Isolation and screening of different isolates of Aspergillus for amylases production
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Citation

Abstract
The diversity of Aspergillus from soil of Kusmi Forest, an Indian subtropical Teak forest at Gorakhpur resulted in characterizing a total of 415 isolates which were then subjected to amylases production. The preliminary amylases production revealed a total of 14 promising isolates of Aspergillus comprising of ten different strains of A. flavus, two strains of A. tamarii, one strain each of A. niger and A. awamori which are characterized further. The optimum temperature of amylases produced from 14 isolates ranged from 30 to 60 °C while thermal stability observed was in the range of 50 to 80 °C for 30 minutes. The pH optima ranged from 5.0 to 8.6 and the Km values calculated ranged from 0.60 to 2.85 mg/ml.

INTRODUCTION
Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, Aspergilli are particularly interesting due to their easy cultivation, and high production of extracellular enzymes with potential industrial exploitation. These enzymes are applied in various industries like detergents, starch, drinks, food, textile, animal feed, baking, pulp and paper, leather, chemical and biomedical products. The use of starch degrading enzymes was in fact the first large-scale application of microbial enzymes in the food industry (Bennett 1998; Deker 2003; Lin et al., 1997; Pandey et al., 2000). Amylases constitute a class of industrial enzyme having approximately 25% share of the enzyme market (Sindhu et al. 1997; Rao et al., 1998). The microbial amylases meet industrial demands with a large number of them available commercially and have almost completely replaced chemical hydrolysis of starch in starch processing industry (Pandey et al., 2000 and James and Lee, 1997). The major advantage of using microorganisms for production of amylases is the ability to produce in bulk and ease at which it can be manipulated for desired products (Lonsane and Ramesh, 1990). Amylase production has been reported from several fungi, yeasts, bacteria and actinomycetes though fungal and bacterial sources are predominant with potential industrial applications (Pandey et al., 2002). Among fungi the Aspergilli are the important producers of enzymes (Haska and Ohta, 1994; Pandey et al., 2000). In the present study an attempt has been made to screen the indigenously isolated Aspergillus from teak dominated forest soil for amylase production.

MATERIALS AND METHODS
MICROORGANISM
The indigenously isolated different isolates of Aspergillus isolated from Kusmi Forest (Ramgarh forest range, 26o45’N and 83o24’20”E INDIA), a teak dominating forest by plating method. The isolate was deposited in National Institute of Interdisciplinary Science and Technology (NIIST), Thiruvananthpuram, India with assigned accession number NIICC-08162 (Table -1).

CHEMICALS
All analytical reagents and media components were purchased from Hi-Media (Mumbai, India), Merk BDH (Germany) and SISCO Research Laboratories Pvt. Ltd, (Mumbai, India).

GROWTH MEDIA
For isolation of Aspergilli, Potato Dextrose Agar and Czapek’s Agar was used (Raper and Fennell,1965).

MEDIA FOR FUNGAL GROWTH AND SPORE PRODUCTION
Media used for fungal growth and spore production was standard Czapek’s agar containing 3.0 g sodium nitrate, 1.0 g dipotassium hydrogen phosphate, 0.5 g potassium chloride, 0.5 g magnesium sulfate, 0.001 g ferrous sulfate, 30.0 g sucrose and 15.0 g agar dissolved in 1.0 liter double
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distilled water, warmed and autoclaved after maintaining pH at 6.5 (Raper and Fennell, 1965).

SCREENING FOR AMYLASE PRODUCTION

Preliminary screening for amylase production from Aspergillus isolates were carried out by starch agar plate assay on standard media (Abe, et al., 1988; Akpan et al., 1999 and Fogarty, 1983) with minor modification. The final constituents of media was 1.5 g yeast extract, 2.0 g soluble starch, 0.5 g peptone, 1.5 g NaCl and 15.0 g agar dissolved in 1.0 liter double distilled water, warmed and autoclaved. After 72 hours of incubation, the inoculated plates containing media supplemented with starch were stained with Gram's iodine reagent. Plates were flooded by Iodine solution for 15 minute and washed with spring warm water to remove the excess color and subsequently photographed using Olympus digital micrographic camera model C-5050, Japan.

