Factors Affecting Cellulases and β-Glucosidases Activities of *Fusarium oxysporum* Isolated from Old Document

\textsuperscript{1}Siham A. Ismail \textsuperscript{2}A.F. Sahab and \textsuperscript{3}Sawsan S. Darwish

\textsuperscript{1}Department of Chemistry of Natural and Microbial Products, National Research Centre, Dokki, Cairo, Egypt
\textsuperscript{2}Department of Plant Pathology, National Research Centre, Dokki, Cairo, Egypt
\textsuperscript{3}Department of Conservation, Faculty of Archeology, Cairo University, Cairo, Egypt

**Abstract:** A strain of *F. oxysporum* isolated from old manuscripts was tested for its ability to produce cellulases and β-glucosidases when growing in a range of soluble and insoluble carbon sources under static and shaking conditions. Except for β-glucosidases, the best conditions for enzyme production and fungal growth of *F. oxysporum* were found to be at 30°C and pH 5. The optimum activity of endoglucanase and β-glucosidase was found to be at pH 4.5-4.8 and 60°C. Both enzymes possessed high stability at pH range 4-6 and at temperature range 40-60°C. Generally, β-glucosidase have higher stability properties than endoglucanase.

**Key words:** *Fusarium oxysporum* · old documents · cellulases · β-glucosidases · contamination · papyrus · linen paper

**INTRODUCTION**

Cellulose is the most abundant biological compound decomposed by a synergetic action of a multienzyme complex known generally as cellulases, which consists of at least, three enzymes that may vary with microorganisms [fungi, bacteria, actinomycetes] and/or substrate [1-5]. These systems comprise, minimally, endoglucanases [1, 4-β-D-glucon, gluconolactonase, EC 3.2.1.4] which cleave internal 1,4-β-linkage of cellulose; exoglucanases [cellobiohydrolase, 1,4-β-D-glucan cellobiohydrolase, EC3.2.1.91] which cleaves cellobiose unit from the non-reducing end of cellulose chains; or exoglucosidase, [1,4-β-D-glucan glucosidase, EC 3.2.1.74] which cleave glucose unit from cellulose chains and β-glucosidases (1,4-β-D-glucoside glucosidase, EC 3.2.1.21) which hydrolyzes cellobiose to glucose [6].

*Fusarium oxysporum* is considered one of the most potent fungi for the degradation of the cellulolytic materials [7, 8]. This microorganism causes damage when it attacks the old valuable manuscripts and art which composed of different cellulose materials [4, 7, 9-11]. The nature and the types of these microorganisms and the relation of their physiology to environmental conditions (pH, temperature, aeration, humidity, light ... etc) must be evaluated in order to adopt the most suitable methods to prevent and control biodeterioration without producing any negative interference with the constituent materials of the objects to be conserved [10].

The present work was carried out on *F. oxysporum* as one of the most active cellulolytic microorganism isolated from the old manuscripts from the view concerning: a) the environmental and nutritional demands as well as physiological behavior under different cultivation conditions and also some properties of the tested enzymes and b) the role of this fungus in the deterioration of papyrus and linen-papers

**MATERIAL AND METHODS**

**Fungal isolate:** An isolate of fungi isolated from deteriorated old manuscripts, obtained from the stores of General Egyptian Book Organization [4] and identified as *Fusarium oxysporum* in the Plant Pathology Dept. of the National Research Center, Cairo, Egypt, according to Barnett and Hunter [12] and Nelson et al. [13]. The identified strain was maintained on potato dextrose agar slants (FDA) supplemented with 5% avicel.

**Substrates:** Different types of the substrates were used individually in these research including: Avicel, carboxymethyl cellulose (CMC), cellulose powder; sugars,
including cellobiose, sucrose and glucose; pulps of cotton, soft wood and wood; papers of papyrus, linen, pega and filter paper. Papers of journals and 40 g papers of different ages (i.e., new, 25, 50 and 100 years old) were also used as substrates. The used papers were cut into small pieces and were used in liquid culture medium without further pretreatments.

**Growth conditions:** The spore suspension of *F. oxysporum* was transferred to 250 ml Erlenmeyer flask containing 50ml of sterilized [14] medium at pH 5 and incubated in a rotatory shaker (180 rpm) at 30°C for three days before use as inoculum. The culture flasks, which contained 50 ml of the above medium was supplemented with different substrates (0.5 g/flask) and inoculated with 5% inoculum (v/v). Unless otherwise stated the culture flasks in all the experiments were incubated at 30°C for 20 days in a rotatory shaker (180 rpm) for shake culture or placed in an incubator for static cultivation [4].

**Enzymes preparation:** Extracellular enzymes were prepared by filtering the culture through Whatman No.1 filter paper. Intracellular enzymes were obtained by grinding the washed, cold mycelium with sand in a minimum volume of citrate-phosphate buffer (0.05 M, pH 4.8). The mixture was then centrifuged and the supernatant was used as the enzyme solution.

