INFLUENCE OF SOME AMINOACIDS ON THE ACTIVITY OF CELLULOLYTIC AND XYLANOLYTIC ENZYMES IN THE FUNGUS *TRICHODERMA REESEI* QM-9414

Mihaela CRISTICA [1], Tamara BARBĂNEAGRĂ [1], Elena CIORNEA [1], Alexandru MANOLIU

e-mail: cristica_mihaela@yahoo.fr

Abstract

Cellulases and hemicellulases are hydrolytic enzymes involved in the conversion of lignocellulose to glucose. Filamentous fungus *Trichoderma reesei* is one of the most known and efficient producers of cellulases and hemicellulases. These enzymes have a huge potential application in the bioconversion of agricultural wastes and production of valuable products that can be used later in different areas. Lignocellulose-degrading enzymes are induced by the presence in the cultivation medium of carbon sources such as cellulose, as well as other culture parameters such as pH, concentration of the inoculation material, nitrogen source used, etc. In this context, this study aims to investigate how different amino acids influence the activity of cellulases and hemicellulases in the fungus *Trichoderma reesei*. Therefore *T. reesei* QM-9414 was grown on medium in which the carbon source was replaced with 30 g / l wheat straw and nitrogen source with a 1 g / l various amino acids: alanine, glutamic acid, methionine, valine, asparagine, histidine and serine. Total cellulase activity, endoglucanase activity, ß-glucosidase and b-xylanase were assayed. The results demonstrate that these enzymes are stimulated by the presence in the culture medium of asparagine and glutamic acid and inhibited by the presence of methionine.

Key words: Trichoderma reesei, cellulase, xylanase, wheat straws

Degradation of lignocellulosic materials is a process which requires the concerted action of various enzymes, acting in a sinergetic manner. Sugars produced through this process can be used as raw materials in a number of biotechnological processes, for instance to obtain ethanol, lactic acid and hydrogen (Lawford, Rousseau, 2003; Hawary şi colab., 2003; Taguchi şi colab., 1996).

Lignocellulosic materials are very cheap and easy available as wastes from different industries and agriculture. Lignocellulose is a rich mixture of carbohydrate polymers (cellulose and hemicellulose), lignin, proteins and other compounds that are found in small amounts (Lee, 1997). *Trichoderma reesei* (syn. *Hypocrea jecorina*), is a filamentous fungus known for its ability to secrete large amount of polysaccharide hydrolizing enzymes (Kubicek et al., 1993).

The cellulase complex in *Trichoderma reesei* QM-9414 consists mainly of three enzymes: endo-β-glucanase (EC 3.2.1.4), cellobiohydrolase (exoglucanase, EC 3.2.1.91) and β-glucosidase (cellobiase, EC 3.2.1.21). Endoglucanases (EGs) randomly cut within the cellulose chains, cellobiohydrolases (CBHS) liberate cellobiose from the ends of cellulose chains and β-glucosidases release glucose from the soluble oligomeric breakdown products.

Also, *Trichoderma reesei* can produce two endo-1,4-β-xylanases (EC 3.2.1.8), known as XYNI and XYNII (Törrönen et al., 1992), both responsable for degrading xylan, one of the most abundant hemicellulose in plants cell walls.

Culture conditions affect significantly the production of cellulases and hemicellulases. One of the factor that plays an important role in enzyme production is the carbon source (Kubicek, Penttilä, 1998). Another factor influencing enzyme activity is the the nitrogen source of the culture medium.

Wheat straws are a cheap carbon source that can be used for cellulase and xylanase synthesis. This byproduct is produced in large quantities in Romania. Chemical composition of wheat straw was analyzed by various authors (Antogiovanni, Sargentini, 1991, Graham, Amman, 1984).

Wheat straws are made up of cellulose (35-45%), hemicellulose (10-30%) and lignin (8-15%; Saha, Cota, 2006). In this context, our study wants to investigate the influence of different amino acids on the activity of cellulase and xylanase from

[1] Faculty of Biology, Al. I. Cuza University, Iaşi
[2] Institute of Biological Research, Iaşi

Trichoderma reesei QM-9414, the fungus was grown on medium in which carbon source is represented by wheat straw.

MATERIAL AND METHODS

Microorganisms. *Trichoderma reesei QM-9414* and was acquired by the Institute of Biological Research, Iaşi from the Institute Scientifique de la Santé Publique, Belgium.

