Chapter 1

Introduction

Objective of the Present study

1.2

Review of Literature

1.2.1
Tannins

1.2.2
Tannase

1.2.3
Microbial source of Tannase

1.2.4
Tannase Production

1.2.4.1
Submerged Fermentation

1.2.4.2
Solid state Fermentation

1.2.5.
Purification and characterization of tannase

1.2.6.
Genetic characterization

1.2.7.
Applications of tannase

1.2.7.1
Industrial

1.2.7.1.1.
Pharmaceutical industry

1.2.7.1.2.
Beverage clarification

1.2.7.1.3.
Instantaneous tea elaboration

1.2.7.1.4.
Animal feed

1.2.7.1.5.
Cell wall digestion

1.2.7.1.6.
Effluent treatment

1.2.7.2.
Environmental

1.2.7.2.1.
Bioremediation and tannin-contaminated waste water

1.2.7.3.
Other applications

Chapter 2

Biosynthesis of Tannase from cashew testa using *Aspergillus niger* MTCC 5889 by Solid state Fermentation.

2.

Introduction

2.1
Materials and methods

2.1.1
Isolation and screening of tannase producing microorganism
2.1.2 Medium used for the secondary screening of tannase production 46
2.1.3 Morphological identification of selected microbes for the production of tannase 46
2.1.4 Ribotyping using partial 18S rRNA gene 47
2.1.4.1 Polymerase chain reaction 47
2.1.4.2 DNA sequencing 48
2.1.4.3 Phylogenetic tree construction 48
2.1.4.4 Growth curve determination 48
2.1.5 Enzyme extraction 49
2.1.5.1 Inoculam preparation 49
2.1.5.2 Inoculation and incubation 50
2.1.5.3 Recovery of enzyme 50
2.1.6 Analytical methods 50
2.1.6.1 Tannase assay 50
2.1.7 Tannin degradation 51
2.1.7.1 Sample collection 51
2.1.7.2 Extraction and Quantification of tannin 51
2.1.7.3 Tannase degradation assay 52
2.1.8 Solid state fermentation 52
2.1.9 Submerged Fermentation 53
2.1.10 Evaluation of tannase production in different substrates 54
2.2 Result 55
2.2.1 Screening and Selection of potential strain 55
2.2.2 Identification of the selected strain 56
2.2.3 Ribotyping using 18S rRNA gene & Phylogenetic tree construction 58
2.2.4 Growth curve 60
2.2.5 Quantification of tannins 61
2.2.6 Enzyme Production 61
2.2.7 Evaluation of natural substrate for the production of tannase 62
2.3.1 Discussion 64
2.4 Conclusion 67
Chapter 3  Statistical optimization of bioprocess variables for the production of Tannase by Response surface Methodology

3  Introduction 68
3.1. Materials methods 71
3.1.1. Optimization of the SSF production parameters of Tannase by A. niger CEPC 11 71
3.1.2. Optimization of Significant Parameters by Box-Behnken Design 73
3.2  Result 75
3.2.1. Screening of dependent factor using Placket-Burman design experiment 75.
3.2.2. Optimization of Significant Parameters using Box-Behnken design 78
3.2.3. Response surface three dimensional plot curves for the production of tannase. 82
3.2.3.1. Three-dimensional response surface for tannase production using Cashew testa and Di-KHPO₄ 82
3.2.3.2. Three-dimensional response for tannase production in relation to Cashew testa and Incubation temperature 83
3.2.3.3. Three-dimensional response for tannase production in relation to Di-KHPO₄ and Incubation Temperature 84
3.2.3.4. Three-dimensional response for tannase production in relation to Cashew testa and Sodium chloride 86
3.2.3.5. Three-dimensional response for tannase production in relation to Sodium chloride and Di-KHPO₄ 87
3.2.3.6. Three-dimensional response for tannase production in relation to Sodium chloride and Incubation temperature. 88
3.2.4. Validation of the model 89
Chapter 4  Purification and characterization of tannase enzyme from *Aspergillus niger* CEPC 11

