Assessment of fungal protease enzyme from French bean using A. niger by Solid State Fermentation

1Akhilesh Upgade*, 2Aashu Nandeshwar and 3Lalit Samant

1Department of Microbiology, Centre for Research & Development, PRIST University, Thanjavur, Tamilnadu, India
2Microbiologist, Quality Control Division, ZIM laboratories Ltd. MIDC, Kalmeshwar, Nagpur, Maharashtra, India
3Department of Biochemistry, Centre for Research & Development, PRIST University Thanjavur, Tamilnadu, India

ABSTRACT

Proteases are an important class of enzymes and account for the major industrial market in the world. There are various sources of proteases as they occur naturally in all organisms and constitute 1 to 55% of the gene content depending upon the more of attach they have been broadly classified as exoproteases and endoproteases French beans are commonly grown in India and this fairly rich in protein content as well as minerals. French bean meals thus can be modified suitably for the growth of fungal species. Our study supports the fact that French beans are a moderate type of solid state fermentation substrate for protease production using Aspergillus spp. The aim of present study was Isolation and identification of protease producing Aspergillus spp., solid state fermentation of French bean meal using isolated Aspergillus spp., Quantification of protease. French bean meal fermentation medium as a solid state system reveals encouraging results. Proteases are very important enzymes and in the present study a maximum amount of enzyme activity of 0.0565 ± 0.00282 µm of tyrosine produced per min. in 48hrs. Although in the initial phase up to 48hrs. A steady increase in enzyme activity can be observed but on prolonged incubation the enzyme activity became lowest in 216 hrs. With an intermittent interval of increase could also be seen at around to 288 hrs. Although French bean are not known to be richest source of nitrogen even then they are supporting solid state fermentation of protease production.

Keywords: - PDA, Aspergillus niger, Protease, Solid State Fermentation.

INTRODUCTION

Enzymes are among the most important products obtained for human needs through microbial sources. A large number of industrial processes in the areas of industrial environments and food biotechnology utilize enzyme at same stage or the other solid state fermentation holds tremendous potential for the production of enzymes. It can be of special interest in those
processes where the crude fermented product may be used directly as the enzyme source. [1] Solid state fermentations [SSF] are known to be superior over submerged fermentation [SMF]. Particularly with respect to high volumetric productivity, higher yields lower effluent generation and simpler equipment’s requirements moreover a wide verity of Microbes can be used for SSF. As far as organisms are concerned, fungal species are more suitable for solid state fermentation. Aspergillus niger has been shown to produce 19 types of enzymes as has been reported[2 – 9]. Proteases (Proteinases, Peptidase or Proteolytic enzymes) are enzymes that break peptide bonds between amino acid of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes especially involved in blood coagulation or digestion. They use a molecule of water for this and are thus classified as hydrolases.[10] 

Proteases, which account for about 60% of total enzyme market and among the most valuable commercial enzymes, are the single largest class of enzymes occupying a pivotal position due to their wide application in the industrial processes. Plants, Animals and Microbial sources are employed for protease production. Microbes serve as the preferred source of protease because of their rapid growth, the limited space required for their cultivation, and the case with which they can be genetically manipulated to generate new enzymes with altered properties. Microbial extracellular alkaline proteases are important enzymes and are mainly used in detergents to facilitate the release of proteinaceous stains such as blood, milk, egg and meat. [11, 12] French beans (Phaseolus vulgaris, Var. Prince), is a good source of proteases and they even have been shown to possess carboxy peptidase like activity. Similarly, it has been shown to contain 42000 glycoprotein with specific inducibility. Various proteases have also been reported to have detergent activities. [13, 14, ] Production of proteases have been done by various means including or most all the types of fermentation techniques involving various types of Microbes since the enzymatic action of proteases are pH dependent hence proteases are also classified as acid neutral or alkaline proteases and all of them have been industrially produced. [15 – 34] A number of substrate has been used for the production of proteases by solid state fermentations. Leguminous pods are very suitable for carrying a solid state fermentation. Soya beans, chickpea and horse bean along with jack beans are major leguminous seeds used in solid state fermentation techniques; Kao et al have reported the fermentation of such beans by solid state fermentation. [35] Various types of modifications have been done by solid state fermentation technique. Rotating drum techniques, Continuous tray techniques and Fix tray techniques have been reported in different fermentations. [36, 37]