The amylase from different isolates were produced under submerged culture conditions using standard media comprising of Czapek solution with yeast extract, starch, peptone and thyme HCl. The final composition of the used media was 0.1 g yeast extract, 0.3 g peptone, 3.0 g NaNO₃, 1.0 g KH₂PO₄, 0.5 g MgSO₄, 0.01 g FeSO₄, 10.0 g soluble starch and 0.001 g Thymine HCl dissolved in 1.0 liter warmed double distilled water. The prepared media was aliquoted as 50 ml dispensed in 150 ml conical flasks prior to sterilization by autoclaving. The spore suspension with final concentration of 10⁶ spore/ml was inoculated aseptically in triplicate sets of aliquoted and sterilized media. After 72 hrs of incubation at 300C under stagnant condition, the production of amylase was assayed by DNSA method (Miller, 1959).

ENZYME ASSAY

Amylase assay was made by using a reaction mixture (1 ml of 2% starch solution mixed with 1.0 ml of 50 mM buffer, pH 6.5) and 0.1 ml of crude enzyme, incubated for 15 min. at 300C. After incubation 2 ml DNA reagents was added and boiled for 10 min. and finally 1.0 ml of chilled 40% K-Na tartrate was added. The resulting colour due to reaction of DNSA and reducing sugar was measured at 540 nm wavelength (Miller 1959). One enzyme unit is equivalent to release of 1.0 μM maltose per unit time per unit volume.

ENZYME KINETICS

The amylase activity subjected to temperature ranging from 20 -100°C was monitored by standard enzyme assay while thermostability of the enzyme was determined by incubating the crude enzyme preparation at temperatures ranging from 40-100°C for 30 minute in a constant-temperature water bath (Lin, et al., 1998; MacKenzie et al., 2000 and Pedersen et al., 1999). The residual enzyme activities were then assayed after each treatment. Optimum pH for amylase activity was determined by subjecting the crude enzyme preparation to three different types of buffers namely citrate buffer (pH range 2.5), Phosphate buffer (pH range 6-9) and Glycine NaOH (pH range 10-12). The assay conditions were similar except variability of pH from 2 to 11 as mentioned above (Ellaiah et al., 2002; Gigras et al., 2002). The Km value was calculated by Michaelis- Menten and double reciprocal graph (Aguilar et al., 2000 and Fogarty, 1983).

RESULTS

A total of 415 isolates of Aspergillus were characterized based on cultural as well as morphological characteristics (Raper and Fennel, 1965) from soil of Kusmi forest with dominated teak vegetation. All the isolates were screened for production of amylase by starch plate method resulting in clear zone of starch hydrolysis in Petridishes after KI/I2 treatment. The preliminary amylase production analysis of all the isolates revealed a total of 119 isolates to be producer of amylases under submerged culture conditions. Out of 119 isolates 14 producing isolates were selected for further study (Figure 1). These strains were deposited in Microbial Type Culture Collection, (MTCC) and Gene Bank, Chandigarh, India and National Institute of Interdisciplinary Science and Technology (NIIST), Thiruvananthapuram, India.

Figure 1

The ten strains of Aspergillus flavus namely, NIICC-08143, NIICC-08145, NIICC-08149, NIICC-08150, NIICC-08151, NIICC-08157, NIICC-08160, MTCC-8835, MTCC-8836, MTCC-8838, two strains of Aspergillus tamarii NIICC-08152 and MTCC-8841 and one of each Aspergillus niger NIICC-08153 and Aspergillus awamori NICC-08161
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were found to be promising producer among all. The production of amylase under submerged culture condition is expressed in terms of IU/ml (Table 1). Based on their production status for amylase secretion, further characterization of enzymatic properties like optimum pH, temperature, Km was carried out (Table 2).

Table 1. Production chart of Amylases, Secreted by selected indigenous isolated Aspergillus strains.

