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Screening 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 such as cellulase, lipase and proteinase. Four fungal cultures were isolated and identified. Among these fungal cultures, three belonged to *ericoid mycorrhizal fungi* sp. and one belonged to *Phialocephala*sp. These fungal cultures were tested on Solid-state fermentation (SSF) to find their ability to produce variable enzymes. The fungal isolate (S2) was noticed to show maximum of lipase production and the fungal isolate (S4) showed maximum production of proteinase and cellulase. These enzymes produced through a low cost methodology can be applied in biological control of several fungal plants.

1. 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[1].In Morocco, the *Ericaceae* family is represented by only three genera and 10 species including *Arbutus* L.*, Calluna vulgaris* L. *and Erica* L[2]. They can establish symbiotic root associations with the group of a distinctive type of mycorrhizae, termed ericoid mycorrhiza[3] and with the most studied group of fungal root endophytes belonging to the group of Dark SeptateEndophytes[4]. Several different enzymatic activities have been detected in ericoid mycorrhizal fungi and dark septate[5]. The selection of the right organism is essential to obtain high yield of desirable enzymes [6]. Among the most important enzymes are proteinases, that are the most widespread in nature[7]; they possess considerable industrial potential due to their biochemical diversity in tannery and food industries [8], medicinal formulations and detergents [9-10]. Besides, the cellulases are considered also important industrial enzymes. They hydrolyze β-1,4 linkages in cellulose chains. They are used in the textile industry [11], in detergents[12], pulp and paper industry and for bioconversion of lignocelluloses to fuel ethanol [13-14].

Another important enzymes for the industry are lipases, they are not involved in the lignin degradation, but they are produced by fungi and theirindustrial application is extensive, they are used in wastewater treatment [15].

The main disadvantage with production of fungi enzyme is the requirement of cost intensive procedures for separation of enzymes from cells [10]. 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) [16-17].

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 [18-19]. 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 indigenousto Morocco, and thereby, be able to select those fungi with the greatest biotechnological potential.

2. Experimental details

2.1. Microorganism

The S1, S2, S3, S4 strains have been isolated from ericaceous roots, and identified through the amplification of the Internal Transcribed Spacer (ITS) region[2]. The results are presented in Table 1.

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

Strains	Best match	Accession	Host species	Region	Ordre
S1	*Ericoid mycorrhizalsp*	AF072301.1	*Calluna vulgaris*	Melloussa	Helotiales
S2	*Ericoid mycorrhizalsp*	AF072296.1	*Ericaumbellata*	Sahel	Helotiales
S3	*Ericoid endophyte sp.*	AF252845.1	*Calluna vulgaris*	Cap spartel	Helotiales
S4	*Phialocephalafortinii*	EU888625.1	*Calluna vulgaris*	Melloussa	Helotiales

2.2. Cultivation media

2.2.1. Solid-state fermentation

Sugarcane bagasse (50%), a solid residue from sugar cane was used as the solid-state fermentationculture 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.

2.2.2. Different media culture

The growth of strains fungi was studied in different media culture such as Potato Dextrose Agar media (PDA), Modified MelinNorkrans (MMN) media and Malt Agar (MA) media.

2.3.pH, enzymatic activity and moisture measurement

1 g of the substrate was ground with an Ultra-Turrax (Ika) in10 ml of distilled water. Therefore, a pH measurement was performed using a pH meter previously calibrated. Observations were made from a dissecting microscope (Zeiss, Stemi 2000- C) and a microscope (Zeiss, ICS Standard 25). It allows assessing the physiological stages of the fungus. The moisture content was used to express the weight loss after fermentation; it was made in triplicate and measured using a dryer (Sartorius MA45). After fermentation, phosphate buffer (100 mM, pH 7.0, 5 mL/g) was added to each flask containing the fermented solids. The enzyme 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 for 5 min [20]. The supernatant was used for enzyme activity determination. Four sampling date were identified: 0; 9; 14; 27, and 35 days.