Culture medium. To determine enzyme activity, the fungus was grown in a liquid medium, distributed in 250 ml Erlenmeyer flasks, each of these containing 100 ml of a modified Mandels medium: (Ferreira et al., 2009), with the following composition: 2.0 g/L KH_2PO_4; 1.4 g/L $(NH_4)_2SO_4$; 0.0027 g/L $FeSO_4.7H_2O$; 0.0016 g/l $MnSO_4.H_2O$; 0.0014 g/l $ZnSO_4.H_2O$; 0.0037 g/L $CoCl_2.6H_2O$; 0.6 g/L $MgS0_4.7H_2O$; 0.4 g/l $CaCl_2.2H_2O$; 0.75 g/l peptone; 2.0 ml/L Tween 80; 0.3 g/L urea, 30g/l glucose. In this medium, the carbon source-glucose was replaced with by 30 g/l of wheat straws (straws were previously grinded in an electric grinder to a size less then 1mm), and the nitrogen- $(NH_4)_2SO_4$, urea and peptone was replaced with a quantity of 1g/l of the following amino acids: alanine, glutamic acid, methionine, valine, asparagine, histidine and serine. Also, a control was made without any nitrogen source.

Before being inoculated in the liquid medium, the fungus was grown on a similar solid medium. Solid cultures were maintained at 28°C for 7 days. 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 a source of enzyme. The experiments were carried out in triplicate and mean values were calculated

The filter paper activity, cellobiase and endoglucanase activity was measured by the method develloped by Ghose (1987). For filter paper assay, a mixture of 50 mg of Whatman No. 1 filter paper, 1 ml of 0.05 M citrat buffer and 0.5 ml of appropriately diluted enzyme solution were incubated for 60 min at 50°C. One unit of filter paper activity was defined as the amount of enzyme that forms 1 µmol glucose per minute under the assay conditions.

Endoglucanase (Carboxymethyl cellulase, CMCase) was determined by measuring reducing sugars released in 30 minutes from a mixture of 0,2 ml of diluted enzyme and 1 ml of solution of 1.0 % CMC (dissolved in 0,05 M citrate buffer pH 4.8), incubated at 50°C.

The β-glucosidase activity was assayed in a reaction mixture containing 1 ml of 0,1 % cellobiose solution (prepared in 0,05 M citrate buffer, pH 4.8) and 0,2 diluted enzyme solution, incubated at 50° for 30 minutes. An enzyme unit is defined as the amount of enzyme that forms 1 µmol of glucose per minute from celobiose.

Endo-1, 4-ß-xylanase (EC 3.2.1.8) activity was assayed according to Bailey et al. (1992) using 1% beechwood xylan as substrate for enzyme reaction.

The reaction mixture contained 1 ml of 1% beechwood xylan (Sigma), dissolved in 0.05 mM citrate buffer and 0, 2 ml of enzyme solution. Blanks were also made. The reaction mixture was incubated at 50°C for 10 min. Both samples and blanks were incubated at 50°C for 10 minutes.

The total amount of reducing sugars released from xylan was estimated according to Miller (1959), using D-xylose as a standard.

RESULTS AND DISCOSSIONS

A dynamic profile of cellulase and xylanase activity of the fungus *Trichoderma reesei* QM-9414 was developed and for this purpose enzyme activity was recorded at different intervals, namely at 3, 6, 9 and 12 days after inoculation. We analyzed the effect of the following amino acids: alanine, glutamic acid, methionine, valine, asparagine, histidine and serine on the activity of endo and exoglucanazei, ß-glucosidase and endo-ß-xylanase.

Figure 1 shows graphically the endoglucanase activity of *Trichoderma reesei* QM-9414, cultivated on medium with wheat straws. Some of the amino acids increased endoglucanase activity: glutamic acid (15.816 IU/ml) and asparagine (15.024 IU/ml). The activity was also influenced by alanine (8.216 IU/ml), histidine (8.068 IU/ml) and serine (7.296 IU/ml).

Endoglucanase activity was low in the medium without a nitrogen source (3.017 IU/ml). Low activity was recorded when methionine was used as a nitrogen source (3.41 IU/ml). The dynamic of the activity during the twelve days cultivation period reflects an increase in endoglucanase activity in all the media used, reaching a miximum at 9 days, especially when glutamic acid, asparagine, serine and histidine is used as the nitrogen source.

Figure 2 illustrates the total cellulase activity recorded in *Trichoderma reesei* QM-9414. Enzyme activity was stimulated by the presence in the culture medium of glutamic acid and asparagine, activity values reaching 0.329 IU/ml and 0.264 IU/ml. Culture media with alanine, valine and histidine showed similar values of 0.184 IU/ml, 0.182 IU/ml and 0.167 IU/ml.

The lowest values were recorded on medium with methionine (0.063 IU/ml). The graph shows an increase of total cellulase activity in the early days, followed by a slight decrease and a maximum in the last interval. This statement is valid for media with glutamic acid, valine, asparagine, histidine and methionine.

