EVALUATION OF SOME PARAMETERS AFFECTING ENDOGLUCANASE ACTIVITY OF THE FUNGUS *TRICHODERMA REESEI* QM9414

EVALUAREA UNOR FACTORI CE INFLUENȚEAZĂ ACTIVITATEA ENDOGLUCANAZEI SPECIFICĂ FUNGULUI *TRICHODERMA REESEI* QM9414

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Abstract: *Trichoderma reesei is a cellulolytic fungus, well known for its ability to degrade cellulose. The fermentation process for cellulase production is affected by various factors. In this context, we analysed some parameters which influence endoglucanase activity of Trichoderma reesei QM9414: the substrate type and its concentration, the pH of the medium, the nitrogen source and the incubation time. Lignocellulosic biomass, resulting from agricultural practices, such as wheat, barley straws and maize stalks, were used for their great potential as cheap substrate for enzyme production. Endoglucanase activity was higher when Trichoderma reesei QM9414 was grown on medium with wheat straws and maize stalks and at substrate concentration of 40g/L. The addition of some amino acids, namely glutamic acid and asparagine improved endoglucanase activity. The initial pH of the medium is an important factor which shapes endoglucanase activity, Trichoderma reesei displaying an optimum in enzyme activity depending on the nutritional substrate used*
Keywords: *Trichoderma reesei, endoglucanase, wheat straws, barley straws, maize stalks*

Rezumat: *Trichoderma reesei este o ciupercă celulozolitică bine cunoscută pentru capacitatea sa de a degrada celuloza. Procesul de fermentație pentru producerea de celulaze este influențat de diverși factori. În acest context, am analizat o serie de parametri ce modulează activitatea endoglucanazei la specia Trichoderma reesei QM9414: tipul substratului și concentrația acestuia, pH-ul mediului, sursa de azot și timpul de incubare. Biomasa lignocelulozică, ce rezultă din diferite practici agricole, precum paiele de grâu, de orz și cocenii de porumb au fost utilizate pentru potențialul mare de substrat ieftin in producerea de enzime. Activitatea endoglucanazei a fost ridicată când Trichoderma reesei a fost cultivată pe mediu cu paie de grâu și coceni de porumb, la o concentrație de 40g/L. Adiția în mediu a unor aminoacizi precum acidul glutamic și asparagina sporesc activitatea endoglucanazei. PH-ul inițial al mediului reprezintă un factor important ce modelează activitatea endoglucanazei, Trichoderma reesei etalând un optim al activității enzimatice în funcție de substratul utilizat*
Cuvinte cheie: *Trichoderma reesei, endoglucanaza, paie de grâu, paie de orz, coceni de porumb*

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INTRODUCTION

Cellulose is the most abundant renewable resource in the world. Chemical and biochemical conversion is needed to transform cellulose to glucose. Through bioconversion, cellulose is hydrolysed with minimal byproduct formation, low energy requirement and minimal operating conditions (Acebal et. al., 1986). Waste cellulose, resulting from various agricultural practices (e.g. sugar cane bagasse, corn stover and stalks, or wheat, barley and rice straws), industries and as forest residues (e.g., sawdust), represents a promising substrate for cheap enzymatic hydrolysis. A variety of filamentous fungi and bacteria are known to produce enzymes, called cellulases, which are capable to hydrolyse cellulose. Most studied organisms known to produse cellulolytic enzymes include fungi belonging to *Trichoderma, Humicola*, *Penicillium, Aspergillius* and bacteria-*Bacillus, Pseudomonas* (Sukumaran et. al., 2005).

