of carboxyl group</td>
<td>15.4</td>
<td>ND</td>
</tr>
<tr>
<td>Esterification of phosphate group</td>
<td>18.7</td>
<td>ND</td>
</tr>
<tr>
<td>NaOH</td>
<td>41.9</td>
<td>6.27</td>
</tr>
<tr>
<td>HCl</td>
<td>10.2</td>
<td>2.87</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>4.3</td>
<td>5.91</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.4</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not detected.

Figure 1. Potentiometric titration curves of heat inactivated biomass of *T. clypeatus*. (A) Determination of acidic sites and functional groups. (B) Determination of basic sites and functional groups.

#### 3.4. Potentiometric titration of the biomass

Test for the determination of acidic and basic sites and functional groups present on cell wall of the biomass was determined by potentiometric titration as developed by Fourest and Volesky [39] in metal biosorption and followed by other researchers [8,19,21,40,41]. The results provided a rough characterization of the fungal biomass, when ionic exchange is one of the prevalent mechanisms in the removal of Cr(VI) from aqueous solution [23]. In order to gain closer inside of fungal biomass surface properties for acidic sites biomass suspension in 0.1 N NaOH (pH > PZC) was potentiometrically titrated with 0.1 N NaOH (Fig. 1A). Likewise, the NaOH suspension of biomass was potentiometrically titrated with 0.1 N HCl for determination of basic sites and the respective curve is present in Fig. 1B. Both the curves displayed, respectively, four and five inflexion points and corresponding pK$_a$ values suggested the binding functional groups present on the cell wall of the biomass. Table 3 showed chemical binding groups, with occurrence in selected biomolecules and their pK$_a$ values [40]. The results provided a rough characterization of the fungal biomass, when ionic exchange is one of the prevalent mechanisms in the removal of Cr(VI) from aqueous solution [23]. The results of titration permitted the qualitative and semi-quantitative determination of the nature and number of active (acidic or basic) sites present on biomass. The curve of Fig. 1A showed four flexion points at approximately pH 3.45, 4.29, 5.98 and 6.75 corresponding to pK$_a$ values of acidic binding groups and five flexion points at approximately pH 8.48, 9.96, 11.12, 11.92, 12.47 corresponding to amine functional groups [17]. It may be inferred that acidic groups were carboxylic (pK$_a$ 1.7–4.7), imidazole (pK$_a$ 5.5–6.0) and phosphate (pK$_a$ 6.1–6.8) and the alkaline groups were comparable to the values reported for amines (pK$_a$ 8.0–11.0), sulfhydryl (thiol) (pK$_a$ 8.0–10.0) and hydroxyl (pK$_a$ 9.5–13), as summarized in Table 3 [17,23].

The curve of Fig. 1B showed flexion points around pH 12.42 and pH 10.13 for hydroxyl (–OH) and amine (–NH$_2$) as alkaline binding groups. The acidic functional groups noted were imidazole (pK$_a$ 12.47), carboxylic (pK$_a$ 4.29), sulfate (pK$_a$ 6.27) and thiol (pK$_a$ 8.27) which are present on fungal biomass surface.
Table 3
Functional groups determination by potentiometric titration.

<table>
<thead>
<tr>
<th>S. no.</th>
<th>pKₐ values</th>
<th>Functional groups</th>
<th>Structural formula</th>
<th>Ligand atom</th>
<th>Occurrence in selected biomolecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.45</td>
<td>Carboxyl</td>
<td>-C=O</td>
<td>O</td>
<td>AA, PS, SPS, peptide bond</td>
</tr>
<tr>
<td>2</td>
<td>4.29</td>
<td>Carboxyl</td>
<td>-C=O</td>
<td>O</td>
<td>AA, PS, SPS, peptide bond</td>
</tr>
<tr>
<td>3</td>
<td>5.98</td>
<td>Imidazole</td>
<td>H-C-N</td>
<td>N</td>
<td>AA</td>
</tr>
<tr>
<td>4</td>
<td>6.75</td>
<td>Phosphate</td>
<td>OH</td>
<td></td>
<td>PL</td>
</tr>
<tr>
<td>5</td>
<td>8.48</td>
<td>Sulfhydryl (thiol) and amine</td>
<td>-S-H</td>
<td>S and N</td>
<td>Cto, AA, SPL</td>
</tr>
<tr>
<td>6</td>
<td>9.96</td>
<td>Sulfhydryl (thiol) and amine</td>
<td>-S-H</td>
<td>S and N</td>
<td>Cto, AA, SPL</td>
</tr>
<tr>
<td>7</td>
<td>11.12</td>
<td>Amine and hydroxyl</td>
<td>-OH, -NH₂</td>
<td>N</td>
<td>Cto, AA</td>
</tr>
<tr>
<td>8</td>
<td>11.92</td>
<td>Amine</td>
<td>-NH₂</td>
<td>N</td>
<td>Cto, AA</td>
</tr>
<tr>
<td>9</td>
<td>12.47</td>
<td>Amine</td>
<td>-NH₂</td>
<td>N</td>
<td>Cto, AA</td>
</tr>
</tbody>
</table>

Phs, polysaccharides; SPS, sulfated PS; Cto, chitosan; AA, amino acids; PL, phospholipids; LPS, lipoPS.

