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Enzymatic activity of the endophytic *Fusarium* species strains isolated from wheat

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Abstract: *Fusarium* is a genus of fungi that cause some of the most important plant diseases affecting agricultural and horticultural crops. Members of the genus establish an endophytic role inside the tissue of plants and produce a wide range of biologically active metabolites and enzymes. In the present study, the enzymatic profiles of several dominant *Fusarium* spp. were determined under solid state fermentation and activities were detected for xylanase, lipase, amylase, polygalacturonase, filterpase, and carboxy-methyl cellulase. Each *Fusarium* spp. showed a wide range of enzyme activities and protein contents. The ability to produce these enzymes was distributed amongst the strains tested, however amylase and xylanase *F. solani* Sy7 was found in a high percentage of strains. This study provides additional information to support future research about the industrial potential of these enzyme-producing species.

1. Introduction

Fusarium is an endophytic genus consisting of an array of species responsible for damping-off, root rot, and vascular wilt in a multitude of economically important plant species (Summerell *et al.*, 2001). The importance of *Fusarium* spp. in the current context is that infection may sometimes occur in developing seeds (Tajehmiri *et al.,* 2014). Because of this unusually wide range of symptoms, cell wall degrading enzymes (CWDEs) could have a range of important functions for the fungus in penetration, in heads, and in saprotrophic growth in dead tissue. This ecological habitat of the fungus implies that *Fusarium* could be a useful resource of extracellular enzymes (Kwon *et al.,* 2007; Bakri *et al.,* 2013).

Extracellular enzymes are thought to be particularly important CWDEs in interactions between *Fusarium* spp. and their hosts (Ahmed *et al.,* 2012). Much research work is still needed to fully understand the degradation process, and particularly the enzymes and other metabolites secreted by the fungi during infection. Information on these aspects could help to elucidate the biochemical mechanisms of wheat infection by *Fusarium* diseases and consequently facilitate fungal strain selection for industrial applications. However, studies of enzyme production by a phytopathogenic fungus are complicated by the presence of the plant, particularly by the presence of plant enzymes and microbial enzyme inhibitors that occur in plants. The most practical way to study the production of enzymes by a fungus is therefore to study the production of its enzymes on artificial growth media that contain no plant or enzyme inhibitors.

Solid-state fermentation (SSF) is considered an attractive alternative method for the production of industrially demanded enzymes that employ microorganisms (Krishna, 2005). SSF was defined by Pandey (2003) as a fermentation process involving solids in the absence (or near absence) of free water. Among the microorganisms that are capable of growing on solid substrates, only filamentous fungi can grow to a significant extent in the absence of free water (Guimaraes *et al.,* 2006).

The present study was undertaken to assess the potentialities of several *Fusarium* spp. in the production of industrially relevant enzymes under SSF conditions.

2. Materials and Methods

Isolation of Fusarium *species*

Fusarium spp. have been isolated from infected wheat seeds showing disease symptoms, and 21 were screened out of 105 strains for their host-pathogen interactions (Alazem, 2007). Seeds were sterilized to remove all microbial epiphytes by soaking them in 1:5 dilutions of NaOCl (sodium hypochlorite) solution for 15 min. They were then rinsed in sterile distilled water and dipped in 70% ethanol for 10 min. Subsequently, the seeds were washed with distilled water, dried between sterilized filter paper, and incubated in 9-cm Petri dishes containing potato dextrose agar (PDA, DIFCO, Detroit, MI. USA) for 10 days, at 23±1°C in the dark to allow mycelial growth. All strains were identified morphologically according to Nelson *et al.*

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(1983). The *Fusarium* spp. strains used in the study are listed in Table 1. The cultures were maintained on silica gel at 4°C until needed.

Extraction of enzymes from solid-state cultures

Enzyme production by the *Fusarium* spp. strains was carried out in 250 ml Erlenmeyer flasks containing 5 g of solid substrate and nutrients (based on 100 ml of liquid medium) plus distilled water to adjust the moisture content to 75%. Fresh fungal spores were used as inoculums and 1 ml spore suspension (containing around 10^6 spores/ml) was added to sterilized medium and incubated at 30°C. Flasks were removed after cultivation and the enzyme was extracted by adding distilled water containing 0.1% Triton x 100 to make the in-flask volume equivalent to 100 ml. Flask contents were stirred for 1.5 h on a magnetic stirrer. The clear supernatant was obtained by centrifugation (5000 x *g* for 15 min) followed by filtration (Whatman no. 1. paper).