2.4. Protein quantification

Protein was estimated by Lowry methods [21]. The O.D and the protein concentration were 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.

2.5. Lipase activity

Lipase activity was measured using p-nitrophenyllaurate (pNP-laurate) as substrate. The hydrolysis reaction was carried out at 30 °C and measured over time up to 10 min at 412 nm. One unit of lipase activity is defined as the amount of enzyme, which releases 1 mole of p-nitrophenol under assay conditions [16]. The specific activity was calculated as the ratio of lipase activity (U g^{-1}).

2.6.Cellulase activity

The estimation of reducing sugars by dinitrosalicylic acid was determined by the colorimetric method of Miller [22] 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 (Ug^{-1}).

2.7.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.

3. Results and Discussion

3.1. Identification of fungal cultures

Fungal cultures characteristics such as color and size of colonies during the growth stage in different culture media were studied. Table 2 gives cultural features and growth rate of 4 distinct non-sporulating fungi.

Table 2. Macroscopic features of fungal cultures.

Fungal isolates	Colony Color	PDA[1] medium (mm/day)	MNM[2] medium (mm/day)	MA[3] medium (mm/day)	Dry Weight (g)
S1	Smokey-grey to black	0.36	0.49	0.41	0.3
S2	Grey to green olive	0.33	0.39	0.2	0.18
S3	Smokey-grey	0.34	0.46	0.33	0.59
S4	Olive to black	0.85	0.5	0.7	0.9

[1] Potato Dextrose Agar
[2] Modified MelinNorkrans
[3] Malt Agar

The studied fungi group was slow growing on the range of media tested, generally producing less than a millimeter of growth per day (0.5 mm/ day) for the three strains S1, S2 and S3 while the other isolate (S4) showed higher growth rate reaching a maximum growth of 0.85 mm/ day. We have observed the difference of the growth rate of the different strains according to the culture medium.

3.2. Protein content

The protein concentrations of isolated strains are presented in Table 3 and Figure 1. The statistical analysis has revealed the significant effect of strains and sampling date on protein content (P<0.05).

Table 3. Protein content (mg/ml) of selected fungi.

Day	**Protein content (mg/ml)**			
	S1	S2	S3	S4
0	0.02cB[(2)] ± 0.01[(1)]	0.02cC ± 0.01	0.03bC ± 0.01	0.04aC ± 0.01
9	0.02 cB ± 0.01	0.02 cC ± 0.01	0.04 bB ± 0.01	0.08 aB ± 0.01
14	0.02 cB ± 0.01	0.02 bcC ±0.00	0.03 bC ±0.00	0.08 aB ± 0.01
27	0.02 cB ±0.01	0.03 bcB ± 0.00	0.04 bB ± 0.00	0.09 aB ±0.01
35	0.04 dA ± 0.00	0.07 cA ± 0.00	0.09 bA ± 0.00	0.12 aA ± 0.00

Significant effect at the P<0.05
[(1)] Standard error.
[(2)] The values of each line followed by the same lowercaseletter and the values of each column followed by the same uppercase 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 the highest protein concentration (0.12 mg/ml) and S1 accountedthe 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.The study on the presence of extracellular protease activity in filamentous fungi were studied mainly in ascomycetes, e.g. in *Neurospora* [23], *Aspergillus* [24] or yeasts [25]. This activity was studied as well in basidiomycete fungi, such as *Trametestroggi* [26] and *Trametesversicolor* [27]. In the present study, the result showed low protein concentration, that may be 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. [28] observed that medium type had the greatest impact on proteinase production. [6]explained that the protease enzyme was highly active and stable from pH 6 to 9 with an optimum at pH 7 and with optimum temperature at 37°C.

