Detection and quantification of enzyme activity of ericoid fungi under solid-state fermentation

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Abstract: Today microbial enzymes are commonly used in many industrial applications and the demand for more stable, highly active and specific enzymes is growing rapidly. The aim of this study was the screening of fungal enzymes. Four fungal cultures were isolated and identified. These fungal cultures were tested on Solid-state Fermentation (SSF). The fungal isolate (S2) was noticed to show maximum of lipase production and the fungal isolate (S4) showed maximum production of protein content and cellulase production.

Keywords: Lipase, Protein content, Cellulase, Enzymes, Solid-State Fermentation SSF

Introduction

Microorganisms are attractive sources for enzyme production because of their rapid growth and the limited space required for their cultivation. The ability to secrete large amounts of enzymes is characteristic of a variety of microorganisms such as bacteria, fungi, yeast and Actinomycetes (Gurung et al. 2013). In Morocco, the *Ericaceae* family is represented by only three genera and 10 species including *Arbutus* L*., Calluna vulgaris* L. *and Erica* L (Hamim et al. 2017). They can establish symbiotic root associations with the group of a distinctive type of mycorrhiza, termed ericoid mycorrhiza (Vohnik et al. 2011) and with the most studied group of fungal root endophytes belonging to the group of Dark Septate Endophytes (Lukešová et al., 2015). Several different enzymatic activities have been detected in ericoid mycorrhizal fungi and dark septate (Bending et al. 1996). The selection of the right organism is essential to obtain high yield of desirable enzymes (Padmapriya et al. 2012). Among the most important enzymes are proteinases, that are the most widespread in nature (Akhtaruzzaman et al. 2012), they possess considerable industrial potential due to their biochemical diversity in tannery and food industries (Punt et al. 2002), medicinal formulations, detergents (Devi et al., 2008) Besides, the cellulases are considered also important industrial enzymes. They hydrolyze β-1,4 linkages in cellulose chains (Zhang et al., 2013). Cellulases are used in the textile industry (Carrasco et al. 2016), in detergents and pulp and paper industry (Kasana et al. 2011). Another important enzymes for the industry are lipases, they are not involved in the lignin degradation, but they produced for fungi and its industrial application is extensive, they are used in wastewater treatment (Senthil Raja et al. 2012). The main draw back with production of fungi enzyme is the requirement of cost intensive procedures for separation of enzymes from cells (Devi et al. 2008). Since these enzymes are a product of industrial interest, their production must be combined with cost reduction, which can be achieved through the use of low cost culture

media (residues) from agro-industry. One way to obtain low cost enzymes is through a process named solid-state fermentation (SSF) (Castilho et al. 2000; Godoy et al. 2011). The SSF process is basically the use of a solid culture medium as a nutrient source and as a support to microorganism growth. That allows to reduce the cost of enzyme production processes and to obtain high enzymatic activity through the optimization of production (Vargas et al. 2008, Azcon et al. 2009).

Currently, there is an ever-increasing interest in the isolation and study of microorganisms, capable of producing enzymes with biotechnological applications and high economic impact. Based on the afore mentioned, the objective of the present study emphasizes on screening of fungal cultures for cellulases, lipases and protein content enzyme secreted by the ericoid fungi collected from root of ericaceous plants indigenous to Morocco, and thereby, be able to select those fungi with the greatest biotechnological potential.

Materials and methods

Microorganism: The strains S1, S2, S3, S4 have been isolated from ericaceous roots, and identified through the amplification of the Internal Transcribed Spacer (ITS) region (Hamim et al. 2007).

Table 1. Sequences of isolated ericoid fungi from the gene bank (NCBI).

Strains	Best match	Accession	Host species	Region	Ordre
S1	*Ericoid mycorrhizal sp*	AF072301.1	*Calluna vulgaris*	Melloussa	Helotiales
S2	*Ericoid ycorrhizal sp*	AF072296.1	*Erica umbellata*	Sahel	Helotiales
S3	*Ericoid endophyte sp.*	AF252845.1	*Calluna vulgaris*	Cap spartel	Helotiales
S3	*Phialocephala fortinii*	EU888625.1	*Calluna vulgaris*	Melloussa	Helotiales

Cultivation media: Sugarcane bagasse (50%), a solid residue from sugar cane was used as the solid-state fermentation culture medium supplemented with 30% of wheat bran, 15% of potato mash, 5% of olive oil and 300 ml of distilled water. The medium was inoculated with 15 fungal plugs (1cm^2) moisturized to 75% and incubated at 30°C. phosphate buffer (100 mM, pH 7.0, 5 mL/g) was added to each flask containing the fermented solids. The supernatant extraction was carried out in a rotary shaker at 35°C and 200 rpm for 20 min. Afterwards solid-liquid separation was done by pressing followed by centrifugation g for 5 min (Gombert et al. 1999). The supernatant was used for enzyme activity determination. Four sampling dates were identified at T0 (0 day); T1 (9 days); T2 (14 days); T3(27 days); T4(35 days).