Carboxy methyl-cellulase and filter paperase (Fpase) activity

Extra cellular enzymes were extracted by filtering the culture through Whatman No. 1 filter paper. The CMCase and Fpase activity were measured using the methods described by Refaz *et al.* (2013). One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1 µmol of glucose per ml per minute.

Amylolytic activity

A-amylase activity was determined as described by Okolo *et al.* (2001). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.25 of 0.1 M acetate buffer (pH5.0), 0.25 ml distilled water and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalent) were estimated by the dinitrosalicylic acid method of Miller (1959). One unit (IU) of α-amylase is defined as the amount of enzyme that releases 1 µmol of glucose equivalent per min under the assay conditions.

Lipolytic activity

Lipase activity was determined using 1 ml sunflower oil, 5 ml of 50 mM phosphate buffer pH 7.0 and 1 ml enzyme solution. The assay was carried out according to the method of Park *et al*. (1988). One unit of lipase activity was defined as the amount of enzyme liberating 1µ mol of fatty acid per min under the experimental conditions.

Pectinolytic activity

Assay of polygalacturonase (PGase) activity was carried out according to Marcia *et al.* (1999). PG activity was determined by measuring the release of reducing groups using the dinitrosalicylic acid reagent (DNS) assay (Miller, 1959). The reaction mixture containing 0.8 ml of 1% citric pectin with 67% of metoxilation in 0.2M citrate-phosphate, pH 6.0 buffer and 0.2 ml of culture superna-

Table 1 - Enzyme activity (U/g) of the 21 strains of *Fusarium* species used in this study

Strain	Xylanase	carboxy-methyl cellulase	Fpase	Polygalacturonase	Amylase	Lipase
F.culmorum SY1	20.3	3.31	2.46	23.52	45.5	61.28
2	96.36	6.67	3.64	38.72	55.36	0.30
3	163.69	3.89	2.03	70.96	54.6	0.90
6	131.93	2.78	2.03	80.4	51.13	117.04
9	12.16	1.76	2.03	15.68	52	65.04
12	115.92	2.32	2.03	74.24	66.8	81.28
13	90.64	2.87	2.03	23.92	47.4	0.50
14	19.52	2.09	2.03	41.04	51.4	0.10
F.solani SY7	757.2	5.27	2.03	67.92	118.35	81.28
11	112.16	2.66	2.03	22.72	40.4	0.70
20	234.96	1.43	2.03	61.04	39.25	0.90
35	125.6	2.39	2.03	43.76	41.7	57.52
F.verticillioides SY15	61.92	1.79	2.03	32	44.8	0.40
16	16.56	3.01	2.03	31.44	54.3	0.20
19	108.56	5.74	2.03	97.92	70.95	87.6
27	129.92	8.92	2.03	35.52	76.13	73.76
29	138.72	2.47	2.03	80	38.7	60.4
31	151.92	2.14	2.03	41.52	43.5	54.8
F.equiseti SY41	93.2	2.41	2.03	29.52	33.6	0.60
42	84.64	4.92	2.03	20.56	45.1	0.40
43	122.24	0	2.03	68	58.8	82.48

LSD= Least Significant Difference at $P < 0.05$.

tant, was incubated at 40ºC for 10 min. One unit of enzymatic activity (U) was defined as 1 µmol of galacturonic acid release per minute.

Xylanolytic activity

Xylanase activity was measured with the optimized method described by Bailey *et al.* (1992), using 1% birchwood xylan as substrate. The solution of xylan and the enzyme at appropriate dilution were incubated at 55°C for 5 min and the reducing sugars were determined by the dinitrosalicylic acid method described by Miller (1959), with xylose as standard. The released xylose was measured spectrophotometrically at 540 nm. One unit of xylanase is defined as the amount of enzyme required to release 1 µmol of reducing sugar as xylose equivalent per min under the above assay conditions.

Protein determination

Total proteins were determined for their importance when purifying an enzyme, since purity depends on the removal of unwanted proteins, and can be assessed by relating the activity to total protein present. The protein content in the enzyme preparation was determined according to the method of Lowry *et al*. (1951).

Statistical analysis

All the experiments were performed in triplicate and the means were analyzed statistically with the analysis of variance (Anonymous, 1988) using the STAT-ITCF computer package to test for differences in enzyme production among *Fusarium* spp. strains.

