ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Screening of Extracellular Enzyme Activities of *Ganoderma* and *Fomes* Species Collected from North East Algeria.

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ABSTRACT

White rot fungi are capable to degrade efficiently a wide range of complex substrates due to their ability to produce highly specific extracellular enzymes. Forty five fungal strains of *Ganoderma* sp. and *Fomes* sp. isolated from basidiocarps harvested from different host trees from El Kala National Park, El Tarf (Algeria) were qualitatively screened for extracellular enzymes such as amylase, cellulases and protease. The results revealed that most of fungal isolates produced extracellular enzyme activities with quite different intensity. All the isolates were positive for cellulases, with an important production of endo glucanases on medium with soluble cellulose (carboxymethyl cellulose), moderate exoglucanase activities were observed on media with insoluble celluloses. The production of protease was different among the isolates, a significant protease activity were recorded in only four fungal isolates. Whereas, all the strains showed weak amylase activity in media supplemented with soluble starch or corn starch.

Keywords: white rot fungi, extracellular enzymes, cellulases, protease, amylase.

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ISSN: 0975-8585

INTRODUCTION

Ganoderma and *Fomes* are Basidiomycetes which belong to the Polyporaceae of the order Aphyllophorales [1-3].These mushrooms have been currently used for medicinal purposes for centuries [4, 5]. For the last decades, *Ganoderma* and *Fomes* were intensively studied for their active compounds, several compounds were elucidated and many of valuable biological activities were revealed [6-9]. *Ganoderma* and *Fomes* species, commonly known as wood decaying fungi, cause white rot on wide variety of trees, mainly hardwoods [1, 2, 10].

Indeed, for surviving; fungi secrete a wide range of enzymes to digest complex materials in the environment into solubilized breakdown products that can be up taken into the hyphae and used as nutrients [11]. Wherefore the white rot fungi are able to degrade efficiently the major components of plant cell walls mainly composed of cellulose, lignin and hemicellulose. These fungi possess hydrolytic enzymes like cellulases, pectinases and xylanases, which typically are induced by their sub strates [12-14]. Some lignocellulose-degrading enzymes have been studied and isolated by many relevant researches [13, 15-19].

Some researchers have investigated production of other extracellular enzymes by these fungi; Ro [20] produced and purified a protease from *F. fomentarius* (Fr.) Kicky (as cited in Jo, Park [21]), Choi and Sa [22] and Kumaran, Palani [23] isolated proteases with fibrinolytic activity from *G. lucidum.* Amylase activity of *Ganoderma* species has been also studied [24, 25].

Fungal enzymes have been extensively exploited for industrial applications for many years, they show also a promising potential for biotechnological and bioremediation applications [12, 16, 26, 27], therefore considerable research efforts are focused on characterizing newer enzymes and selecting hyper-producing strains.

This work was aimed to screen extracellular enzymes produced by *Ganoderma* and *Fomes* isolates collected from Kala National Park, El Tarf (ALGERIA), hence qualitative methods were chosen to assess different enzyme activities. Such tests give a positive or negative indication of enzyme production. Qualitative tests are powerful tools and particularly useful in screening large numbers of fungal isolates for several classes of enzyme, where definitive quantitative data are not required [28].

MATERIALS AND METHODS

Isolation of fungi

The fungi used in qualitative tests were isolated from basidiocarps of *Ganoderma* and *Fomes* harvested from different host trees from El Kala National Park, El Tarf (Algeria). Small pieces of fruiting body were suspended in sterile distilled water, after shaking (two minutes), 0.1 ml of suspension was aseptically transferred into Petri dishes containing Potato Dextrose Agar (PDA), or PDA supplemented with Rose Bengal (50mg.l^{-1}). The incorporation of Rose Bengal in the medium was to inhibit the rapidly spreading of fungal colonies [29, 30] and to suppress most of bacteria [31].The plates were incubated at 30°C until fungal growth. After confirmation of purity, the isolates were routinely maintained on PDA slants at 4°C. Then the isolates were identified on the basis of their macroscopic and microscopic features.

Qualitative detection of Cellulase Enzymes

In the order to investigate extracellular cellulases production, two different methods proposed by Pointing, Vrijmoed [32] and Pointing [28] were chosen, therefore a cellulolytic basal medium (CBM) was prepared and containing the following compounds (g.l^{-1}): $C_4H_{12}N_2O_6$5, $KH_2PO_4$1, $MgSO_4.7H_2O$ 0.5, Yeast Extract 0.1, $CaCl_2.2H_2O$ 0.001.

