Screening of some *Zygomycetes* for cellulase activity

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This study was aimed to screen the cellulytic ability of some genera of *Zygomycetes* under laboratory conditions. Carboxy methyl cellulose (CMC) and Wheat straw (WS) were used as the only carbon source in a minimal culture medium. Four days after inoculation, released proteins and sugars were assayed with related reagents and repeated each 3 days up to the 31\(^{\text{st}}\) day. Statistical analysis showed significant variation in released sugars but no significant variation in released proteins among tested genera. *Mucor hiemalis f. corticola* had highest and *Mucor circinelloides f. circinelloides* had lowest sugar levels. Glucose levels for *M. hiemalis f. corticola* increased until 16 days after inoculation, then decreased until 25\(^{\text{th}}\) day, but had no variation until 30\(^{\text{th}}\) day. These results showed that isolates belonging to the same forms had no significant difference in cellulase activity, but the ability of different genera and species were noticeable. This study also showed that WS medium can be effectively used for cellulase production by fungi.

**Key words:** Cellulase activity, carboxy methyl cellulose, wheat straw, protein assay, sugar assay.

**INTRODUCTION**

Plant cell walls are the most abundant renewable source of fermentable sugars on earth (Himmel et al., 1999; Saleem et al., 2008) and are the major reservoir of fixed carbon in nature (Yang et al., 2007). The major components of plant cell walls are cellulose, hemicellulose and lignin, with cellulose being the most abundant component (Han et al., 2003). Cellulose consists mainly of long polymers of β 1-4, linked glucose units and forms a crystalline structure (Shallom and Shoham, 2003). Cellulase enzymes, which can hydrolyze cellulose forming glucose and other commodity chemicals, can be divided into three types: endoglucanase (endo-1, 4-β-D-glucanase, EG, EC 3.2.1.4); cellobiohydrolase or exoglucanase (exo-1, 4-β-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1, 4-β- D-glucosidase, BG, EC 3.2.1.21) (Li et al., 2006; Gao et al., 2008). Cellulases are important industrial enzymes and find applications in several industrial processes (Hanif et al., 2004).

Researchers have strong interests in cellulases because of their applications in industries for starch processing, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry (Gao et al., 2008; Zhou et al., 2008). One of the potential applications of cellulases is the production of fuel ethanol from lignocellulosic biomass (Duff and Murray, 1996), which is a good substitute for gasoline in internal combustion engines. The most promising technology for the conversion of the lignocellulosic biomass to ethanol biofuel is based on the enzymatic breakdown of cellulose using cellulase enzymes (Holker et al., 2004; Ahamed and Vermette, 2008). Many fungal strains secrete higher amounts of cellulases than bacterial ones, with *Trichoderma* as the leading one (Amouri and Gargouri, 2006). Most commercial cellulases are mesophilic enzymes produced by the filamentous fungus *Trichoderma reesei* and *Aspergillus niger*. This process reflects well the fact that filamentous fungi are naturally excellent protein secretors and can produce industrial enzymes in feasible amounts (Bergquist et al., 2002). Considering the importance and application of the cellulases, this study was aimed to screen the indigenous fungal isolates of...
some genera of zygomycetes for cellulase activity in laboratory condition.

MATERIALS AND METHODS

Mycological isolates and maintenance

Sixteen isolates of Zygomyceetes belonging to Mucor circinelloides f. circinelloides, Mucor circinelloides f. griseocyanus, Mucor circinelloides f. janssenii, Mucor circinelloides f. lusitanicus, Mucor hiemalis f. corticola, Mucor hiemalis f. luteus, Mucor hiemalis f. silvicatus, Mucor genevensis, Mucor strictus, Mucor plumbeus, Cunninghamamella echinulata, Rhizomucor pusillus and Rhizopus stolonifer which were isolated from cultural soils of different regions of Iran were selected for the experiments. The isolates were grown on PDA slants and stored at 4°C.

Culture medium

Wheat straw (WS) medium containing 1 g wheat straw that were cut to 1 cm pieces per 50 ml distilled water and carboxy methyl cellulose (CMC) medium containing 0.05 g FeSO₄ 7H₂O, 0.25 g MnSO₄ H₂O, 0.25 g CoCl₂, 0.25 g ZnSO₄, 0.25 g (NH₄)₂SO₄, 2 g KH₂PO₄, 0.25 g MgSO₄ 7 H₂O, 0.4 g CaCl₂, 0.3 g urea, 0.2 ml Tween 80 and 10 g carboxy methyl cellulose per liter were prepared for cellulose degradation experiments. 50 ml of broth were distributed in 250 ml erlenmeyers and then both media were autoclaved at 120°C for 20 min.