3. Results

All 21 strains of endophytic *Fusarium* spp. tested were able to produce one or the other extracellular enzymes (Table 1); some of the strains were able to produce all six enzymes tested. The profiles of extracellular enzymatic activities varied among *Fusarium* spp., but some general features were noted. Significant differences ($P<0.05$) in the mean yield values were detected among strains, with high values being consistently higher in the strain *F. solani7* for xylanase and amylase activities with mean values of 757.2 and 118.35 U/g, respectively. Some authors have suggested that the absence of catabolic repression in SSF systems is due to several factors collectively, including the slow and low processes of diffusion in solid state cultures due to low water activity (Krishna, 2005).

On the other hand, CMCase and Fpase activity were detected in the lowest quantity, in terms of total units per culture (Table 1). Whereas, *F. verticillioides* strain *SY19*, showed the highest PGase activity with a mean value 97.92 U/g. Hoondal *et al.* (2002) reported that degradation of host tissue by phytopathogens generally begins with the production of pectinolytic enzymes, which are the major enzymes involved in plant attack. The effect of different carbon sources on pectinase synthesis by fungi in SSF have been studied and it is generally agreed that the optimum medium for the enhanced production of extracellular pectinase contains pectic materials as an inducer (Yadav *et al.,* 2005).

Additionally, the results show that *Fusarium* spp. strains varied in lipase activity (Table 1), which could be related to a differential capacity to infect wheat. Pritsch *et al.* (2000) suggested that lipases might have participated, to a certain extent, in prior degradation of the cuticle. On the other hand, protein contents also showed significant ($P<0.05$) differences among *Fusarium* spp. (Fig. 1). The increase in protein content in some strains - *F. solani Sy7*, *F. culmorumSy2* and *F. verticillioides Sy27* - that produced high enzymatic activity suggests the growth of fungi contributing to the fungal protein by utilizing available nutrients in the substrate, making it possible to metabolize the available protein in the substrate. Consumption of substrate protein the production of biomass protein are reported by Scopes (1993).

4. Discussion and Conclusions

The *Fusarium* spp. strains studied here produced significant levels of enzyme activity *in vitro.* The results might indicate that this activity influences the aggressiveness of the strains of *Fusarium* spp. towards wheat plants. Kang and Buchenauer (2000) showed that *Fusarium culmorum* infects the wheat ovary usually through the junctions between the epidermal cell walls. These junctions may be a more preferable site for entry of the pathogen, allowing a quicker establishment of infection. However, Priest (1984) showed that there are several possible regulatory mechanisms in enzyme production including induction. The action of such enzymes gives rise to the possibility that the "genetic recombination" of the endophyte with the host which occur in evolutionary time. This may be the reason why some endophytes *Fusarium* spp. can produce some photochemicals originally characteristic of the host.

Promputtha *et al.* (2007) provided phylogenetic evidence indicating that the endophytes produced the same degrading enzymes as their saprobic counterparts. However, the capability of studied endophytes, such as the *Fusarium spp.* in this work, to produce different enzymes should have an important role as saprobes since they do not decompose the host living tissue. These degrading en-

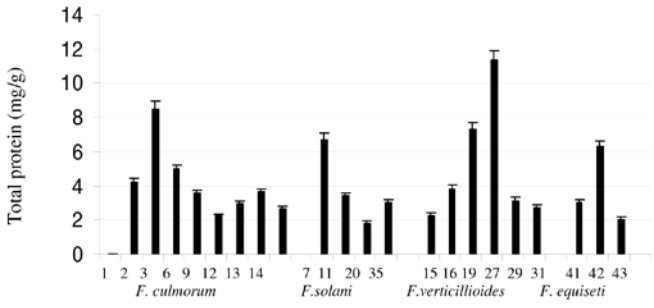

Fig. 1 - Total protein of *Fusarium* spp. strains under SSF.

zymes are important factors which affect the lifestyle of these species to become pathogens on wheat plants. Indeed, *Fusarium* species strains produced *in vitro* enzymes, a good indication that they may also do so under natural conditions. Schwarz *et al.* (2002) reported that *Fusarium* can produce various CWDEs *in vitro* and analyzed their regulation, suggesting that the initial infection depends of the secretion of these enzymes.

The present study demonstrates that *Fusarium* spp. are able to produce quite a good source of different types of industrially important enzymes. Knowledge of the types and amounts of enzymes produced by these species would be useful for the selection of strains best suited for industrial requirements. There are currently no reported studies on xylanase, lipase, amylase, polygalacturonase, filterpase, and carboxy-methyl cellulase enzymes to the authors' knowledge and thus there is a need for further in-depth studies on these isolated bioactive *Fusarium* spp. strains.

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