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Designated Strain No.</th>
<th>Aspergillus identified (Assigned accession number)</th>
<th>Enzyme Production IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AF 005</td>
<td><em>A. flavus</em> (NIICC-08143)</td>
<td>211.6</td>
</tr>
<tr>
<td>2</td>
<td>AF 009</td>
<td><em>A. flavus</em> (NIICC-08145)</td>
<td>198.5</td>
</tr>
<tr>
<td>3</td>
<td>AF 019</td>
<td><em>A. flavus</em> (NIICC-08149)</td>
<td>189.5</td>
</tr>
<tr>
<td>4</td>
<td>AF 020</td>
<td><em>A. flavus</em> (MTCC 8835)</td>
<td>202.4</td>
</tr>
<tr>
<td>5</td>
<td>AF 024</td>
<td><em>A. flavus</em> (NIICC-08150)</td>
<td>186.0</td>
</tr>
<tr>
<td>6</td>
<td>AF 025</td>
<td><em>A. flavus</em> (MTCC 8836)</td>
<td>214.5</td>
</tr>
<tr>
<td>7</td>
<td>AF 032</td>
<td><em>A. flavus</em> (NIICC-08151)</td>
<td>221.5</td>
</tr>
<tr>
<td>8</td>
<td>AF 074</td>
<td><em>A. tamarii</em> (NIICC-08152)</td>
<td>208.6</td>
</tr>
<tr>
<td>9</td>
<td>AF 080</td>
<td><em>A. niger</em> (NIICC-08153)</td>
<td>213.2</td>
</tr>
<tr>
<td>10</td>
<td>AF 081</td>
<td><em>A. flavus</em> (MTCC 8838)</td>
<td>198.5</td>
</tr>
<tr>
<td>11</td>
<td>AF 170</td>
<td><em>A. tamarii</em> (NIICC-08151)</td>
<td>199.5</td>
</tr>
<tr>
<td>12</td>
<td>AF 220</td>
<td><em>A. flavus</em> (NIICC-08157)</td>
<td>206.5</td>
</tr>
<tr>
<td>13</td>
<td>AF 325</td>
<td><em>A. awamori</em> (NIICC-08161)</td>
<td>195.5</td>
</tr>
<tr>
<td>14</td>
<td>AF 361</td>
<td><em>A. awamori</em> (NIICC-08161)</td>
<td>193.3</td>
</tr>
</tbody>
</table>

The amylases from different isolates were characterized for their basic properties and are shown in figure 2 to 16 for *A. flavus* (NIICC-08143), *A. flavus* (NIICC-08145), *A. flavus* (NIICC-08149), *A. flavus* (MTCC 8835), *A. flavus* (NIICC-08150), *A. flavus* (MTCC 8836), *A. flavus* (NIICC-08151), *A. tamarii* (NIICC-08152), *A. niger* (NIICC-08153), *A. flavus* (MTCC 8838), *A. tamarii* (MTCC 8841), *A. flavus* (NIICC-08157), *A. flavus* (NIICC-08160) and *A. awamori* (NIICC-08161). The amylase secreted by different isolates shows their pH optima in the range of 5.0 to 8.6 (Table 2). The secreted amylases from *A. niger* (NIICC-08153), *A. flavus* (NIICC-08160), and *A. awamori* (NIICC-08161) have pH optima 5.0 while pH optima of amylase secreted by *A. flavus* (NIICC-08145), *A. tamarii* (MTCC 8841) and *A. flavus* (MTCC 8838) were 5.4, 5.6 and 5.8 respectively. The pH optima was found 6.2 for amylase secreted by *A. flavus* (MTCC 8835) *A. flavus* (NIICC-08151) and *A. tamarii* (NIICC-08152). The amylase secreted by *A. flavus* (NIICC-08143) *A. flavus* (NIICC-08150) and *A. flavus* (NIICC-08157) shows optimum activity at pH 8.2 while Amylase from *A. flavus* (MTCC 8836) and *A. flavus* (NIICC-08149) shown optimum pH at 7.2 and 8.6 respectively. The amylase secreted by different isolates shown the optimum pH activity and range of pH on which they are active also supported by previous reports. The amylases secreted from above strains may be useful for enhanced starch hydrolysis and alcohol production (Hattori, 1984). Amylases from *A. flavus* (NIICC-08143), *A. flavus* (NIICC-08149), *A. flavus* (NIICC-08150) and *A. flavus* (NIICC-08157) shows pH optima in alkaline range and also active at higher pH (Snadhya et al., 2005).
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Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