One of the most studied cellulase system is the one produced by *Trichoderma reesei*, a filamentous fungus known for its ability to produce a full set of cellulases involved in the hydrolysis of cellulose to glucose monomers. *T. reesei* produces two cellobiohydrolases, Cel6A (CBH II) (Teeri T. Et. al., 1987) and Cel7A (CBH I) (Shoemaker et. al., 1983a; Teeri et. al., 1983). Also, *T. reesei* produces five endoglucanases, the most studied are Cel7B (EG1) (Penttilä et. al., 1986) and Cel5A (EG II) (Saloheimo et. al., 1988), and also Cel12A (EG III), Cel45A (EG5), and last but not least Cel61A (EG IV). Endoglucanases hydrolyse the cellulase chains internally, providing new chain ends for cellobiohydrolases (Miettinen-Oinonen et. al., 2005). In the fermentation process for cellulase production, environmental and nutritional factors are essential. For example, cellulases are repressed when glucose is present and expressed when the organisms starves or certaing inducers are present (Ilmén, 1997).

In this context, our study aims to analyse various factors that may increase endoglucanase activity of the fungus *Trichoderma reesei*, factors such as: initial pH of the growth environment, nitrogen source (e.g. amino acids), cultivation period and concentration of substrate used in the growth environment.

MATERIAL AND METHOD

Trichoderma reesei was maintained on potato dextrose agar (PDA). To determine endoglucanase activity, the fungus was grown on a liquid medium, distributed in 250 ml Erlenmeyer flasks, each of these containing 100 ml of a modified Mandels medium (Ferreira et. al., 2009).

The influence of the substrate was analyzed by replacing the carbon source-glucose with different concentration of grinded wheat straws, barley straws and maize stalks, resulting four working variants: V1-10 g/L, V2-20 g/L, V3-30 g/L and V4-40 g/L. The influence of the initial pH was analyzed by changing the carbon source-glucose with 30g/L of grinded wheat straws, barley straws and maize stalk, and the initial pH of the medium was modified to 3,4,5,6, and 7, respectively. The influence of amino acids on endoglucanase activity was assayed by replacing the nitrogen source of the Mandels medium, e.g. peptone, urea and ammonium sulfate with the following amino acids: alanine, asparagine, glutamic acid, serine, methionine, histidine, serine and a

control variant without any nitrogen source. To determine the importance of pretreatment on endoglucanase activity, the agro wastes were treated with 1% H_2SO_4, 2% H_2SO_4, 1% NaOH and 2% NaOH, then placed in an autoclave for 30 minutes at 121°C. A control variant was made in which no pretreatment was performed.

Liquid medium was inoculated with 8 mm in diameter discs from the solid medium and incubated at 28°C for 12 days. During this period, 3 ml of culture liquid were withdrawn every three days, and used as enzyme solution. The experiments were carried out in triplicate and mean values were calculated. To estimate endocellulase activity within the collected supernatant carboxymethyl cellulase assay was used, as described by Cojocaru (2008). One unit of cellulase activity (EU) was defined as the amount of enzyme required for liberating 1 mg of reducing sugar per mililiter per minute.

RESULTS AND DISCUSSIONS

It is well known, that several environmental factor affect enzymatic activity of filamentous fungi, thus we assayed how factors such as substrate type and concentration, pH of the growth environment, the nitrogen source represented by several amino acids and chemical pretreatment of the agro wastes used in the experiment influence endoglucanase activity of *Trichoderma reesei*.