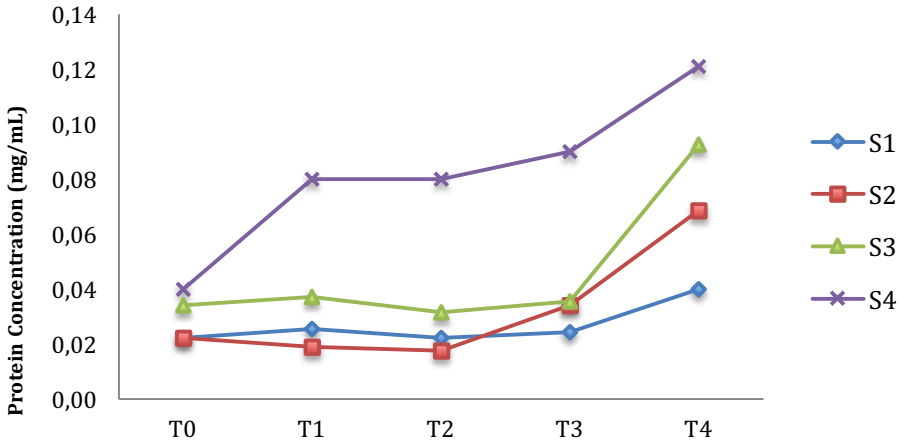

Figure 1: Protein concentration of fungal cultures.

3.3: Cellulase Activity

The cellulase activity results of selected fungal are presented in Table 4 and Figure 2**.** The cellulase concentration was calculated from the standard curve as mg of cellulase per ml of test samples. Linear regression had coefficient of correlation R (0.99).

Table 4. Cellulase activity (Ug^{-1}) of fungal isolates.

Day	S1		S2		S3		S4	
	pH	Cellulase activity (Ug^{-1})	pH	Cellulase activity (Ug^{-1})	pH	Cellulase activity (Ug^{-1})	pH	Cellulase activity (Ug^{-1})
0	6.39	7.20 b[(2)] ± 0.01[(1)]	6.02	7.23 a ± 0.01	6.33	7.18 c ± 0.01	6.23	7.23 a ± 0.01
9	6.08	7.14 d ±0.01	6	10.03 c ±0.05	6.12	11.41 b ±0.02	6.04	12.34 a ±0.02
14	6.74	9.69 d ±0.04	6.17	11.84 a ±0.07	6.57	10.85 b ±0.01	6.46	10.12 c ±0.01
27	6.37	10.93 c ±0.00	6.88	11.67 b ±0.01	7.13	7.31 d ±0.01	7.2	15.15 a ±0.01
35	6.67	5.88 d ±0.05	6.58	12.07 b ±0.00	6.88	10.08 c ±0.01	6.88	17.34 a ±0.01

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).

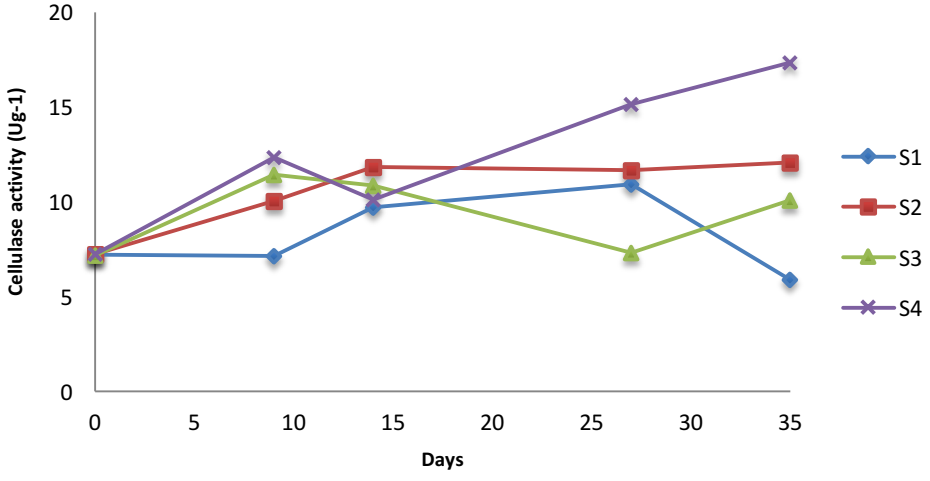

Figure 2: Cellulase activity for fungal strains.

