RAPID BIOASSAY FOR EVALUATING ENZYME PRODUCTION IN FUNGAL ISOLATES FROM ENVIRONMENTAL SOURCES

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Introduction

Fungal hydrolytic enzymes have a great potential due to the rapid development of enzyme technology and their industrial applications.
To screen hydrolase-producing microbial strains the fluorimetric method could constitute an effective alternative to the spectrophotometric method.
In fact the substrates used are conjugates of the highly fluorescent compounds 4-methylumbelliferone (MUB) and thus product formation can be measured directly in the microplate without previous extraction and purification of the product.

Objective

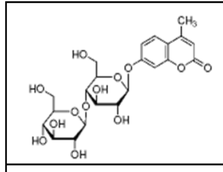
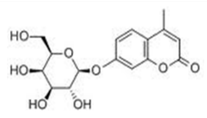
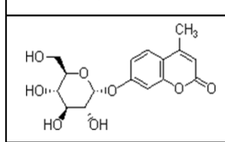
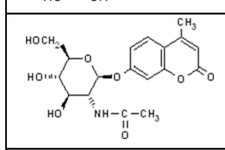
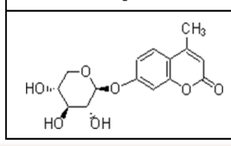
The objective of this work was to develop a rapid method for measuring activity of β-D-exoglucanase, β-D-glucosidase, α-D-glucosidase, β-N-acetyl-exosaminidase and β-D-xylosidase in culture broth samples of nine fungal strains isolated from shrub species *(Tamarix*) that can be used in phytoremediation.

Culture maintanance and enzyme production

Isolates were maintained on a potato-dextrose agar at 30 C, while two liquid media, named (1) and (2), containing Avicell PH 101 as inducer were used for enzymatic production. After inoculum cultures were incubated at 30 C in a rotary shaker at 180 rpm.

Microplate Fluorimetric Enzymatic Assay

Enzymatic activities were measured using microplate fluorimetric enzymatic assays on culture filtrates periodically collected from the broths. Five enzyme substrates based on 4-methylumbelliferone (MU) were used for the detection of five extracellular hydrolytic enzymatic activity. The substrates were dissolved in 20 ul of dimethyl sulfoxide and diluted in sodium acetate trihydrate 0.5 M pH 5.5. The resulting working solutions of 10 µM were kept at 4 C. Each well of the microplate contained: substrate solution, 100 µl; an appropriate dilution of culture filtrate, 20 ul; sodium acetate trihydrate 0.5 M pH 5.5 to 250 µl. Standard curves construction, fluorescence readings and processing data were obtained as described from Marx *et al*. 2001.

Fluorogenic substrate	Target enzyme
 4-methylumbelliferyl β-D-cellobioside (EC 276-744-7)	β-D-exoglucanase
 4-methylumbelliferyl-β-D-glucopyranoside (EC 242-736-7)	β-D-glucosidase
 4-methylumbelliferyl α-D-glucopyranoside (EC 241-794-0)	α-D-glucosidase
 4-methylumbelliferyl N-acetyl-β-D-glucosaminide (EC 253-333-0)	β-N-acetylexosaminidase
 4-methylumbelliferyl-β-D-xylopyranoside (EC 229-784-4)	β-D-xylosidase

Substrate saturation curves

For the choice of the appropriate dilution of culture filtrates were constructed substrate saturation curves using a commercial cellulase purified from *Aspergillus niger*. Six serial dilutions was obtained from a solution containing 2 mg of the enzyme dissolved in 10 ml of sterile water. The dilutions were analyzed through microplate fluorimetric assay as described on the left.

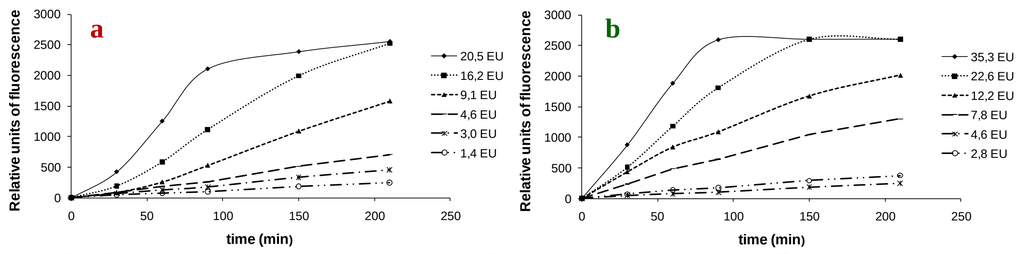


Fig.1 Substrate saturation curves for β-D-exoglucanasic (a) and β-D-glucosidasic (b) activity in solutions with different enzymatic activity obtained using a commercial cellulase purified from *Aspergillus niger*. EU= Enzymatic Units (µmol ml-1 min-1)

