Molecular characterization and phylogenetic analysis of protease producing *Streptomyces* sp. isolated from mangrove sediments

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Introduction

- Microbial enzymes have great utility in industries.
- Protease, Lipase, Amylase, Laccase, Cellulase, L-asparaginase have enormous potential
- Source of Microbial enzymes: Bacteria, actinomycetes and fungi
Introduction

• Actinomycetes are the Gram-positive filamentous bacteria.

• Actinomycetes form fungus-like branched networks of hyphae.

• Actinomycetes contribute to around 70% of the source of antibiotics, non-antibiotic bioactive metabolites, such as enzyme, enzyme inhibitors, immunological regulators, anti-oxidation reagents and so on.
Proteases

- Proteases are mainly obtained from microbial sources for industrial purposes.

- Proteases are the most important group of the enzymes produced commercially and industrial purpose.

- Proteases has extensive applications in a range of industrial products and processes including food, detergent, pharmaceuticals and leather.

- Proteases may be classified as two major groups; exopeptidase and endopeptidase based on their ability to degrade N- or C- terminal peptide bond.

- Acid Protease, Neutral Protease and Alkaline Protease
METHODOLOGY

Collection of Soil Samples

Processing of Soil Samples

Isolation of Pure Culture of Actinomycetes

Characterization of Actinomycetes
- Morphological Characterization
- Biochemical Characterization
- Microscopical Characterization

Qualitative & Quantitative Screening

Molecular Characterization
Sample Collection

- Soil and sediment samples were collected from the mangrove forest, Pichavaram, Chidambaram.

- Soil and sediment samples were collected from various depths of the earth surface.

- Fifty soil samples were collected within a period of ten months (Feb, 2015–November, 2015).
Pichavaram Mangrove Forest
Pichavaram Mangrove Forest
Processing of Soil Samples

• The collected soil samples were shade dried and pulverized well into a fine powder using a mortar and pestle.

• The powdered soil and sediments were sieved by using a filter and stored in a plastic container until examined.
Isolation of Pure Culture of Actinomycetes

• Actinomycete strains were isolated from mangrove soil and sediments by using serial dilution technique

• The medium used for isolation was Starch-casein-agar medium supplemented with Nalidixic (25 μg/mL), Actidione (50 μg/mL) and Nystatin (50 μg/mL) on petridishes.

• The following Pre-treatments had been followed to isolate the rare actinomycetes:
  1. Air Dying.
  2. Dry Heating.
  3. Phenol Treatment
  4. Calcium Carbonate Treatment

• The total of 75 actinomycetes strain was isolated from mangrove soil and sediments by using standard microbiological method.
Characterization of Actinomycetes

The Actinomycetes were characterized by

1. Morphological Characterization.
2. Biochemical Characterization and
3. Microscopical Characterization

Morphological characterization

Actinomycetes colonies were characterized morphologically following the direction given by the International Streptomyces Project (ISP)

Colour, texture, aerial mycelium and substrate mycelium were observed for characterization
Biochemical Characterization

- The 75 actinomycete isolates were studied using these biochemical tests.

- Various biochemical tests were performed for the identification of the potent isolates are as follows Indole, Methyl Red, Voges Proskauer, Citrate, Urease, Triple Sugar Ion, Starch, Gelatin, Nitrate, Catalase and Oxidase.
Biochemical Characterization

- methyl red: 11
- citrate: 45
- urease: 72
- TSI: 62
- starch: 19
- nitrate: 37
- catalase: 69
Microscopical Characterization

- Gram staining techniques were followed for the identification for all 75 actinomycete isolates

- Gram positive isolates were observed by microscopical identification

- Spore morphology of the isolates were identified.
Qualitative screening for protease production

- Qualitative screening was done for all the 75 isolates.

- The isolates were screened for protease production using skim milk agar medium.

- After 5 days of incubation the plates were observed for clear lysing zones around the colonies.
Qualitative Screening for Protease production

- 31 High protease activity
- 18 Low protease activity
- 16 Moderate protease activity
- 10 No protease activity

Total strains 75

Zone formation best protease producing isolates
Quantitative screening for protease production

- The isolate strains was added to a 250mL flask containing 50mL of protease production broth containing (g/L) peptone -10, sucrose -10, K₂HPO₄ - 0.5, MgSO₄·7H₂O - 0.5, NaCl - 0.5, CaCl₂ - 0.5 and pH7.