French beans are commonly grown in India and this fairly rich in protein content as well as minerals. The beans contain all the major minerals a required for the growth of Aspergillus species French bean meals thus can be modified suitably for the growth of fungal species. [38] Study of activity of enzymes discussed earlier but effect of various parameters have also been reported which clearly shows metal ions enhanced the activity of the enzymes.[39]

**MATERIALS AND METHODS**

**Sample collection and isolation of Aspergillus species**

Various vegetables and fruits were collected from the market showing spoilage to the extent that the integuments and the outer skin damaged extensively. The sample was examined macroscopically after staining fungal infected samples were screened and scrapings were inoculated on Potato Dextrose Agar (PDA) and grown at room temperature for 7 days. The PDA plates showing intense mycelia growth with profuse brown to black spores were selected and repeatedly purified over PDA to get axenic cultures. [40]
Characterization and confirmation of isolates
The axenic fungal culture showing brown to black spores were subjected to Microscopic examination by lacto phenol cotton blue staining. A wet mount was prepared on the slide with the culture from the PAD plates the mount was stained with a few drops of cotton blue and covered with a coverslip.[41,42]The typical morphology of Aspergillus niger was observed under microscope for confirming Aspergillus species. [43]

The species of Aspergillus niger confirmed by subjecting it to standard slide culture technique. A thick layer of PDA was laid over the slide aseptically and then they were inoculated with the fungal isolates. The slides were put in sterile Petri plates and incubated at room temperature for development of vertical hyphae, microscopic examination of the slides were done to confirm Aspergillus species. [44]

Solid state fermentation of French bean and estimation of protease activity
Media was developed with some modifications including Dehouled seeds were converted into a semisolid pest using a varying grinder. The resulted pest was mixed with phosphate buffer and mineral solution. Protease was estimated by Folin’s reagent using tyrosine as standard as per the method described in standard methods Enzyme activity was reported as micrograms (µgs) of tyrosine produced.

Examination of spoiled vegetables and fruits
Samples consisting of Onion, Tomato, Lemon, and Jaggery which were extensively damaged were collected and the spoiled parts were scrapped on a clean slide over drop of water. The mount was spread into a smear and stained with lactophenol cotton blue and Gram stained.

Production of protease synthetic French bean meal Fermentation media:
The axenic culture from the PDA plates were transferred to fresh PDA plates and allowed to form spores by incubating the plates at room temperature. The spores were suspended in 10ml buffer mixed and poured into fermentation flask containing synthetic French bean meal fermentation medium. Flasks were grown at room temperature at static conditions for a prolonged period of 360 hrs. The sample were intermittently collected in an aliquot of 10ml and centrifuged at 5000 rpm for 10min. The supernatant was collected and considered as crude enzyme it will also suitably diluted and referred to as diluted enzyme. The amount of enzyme activity was determinant as further procedure described in methodology. [45]

Production of protease by altered synthetic Fermentation media
Fermentation medium condition was altered by addition of sodium chloride as described in methodology. The Aspergillus isolate was grown on PDA as described earlier and 10 ml suspension of the spores were inoculated into modified synthetic fermentation media. The flasks were incubated at room temperature for 72hrs. The fermented matter was centrifuged, as described earlier, and the supernatant was used for enzyme estimation. The 72hrs. Production of enzyme in synthetic fermentation medium was compared.

Variation in Fermentation condition
The composition of the fermentation media was altered by incorporating sodium chloride in the medium with the supplementing NaCl at 2.5 gm. / 250 ml media, other growth condition were maintain identical enzymes was estimated.
RESULTS AND DISCUSSION

Table 1: Microscopic Examination of Spoiled sample

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Vegetable / fruit</th>
<th>Nature of organism seen.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Onion</td>
<td>Multipolar budding yeast, Mycelium spores.</td>
</tr>
<tr>
<td>2.</td>
<td>Lemon</td>
<td>Gram Negative Bacilli</td>
</tr>
<tr>
<td>3.</td>
<td>Tomato</td>
<td>Gram Negative Bacilli</td>
</tr>
<tr>
<td>4.</td>
<td>Jaggery</td>
<td>Yeast cells</td>
</tr>
</tbody>
</table>