Results shown that enzymatic activities higher than 20 EU cause substrate saturation and so must be diluted to be precisely detected from the instrument (Fig.1).

Enzymatic activities in medium (1)

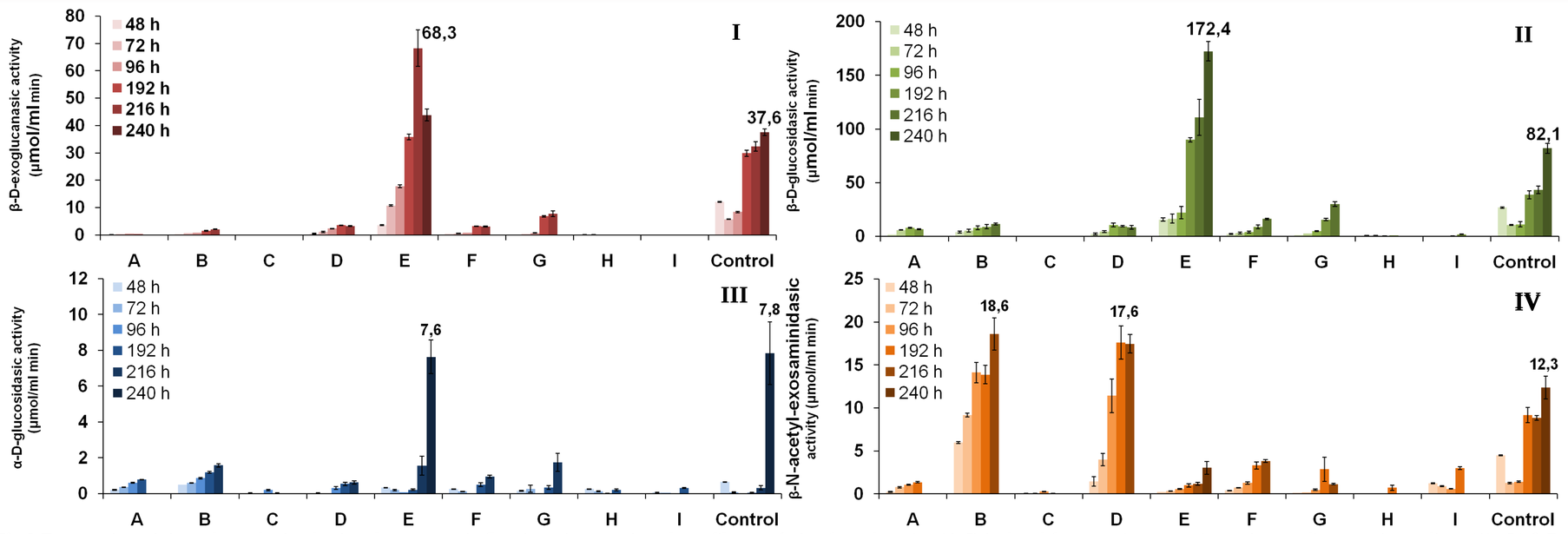


Fig.2 Enzymatic activity values obtained using the enzymatic fluorimatric assay in colture filtrate of nine fungal strains (A-I) isolated from environment (tamarix wood) grown in medium (1). .I) β-D-exoglucanase, II) β-D-glucosidase, III) α-D-glucosidase, IV) β-N-acetyl-exosaminidase (A-I) Control: *Trichoderma viride*; h: hours from inoculum (values reported are the average S.D. of the values obtained in six different experiments)

The composition of the first medium used for enzyme production (modified from Tansey, 1971) was as follows: NH_4NO_3, 2.0 g; K_2HPO_4, 0.4 g; KH_2PO_4, 0.6 g; $MgSO_4$ $7H_20$, 0.8 g ; thiamine, 0.01 g; adenine, 0.004 g; yeast extract, 0.5 g; Avicell PH 101, 5.0 g; distilled water to 1 liter. The final pH was adjusted to 6.5. Results shown that strain E was the most producer of β-D-exoglucanasic, β-D-glucosidasic and α-D-glucosidasic activity, with values higher than the control. For β-N-acetyl-exosaminidasic activity the higher producers resulted instead the strain B and D (Fig.2).