- The mixture was incubated under shaking conditions at 30°C and 120 rpm.

- At every 2 days interval, the cultures were harvested and the culture medium was centrifuged to remove mycelia and medium debris.

- The protease activity of culture supernatants was measured and determined by quantitative enzyme assay.
Best Protease producing strains on 10\textsuperscript{th} Day

Protease Production U/mL

Strains
Molecular Characterization

Sequencing of 16S rRNA region using universal primers

1. Genomic DNA Isolation
2. Agarose Gel Electrophoresis for DNA Quality and Quantity check
3. PCR Analysis
4. Agarose Gel electrophoresis of PCR products
5. ExoSAP-IT Treatment
6. Sequencing using BigDye Terminator v3.1
Molecular Characterization

• Molecular identification by means of 16S rRNA sequencing was employed for the identification of the isolates. PCR amplification of the genomic DNA at the ITS region of the rRNA was carried out using the universal primers

• The best 8 promising protease producers are sequenced which are identified up to genus levels. All the 8 isolates belong to *Streptomyces* genus.
<table>
<thead>
<tr>
<th>TARGET</th>
<th>Primer Name</th>
<th>Direction</th>
<th>Sequence (5’ → 3’)</th>
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<tbody>
<tr>
<td>16S rRNA</td>
<td>16S-27F</td>
<td>Forward</td>
<td>AGAGTTTGATCMTGGCTCAG</td>
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<td></td>
<td>16S-1492R</td>
<td>Reverse</td>
<td>GTTACCTTGTACGACTTT</td>
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</table>
Phylogenic tree for *Streptomyces sp.* LCJ1A and LCJ2A
Phylogenic tree for *Streptomyces sp.* LCJ3A and LCJ4A
Phylogenic tree for *Streptomyces sp.* LCJ17A and LCJ18A
Effect of Medium on Protease Production

- Protease production by *Streptomyces* sp. LCJ17A and *Streptomyces* sp. LCJ1A was studied using six different media.
- The mediums used were PPB, MNGA, GB, SCB, GYEB and MYEB.
- All the six culture medium was inoculated with two culture discs (4mm) each under sterile conditions.
- The inoculated mediums were kept for incubation on an orbital shaker at 30°C with an agitation of 120 rpm.
- Aliquots of the culture filtrate were collected for determination of protease activity.
### Protease Activity of Actinomycetes isolate on six various medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>PPB</th>
<th>MNGA</th>
<th>GB</th>
<th>SCB</th>
<th>MYEB</th>
<th>GYEB</th>
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<tbody>
<tr>
<td>LCJ17A</td>
<td>27.28</td>
<td>70.59</td>
<td>33.46</td>
<td><strong>80.10</strong></td>
<td>20.40</td>
<td>32.32</td>
</tr>
<tr>
<td>LCJ1A</td>
<td>32.66</td>
<td>40.00</td>
<td>43.09</td>
<td><strong>70.59</strong></td>
<td>40.80</td>
<td>79.41</td>
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<tr>
<td>LCJ3A</td>
<td>22.46</td>
<td>32.20</td>
<td>37.93</td>
<td>56.50</td>
<td>17.65</td>
<td>16.16</td>
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<tr>
<td>LCJ2A</td>
<td>24.76</td>
<td>25.33</td>
<td>41.60</td>
<td>62.34</td>
<td>50.31</td>
<td>19.60</td>
</tr>
<tr>
<td>LCJ6A</td>
<td>22.81</td>
<td>41.71</td>
<td>21.32</td>
<td>44.69</td>
<td>31.17</td>
<td>26.93</td>
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<tr>
<td>LCJ8A</td>
<td>21.20</td>
<td>37.13</td>
<td>40.91</td>
<td>21.32</td>
<td>26.02</td>
<td>46.53</td>
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<tr>
<td>LCJ4A</td>
<td>25.79</td>
<td>43.66</td>
<td>22.58</td>
<td>25.79</td>
<td>38.85</td>
<td>20.75</td>
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<tr>
<td>LCJ18A</td>
<td>21.20</td>
<td>32.32</td>
<td>17.31</td>
<td>21.20</td>
<td>25.90</td>
<td>47.56</td>
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</tbody>
</table>
Effect of Carbon source on Protease Production