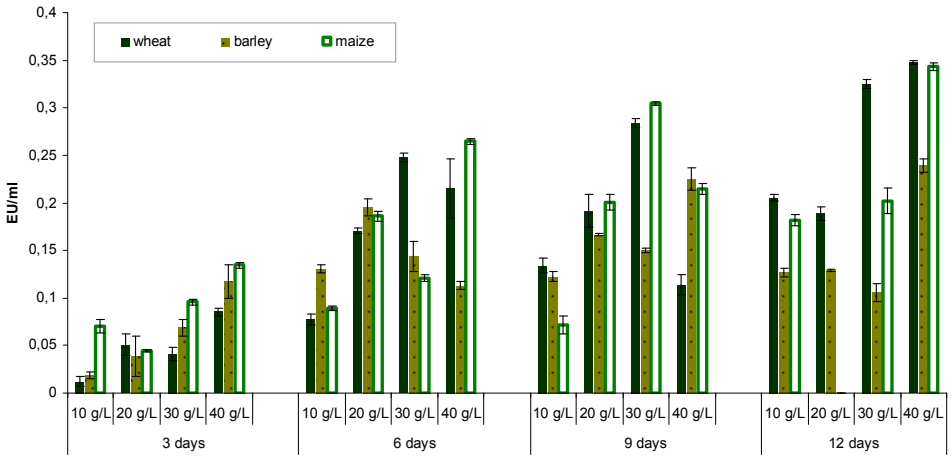

Fig. 1 - The influence of nutritional substrate on endoglucanase activity of *Trichoderma reesei* grown on media with wheat straws, barley straws and maize stalks.

Endoglucanase activity of *Trichoderma reesei* is presented graphically in Figure 1. Enzyme activity shows a variation during the incubation of the fungus. Thus, in the first 3 days endoglucanase activity was lower, increasing progressively in the next 9 days. Maximum activity was reported at a substrate concentration of 40 g/L wheat straws (0,3474 UE/ml) and 40 g/L maize stalks(0,3431 UE/ml).

Depending on the nature of the carbon source, used to improve cellulase activity in *Trichoderma* reesei, different initial pH values of the growth medium may be optimal to induce maximum cellulase yield. As shown in figure 2,

endoglucanase activity was increased at pH values ranging between 3.0-5.0, these values can be correlated with the optimum pH for fungal growth for *Trichoderma* species (Harman and Kubicek, 1998).

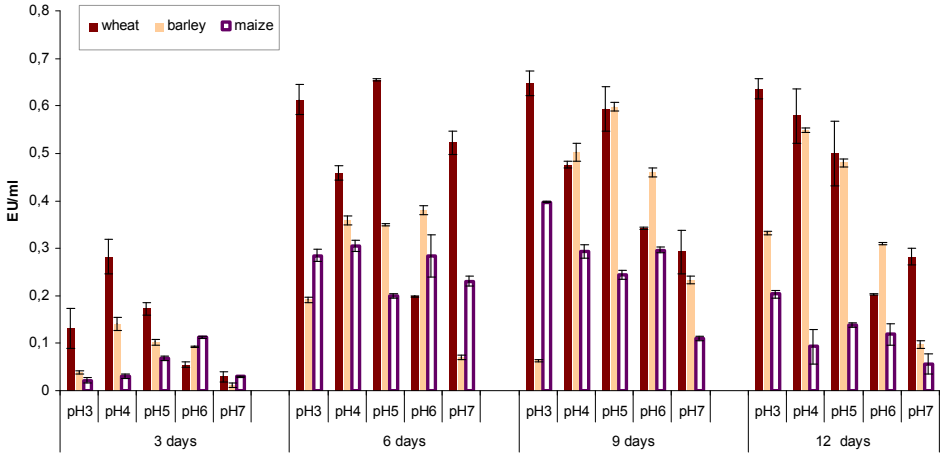

Fig. 2 - The influence of the initial pH of the cultivation medium on endoglucanase activity of *Trichoderma reesei* grown on media with wheat straws, barley straws and maize stalks.