**Enzyme assay:** Avicelase (1, 4-β-D-glucan cellobiohydrolase) was measured according [15] using avicel cellulose as substrate. Endoglucanase (1, 4, 5-β-D-glucanohydrolase) was assayed as carboxymethyl cellulase (CMCase) according to the method of Mandels and Webbers [14]. The resulting reducing sugar, in both case, was measured by, Somogyi reagent [16] using glucose as standard.

Cellobiase (1,4-β-glucosidase) was measured by a modification of the method of Bergham and Pettersson [17] where 0.5 ml of enzyme solution was incubated with 0.5 ml of 0.4% cellobiose in 0.05 M citrate-phosphate buffer at pH 4.8 for 30 min at 50°C. The reaction was stopped by heating the reaction mixture in a boiling water bath for 5 min. The enzyme activity was determined by measuring the concentration of the released glucose using glucose-oxidase kit (Bioanalytical laboratories-Palm City USA). Enzyme assays were performed in duplicate at the condition specified. Filtrate and substrate controls were included in all assays. One enzyme unit was considered as the amount of enzyme necessary to liberate one μ mol of the reducing sugar under assays conditions specified above.

The protein contents of both culture filtrate and intercellular solution were estimated calorimetrically according to the method of Lowry et al. [18] using bovine serum albumin as standard.

**Effect of pH and temperature of culture media on growth and enzyme production:** To predicate the effect of pH and temperature, three separate experiments were done using papyrus and linen-papers in Mandels and Webers medium. In the first experiment the pH of the culture medium was initially adjusted to pH 5. In the second experiment, the pH of the culture media was readjusted daily to the initial pH of 3, 4, 5, 6, 7 and 8. In the third, the cultures of papyrus and linen-papers at their optimum pH for enzyme production were subjected to different temperatures (20, 30 and 40°C) during the incubation periods.

**Enzymes properties:** The effect of the pH (3-7) and the temperature (30-80°C) on the activity and the stability of the tested enzymes were predicted for the cultures of avicel, papyrus and linen papers.

**RESULTS**

**Effect of different substrates and cultural conditions on cellulas and cellobiase production:** The results generally showed that the extracellular enzymes in shaken cultures were higher than that in the static cultures (Table 1). The effect of the culture condition on enzyme production varied from one enzyme to another. For example, the highest activity of avicelase (13.88 IU g⁻¹) was obtained from papyrus paper cultured under static conditions, but the highest activities of CMCase (13.28 IU g⁻¹) and β-glucosidase (70.45 IU g⁻¹) were recorded for the linen and papyrus papers respectively under shaken conditions.

The paper cellulase activity was in the range of 0.001-0.034 IU ml⁻¹ under shaken conditions and 0.001-0.005 IU ml⁻¹ under static conditions. The final pH's were in the range of 4.98-6.93 for shaken cultures and in the range of 6.82-7.19 for static cultures.

**Effect of different temperatures and pH's on growth and enzyme production:** Results in Table 2 indicated that 30°C was the best temperature for the fungal growth and the enzyme production. Further investigation using linen and papyrus-papers indicate that the pH of the culture medium had a great influence on the levels of the produced enzymes and its effect related to the type of the cultures (either shaken or static).
### Table 1: Influence of different substrates on the production of cellulases and cellobiase of *F. oxysporum* under static and shaken conditions