- Starch Casein Broth, is the Medium used for optimization of carbon source.
- The starch contained in the production medium was replaced with the Glycerol, Maltose, Sucrose, Fructose, Lactose, Mannitol and Glucose.
- Isolates were inoculated in the medium and kept in shaker at 120 RPM.
- Every alternative days reading were taken at 660 nm.
- Among the various carbon sources tested, Glycerol and Glucose stimulated protease production maximally *Streptomyces* sp. LCJ17A.
- In *Streptomyces* sp. LCJ1A, Maltose and Mannitol produced higher amount of protease.
Effect of different carbon source on protease production by *Streptomyces* sp. LCJ17A

<table>
<thead>
<tr>
<th>Carbon Sources</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>12.27</td>
<td>17.42</td>
<td>12.84</td>
<td><strong>34.40</strong></td>
<td>9.52</td>
<td>15.36</td>
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<tr>
<td>Glucose</td>
<td>12.50</td>
<td>20.40</td>
<td>20.52</td>
<td><strong>49.96</strong></td>
<td>18.34</td>
<td>21.66</td>
</tr>
<tr>
<td>Sucrose</td>
<td>8.94</td>
<td>16.05</td>
<td>11.24</td>
<td>33.69</td>
<td>12.50</td>
<td>21.89</td>
</tr>
<tr>
<td>Starch</td>
<td>17.0</td>
<td>17.42</td>
<td>11.35</td>
<td>42.17</td>
<td>8.47</td>
<td>13.76</td>
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<tr>
<td>Fructose</td>
<td>12.50</td>
<td>20.06</td>
<td>15.13</td>
<td>30.83</td>
<td>12.27</td>
<td>27.16</td>
</tr>
<tr>
<td>Mannitol</td>
<td>8.94</td>
<td>16.74</td>
<td>11.01</td>
<td>24.64</td>
<td>27.39</td>
<td>14.10</td>
</tr>
</tbody>
</table>
Effect of different carbon source on protease production by *Streptomyces* sp. LCJ1A

<table>
<thead>
<tr>
<th>Carbon Sources</th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>Day 8</th>
<th>Day 10</th>
<th>Day 12</th>
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</thead>
<tbody>
<tr>
<td>Glycerol</td>
<td>23.61</td>
<td>52.60</td>
<td>42.40</td>
<td>10.78</td>
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<td>Maltose</td>
<td>25.21</td>
<td>42.17</td>
<td><strong>49.96</strong></td>
<td>13.99</td>
<td>18.91</td>
<td>13.30</td>
</tr>
<tr>
<td>Glucose</td>
<td>26.59</td>
<td>32.66</td>
<td>18.11</td>
<td>10.66</td>
<td>16.85</td>
<td>20.75</td>
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<tr>
<td>Sucrose</td>
<td>20.17</td>
<td>42.98</td>
<td>34.61</td>
<td>8.37</td>
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<td>Starch</td>
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<td>24.18</td>
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<td>17.74</td>
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<tr>
<td>Fructose</td>
<td>15.25</td>
<td>34.27</td>
<td>19.03</td>
<td>10.77</td>
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<td>Lactose</td>
<td>14.56</td>
<td>30.71</td>
<td>30.14</td>
<td>10.89</td>
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<tr>
<td>Mannitol</td>
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<td>30.60</td>
<td><strong>52.71</strong></td>
<td>11.13</td>
<td>16.80</td>
<td>9.86</td>
</tr>
</tbody>
</table>
Conclusion

• The actinomycetes culture namely LCJ1A (GenBank accession number KU870428) and LCJ17A (GenBank accession number KU870434) produced significantly higher amount of protease enzymes.

• Highly stable at neutral pH and alkaline pH.

• It has the potential for use in industrial purpose.
THANK YOU