Cellulose agar clearance (cellulose agar)

This assay is based on the insolubility of the cellulose. Clearance indicates cellulolysis, although rates of clearance vary according to substrate. CBM medium was prepared by incorporating 2% w/v cellulose and 1.6% w/v agar. In this survey; insoluble cellulose from Sigma® (C6288) and fine-milled filter paper (almost

100% cellulose) were used. The test fungi were seeded by streaking centrally on the surface of sterile solidified medium using a fine needle. The plates were incubated at 30°C and examined daily for 10 days. Cellulolysis assessment is based on clearance zones of the opaque agar around growing colonies. A CBM medium supplemented only with 1.6% w/v agar was used as negative control. Inoculated dishes were kept in the same conditions of the test medium.

Dye staining of Carboxymethyl cellulose agar (CMC agar)

Carboxymethyl cellulose (CMC) is soluble cellulose and a substrate for endoglucanase [12, 28], The CBM medium was supplemented with 2% w/v viscosity CMC and 1.6% w/v agar. Subsequently the medium was inoculated with test fungi then incubated at 30°C for 3 days; the agar plates were stained by flooding with 2% w/v aqueous Congo red and left for 15 minutes. The stain was poured and the agar surface was washed with distilled water, the plates were flooded again with 1M NaCl solution to destain and left for another 15 minutes. The activity was observed as yellow opaque area against a red color of undegraded CMC.

Detection of proteolytic activity

Protease production was detected in skimmed milk agar prepared as described by Cruz da Silva, Camilo de Souza [33] with a little modification. Commercial liquid skimmed milk (Candia®) was solidified with 1% w/v agar. The plates were inoculated, and then incubated at 30°C for 10 days. The formation of transparent zone around the growth was used as criterion of protease production. Diameters of hydrolysis zone were daily measured and recorded [33, 34].

Detection of Amylase production

Screening for amylase production efficiency was carried out on starch agar medium composed of ($g.l^{-1}$) ; yeast extract 1.5, peptone 0.5, sodium chloride 1.5, starch 10, agar 15, pH 5.6 [35]. In this work, two starches were tested; soluble starch (Sigma®) and commercial cornstarch. Seeded plates were incubated at 30°C for 3 days, and then flooded with a dilute iodine solution (Lugol's iodine). After flooding with iodine, the starch stains blue-black and the zone of degradation around the colonies is either stained brown or remains colorless [34, 35].

RESULTS AND DISCUSSION

Isolation of fungi

45 basidiomycete strains were collected by using PDA and PDA supplemented with rose Bengal. The addition of Rose Bengal in PDA medium was suitable and reduced the overgrowth of fungi, which aided to select the wanted colonies. However, the rose Bengal was not effective against bacterial development as described by Ottow and Glathe [36] and Ottow [31]. The 45 obtained isolates were identified as *Ganoderma* sp. and *Fomes* sp., *Ganoderma* was the most predominated genus. The test results are presented in Table.1.

Qualitative detection of Cellulase Enzymes

Cellulose agar clearance (cellulose agar)

All the 45 isolates grew in medium test with different growth rates and quite diverse colony aspects; from very thin to fluffy and dense hyphae, although; better growth rates were observed in medium with milled filter paper than medium with crystalline cellulose for most of strains. Generally, insoluble cellulose is attacked slowly by cellulolytic enzymes [1, 28].

The clearance of media was not easy to assess in both of medium, only 3 isolates showed an obvious positive reaction; Gsp22, Fsp1 and Fsp2, whereas Gsp3, Gsp29 and Gsp38 exhibited positive reaction in only filter paper medium. In fact, cellulolysis of heterogeneous cellulose substrates like filter paper and crystalline cellulose is due to simultaneous action of all cellulolytic enzymes [28, 37], but recording clearance of cellulose within the growth medium can be difficult to assess, particularly with dense or dark hyphal growth [28].

ISSN: 0975-8585

White rot fungi are able to fully solubilize pure crystalline cellulose substrates, because they possess the synergistic endo/exoglucanases system [1, 12, 37].

