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Evaluation of Various Extracellular Enzymes of Ectomycorrhizal Mushrooms

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Abstract

Fungi are characterized by their abilities to secrete enzymes to the external environment known as extracellular enzymes. In ectomycorrhizal fungi these extracellular enzymes plays an important role in nitrogen and phosphorus uptake. In the present investigation protease, acid phosphatase, chitinase and β-glucosidase activity was estimated by using standard protocols. Maximum acid phosphatase (69.2 ± 4.64 μM $g^{-1}h^{-1}$) and protease (2.34 ± 0.15 μM $g^{-1}min^{-1}$) activities were exhibited by *Suillus triacicularis* isolate DMRX-1625, whereas minimum acid phosphatase (16.99 ± 1.97 μM $g^{-1}h^{-1}$) and protease activities (0.94 ± 0.23 μM $g^{-1}min^{-1}$) were found in *Lyophyllum decastes* isolate DMRX-1593 and *Cortinarius* sp. isolate DMRX 1628, respectively. In case of chitinase, *Rhizopogon* sp. isolate DMRX-1627 showed maximum activity (6.31 ± 0.67 mM $g^{-1}min^{-1}$) and *Suillus triacicularis* isolate DMRX-1600 exhibited minimum activity (1.30 ± 0.14 mM $g^{-1}min^{-1}$). α-glucosidase activity was found maximum in *Suillus sibricus* isolate DMRX-1626 (10.49 ± 0.68 μM $g^{-1}min^{-1}$), whereas β-glucosidase activity was found maximum in *Suillus triacicularis* isolate DMRX-1625 (10.99 ± 3.44 μM $g^{-1}min^{-1}$). *Suillus triacicularis* isolate DMRX-1600 showed the minimum α-glucosidase activity (3.8 ± 0.28 μM $g^{-1}min^{-1}$) as well as β-glucosidase activity (5.54 ± 1.08 μM $g^{-1}min^{-1}$). Present study concludes that ectomycorrhizal mushrooms are potent source of extracellular enzymes.

Keywords: Ectomycorrhizal Mushrooms; Extracellular Enzyme; Acid Phosphatase; Protease; Chitinase

Abbreviations: ECM: Ectomycorrhizal; pNPP: p-Nitrophenyl Phosphate; pNP: p-Nitrophenol; TCA: Trichloroacetic Acid; ANOVA: Analysis of Variance.

Introduction

Fine roots are important compartments of the trees, especially in terms of nutrient and water uptake. Ectomycorrhizal (ECM) fungi symbiotically colonise in these fine roots. Ectomycorrhizal roots are mostly located in nutrient rich top soil upto 20 cm [1]. Ectomycorrhizal fungi also empower nutrient utilization from complex organic sources, which the plant does not have direct access [2,3]. These fungi are able to mobilize nutrients from organic substrates (proteins, amino acids, chitin, phosphomonoesters and phosphodiesters) or nutrients linked to organic residues by secreting extra-cellular enzymes [4-7]. Enzymatic activities of ECM roots play a key role in nutrient mobilisation and transfer them from forest soil and litter via ECM hyphae to their plant host. The extracellular enzymes present in

them directly interact with the environment to which they are released. In forest soils, decaying plant material and humus is the main nutrient source for all soil organisms and the fungi are the most important organisms involved in turnover, degradation and mineralisation of carbon, nitrogen, phosphorous and other mineral elements, making these nutrients available for plants and the soil microflora [8]. The ability to secrete extra-cellular enzymes differs within ECM fungal isolates. Bue´e M, et al. [9] and Courty PE, et al. [10] have also reported such activities in winter, when deciduous trees are leafless. Higher the extracellular enzymes more nutrients are available to trees and better the growth. Considering the above facts, this study was aimed to screen extracellular enzyme production by isolates of different ectomycorrhizal mushrooms.

Materials and Methods

Culture Source and Growth Conditions

Five strains of ectomycorrhizal mushroom *Suillus sibiricus* (Singer) Singer, *S triacicularis* B. Verma & M.S. Reddy, *Rhizopogon* sp., *Lyophyllum decastes* (Fr.) Singer and *Cortinarius* sp. isolated from wild mushrooms collected from the forests of Himachal Pradesh. All the cultures have been deposited in the gene bank of ICAR-DMR Solan under the Acc. No. *S. triacicularis* (DMRX-1625), *S. sibiricus* (DMRX-1626), *Rhizopogon* sp. (DMRX-1627), *Cortinarius* sp. (DMRX-1628), *L. decastes* (DMRX-1593). All the strains were maintained on Modified Melins Nokran's medium with a single mycelial bit and maintained at 25°C in dark for 20 days. After 20 days mycelial mats were collected on preweighed filter paper and dried in hot air oven to obtain a constant weight. The filtrate part was used for enzyme assay using Perkin Elmer-UV/VIS spectrophotometer Lambda 12. Enzyme activity was expressed as $\mu Molg^{-1}min^{-1}$ except phosphatase $\mu Molg^{-1}h^{-1}$ chitinase in $mMolg^{-1}min^{-1}$.