6.05), phosphates (pKₐ 6.57) and carboxylic (pKₐ 4.01, 3.13 and 2.24). According to the experimental data, the acidic and basic sites for heat inactivated biomass of T. clypeatus were estimated as 7.75 and 3.25 mmol/g, respectively, it was observed that the concentration of acidic sites were higher than those of the basic sites, hence the surface of the biomass was acidic [37,42].

3.5. Chemical modification of functional groups of the biomass

Functional groups on binding sites of the heat inactivated biomass were investigated through chemical modification, where the potential site was altered to either block the site from metal binding or enhanced by introducing a new functional group that might have higher affinity for metal binding. The metal binding experiments were carried out at pH 5.0 and 7.0 and the effects of chemical modification of the biomass were presented in Fig. 2. The biosorption and Cr(VI) uptake (qₑ) of control biomass were 79.65% and 9.92 mg/g at pH 5.0 and 74.98% and 9.55 mg/g at pH 7.0, respectively. The effects of functional group modification of the biomass are discussed below.

3.5.1. Methylation of amines

As shown in Fig. 2 the methylation of amino groups enhanced the biosorption efficiency by approximately 42–45% at first 4h and at equilibrium phase it became 34.7% at both the assayed pH as compared with the heat inactivated biomass. At late stage (bioaccumulation phase at 48h) of adsorption the biomass showed about 20–25% enhancement in Cr(VI) biosorption (Table 4). In this study, increase in biosorption may be due to the acidic condition, which might have caused unmasking and hence availability of other binding sites (those other than the amine groups) on the cell surface, which may not have been able to participate in the sorption process without the treatment. Generally methylation of amino groups present on cell wall significantly reduced biosorption capacity of Cr(VI) [2,40] and recommended that positively charged amino groups of cell wall contributed to chromium biosorption.

3.5.2. Esterification of carboxylic acids

It was observed from the adsorption experiments that the amount of Cr(VI) bound was reduced from 79.65 to 60.88% at pH 5.0 and 74.98 to 48.28% at pH 7.0 by esterification of the carboxyl groups in the biomass (Fig. 2 and Table 4). Slight enhancements (9–12%) in biosorption efficiency were recorded during primary phase, however reduced (by 3–10%) at equilibrium stage of biosorption (24h). The results suggested the contribution to the binding of the Cr(VI) ions to carboxyl groups present on the cell wall of
3.5.3. Esterification of phosphates

Biosorption efficiency reduced by 27.88% at pH 5 and 23.60% at pH 7 by modification of the phosphate groups in the biomass, indicating their active role in Cr(VI) removal. Phosphates in the yeast 
S. cerevisiae provided binding sites for chromate ions [19].

3.5.4. Modification of sulphydryl group

Modification of sulphydryl group also showed a negative effect on chromium biosorption and reduction efficiency of 19.48% at pH 5 and 10.66% at pH 7 was noted (Fig. 2 and Table 4), which was not as prominent as compared to other treatments/modifications. The results indicated that sulphydryl groups were involved though not as the major binding sites of chromium. Marginal decrease of 23.5% was observed for the binding of lead after above treatment [40] and insignificance difference in uptake of alkynes and alkyl (–CH3 and –CH2) groups of functional groups in biosorption process. It should be noted that probable structural changes that may have occurred due to the harsh condition of the extraction process reduced the biosorption of the metal. Reduction of 41.54% efficiency of chromium biosorption was reported after lipids extraction from S. cerevisiae [19].

3.5.5. Extraction of lipids

Removal of lipids from the biomass exhibited a negative effect on Cr(VI) biosorption and 13.19% and 19.99% reduction was observed for the binding of lead after above treatment [40] and insignificance difference in uptake of alkynes and alkyl (–CH3 and –CH2) groups of functional groups in biosorption process. It should be noted that probable structural changes that may have occurred due to the harsh condition of the extraction process reduced the biosorption of the metal. Reduction of 41.54% efficiency of chromium biosorption was reported after lipids extraction from S. cerevisiae [19].

3.6. Characterization using FTIR spectroscopy

FTIR analysis of pretreated biomass (e) was taken before and after Cr(VI) uptake. Table 5 listed band assignments and the typical functional groups present in heat inactivated biomass and Cr(VI) loaded biomass of T. clypeatus. The respective FTIR spectra are shown in Fig. 3A. The functional groups present on surface of heat inactivated T. clypeatus biomass were amino, carboxylic, phosphate, sulfonyl and carbonyl.