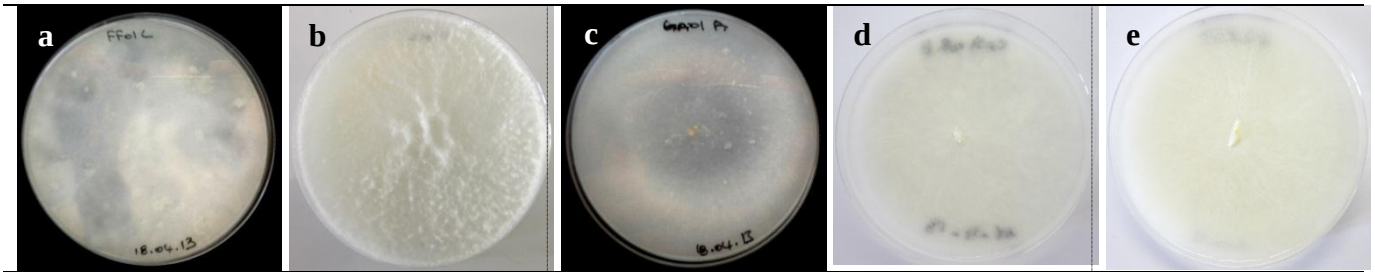
Little or no growth was observed when the fungi were grown on solidified CBM (negative control medium). These results confirm that the growth in cellulose media implies the production of cellulolytic enzymes. Thus, the difficult assessment of cellulolysis is an inherent problem in this method.

Table 1: Detection of extracellular enzyme activities in *Ganoderma* and *Fomes* species

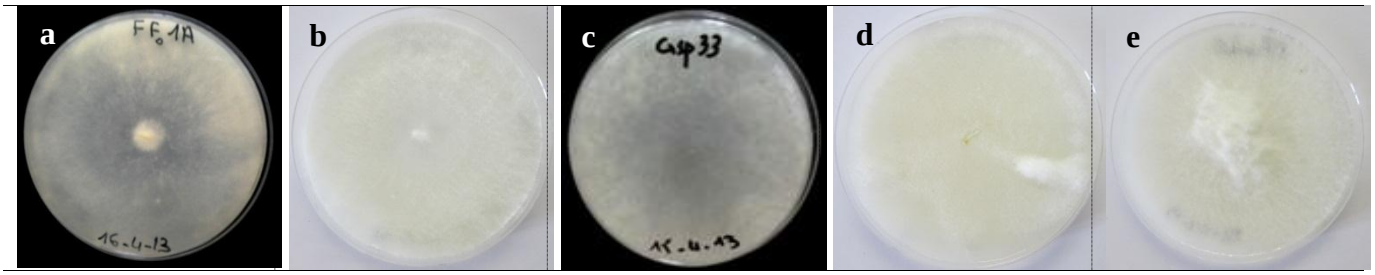
Strain	*Enzymatic activity*					
	Cellulases			**Proteases**	**Amylases**	
	Crystalline Cellulose	**Filter paper**	**CMC**	**Milk agar**	**Cornstarch**	**Soluble starch**
Gsp01	+	+	+	+	- (*)	- (*)
Gsp02	+	+	+++	+++	- (*)	- (*)
Gsp03	+	++	++	++	- (*)	- (*)
Gsp04	+/-	+/-	++	+	- (*)	- (*)
Gsp05	+	+	++	+	- (*)	- (*)
Gsp06	+	+	++	+	- (*)	- (*)
Gsp07	+	+/-	+++	+	- (*)	- (*)
Gsp08	+	+	++	+	- (*)	- (*)
Gsp09	+	+	++	++	- (*)	- (*)
Gsp10	+	+	+++	+	- (*)	- (*)
Gsp11	+	+	+	++	- (*)	- (*)
Gsp12	+	+	++	+++	- (*)	- (*)
Gsp13	+	+	++	- (*)	- (*)	- (*)
Gsp14	+	+	+	- (*)	- (*)	- (*)
Gsp15	+/-	+	+	- (*)	- (*)	- (*)
Gsp16	+	+	++	+	- (*)	- (*)
Gsp17	+/-	+	+	+	- (*)	- (*)
Gsp18	+/-	+/-	++	+	- (*)	- (*)
Gsp19	+	+	+	- (*)	- (*)	- (*)
Gsp20	+	+	++	+	- (*)	- (*)
Gsp21	+	+	+++	- (*)	- (*)	- (*)
Gsp22	++	++	+++	+	- (*)	- (*)
Gsp23	+	+/-	+++	++	- (*)	- (*)
Gsp24	+	+	+++	+	- (*)	- (*)
Gsp25	+	+	++	+	- (*)	- (*)
Gsp26	+/-	+	+	++	- (*)	- (*)
Gsp27	+	+	+	+	- (*)	- (*)
Gsp28	+	+	++	+	- (*)	- (*)
Gsp29	+	++	++	+	- (*)	- (*)
Gsp30	+	+	++	+	- (*)	- (*)
Gsp31	+	+	+	+	- (*)	- (*)
Gsp32	+	+	+++	+	- (*)	- (*)
Gsp33	+	+	++	- (*)	- (*)	- (*)
Gsp34	+	+	+	+	- (*)	- (*)
Gsp35	+	+	++	+	- (*)	- (*)
Gsp36	+	+	++	+++	- (*)	- (*)
Gsp37	+	+	++	- (*)	- (*)	- (*)
Gsp38	+	++	+++	+	- (*)	- (*)
Gsp39	+	+	+	- (*)	- (*)	- (*)
Gsp40	+	+	++	+	- (*)	- (*)
Gsp41	+	+	+	+	- (*)	- (*)
Gsp42	+	+	+++	+	- (*)	- (*)
Gsp43	+	+	++	+	- (*)	- (*)
Fsp01	++	++	++	+++	- (*)	- (*)
Fsp02	++	++	++	- (*)	- (*)	- (*)