Enzyme Activity Estimation

Acid Phosphatase (ACPase) (EC 3.1.3.2)

Acid phosphatase activity was measured by following the method of Tibbett M [11]. Reaction mixture contained 1 ml culture filtrate and 4 ml of modified universal buffer. These were incubated at 37°C for 5 min prior to the addition of 1 ml substrate [0.115 M disodium p-nitrophenyl phosphate (pNPP) in modified universal buffer (pH 5.5 ± 5)]. Control contained the same volume of buffer without culture filtrate. Assays mixture were incubated for 1 h in water bath at 37°C, after which reactions were stopped by addition of 4 ml of 1 N NaOH to bring the final test tube volumes to 10 ml. The absorbance of liberated p-nitrophenol (pNP), yellow coloured, was measured at 410 nm, against standards made from 0 to 10 mg.

Acid Protease (EC 3.4.23.18)

Protease activity was measured by degradation of casein, 1ml of filtrate was added to 1 ml of 1% (w/v) casein (pH-5.5) and incubated for 1 hour at 30°C. The reaction was stopped by adding protein precipitating agent, 3 ml of 0.5 M trichloroacetic acid (TCA). Solutions were centrifuged at speed of 5000 rpm for 10 minutes and absorption of filtrate was measured at 275 nm [12].

Chitinase (EC 3.2.1.14)

Colloidal Chitin Preparation: Ten gram of commercial chitin was grounded in a mortar and pestle and sieved through a # 40 mesh. The obtained powder was added to 100 ml of 85% phosphoric acid and kept in a refrigerator (5°C) for 24 h. Thereafter, 2 l of tap water was added and the gelatinous white material formed was separated by filtration. The retained cake was washed with tap water until the filtrate had a pH of 6.5. The colloidal chitin obtained had a soft, pasty consistency, with 90–95% moisture [13].

For chitinase estimation, the reaction mixture contained 0.1 ml of culture filtrate and 2.9 ml of a 2% (w/v) suspension of colloidal chitin, in 50 mM acetate buffer (pH 5.5) incubated at 30°C for 10 min. 3 ml of DNS reagent was added to the reaction mixture and incubated at 90°C for 15 min. After incubation, 1 ml of 40% (w/v) potassium sodium tartrate solution was added immediately to stabilize the colour. The mixture was cooled to room temperature, centrifuged at 4000 rpm for 10 min. to remove insoluble chitin, and the colour intensity of resulting supernatant was estimated spectrophotometrically at 575 nm [14].

α- Glucosidase (EC 3.2.1.20)

α- glucosidase activity was done following the method of Bramono K, et al. [15]. Reaction mixture consists of 125 µl of culture filtrate, 500 µl 5 mM pNPG prepared in 1 ml of 0.1M phosphate citrate buffer, incubated at 37°C for 30 min. The reaction was terminated by addition of 2 ml of 0.1 M Na_2CO_3. The colour formed as the result of pNP liberation was measured at 410 nm.

β-Glucosidase (EC 3.2.1.21)

Determination of β-glucosidase activity was done using the method of Cai YJ, et al. [16]. Reaction mixture consists of 0.125 ml of culture filtrate, 0.5 ml pNPG, Incubated at 45°C for 30 min. Reaction was stopped by addition of 1.25 ml sodium carbonate solution. The colour librated was measured at 410 nm.

Statistical Analysis

Three replicates were used for each experiment. The data were analyzed by analysis of variance (ANOVA) and the

means were compared with Tukey's test at $p < 0.05$. All the analyses were performed using Microsoft excel.

Results

Acid Phosphatase Activity: Acid phosphatase activity of different ECM isolates ranged from 16.99 ± 1.97 to 69.2 ± 4.64 µM $pNPg^{-1}h^{-1}$ (Figure 1 & Table 1). *Suillus triacicularis* isolate DMRX-1625 (69.2 ± 4.64 µM $pNPg^{-1}h^{-1}$) showed significantly higher level of ACPase activity followed by *Rhizopogon* sp. isolate DMRX-1627 (52.8 ± 3.86 µM $pNPg^{-1}h^{-1}$), *S. triacicularis* isolate DMRX-1600 (40.55 ± 1.92 µM $pNPg^{-1}h^{-1}$) and *S. sibiricus* isolate DMRX 1626 (39.97 ± 2.78 µM $pNPg^{-1}h^{-1}$). *Cortinarius* sp. isolate DMRX-1628 (17.47 ± 1.74 µM $pNPg^{-1}h^{-1}$) and *L. decastes* isolate DMRX-1593 (16.99 ± 1.97 µM $pNPg^{-1}h^{-1}$) showed significantly lower ACPase activity.