After contact with Cr(VI) solutions the heat inactivated T. clypeatus biomass exhibited FTIR spectra with clear appearance of a band ranging from 1500 to 1650 cm⁻¹, which specified primary, secondary and tertiary amines and ammonium salts of carboxylic acid compounds in solid state samples. This could be attributed to an interaction between Cr(VI) species and N-containing bio-ligands. There was a clear disappearance of the peak at 1403.92 cm⁻¹ corresponding to sulfonyl, sulfonamide and phosphate groups in Cr(VI) loaded fungal biomass (Fig. 3A and Table 5). This shift is typical for the complexation of sulfonyl and phosphate group’s coordination with metal ions [11,20]. The peaks of 1299.79 cm⁻¹ and 1078.01 cm⁻¹ represented P=O and C=O bands of polysaccharides, respectively. There was also clear frequency increase of 1633.41–1741.41 cm⁻¹ and 545.76–566.01 cm⁻¹ when compared with that of Cr(VI) loaded biomass and groups involved were –NH, carbonyl (–CO) and disulfide. These results indicated the involvement of these functional groups in biosorption process. It should be noted that the intensity peak of alkenes and alkyl (–CH3 and –CH2) groups of carbon skeleton peak at 2929.34 decreased to 2921.63 cm⁻¹ in Cr(VI) loaded biomass.

Table 5 and Fig. 3 (B–F and G–J; additional details for FTIR spectra (G–J) are provided in Fig. 3 (continued) as Supplementary data) showed a comparison of FTIR spectra of all the treated biomass of T. clypeatus and the changes in appearance and disappearance in transmittance peaks. The changes on functional groups occurred after Cr uptake of methylated amino group, esterified carboxyl and...
phosphate group biomass. The clear appearance of peaks ranging from 1500 to 1200 cm\(^{-1}\) and 1200 to 1000 cm\(^{-1}\) which are assigned to \(\text{C=O}\) stretching in carbonyl or amide I band, N–H banding in amide II and C–N stretching in –CO–NH–, and C–OH stretching was noted. The increase of these bands indicated the improvement in biosorption and involvement of N–H of amines, \(\text{C=O}\) of amides, carboxyl and phosphate groups. Modification of sulfhydryl groups and lipid extraction in the biomass did not show significant influence on transmittance peaks.

3.7. SEM–EDX analysis

SEM micrographs (Fig. 4A and B) and EDAX (Fig. 5A and B) spectra were obtained before and after Cr(VI) biosorption onto heat treated biomass. The SEM–EDX analysis made the Cr(VI) adsorption more visual between biosorbents and metal ions. It could be clearly observed from SEM pictures that the surface of the biomass becomes rougher and shows the presence of new shiny bulky particles over the surface of the biomass saturated with Cr(VI) than in case of before adsorption. This observation was confirmed by EDX analysis which revealed Cr peaks in the spectra (Fig. 5B) while no such a peak could be found on the biomass cell surface (Fig. 5A).

4. Removal mechanism by the heat inactivated (non living) biomass

Higher fungi (mushrooms) are abundantly available in nature and can be exploited as low cost materials for their biosorption properties. Studies on biosorption process characteristics of the heat inactivated \(T.\) clypeatus biomass such as pH profile, kinetics and biosorption isotherms concluded that at pH 2.0 the rate of biosorption was faster than at pH 5.0 and 7.0 and with increasing contact time similar biosorption efficiency was reported [44]. The biosorption complied with both the Langmuir and Freundlich isotherm models and it appeared that monolayer as well as heterogeneous surface conditions biosorption co-existed. Hence the overall adsorption phenomenon of Cr(VI) on the biomass was complex, involving more than one mechanism, such as ion exchange, surface complexation and electrostatic attraction. The kinetic biosorption process of Cr(VI) consisted of a rapid phase in first 4 h (30%) in which near saturation of adsorption was attained and reached to equilibrium phase (24 h, 71%) and finally a slow phase (48 h, 84%) that followed the second order kinetic model. The rapid phase of biosorption may be principally dependent on the surface nature of cell wall, which was substantially related to functional groups present in cell components and the charge density on the biomass surface. The protonated active sites were bound to Cr(VI) ions (\(\text{CrO}_4^{2-}\) or \(\text{Cr}_2\text{O}_7^{2-}\)) by electrostatic force [11]. The slow phase of the process depended on the diffusion rate of ions through the cell skeleton where the reductive group of the biomass reduced Cr(VI) to Cr(III), in which the topography and surface structure of the cells acted as the major factor. Cr(VI) was completely reduced to Cr(III) by contact with brown seaweed \(Ecklonia\) biomass [45]. The hydroxyl groups [46] and carbonyl groups [47] contained in the cell wall of the biomass (analyzed through FTIR) played a role as electron donor for the reduction reaction.