<table>
<thead>
<tr>
<th>Cultivation condition</th>
<th>Static Avicelase (U mL⁻¹)</th>
<th>Static CMC-ase (U g⁻¹)</th>
<th>Static Cellobiase (U mL⁻¹)</th>
<th>Shaking Avicelase (U mL⁻¹)</th>
<th>Shaking CMC-ase (U g⁻¹)</th>
<th>Shaking Cellobiase (U mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicel</td>
<td>0.010</td>
<td>0.16</td>
<td>0.12</td>
<td>0.66</td>
<td>0.06</td>
<td>2.95</td>
</tr>
<tr>
<td>Control J. paper</td>
<td>0.002</td>
<td>N.d.</td>
<td>0.16</td>
<td>0.26</td>
<td>0.06</td>
<td>2.00</td>
</tr>
<tr>
<td>J. paper 25 years</td>
<td>0.001</td>
<td>N.d.</td>
<td>0.15</td>
<td>0.77</td>
<td>0.05</td>
<td>3.37</td>
</tr>
<tr>
<td>J. paper 50 years</td>
<td>0.005</td>
<td>N.d.</td>
<td>0.18</td>
<td>1.11</td>
<td>0.06</td>
<td>2.25</td>
</tr>
<tr>
<td>J. paper 100 years</td>
<td>0.005</td>
<td>N.d.</td>
<td>0.17</td>
<td>2.97</td>
<td>0.07</td>
<td>2.99</td>
</tr>
<tr>
<td>Control 40 g paper</td>
<td>0.006</td>
<td>N.d.</td>
<td>0.11</td>
<td>1.33</td>
<td>0.05</td>
<td>2.02</td>
</tr>
<tr>
<td>40 g paper 25 years</td>
<td>0.003</td>
<td>N.d.</td>
<td>0.12</td>
<td>0.31</td>
<td>0.47</td>
<td>2.55</td>
</tr>
<tr>
<td>40 g paper 50 years</td>
<td>0.000</td>
<td>4.32</td>
<td>0.12</td>
<td>6.69</td>
<td>0.05</td>
<td>10.3</td>
</tr>
<tr>
<td>40 g paper 100 years</td>
<td>0.006</td>
<td>1.45</td>
<td>0.13</td>
<td>4.11</td>
<td>0.05</td>
<td>2.47</td>
</tr>
<tr>
<td>CMC</td>
<td>0.006</td>
<td>0.13</td>
<td>0.12</td>
<td>0.18</td>
<td>0.06</td>
<td>0.97</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>0.010</td>
<td>7.49</td>
<td>0.09</td>
<td>6.69</td>
<td>0.05</td>
<td>10.3</td>
</tr>
<tr>
<td>Linen paper</td>
<td>0.000</td>
<td>0.04</td>
<td>0.08</td>
<td>0.17</td>
<td>0.06</td>
<td>2.05</td>
</tr>
<tr>
<td>Papyrus paper</td>
<td>0.005</td>
<td>13.9</td>
<td>0.19</td>
<td>4.11</td>
<td>0.13</td>
<td>8.24</td>
</tr>
<tr>
<td>Cotton pulp</td>
<td>0.001</td>
<td>0.03</td>
<td>0.10</td>
<td>0.24</td>
<td>0.05</td>
<td>0.69</td>
</tr>
<tr>
<td>Soft wood pulp</td>
<td>0.000</td>
<td>N.d.</td>
<td>0.10</td>
<td>N.d.</td>
<td>0.06</td>
<td>1.23</td>
</tr>
<tr>
<td>Wood pulp</td>
<td>0.003</td>
<td>0.04</td>
<td>0.09</td>
<td>0.13</td>
<td>0.05</td>
<td>1.26</td>
</tr>
<tr>
<td>Pergamitine</td>
<td>0.005</td>
<td>0.04</td>
<td>0.14</td>
<td>0.41</td>
<td>0.05</td>
<td>1.52</td>
</tr>
<tr>
<td>Filter paper</td>
<td>0.000</td>
<td>N.d.</td>
<td>0.09</td>
<td>0.01</td>
<td>0.05</td>
<td>1.11</td>
</tr>
<tr>
<td>Cellobiase</td>
<td>0.000</td>
<td>N.d.</td>
<td>0.10</td>
<td>0.14</td>
<td>0.10</td>
<td>11.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0.004</td>
<td>N.d.</td>
<td>0.09</td>
<td>4.93</td>
<td>0.07</td>
<td>12.3</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.006</td>
<td>0.32</td>
<td>0.11</td>
<td>0.87</td>
<td>0.08</td>
<td>9.65</td>
</tr>
</tbody>
</table>

N.d. = Not detected

### Table 2: Effect of culture temperature on the growth and the production of endoglucanase and β-glucosidase of *F. oxysporum*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Protein content</th>
<th>CMC-ase</th>
<th>β-glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C.F. (mg ml⁻¹)</td>
<td>My. ext. (mg g⁻¹)</td>
<td>C.F. (IU g⁻¹)</td>
</tr>
<tr>
<td>Linen paper</td>
<td></td>
<td>3.75</td>
<td>0.01</td>
<td>1.11</td>
</tr>
<tr>
<td>Static</td>
<td>5.65</td>
<td>0.02</td>
<td>0.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Papyrus paper</td>
<td></td>
<td>18.94</td>
<td>0.53</td>
<td>0.63</td>
</tr>
<tr>
<td>Static</td>
<td>20.00</td>
<td>0.53</td>
<td>0.63</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Fig. 1: Effect of different pH's of the culture of the linen (I) and papyrus (II) papers on the production of endoglucanase (A) and β-glucosidase (B). Extracellular under static condition (IU ml⁻¹), Extracellular under shaking condition (IU ml⁻¹), Intracellular under static condition (IU g⁻¹), Intracellular under shaking condition (IU g⁻¹)

Fig. 2: Effect of different pH's on the activity of (A) endoglucanase and (B) β-glucosidase of the cultures of avicel, papyrus and linen-papers. The reactions were carried out for 30 min at 50°C at the pH's indicated.