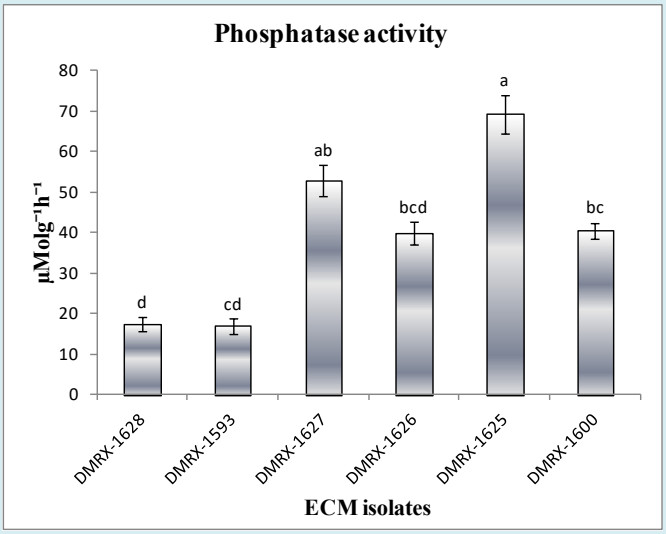

Figure1: Acid phosphatase activity of different ectomycorrhizal fungi in Melin Nokran's broth medium.

Isolate	Acid phosphatase (µMolg^{-1}h^{-1})	Protease (µMolg^{-1} min^{-1})	Chitinase (mMolg^{-1} min^{-1})	α-glucosidase (µMolg^{-1} min^{-1})	β-glucosidase (µMolg^{-1} min^{-1})
DMRX-1628	17.47±0.87d	0.94±0.23d	2.77±0.47b	8.98±1.81ab	6.29±0.72bc
DMRX-1593	16.99±1.97cd	1.1±0.18c	2.27±0.45b	5.8±0.82cd	6.03±1.03bc
DMRX-1627	52.8±3.86ab	1.87±0.29b	6.31±0.67a	6.29±1.39bcd	7.44±0.37abc
DMRX-1626	39.97±2.78bcd	1.46±0.24c	2.52±0.42b	10.49±0.68a	9.44±1.68ab
DMRX-1625	69.2±4.64a	2.34±0.15a	2.46±0.2b	8.24±0.6abc	10.99±3.44a
DMRX-1600	40.55±1.92bc	1.53±0.11c	1.3±0.14b	3.8±0.28d	5.54±1.08c

Table 1: Extracellular enzyme activities of different ectomycorrhizal isolates.

Protease: Protease activity varied form 0.94 ± 0.23 to 2.34 ± 0.15 µM $g^{-1}min^{-1}$ (Figure 2 & Table 1). Protease activity was significantly higher in *S. triacicularis* isolate DMRX-1625 (2.34 ± 0.15 µM $g^{-1}min^{-1}$) followed by *Rhizopogon* sp. isolate DMRX-1627 (1.87 ± 0.29 µM $g^{-1}min^{-1}$) and *S. triacicularis* isolate DMRX-1600 (1.53 ± 0.11 µM $g^{-1}min^{-1}$). *Suillus sibiricus* isolate DMRX-1626 (1.46 ± 0.24 µM $g^{-1}min^{-1}$) and *L. decastes* isolate DMRX-1593 (1.1 ± 0.18 µM $g^{-1}min^{-1}$) exhibit intermediate protease activity. Significantly lower activity was observed in *Cortinarius* sp. isolate DMRX-1628 (0.94 ± 0.23 µM $g^{-1}min^{-1}$).