Inoculation and sampling

Each flask was inoculated with 1 ml spore suspension in three replicates for each species. The flasks were treated at 25°C for 31 days. Sampling was started four days after inoculation and repeated every two days for protein and sugar assays.

Protein and sugar

Five hundred µl of broth medium in each clean test tube were subjected to protein and released sugars assays. Released fungal extracellular proteins and produced sugars concentrations were determined using Bradford method and Arsenate-Molybdate reagent, respectively (Bradford, 1976; Kossem and Nannipieri, 1995).

Statistical analysis

The results obtained in the present study were analysed by SAS (v. 9.1) software.

RESULTS

The growth process started at least 12 h after inoculation. Table 1 shows the significant differences for measurements of sugar assays. No statistical variations were detectable between released proteins of different isolates. No significant differences for protein and sugar assays were observed between both CMC and WS media.

Sugar assay

Released extracellular enzymes of different species caused some increase and decreases in sugar levels produced from cellulose degradation. M. hiemalis f. corticola showed the highest and M. circinelloides f. circinelloides and R. pusillus showed the lowest potential of glucose production (Table 2). M. hiemalis f. corticola showed the highest released sugar content in the CMC medium and M. plumbeus and M. circinelloides f. lusitanicus showed the highest released sugar content in the wheat straw medium (Figure 1). Nevertheless, these several superior isolates (M. hiemalis f. corticola, M. plumbeus and M. circinelloides f. lusitanicus) produced the highest amount of released sugar in 12, 9 and 18 days after inoculation, respectively, however, the trend dropped down until days 15th and 21st (Figure 2). There was a significant difference in the released sugar content between different genera and species in this study but no significant statistical variation in sugar production was observed (Table 1).

Protein assay

Protein assays during the experiments showed some gradual changes in released proteins concentration. According to Tables 1 and 2, there were no significant statistical variations between different isolates also between CMC and wheat straw media in released protein content. Levels of released protein of tested isolates in CMC and wheat straw are shown in Figure 3. M. hiemalis f. corticola , M. plumbeus and M. circinelloides f. lusitanicus which showed the highest amount of released sugar content, had the highest levels of released protein in 12

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Table 1. Analysis of variance of protein and sugar assay on CMC and wheat straw media.

<table>
<thead>
<tr>
<th>Source variations</th>
<th>DF</th>
<th>Protein assay (mg/l)</th>
<th>Sugar assay (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate</td>
<td>15</td>
<td>0.000223**</td>
<td>0.001917**</td>
</tr>
<tr>
<td>Culture medium</td>
<td>1</td>
<td>0.000776**</td>
<td>0.000195**</td>
</tr>
<tr>
<td>Isolate* Culture medium</td>
<td>15</td>
<td>0.000287**</td>
<td>0.001617**</td>
</tr>
<tr>
<td>Error total</td>
<td>926</td>
<td>0.000287</td>
<td>0.0001834</td>
</tr>
</tbody>
</table>

** = Significant at 1% probability; ns = non-significant at 5% probability.
Table 2. Means comparison of sugar and protein assay in different tested isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Protein assay (mg/l)</th>
<th>Sugar assay (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucor hiemalis f. corticola</td>
<td>0.021a</td>
<td>0.0329a</td>
</tr>
<tr>
<td>M. hiemalis f. corticola</td>
<td>0.02a</td>
<td>0.0323a</td>
</tr>
<tr>
<td>M. plumbeus</td>
<td>0.0171a</td>
<td>0.0237b</td>
</tr>
<tr>
<td>M. circinelloides f. lusitanicus</td>
<td>0.0148a</td>
<td>0.0234b</td>
</tr>
<tr>
<td>Cunninghamella echinulata</td>
<td>0.0137a</td>
<td>0.0232b</td>
</tr>
<tr>
<td>M. circinelloides f. janssenii</td>
<td>0.0225a</td>
<td>0.0230b</td>
</tr>
<tr>
<td>M. circinelloides f. griseocyanus</td>
<td>0.0164a</td>
<td>0.0227bc</td>
</tr>
<tr>
<td>Rhizopus stolonifer</td>
<td>0.0169a</td>
<td>0.0216bc</td>
</tr>
<tr>
<td>M. genevensis</td>
<td>0.0192a</td>
<td>0.0207bc</td>
</tr>
<tr>
<td>C. echinulata</td>
<td>0.0132a</td>
<td>0.0202bc</td>
</tr>
<tr>
<td>M. hiemalis f. luteus</td>
<td>0.0176a</td>
<td>0.0201bc</td>
</tr>
<tr>
<td>M. strictus</td>
<td>0.0160a</td>
<td>0.0184bc</td>
</tr>
<tr>
<td>M. hiemalis f. silvaticus</td>
<td>0.0202a</td>
<td>0.0173cd</td>
</tr>
<tr>
<td>Rhizomucor pusillus</td>
<td>0.0217a</td>
<td>0.0145de</td>
</tr>
<tr>
<td>M. circinelloides f. circinelloides</td>
<td>0.0179a</td>
<td>0.0137de</td>
</tr>
<tr>
<td>M. circinelloides f. circinelloides</td>
<td>0.0178a</td>
<td>0.0125e</td>
</tr>
</tbody>
</table>