Protein quantification was estimated as described by (Lowry 1951). Then O.D and concentration was measured at visible range 750 nm by a spectrophotometer. The amount of the soluble protein was calculated from the standard curve as mg of protein per ml of test samples.

Lipase activity: Lipase activity was measured using p-nitrophenyl laurate (pNP-laurate) as substrate. The hydrolysis reaction was carried out at 30 °C and measured over time up

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to 10 min at 412 nm. The specific activity was calculated as the ratio of lipase activity (U g^{-1}).

Estimation of reducing sugars by dinitrosalicylic Acid method was determined by the colorimetric method of Miller (Miller 1959) using the DNS-reagent. The O.D of the samples was immediately measured at 575 nm. One enzyme unit was defined as 1 µmol of glucose equivalents released per min. The specific activity was calculated as the ratio of cellulase activity (U g^{-1}).

Stastical analysis

The data are reported as means ± SD (standard deviation) for 3 replications. The results were subjected to analysis of variance (ANOVA) according to LSD test (P<0,05) using the stat-graphics plus version 4.0.

Results and discussion

Protein content of isolated strains is presented in Table 1 and Figure 1. The statistical analysis has revealed the significant effect of strains and sampling date on protein content (P<0.05).

Table 1. Protein content (mg/ml) of selected fungi

Day	Protein content (mg/ml)			
	S1	S2	S3	S4
T0	0.02c[(2)] ± 0.01[(1)]	0.02bc ± 0.01	0.03b ± 0.01	0.04a ± 0.01
T1	0.02 c [(2)] ± 0.01 [(1)]	0.02 c ± 0.01	0.04 b ± 0.01	0.08 a ± 0.01
T2	0.02 c ± 0.01	0.02 bc ±0.00	0.03 b ±0.00	0.08 a ± 0.01
T3	0.02 c ±0.01	0.03 bc ± 0.00	0.04 b ± 0.00	0.09 a ±0.01
T4	0.04 d ± 0.00	0.07 c ± 0.00	0.09 b ± 0.00	0.12 a ± 0.00

Significant effect at the P<0.05 [(1)] Standard error. [(2)] The values of each line followed by the same letter are not significantly different according to LSD test (p >0.05).

During the experimentation, the concentration of soluble protein ranged between 0.02 to 0.12 mg/ml. The S4 showed highest protein concentration (0.12 mg/ml) and S1 accounted lowest concentration (0.02 mg/ml). The maximum content protein was 0.12 mg/ml; 0.09 mg/ml; 0.07 mg/ml; observed for S4, S3, and S2 respectively obtained at T4. In the present study, the result showed low protein concentration, that may explained by the inability of strains to degrade protein well, this finding may be explained as well by the composition and volume of medium. Reports in the literature suggested that the protein production varied according to different factors.

Estimation of reducing sugars of selected fungal is presented in Table 2**.** During the experimentation, different sampled cultures fungal showed varied cellulase activity. This concentration ranged between 5.88 to 17.34 Ug^{-1}. For instance, S2 have showed a

continuous increase of cellulase activity form 7.23 to 12.07 Ug^{-1}; however S4, S3 have showed a slight decrease of cellulase activity at T2 and T3 respectively. At T4, the S4 showed highest cellulase activity (17.34 Ug^{-1}) While, S1 have showed an unexpected decline to reach the value of 5.88 Ug^{-1}.