During the experimentation, different sampled fungal cultures showed varied cellulase activity. This concentration ranged between 5.88 and 17.34 Ug^{-1}. For instance, S2 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 35 incubation days, the S4 showed the highest cellulase activity (17.34 Ug^{-1}) while, S1 has showed an unexpected decline to reach the lowest value of 5.88 Ug^{-1}.

3.4. Lipase Activity

Lipase activity produced by selected fungi is presented in Table 5 and Figure 3.

Table 5. Lipase activity (Ug^{-1}) produced by selected fungi.

Lipase Activity (Ug^{-1})				
Day	**S1**	**S2**	**S3**	**S4**
0	0.00c[(2)] ±0.00[(1)]	0.35b ±0.01	1.01a ±0.05	0.35b ±0.03
9	0.00 d ±0.00	1.13 c ±0.01	2.29 a ±0.08	1.24 b ±0.01
14	0.00 d ±0.00	1.98 c ±0.01	2.79 a ±0.01	2.55 b ±0.01
27	0.16 d ±0.01	9.60 a ±0.01	4.52 c ±0.02	6.11 b ±0.01
35	2.63 d ±0.01	18.03 a ±0.01	5.72 c ±0.11	10.22 b ±0.01

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 ($P>0.05$).

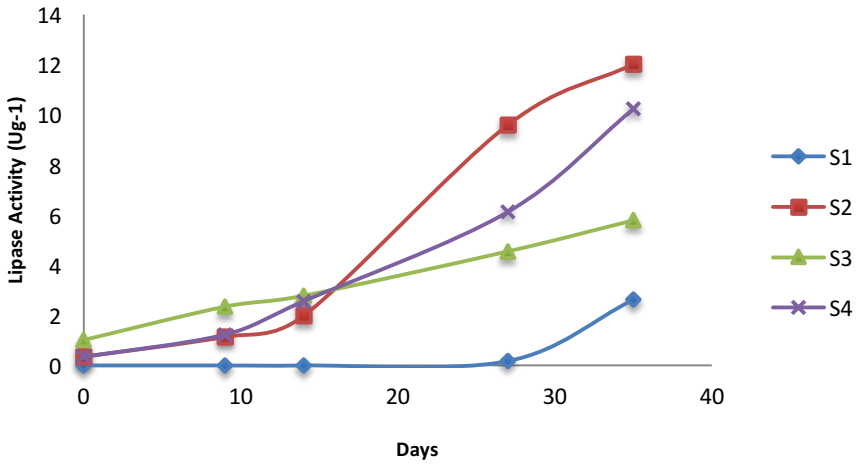

Figure 3: lipase activity of selected strains.

The lipase production varied significantly between strains during the different sampling times. This concentration ranged between 1.13 and 18.03 Ug^{-1}. We have noticed that lipase activity maintain continuous production level during the experimentation. At T4, the maximum of lipase activity have been recorded 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.

Solid-state fermentation uses agro-industrial solid residues as a nutrient source and as a support to microorganism growth. The microorganisms that develop in this type of process need to produce several enzymes able to degrade and make available the nutrients present in the culture medium. Among them proteases or proteinases [29], Lipase [17] and cellulase[30]. Depending on the application, those enzymes may be desirable, as in wastewater treatment [31] or in the leather industry, food processing, detergent formulation and other requirements for bioconversion to fuel ethanol [32]. 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 *phialocephalafortinii*. Those fungi are identified as specific to ericaceous plants indigenous to the north of Morocco [2; 33].