Means with the same letter are not significantly different (p = 0.01).

Figure 1. Variations of released sugar in different isolates in CMC and wheat straw media.

and 15 days after inoculation, respectively. However, these levels gradually decreased by day 18th (Figure 4).

DISCUSSION

Cellulase complex enzymes have a series of industrial applications that increase their commercial importance. The fungi have been described as the best sources of cellulase extraction. Different fungi belonging to Zygo-
mycetes, Ascomycetes, Basidiomycetes and Deutromycetes can degrade cellulose and hemicelluloses (Schulein, 2000). Screening of different genera, species and even isolates is the first step for finding acceptable enzyme producer isolates. Macris (1983) in a survey on some genera like Trichoderma, Fusarium, Aspergillus, Phan-
erochaete, Chrysosporium and Sclerotium showed some differences in their cellulase activity. Sazci et al. (1986) in surveys on cellulase activity of different genera reported that Trichoderma harzianum and A. niger showed highest
Variations of released sugar in three isolates with highest sugar production in different days after inoculation.

Variations of released protein in different isolates in CMC and wheat straw media.

and *Trichotheccium roseum*, *T. reesei*, *Aspergillus ochraceus* and *Penicillium italicum* exhibited lower activity against CMC. In another research, Jahangeer et al. (2005) on the measurement of cellulase activity among 115 isolated fungal strains showed that *Trichoderma*, *Aspergillus* and *Fusarium* strains had the highest activity. Oyeleke et al. (2008) reported cellulose degradation ability in *Mucor* species. Endoglucanase production were reported by Boyce and Walsh (2007) for *Rhizomucor mehei* and Moria et al. (2004) in *Rhizopus oryzae*. Results of Yeoh et al. (1984) showed that Cunninhamella sp. did not show any cellulolytic activity when cultured on medium containing cellulose.

The results showed that there were detectable significant statistical variations in released sugar between tested genera and species but no significant difference between
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Figure 4. Variations of released protein in three isolates with highest sugar production in different days after inoculation.

*M. circinelloides* and *M. hiemalis* was shown. Results on protein assay showed that there was no significant difference in released protein between tested genera and species. Results of sugar assays had significant difference together but there was no significant difference between results of protein assays. Consequently, cellulase production of the isolates was similar but the type and amount of enzyme was different. According to the results, among treated isolates, *M. hiemalis* f. *corticola* had the highest released sugars and *M. circinelloides* f. *circinelloides* and *R. pusillus* showed the lowest cellulase activity. A relatively high protein production took place after CMC inoculation, probably because of the initial fungal growth (Jahangeer et al., 2005). Decrease in protein levels after initial growth might be due to the feedback process of CMC degradation and some protease secretion that reduced the amount of released sugars (Rad et al., 2005; Yeoh et al., 1984). Finally, different *Zygomycetes* had unsimilar behavior in cellulose degradation and their rate was variable. Other sugar consuming microorganisms specially soil bacteria may change in vitro results. Soil microbiota may compete with *Zygomycota* with an increase in cellulase production until the substrate is present. Interaction between soil inhabitant microorganisms is an interesting subject for more researches. In the present study, only one highlighted biochemical process from a few species of *Zygomycetes* were tested using laboratory conditions.

REFERENCES