4  Introduction 94
4.1  Materials and Methods 95
4.1.1  Enzyme purification 95
4.1.1.1  Ammonium sulphate and Acetone precipitation of crude enzyme preparation 95
4.1.1.2  Dialysis 97
4.1.1.3  Gallic acid estimation 97
4.1.1.4  Protein estimation 98
4.1.1.4.1  Estimation 99
4.1.1.5  Concentration of tannase by ultra filtration 99
4.1.1.6  Gel Filtration chromatography 100
4.1.1.6.1  Preparation of column 100
4.1.1.6.2  Sample preparation and application on the column 100
4.1.1.7  Calculation of Yield of Protein, Yield of Enzyme Activity, Fold of Purification 101
4.1.2  Characterization of tannase from *A. niger* CEPC 11 101
4.1.2.1  Determination of the molecular weight of tannase from *A. niger* CEPC 11 102
4.1.2.2  Sample Preparation of Native PAGE 102
4.1.2.2.1  Procedure 102
4.1.2.2.2  Sample Preparation of SDS-PAGE 103
4.1.2.2.3  Procedure 103
4.1.2.3  Identification of tannase by MALDI-TOF-MS 104
4.1.3  Gallic acid Confirmation 104
4.1.3.1  Fourier-Transform Infra red Spectroscopy (FT-IR Spectroscopy) 104
4.1.3.2  High Performance Thin Layer Chromatography (HPTLC) 105
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1.3.3</td>
<td>High Performance Liquid Chromatography (HPLC)</td>
</tr>
<tr>
<td>4.1.4.</td>
<td>Determination of the optimal temperature of activity for the <em>A. niger</em> from tannase enzyme.</td>
</tr>
<tr>
<td>4.1.5</td>
<td>Determination of the optimal pH of activity for the <em>A. niger</em> from tannase enzyme.</td>
</tr>
<tr>
<td>4.1.6</td>
<td>Temperature stability of tannase enzyme of <em>A. niger</em></td>
</tr>
<tr>
<td>4.1.7.</td>
<td>Carbohydrate content</td>
</tr>
<tr>
<td>4.1.8.</td>
<td>Substrate Specificity and Kinetic Constants</td>
</tr>
<tr>
<td>4.1.9.</td>
<td>Effect of various metal salts, detergents and organic solvents, reducing agents and inhibitors on enzyme activity</td>
</tr>
<tr>
<td>4.1.9.1</td>
<td>Effect of various metal salts on tananse enzyme activity from <em>A.niger</em>.</td>
</tr>
<tr>
<td>4.1.9.2</td>
<td>Effect of various detergents on tananse enzyme activity from <em>A.niger</em>.</td>
</tr>
<tr>
<td>4.1.9.3</td>
<td>Effect of organic solvents on tananse enzyme activity from <em>A.niger</em>.</td>
</tr>
<tr>
<td>4.1.9.4</td>
<td>Effect of various Inhibitors on tananse enzyme activity from <em>A.niger</em>.</td>
</tr>
<tr>
<td>4.1.9.5</td>
<td>Effect of various reducing agents on tananse enzyme activity from <em>A.niger</em>.</td>
</tr>
<tr>
<td>4.1.9.6</td>
<td>Residual Activity.</td>
</tr>
<tr>
<td>4.1.10</td>
<td>Thermal stability of cashew testa by TGA analysis.</td>
</tr>
<tr>
<td>4.2.</td>
<td>Results</td>
</tr>
<tr>
<td>4.2.1.1</td>
<td>Ammonium sulphate precipitation</td>
</tr>
<tr>
<td>4.2.1.2</td>
<td>Solvent Precipitation</td>
</tr>
<tr>
<td>4.2.1.3</td>
<td>Dialysis</td>
</tr>
<tr>
<td>4.2.1.4</td>
<td>Gel filtration chromatography</td>
</tr>
<tr>
<td>4.2.2.</td>
<td>Characterization of tannase enzyme from <em>A. niger</em> CEPC 11.</td>
</tr>
<tr>
<td>4.2.2.1</td>
<td>Determination of the molecular weight of purified tannase enzyme.</td>
</tr>
<tr>
<td>4.2.2.2</td>
<td>Native PAGE analysis of purified tannase.</td>
</tr>
</tbody>
</table>
4.2.3. Confirmation of gallic acid 114
4.2.3.1 FTIR 114
4.2.3.2 HPTLC 115
4.2.3.3 HPLC 117
4.2.4. Carbohydrate content 118
4.2.5. Optimal temperature for tannase activity 118
4.2.6. Thermo stability of tannase at different temperatures 120
4.2.7. Optimal pH for tannase activity 121
4.2.8. Kinetic Studies 123
4.2.9 Substrate specificity 124
4.2.10. Effect of various metal salts, detergents, oxidising and reducing agents and inhibitors on enzyme activity. 125
4.2.10.1. Effect of various metal salts on tannase activity 125
4.2.10.2. Effect of various detergents on enzyme activity 127
4.2.10.3. Effect of organic solvents on enzyme activity. 127
4.2.10.4. Effect of Reducing agent and inhibitors on enzyme activity 128
4.2.11. Thermal stability of cashew testa by TGA analysis 129
4.3. Discussion 131
4.4. Conclusion 139