Our SSF is composed of sugarcane bagasse as precursor to cellulase production; the wheat bran contains essentially cellulosic fibers as well as proteins. It is the main source of nitrogen for the fungus that it recovers via the production of proteinase, besides, olive oil, rich in lipids, stimulates the production of lipases. Through this experimental design technique, and use of solid-state SSF as nutrient medium, it was possible to determine the enzymatic activities of selected ericoid fungi. In the present investigation, all studied fungi were able to grow and produce cellulase, lipase and proteinase activity using sugarcane bagasse as nutrient, which was confirmed quantitatively with different earlier reagent methods.

Our study demonstrated the ability to produce enzyme activity obviously varied from strain to strain, some being much more efficient than others. Among the four strains, the ericoid mycorrhizal fungi related strain (S2) and *phialocephalafortinii* 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 *Trichodermaharzianum* at SSF that varied from 11 to 50 Ug^{-1}[34]. Reports in the literature suggest that *Trichodermareesei*has the strongest cellulose-degrading activity [35- 36]. Meanwhile, a wide range of *Aspergillus* sp. and *Penicillium*sps have been identified to possess all components of cellulases complex [37-38]. In addition, [39] have shown that the most active producer of cellulolytic enzymes was *Aspergillus. terreus* compared to *Penicillium. tigrinus*, *Penicillium. ostreatus*, *Fusarium. fomentarus*.

Furthermore, the ericoid mycorrhizal fungi related strain (S2), has a significantly better ability to produce lipase activity than other strains, suggesting good adaptation of this strain to the experimental conditions. Besides, the DSErelated 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 [40-41-42-43]. Furthermore, [44-45] 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 (DSE) [46] however, they varied drastically between strains. Furthermore, [47] reviewed observations of enzymatic capabilities of Dark Septate endophyte (DSE) fungi. They reported activities included amylase, cellulase, lipase, pectinase, polyphenol oxidases, protease and xylanase. Therefore, the different aptitudes for enzymatic activity might vary according to different factors. In this context, [48] examined the enzyme activities of *Cenococcumgeophilum*isolates on solid media. They observed variations in enzyme activities of amylase, caseinolysis, gelatinase, and lipase, among isolates whereas cellulase was not detected in isolates.

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*spstrains commonly used in state-solid fermentation or by the composition of the culture medium. Our finding is in agreement with [49] they confirmed that cellulase production depends on many factors. Among them the genes that encode these enzymes, which are strongly suppressed in the presence of a large amount of glucose in the medium. Moreover, [10] showed that many factors such as the pH and some activators like $CaCl_2$ or EDT have a significant effect on alkaline protease activity isolated from *Aspergillusniger* in SSF.

Furthermore, [50- 20] showed that different parameters in SSF such as pH and temperature affect the enzyme activity. [51]added that the fungi group grows well under low moisture condition. [17]showed the interesting feature of the particle size of the medium, the moisture and the water activity that affect negatively fungal growth in SSF. They demonstrated as well the possibility to optimize the production of lipase of *Penicilliumsimplicissimum*from to 23 to 155 Ug^{-1} leading to an increase of 340 % in lipase production using efficient experimental design technique and respecting the optimum culture conditions. It is interesting to note that the enzymes such as the lipase, cellulase have potential applications in agriculture for controlling plant disease and they are commonly cited in the literature as antagonists of plant-pathogenic nematods[52-54], those enzymes are as well involved in enhancing plant growth and development [55].

Conclusions

In the present study, the selected fungal isolates from ericaceous root plants posses enzymatic activities; among these fungal S2 and S4 isolated were noticed to show maximum enzyme activity and they are of interest as producers of lipase activity, which has been scarcely studied for ericaceous plants indigenous to the north of Morocco.

In all cases, the enzymatic activity measured in this work is considered as basal level and important to point out the interesting features of the optimum culture conditions of the obtained isolates which are important characteristics for possible biotechnological applications for the biological control against for instance nematodes. Further research is vital to unravel the full potential of these microorganisms in agriculture.

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