+++ Strong reaction, ++ moderate reaction, + weak reaction, +/- suspicious reaction,
- (*) no halo around the growth with hydrolysis inner zone.

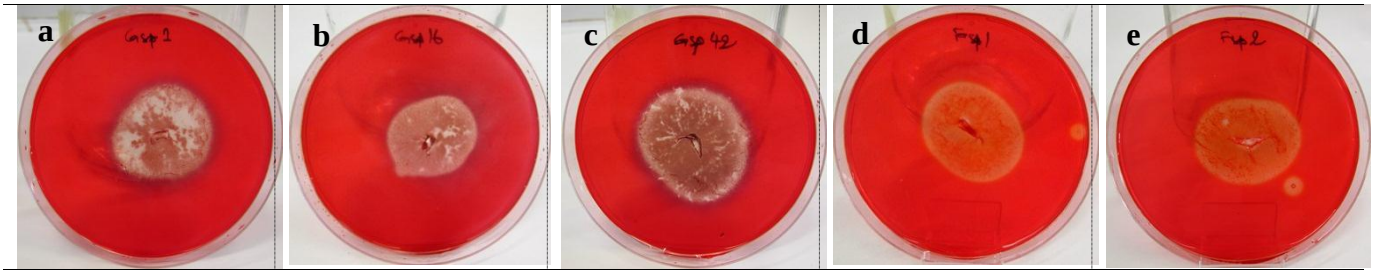
ISSN: 0975-8585



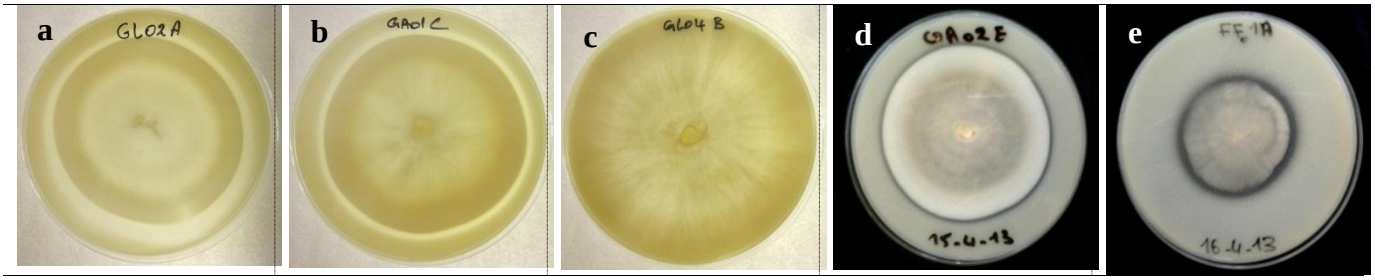
1. Examples of growth and clearance on crystalline cellulose medium