Acid and alkali pretreatment (Section 2.3) on heat inactivated biomass showed the dependence of pH, indicating that adsorption through ion exchange was responsible for the observed chromium binding. The observed increased in biosorption efficiency with acid pretreatment was due to the over all surface charge on the biomass that may become positive or less negative and promoted the approach of negatively charged metal ion like \(\text{CrO}_4^{2-}\) and \(\text{Cr}_2\text{O}_7^{2-}\). At low pH values the protonation of functional groups (carboxyl and amino groups) caused increase in over all positive charge of the biomass making adsorption of negatively anion metal ions effective [48].

Dead cells sequestered the metal through chemical functional groups of the material comprising the cell wall and the passive metal uptake continued by the metabolically inactive cell...
The anionic Cr(VI) ion species were bound to the positively charged groups of nonliving cells and amino and carboxyl groups were involved in the removal of chromium. Although increase in biosorption efficiency was observed in the present study with the methylation of amino group it may be noted that the methylation process was carried out at acidic conditions which also enhanced the biosorption efficiency (Section 2.6.3.1). The FTIR analyses confirmed the role of positively charged amino group in the biosorption process, which allowed binding through ion exchange of negatively charged chromium ions. Cr(VI) binding was reduced when the negative charge of carboxyl and phosphoryl groups were neutralized by chemical modification, indicating that both groups had a significant role in binding of the metal at the sites, where electrostatic interaction occurred. The reduction of biosorption efficiency with modification of sulfhydryl group and lipid extraction indicated their involvement for the observed chromium binding due to complex formation. In nutshell, surface characterization techniques were helpful in identification of Cr binding groups on the biomass. Accordingly it was concluded that the functional groups taking part in biosorption were carboxyl (–COOH), amide (–NH2), thiol (–SH), phosphate (PO4^{3−}) and hydroxyl (–OH). Enhanced biosorption with acid pretreatment recommended that electrostatic attraction and ion exchange mechanism were involved. The FTIR analysis confirmed the role of positively charged amino group in the biosorption process, due to ion exchange binding of negatively charged chromium ions. It became imperative that the biosorption of Cr(VI) involved two successive steps, the biosorption of CrO4^{2−} and Cr2O7^{2−} ions by electrostatic attraction at the protonated active sites followed an additional step of complexation as reduction of Cr(VI) to Cr(III) by the reductive groups on the surface of the biomass.

5. Concluding points/remarks

The following conclusions were reached based on the study:

- Chemical pretreatment of the biomass by acid and CaCl2 showed improvement of Cr(VI) removal rate at pH 5.0 and 7.0 while reverse was true for alkali and NaCl treatments.
- The mass loss recorded after each chemical pretreatment due to cleaning of the cell wall of the biomass, which indicated that degradation and solubilization of the biomass caused by the alkali treatment and thereby exhibited negative effect on Cr(VI) biosorption.
- Identification of the active sites involved in the sequestration of Cr(VI) was derived using biochemical techniques like potentiometric titration, PZC, modification of functional groups and instrumental analysis like FTIR.
- The acidic and alkaline functional groups, carboxylic, imidazole, phosphate, amine, sulfhydryl and hydroxyl were present on the cell wall of the biomass as inferred from their pK_a values derived from potentiometric titration curve.
- Total acidic sites estimated were higher than the basic sites suggesting that the surface of the biomass was acidic.
- Non-living cell surface sorption for Cr(VI) removal by the heat inactivated fungal biomass showed involvement of more than one mechanisms such as physical adsorption, ion exchange, complexation and electrostatic attraction.
Low pH and acid treatment rendered the biomass surface more positive and showed faster the removal rate of Cr(VI) in the aqueous phase, since the binding of anionic Cr(VI) ion species with positively charged groups was enhanced.

Amino, carboxyl and phosphate groups were involved in Cr(VI) biosorption process, as established after chemical modification of those functional groups.

FTIR analysis confirmed the involvement of amino, carboxylic, phosphoric, sulfonic and carboxyl groups in Cr(VI) biosorption by lignocellulosic biomass of T. Cypreus.

Integrative analyses of kinetic studies, surface chemistry, the effect of pH values on adsorption behavior of Cr(VI) and the results of FTIR showed that the biosorption of Cr(VI) followed two subsequent steps, biosorption of CrO4^2− by electrostatic force at the protonated active sites (amino, carboxyl and phosphate groups) and reduction of Cr(VI) to Cr(III) by reductive groups (hydroxyl and carboxyl groups) on the surface of the biomass.

Acknowledgment

Funding to LR by CSIR, India is duly acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cej.2011.05.002.

References


[39] ...