Figure 1 indicates that, except for β-glucosidase in papyrus culture, the adjustment of the pH of the culture medium was not necessary to produce high levels of the tested enzymes. The highest activity of β-glucosidase (136.86 U g⁻¹) was recorded for the papyrus paper culture adjusted to pH 7 under shaken conditions. The visual observation and the protein contents indicated feeble growth of the fungus at pH 3 and 8.

Enzymes properties: The effect of the pH and the temperature on the activity and the stability of endoglucanase and β-glucosidase on the cultures of
Fig. 3: Effect of different temperatures on the activity of (A) endoglucanase and (B) β-glucosidase of the cultures of avicel, papyrus and linen-papers. The reactions were carried out at the optimum pH of each enzyme (and substrate) for 30 min at the temperature indicated.

Fig. 4: Effect of different pH's on the stability of (A) endoglucanase and (B) β-glucosidase of the crude cultures of avicel, papyrus and linen-papers. The enzymes were held for 1 hr at the pH's indicated before assay ing at the optimum condition for each one. The activity at zero time incubation was taken as 100%.

Fig. 5: Effect of different temperatures on the stability of (A) endoglucanase and (B) β-glucosidase of the cultures of avicel, papyrus and linen-papers. The enzymes were held for 1 hr at the temperature indicated before assay ing at their optimum conditions. The activity at zero time incubation was taken as 100%.

avicel, linen and papyrus papers are illustrated in Fig. 2-5. The results indicate that there is no significant difference in the optimum pH and temperature of the enzyme activities of all cultures. However there was a little difference in the pH and temperature stability of the tested enzymes from one culture to another. As an example the relative residual activity of β-glucosidase of papyrus culture, at different temperatures, was quite different from that of avicel and linen cultures.
DISCUSSION

The growth of the microorganism as well as the levels of the produced enzymes was dependent on many factors including: the type of the substrate, the culture condition, the pH and the temperature of the culture medium. The results of this work indicate that the isolated strain of *F. arxysporum* was able to utilize a wide range of carbon sources for growth and that the production of cellulolytic enzyme by this strain was substrate dependent as reported previously [3, 5, 19, 20].

Although soluble substrate generally yielded lower level of cellulolytic enzymes, the fungal strain used in this research produced noticeable quantities of the tested enzymes in the soluble medium. Furthermore, glucose, which is recorded by many authors as an inhibitor of β-glucosidase [21-23] did not prevent enzyme production.

The higher values of the tested enzymes in the mycelial extract may be attributed to the solubilization of the enzymes bonded to the cell wall with soluble intracellular fractions [24]. The enzymes may also be adsorbed on the microcrystalline surface of cellulose or papers in the medium [5, 25, 26] which are then desorbed during the extraction process.

The data indicate that not only the difference of the substrate produced different levels of the tested enzymes but that any change in the structure of the substrate (such as with the paper of different age) lead to the variation in the levels of these enzymes. Generally, the new paper exhibited more durability to fungal invasion than the old one. However, the results of this research not always reflect this fact; where the level of enzymes produced on paper cultures was not regularly increase with increasing papers age; this is may be due to the short period of incubation, where fungal growth causing at first in closely connection of paper than the fungal mycelia get to self autolysis. The released enzymes adsorbed to the surface of the insoluble substrate and became responsible for cellulose hydrolysis. Some authors reported that, the crucial factor in the degradation of cellulose by bacteria and fungal cellulases is the binding of these enzymes to the insoluble substrate [27, 28].

Further investigation on the cultures of linen and papyrus papers indicated the great influence of aeration, pH and temperature of the culture medium on the fungal growth and the levels of the produced enzymes. The results indicated that, except for β-glucosidase of papyrus paper culture, the adjustment of the pH of the culture medium was not necessary to achieve higher levels of the tested enzymes. The data revealed that the growth in different culture conditions (aeration, temp., pH, or different substrate) lead to the variation in the distribution of the tested enzymes between extra and intracellular fractions. The optimum pH for cellulases synthesis was recorded to be 4.5-5 [19, 29]. The irregularity of the produced enzymes with the changing of the pH may be attributed to the multiplicity of the enzymes produced [19, 30].

The temperature of the culture medium also have a great effect on fungal growth and on enzymes production as indicated in Table 2 which illustrates that 30°C is the suitable temperature for growth and enzyme production. This is in agreement with that is reported by other researchers [31-33]. The difference in the distribution of tested enzymes between extra-and intra-cellular fraction, in the pH and temperature experiments, may be due to the autolysis of the cells [24].

The optimum pH and temperature of the activity of the tested enzymes were within the range that has been recorded by other researchers [19, 23, 30]. The differences in the value of these properties from one culture to another may be due to the microsite differences around the tested enzymes. The different substrates may also produce enzymes with different properties. Further research is needed to prove the last point.

REFERENCES