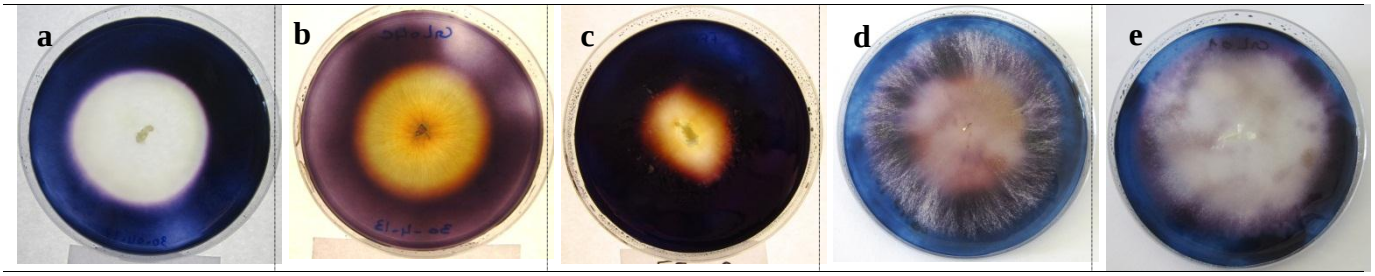
2. Examples of growth and clearance on Filter Paper medium



3. Examples of Endoglucanase activity on CMC medium



4. Examples of different proteolytic activity on milk agar medium



5. Examples of Amylase activity on Starch medium and Cornstarch medium

Figures: Enzyme Activities of *Ganoderma* and *Fomes* species.

Figs (1a and 1b): Fsp2; Fig 1c: Gsp22: Figs. (1d and 1e); *Ganoderma* sp.
Fig2a: Fsp1; Fig 2b: Fsp2; Fig 2c: Gsp33; Figs (2d and 2e): *Ganoderma* sp.
Figs (3a, 3b and 3c): *Ganoderma* sp., Figs (3e and 3d):*Fomes* sp.
Figs (4a, 4b, 4c and 4d): *Ganoderma* sp., Fig4e: Fsp1.
Figs (5a and 5b): *Ganoderma* sp. and Fig 5c: Fsp1 on soluble starch medium,
Figs (5d and 5e): *Ganoderma* sp. on cornstarch medium

Dye staining of Carboxy methylcellulose agar (CMC agar)

All tested fungi exhibited a positive reaction in this essay, with slight different intensities between strains. Highest activity was observed with Gsp21, Gsp22, Gsp24 and Gsp38. Many fungi have been shown to produce endoglucanase activities [38-41], thus; many of them degrade successfully cellulose in wood produce no detectable exoglucanases (Eaton and Hale [42] as cited in Pointing [28]). Although, purified endoglucanases do not show significant activity on crystalline cellulose [12]. This assay is a good indicator of cellulolytic ability, positive reactions are easy to assess.

Detection of proteolytic activity

The 45 isolates grown on skimmed milk agar showed different reactions with different growth rates. Strains exhibited the highest protease activity are Gsp36, Gsp2 and, followed by Gps12, Fsp1 exhibited a significant proteolytic reaction. Most of isolates showed weak proteolytic activity; by forming reduced hydrolysis zones (< 0.2cm) or without halos, but with clearance zone within the colony. Jo, Park [21] reported weak protease production of *Ganoderma* species. The inner clearance zone indicates proteolysis, because milk proteins (mainly caseins) in this medium are the sole source of nitrogen; therefore the growth itself involves the ability of fungi to digest caseins by producing proteolytic enzymes.

Detection of Amylase production

In this test, *Ganoderma* sp. and *Fomes* sp. showed weak reactions. No clear zone around the colonies, just limited brownish or colorless zone below the colony. Choi, Hodgkiss [43] considered this kind of reaction an indicative of intracellular amylase production. Hydrolysis zones in cornstarch medium were mostly irregular and spotted with blue-purple, which indicating the presence of an undegraded starch under the growing hyphae. That could be explained by the heterogeneous nature of starch granules, which composed of two constituents; amylose and amylopectin. The ratio of amylose/amylopectin contents in starch varies depending on botanical source, Amylose is a relatively long, linear α-glucan containing around 99% α(1→4) and α(1→6) linkages, whereas amylopectin has a heavily branched structure built from about 95% α(1→4) and 5% α(1→6) linkages [44-46], α-amylases degrade mainly α(1→4) links bonds, the enzymes have a weak α(1→6) hydrolyzing activity [12, 47]. Lacerda, Silva Carvalho Filho [48] have investigated the hydrolysis of cornstarch granules with fungal α-amylase, this latter; acts preferentially at the amorphous regions of granules, the crystalline parts were less altered by the enzymatic attack. The crystallinity of the starch is related to amylopectin fraction [44, 45, 49]. Therefore, high amylopectin content cornstarch can explain the obtained results.

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