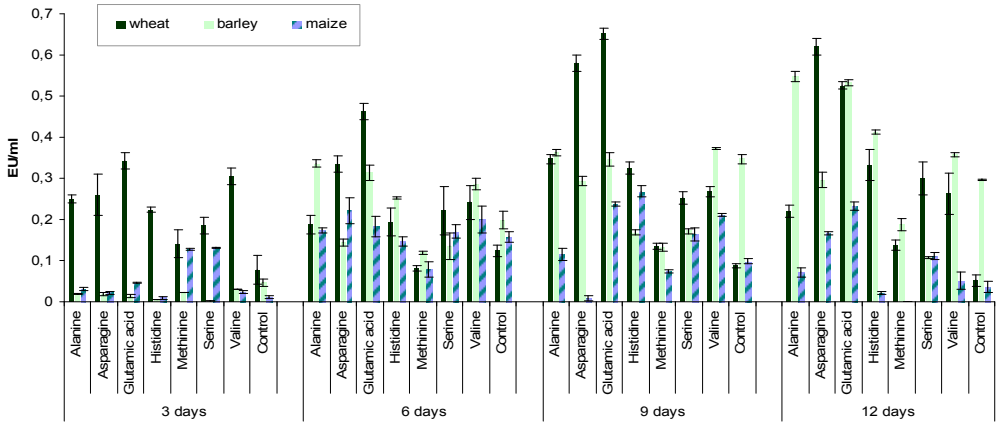

Fig. 3 - The influence of amino acids on endoglucanase activity of *Trichoderma reesei* grown on media with wheat straws, barley straws and maize stalks.

It is known that commercially available endoglucanases produced by *T. reesei* display a optimum activity between a pH of 4.0 to 6.0, and these enzymes are inactive at a higher alkaline pH (Wang et. al., 2005). Since *T.reesei* produces more then one endoglucanase, each has a different pH optimum, ranging between 3.0 and 5.0. Endoglucanase activity decreases by about 50% at pH 7.0 (Karlsson et. al., 2002). In agitated cultures, pH control is essential, and is achieved by adding phosphate buffer solutions. Thus, maintaining a pH greater than 4.0 enhances

cellulase activity in *Trichoderma reesei* RUT C-30 and growing this organisms at pH 5.0 increases endoglucanase activity (Tagnu et. al., 1981).

Cellulase synthesis in *Trichoderma reesei* is reduced unless urea, peptone or ammonium sulphate are added to the growth medium, these substances are known for their ability to stimulate enyzme productivity (Haapala et. al., 1996). We examined if other nitrogen sources, such as amino acids can stimulate endoglucanase activity. Results depicted in Figure 3 underline that by adding in the medium amino acids such as glutamic acid, asparagine, histidine and alanine, endoglucanase activity is increased. In contrast, the addition of methinonine inhibits endoglucanase activity in *Trichoderma reesei*.

Lignocellulosic materials, such as agro wastes, are composed of cellulose, and hemicellulose held together by lignin, which is known to form a barrier against enzymatic attack (Fan et. al., 1982). Through pretreatment, the lignin content and crystallinity of the cellulose is reduced and the surface area is increased (Hatakka 1983). In our study we analised two pretreatment methods: acid hydrolysis, using 1% and 2% H_2SO_4 and alkaline hydrolysis using 1% and 2% NaOH.

Endoglucanase activity was higher when wheat, barley and maize residues were treatead with 2% sulfuric acid instead of 1% (table 1). In a similar manner endoglucanase activity was higher when 2% NaoH was used, compared to 1% NaoH.

Table 1

The influence of some chemical pretreatments applied to the substrate on endoglucanase activity of *Trichoderma reesei*

Treatment	wheat	barley	maize
H_2SO_4 1%	0,2143	0,2957	0,4354
H_2SO_4 2%	0,2628	0,3837	0,5104
NaOH 1%	0,2567	0,2753	0,2764
NaOH 2%	0,2999	0,2787	0,2758
Control	0,3248	0,1499	0,3045

CONCLUSIONS

1. Endoglucanase activity is profoundly influenced by environmental factors. The nutritional substrate, represented by three agro wastes (wheat straws, barley straws, maize stalks) proved to be effective in stimulating endoglucanase activity, of which wheat straws are the most effective, at a concentration of 40 g/L.

2. Favorable pH range for endoglucanase activity in *Trichoderma reesei* is set between 3.0 and 5.0.

3. Some amino acids increase endoglucanase activity, such as glutamic acid, asparagine, histidine and alanine, while methionine causes the opposite effect.

4. Chemical pretreatment of the agro wastes with 2% NaOH and 2% NaOH increases endoglucanase activity.

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