Enzymatic activities in medium (2)

The second medium (modified from Olama *and* Sabry, 1989) was used to further characterize the strain E, resulted the most interesting in previous analysis.
The composition of the medium was as follows: $NaNO_3$, 3.0 g; KCl, 0.5 g; K_2HPO_4, 1.0 g; $MgSO_4$ $7H_20$, 0.5 g ; Fe SO_4, 0.01 g; $CaCl_2$, 1.0 g; yeast extract, 5.0 g; Avicell PH 101, 25.0 g; distilled water to 1 liter. The final pH was adjusted to 7.0. Medium (2) resulted more adapted for enzyme production by strain E since all its enzymatic activities were higher respect to the control and respect to the same strain grown on medium (1) (Fig.3).

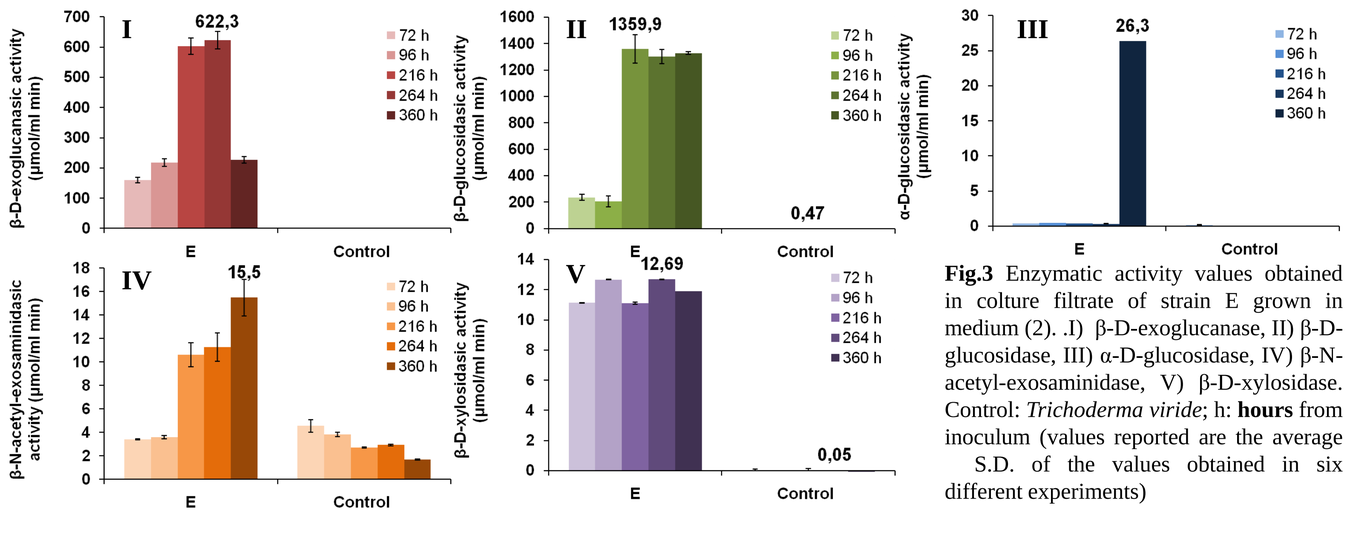


Fig.3 Enzymatic activity values obtained in colture filtrate of strain E grown in medium (2). .I) β-D-exoglucanase, II) β-D-glucosidase, III) α-D-glucosidase, IV) β-N-acetyl-exosaminidase, V) β-D-xylosidase. Control: *Trichoderma viride*; h: **hours** from inoculum (values reported are the average S.D. of the values obtained in six different experiments)

CONCLUSIONS

The microplate fluorimetric enzymatic assay constitutes a rapid and sensitive method for evaluating enzyme production in fungal isolates. Its use allowed to select a fungal strain able to produce high levels of five specific hydrolases respect to the control *Trichoderma viride.* The selected strain is potentially interesting for industrial applications.

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