Table 2. Cellulase activity (Ug^{-1}) of fungal isolates

Day	Cellulase activity (Ug^{-1})			
	S1	**S2**	**S3**	**S4**
T0	7.20 b[2] ± 0.01[1]	7.23 a ± 0.01	7.18 c ± 0.01	7.23 a ± 0.01
T1	7.14 d ±0.01	10.03 c ±0.05	11.41 b ±0.02	12.34 a ±0.02
T2	9.69 d ±0.04	11.84 a ±0.07	10.85 b ±0.01	10.12 c ±0.01
T3	10.93 c ±0.00	11.67 b ±0.01	7.31 d ±0.01	15.15 a ±0.01
T4	5.88 a ±0.05	12.07 b ±0.00	10.08 c ±0.01	17.34 d ±0.01

Significant effect at the P<0.05 [1] Standard error. [2] The values of each column followed by the same letter are not significantly different according to LSD test (p >0.05).

Lipase Activity: Lipase activity produced by selected fungi is presented in Figure 1. During different sampled time, the lipase production varied significantly between strains. This concentration ranged between 1.13 to 18.03 Ug^{-1}. We have noticed that lipase activity maintain continuous production level during the experimentation. T4 have recorded the maximum of lipase activity for all fungi. Therefore, S2 showed highest lipase activity at T4 (18.03 Ug^{-1}) However S3 (5.72 Ug^{-1}) and S4 (10.22 Ug^{-1}) have showed a moderate lipase activity, while S1 (2.63 Ug^{-1}) had lowest lipase activity.

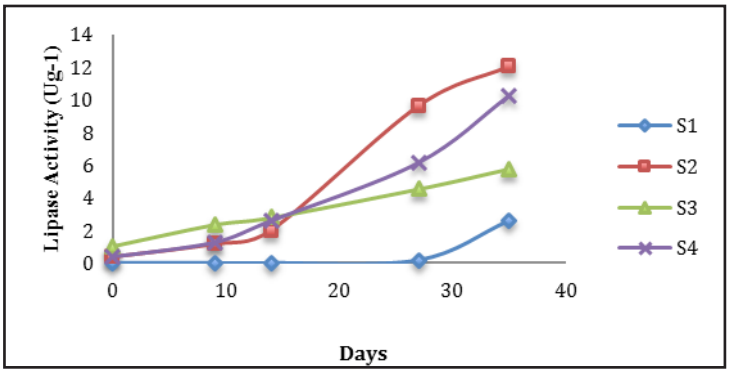

Figure 1. Lipase activity of selected strains.

The current study is the first report on cellulase, lipase enzymatic activity and protein content of selected ericoid fungal related to *ericoid mycorrhizal fungi* and to *phialocepha fortinii*. Those fungi are identified as specific to ericaceous plants indigenous to the north of Morocco (Hamim et al. 2017). Our study demonstrated the ability to produce enzyme activity obviously varied from strain to strain. Among the four strains, the ericoid

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mycorrhizal fungi related strain (S2) and *phialocephala fortinii* related strain (S4) have a significantly better ability to produce cellulase activity during the experimentation. However, this cellulase activity was lower to that reported for *Trichoderma harzianum* at SSF which varied from 11 to 50 Ug^{-1} (Berne et al. 2013). Furthermore, the ericoid mycorrhizal fungi related strain (S2), has a significantly better ability to produce lipase activity than other strains.. Besides, the DSE related strain (S4), has a significantly better ability to produce protein.

Previous studies suggest enzymatic capabilities of symbiotic fungi such as ecto-ericoid mycorrhizal fungi to produce lignolytic and cellulolytic enzymes (Wagner 2015). Furthermore, (Jumpponen and Trappe; 1998) have reported that the enzymatic capabilities of mycorrhizal fungi require lignolytic and cellulolytic enzymes to facilitate penetration through host cell walls. Likewise, Several different enzymatic activities have been detected in dark septate endophyte (DES) (Ahlich 1997) however, they varied drastically between strains.

Our study demonstrated low level of enzymatic activity content in this experimentation when compared to others in SSF, this low level production might be explained by the slow growth rate for the selected strains compared to *Aspergillus* sp., *Penicillium* sp. or *Trichoderma* sp. strains commonly used in state-solid fermentation or by the composition of the culture medium. Our finding is in agreement with (Lynd et al. 2002) they confirmed that cellulase production depends on many factors such as the fungal species.

Conclusion

In the present study, the selected fungal isolates from ericaceous root plants posses enzymatic activities; which has been scarcely studied for ericaceous plants indigenous to the north of Morocco. The enzymatic activity measured in this work is important characteristics for possible biotechnological applications for the biological control. Further research is vital to unravel the full potential of these microorganisms in agriculture.

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