Figure 10
Isolation and screening of different isolates of Aspergillus for amylases production

Figure 11

Figure 12

Figure 13

Figure 14

Figure 15

Figure 16
The temperature optima of amylases produced by 14 isolate ranged from 30 to 60 while thermal tolerance of 50 to 80 was observed (Table 2). The amylase from A. flavus (NIICC-08145), A. flavus (NIICC-08149), A. flavus (MTCC 8838) and A. tamarii (MTCC 8841) has temperature optima 30 oC while amylase from A. tamarii (NIICC-08152) and A. niger (NIICC-08153) shows optimum temperature for maximum activity at 35 oC. During the same study the isolates A. flavus (MTCC 8835), A. flavus (NIICC-08157), A. flavus (NIICC-08160) and A. awamori (NIICC-08161) produces the amylase having maximum enzyme activity at 40 oC while amylases from A. flavus (MTCC 8836) and A. flavus (NIICC-08151) shows maximum activity at 45 oC. Eight isolates namely A. flavus (NIICC-08143), A. flavus (NIICC-08149), A. flavus (MTCC 8835), A. tamarii (NIICC-08152), A. niger (NIICC-08153), A. flavus (MTCC 8838), A. flavus (NIICC-08160) and A. awamori (NIICC-08161) having thermal stability up to 60 oC while A. flavus (NIICC-08145) and A. tamarii (MTCC 8841) secretes amylase with stable up to 50 oC. Four isolates of Aspergillus flavus were found stable at high temperature, among them two namely A. flavus (MTCC 8836) and A. flavus (NIICC-08151) were produced amylase with considerable activity up to 70 oC while amylase secreted by A. flavus (NIICC-08150) and A. flavus (NIICC-08157) was stable up to 80 oC.

The Km values determined for amylase produced by all 14 isolates ranged from 0.6 to 2.85 mg/ml (Table 2) which is similar to what has been reported in literature (Kekos and Macris, 1983 and Kundu and Das 1970).

**DISCUSSION**

Though the conditions of selected site for study is favorable for the growth of fungi and better niche for moderate mesophillic fungi but the results with screening of Amylases production shows the variability and potentiality among isolated Aspergilli. The basic properties of amylases may further exploit in various starch based industries with bests of possibilities. The isolation of amylolytic Aspergillus from soil is reported earlier by various workers (Adebiyi and Akinyanju 1998, Gupta et al., 2008, Abe et al., 1988 ). Aspergillus requires no flooding, no prior replication of colonies on slants (Akpan et al., 1999). Different properties of Amylases secreted by isolates shows their probable applicability in different industries. As it is important to evaluate the enzymatic properties of enzyme for further exploitation in different industries. The results shows the pH optima among A. flavus strains ranges from 5.0 to 8.6 which suggests their wide applicability from acidic to alkaline.
These amylases may serve better in detergent and textile industries. Rest of amylases may useful for bakery industry (Aiyer 2005). Production of anomalously linked oligosaccharides mixture; Removal of starch sizer from textile; Treatment of starch processing waste water and Manufacturing of maltose, high fructose containing syrups, oligosaccharides mixture, maltotetraose syrup, and high molecular weight branched dextrins (Aiyer 2005).

The results show that all Aspergilli isolates possessed a high potential for Amylase production. Though the production of these enzymes has been improved significantly by the utilization of hyper-producing strains of fungi as well, efforts are still being done to find newer sources of enzymes. In conclusion, 415 isolates of Aspergilli were obtained from soil of Kusmi Forest, an Indian subtropical Teak forest at Gorakhpur. Among these Aspergilli, 14 exhibited enzymatic potential for industrial uses. Remarkable production was verified for Aspergillus flavus (Strains NIICC-08143, NIICC-08145, NIICC-08149, NIICC-08150, NIICC-08151, NIICC-08157, NIICC-08160, MTCC-8835, MTCC-8836, MTCC-8838), Aspergillus tamarii (NIICC-08152 and MTCC-8841) and Aspergillus niger (NIICC-08153) also were excellent producers of the enzymes studied. Some studies have already been carried out with fungi from our collection, but we consider that these new isolates may have more potential for industrial uses.

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