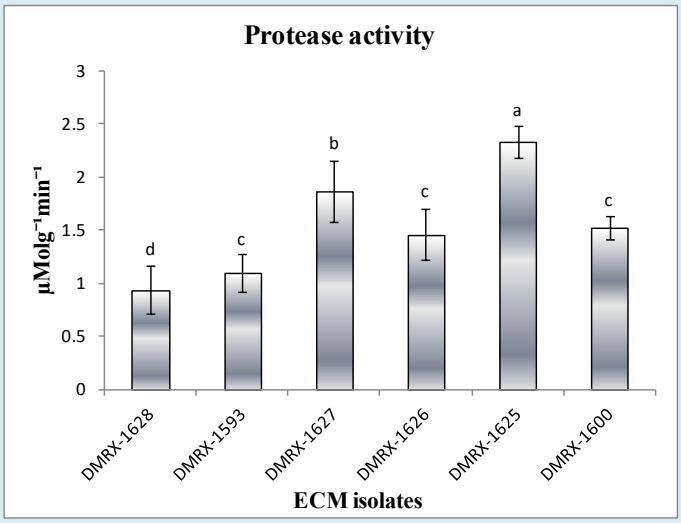

Figure 2: Acid protease activity of different ectomycorrhizal fungi in Melin Nokran's broth medium.

Chitinase Activity: Chitinase activity ranged from 1.3 ± 0.14 mM $g^{-1}min^{-1}$ in *S. triacicularis* isolate DMRX-1600 to 6.31 ± 0.67 mM $g^{-1}min^{-1}$ in *Rhizopogon* sp. isolate DMRX-1627 (Figure 3&Table 1). Among the *Suillus* spp., *S. sibiricus* isolate DMRX-1626 showed maximum activity (2.52 ± 0.42 mM $g^{-1}min^{-1}$) followed by *S. triacicularis* isolate DMRX- 1625 (2.46±0.20 mM $g^{-1}min^{-1}$) and minimum in *S. triacicularis* isolate DMRX-1600 (1.3 ± 0.14 mM $g^{-1}min^{-1}$). *Cortinarius* sp. isolate DMRX 1628 and *L. decastes* isolate DMRX-1593 showed 2.77 ± 0.47 mM $g^{-1}min^{-1}$ and 2.27 ± 0.45 mM $g^{-1}min^{-1}$ chitinase activity, respectively.

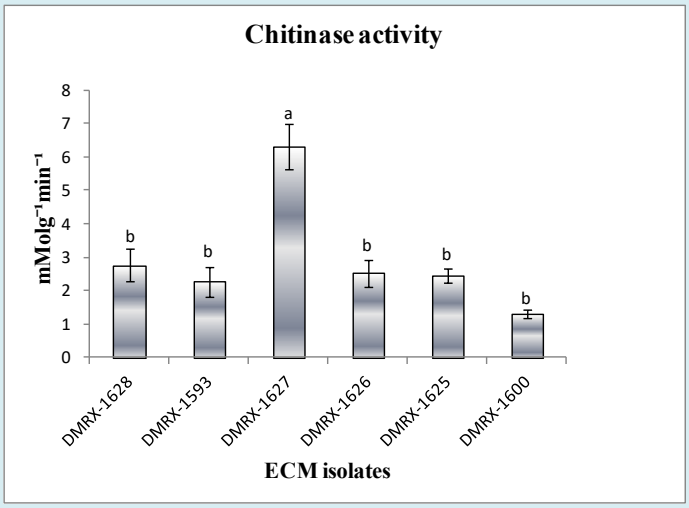

Figure 3: Chitinase activity of different ectomycorrhizal fungi in Melin Nokran's broth medium.

α-Glucosidase Activity: α-glucosidase activity ranged from 3.8 ± 0.28 to 10.49 ± 0.68 µM $pNPg^{-1}min^{-1}$ (Figure 4 & Table 1). The significantly higher activity was observed in *S. sibiricus* isolate DMRX 1626 (10.49 ± 0.68 µM $pNPg^{-1}min^{-1}$) followed by *Cortinarius* sp. isolate DMRX 1628 (8.98 ± 1.81 µM $pNPg^{-1}min^{-1}$), *S. triacicularis* isolate DMRX- 1625 (8.24 ± 0.6 µM $pNPg^{-1}min^{-1}$), *Rhizopogon* sp. isolate DMRX-1627 (6.29 ± 1.39 µM $pNPg^{-1}min^{-1}$) and *L. decastes* isolate DMRX-1593 (5.8 ± 0.82 µM $pNPg^{-1}min^{-1}$). The significantly least activity was observed in *S. triacicularis* isolate DMRX-1600 (3.8 ± 0.28 µM $pNPg^{-1}min^{-1}$).