Chapter 5 Genetic characterisation of Tannase from *Aspergillus niger* and structural elucidation by Molecular docking

5.1 Materials and Methods 141
5.1.1. Cultivation of fungus 141
5.1.2. Isolation of Genomic DNA 142
5.1.3. Primer designing 143
5.1.4. PCR Amplification of partial gene sequences of tannase. 144
5.1.5. DNA Sequencing 145
5.1.6. Three Dimensional Modelled structure of tannase (3D model). 145
5.2 Results 146
5.2.1. Genomic DNA isolation 146
5.2.2. PCR amplification of tannase gene 146
5.2.3. Sequencing and BLAST analysis of partial genes of tannase. 147
5.2.4. Secondary structure of tannase 151
5.2.5. 3D model Structure of tannase. 151
5.2.5.1 Ramachandran plot. 152
5.2.5.2. The galloyl binding site 152
5.3. Discussion 154
5.4. Conclusion 156

Chapter 6 Studies on the application of Aspergillus niger Tannase. 157
6 Introduction 158
6.1 Materials and Methods 158
6.1.1. Juice Clarification. 158
6.1.1.1. Juice Preparation. 158
6.1.1.2. Treatment of Juice with Tannase 158
6.1.2. Tea cream solubilization. 159
6.1.2.1. Preparation of Tea Extract. 159
6.1.2.2. Estimation of solid content. 159
6.1.2.2.1. Total Solid content 159
6.1.2.2.2. Cream solid 159
6.1.2.2.3. Treatment of tea extract with Tannase. 159
6.1.2.2.4. Determination of cream content 160
6.1.2.2.5. Determination of % Cream solubilized 160
6.1.3. Determination of Heavy Metals in Tannery effluent by AAS. 160
6.1.3.1. Determination of Elements by AAS 160
6.1.3.2 Characterization of effluent. 161
6.1.3.2.1. Analytical methods. 161
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1.3.2.2.</td>
<td>pH</td>
<td>161</td>
</tr>
<tr>
<td>6.1.3.2.3.</td>
<td>Biological Oxygen Demand (BOD)</td>
<td>161</td>
</tr>
<tr>
<td>6.1.3.2.4.</td>
<td>Chemical Oxygen Demand (COD)</td>
<td>162</td>
</tr>
<tr>
<td>6.2.</td>
<td>Results</td>
<td>163</td>
</tr>
<tr>
<td>6.2.1.</td>
<td>Fruit Juice Clarification</td>
<td>163</td>
</tr>
<tr>
<td>6.2.2.</td>
<td>Estimation of solid content</td>
<td>164</td>
</tr>
<tr>
<td>6.2.3.</td>
<td>Reduction of Heavy Metals in Tannery effluent</td>
<td>165</td>
</tr>
<tr>
<td>6.3.</td>
<td>Discussion</td>
<td>167</td>
</tr>
<tr>
<td>6.4.</td>
<td>Conclusion</td>
<td>170</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td><strong>Summary and conclusion</strong></td>
<td>171</td>
</tr>
<tr>
<td></td>
<td><strong>Bibliography</strong></td>
<td>178</td>
</tr>
<tr>
<td></td>
<td><strong>Appendix</strong></td>
<td>205</td>
</tr>
<tr>
<td></td>
<td><strong>Publications</strong></td>
<td>210</td>
</tr>
</tbody>
</table>