Table 2: Morphological characters of axenic culture

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Staining</th>
<th>Observation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cotton blue staining on plate</td>
<td>Brown to black conidiospores arranged in typical Aspergillus pattern hyphae accepted conidiospores short</td>
</tr>
<tr>
<td>2.</td>
<td>Cotton blue staining on slide</td>
<td>Brown to black conidiospores arranged in typical Aspergillus pattern hyphae accepted conidiospores short</td>
</tr>
</tbody>
</table>

Table 3: Production of protease using French bean fermentation media

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Duration</th>
<th>µg / ml</th>
<th>µm /ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>24 hrs</td>
<td>62 ± 12.72</td>
<td>0.03434 ± 0.0067</td>
</tr>
<tr>
<td>2.</td>
<td>48 hrs</td>
<td>102.385 ± 5.12</td>
<td>0.0565 ± 0.0028</td>
</tr>
<tr>
<td>3.</td>
<td>72 hrs</td>
<td>74.195 ± 42.70</td>
<td>0.0407 ± 0.023</td>
</tr>
<tr>
<td>4.</td>
<td>144 hrs</td>
<td>63.005 ± 48.09</td>
<td>0.0347 ± 0.083</td>
</tr>
<tr>
<td>5.</td>
<td>216 hrs</td>
<td>49 ± 21.21</td>
<td>0.0270 ± 0.011</td>
</tr>
<tr>
<td>6.</td>
<td>288 hrs</td>
<td>88.82 ± 78.94</td>
<td>0.049 ± 0.043</td>
</tr>
<tr>
<td>7.</td>
<td>360 hrs</td>
<td>57.225 ± 14.46</td>
<td>0.0341 ± 0.0115</td>
</tr>
</tbody>
</table>

Table 4: Production of protease using French bean by altered Fermentation media.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Duration</th>
<th>µg /ml</th>
<th>µm /ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>72 hrs</td>
<td>62 ± 9.89</td>
<td>0.0342 ± 0.0054</td>
</tr>
</tbody>
</table>

Graph 1: shows the production of proteases with respect to time
Comparision of Protease Production at Different fermentation composition

Graph 2: Shows the comparison of protease production at different composition

Table 5: Composition of tyrosine produced at different fermentation composition.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Duration</th>
<th>µg /ml</th>
<th>µm /ml/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>72hrs fermentation composition</td>
<td>74.195</td>
<td>0.0407</td>
</tr>
<tr>
<td>2.</td>
<td>Fermentation composition with NaCl</td>
<td>62 + 9.89</td>
<td>0.0342 + 0.0054</td>
</tr>
</tbody>
</table>

The present project related to fungal protease production by Aspergillus species using synthetic French bean meal fermentation medium as a solid state system reveals encouraging results.

Proteases are very important enzymes and in the present study a maximum amount of enzyme activity of $0.0565 \pm 0.00282$ µm of tyrosine produced per min. in 48hrs. Although in the initial phase up to 48hrs. A steady increase in enzyme activity can be observed but on prolonged incubation the enzyme activity became lowest in 216 hrs. With an intermittent interval of increase could also be seen at around to 288 hrs. as can be seen from graph 1.

An important aspect which is discussed recently that glucose can be used as a carbon source, which can regulate the protease production along with substrates like soya bean meal. This may open door for further research in the field of fermentation technology.[46]

NaCl addition has been shown to decrease protease activity in the modified synthetic French bean meal fermentation medium. The excessive NaCl may be interfering with the protease release and its concomitant coagulation by salting out. Although French bean is not known to be richest source of nitrogen even then they are supporting solid state fermentation of protease production. It is also reported that molasses and cheese whey found to be useful substrate for protease production in submerge fermentation by Aspergillus spp.[47]

CONCLUSION

It is clear the fact that French beans are a moderate type of solid state fermentation substrate for protease production using Aspergillus species. The outcomes of the present study includes French bean meal media is a fermentative system and can be used for solid state fermentation, Maximum yield is obtained at 48hrs of incubation, NaCl modification of the medium decrease protease production and modification of fermentation may lead to better yield of the enzyme.
REFERENCES