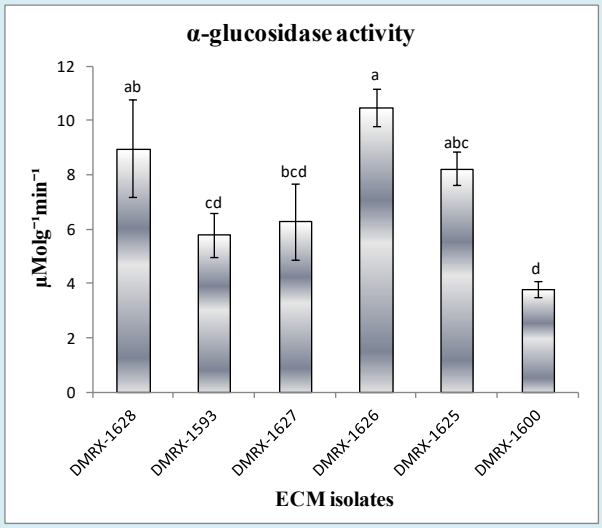

Figure 4: α-glucosidase activity of different ectomycorrhizal fungi in Melin Nokran's broth medium.

β-Glucosidase: Activity of β-glucosidase varied form 6.03 ± 1.03 µM $pNPg^{-1}min^{-1}$ to 10.99 ± 3.44 µM $pNPg^{-1}min^{-1}$ (Figure 5 & Table 1). *Suillus triacicularis* isolate DMRX-1625 exhibited significantly higher activity (10.99 ± 3.44 µM $pNPg^{-1}min^{-1}$) followed by *S. sibiricus* isolate DMRX-1626 (9.44 ± 1.68 µM $pNPg^{-1}min^{-1}$) and *Rhizopogon* sp. isolate DMRX-1627 (7.44 ± 0.37 µM $pNPg^{-1}min^{-1}$). *Cortinarius* sp. isolate DMRX-1628 (6.29 ± 0.72 µM $pNPg^{-1}min^{-1}$) and *L. decastes* isolate DMRX-1593 (6.03 ± 1.03 µM $pNPg^{-1}min^{-1}$) showed intermediate β-glucosidase activity. Least β-glucosidase activity was observed in *S. triacicularis* isolate DMRX-1600 (5.54 ± 1.08 µM $pNPg^{-1}min^{-1}$).

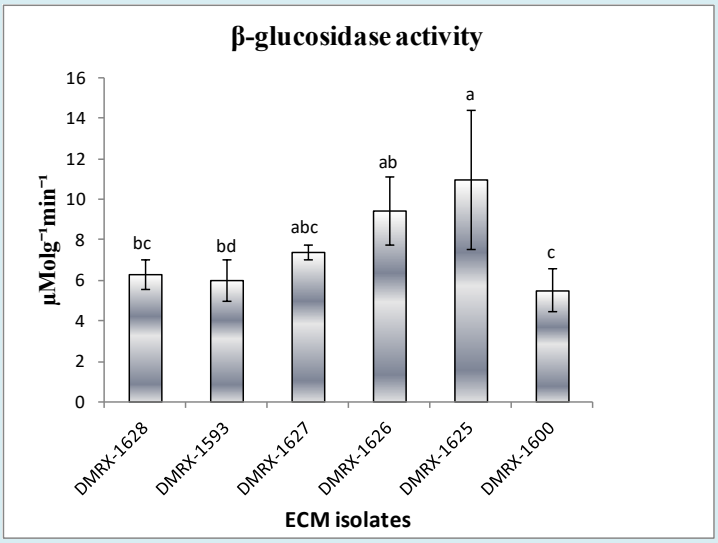

Figure 5: β-glucosidase activity of different ectomycorrhizal fungi in Melin Nokran's broth medium.

Discussion

The findings on the enzymatic activity not always concur because the enzymes were analyzed using different type of samples (fruit bodies, spores, vegetative mycelium, and cultural filtrate) experimental conditions (use of substrate, solid or liquid culture media, incubation temperature, incubation period, indicators, and different pH range) and

different means of determination. The highest phosphatase, protease and β-glucosidase activity was observed in *S. triacicularis* isolate DMRX-1625 while *Rhizopogon* sp. showed maximum activity of chitinase. *S. sibiricus* produced maximum α-glucosidase activity. Ho I, et al. [17] showed the acid phosphatase activity in *Rhizopogon vinicolor* which was 51.7 µmol/g dry weight. Pérez-de-Mora A, et al. [18] worked with enzyme activity of three ECM fungi *Rhizopogon roseolus*, Paxillus involutus and Piloderma croceum using different concentration of phosphorus and activity was observed after 15 days. The activity of phosphatase ranged from 92.9 to 112 µmol/g dry weight for different concentrations. Ho I, et al. [19,] observed the acid and alkaline phosphatase activity on six isolates of Laccaria laccata and showed that the acid phosphatase activity is (36–406 µmol/g dry weight) much higher than alkaline phosphatase activity (2–14µmol/g dry weight) in all the isolates [20].

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