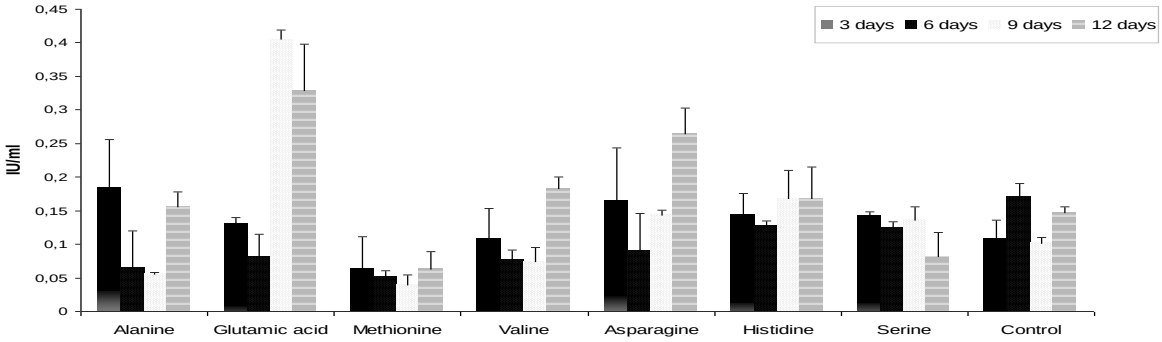

Figure 1 **Endoglucanase activity in fungus *Trichoderma reesei* QM-9414**

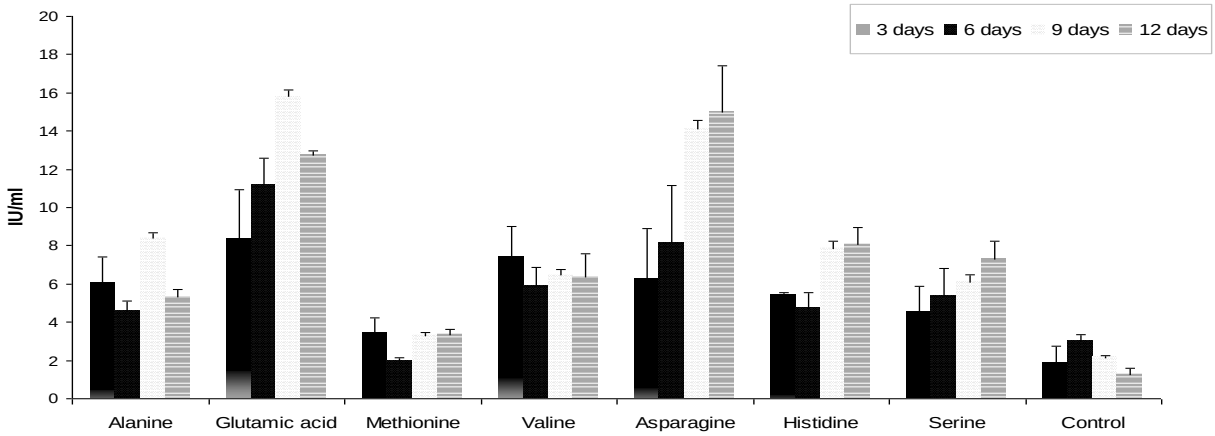

Figure 2 **FPase activity of *Trichoderma ree*sei QM-9414**

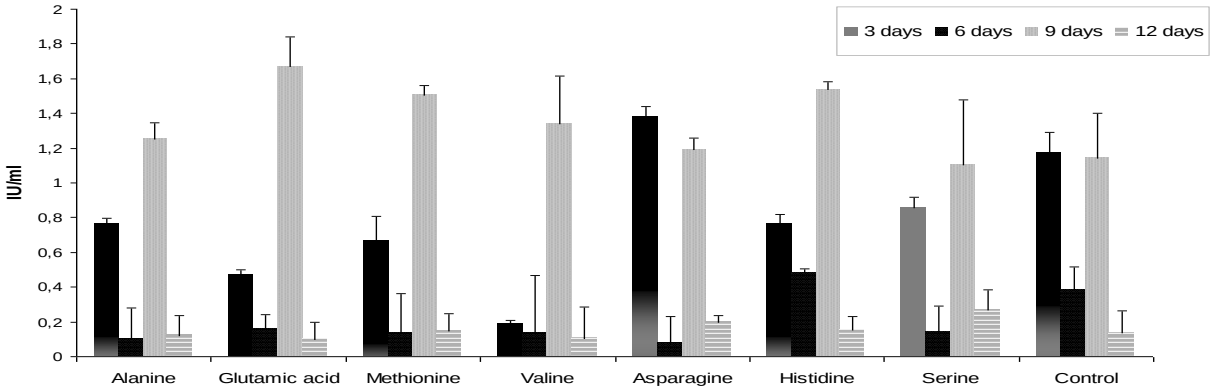

Figure 3 **ß-glucosidase activity in the fungus *Trichoderma reesei* QM-9414**

The influence of amino acids on ß-glucosidase activity in *Trichoderma reesei* QM-9414 is depicted in figure 3. Enzyme activity was stimulated by the presence in the medium of histidine and glutamic acid, both reached values of 1.668 IU/ml and 1.541 IU/ml. Enzyme activity was also high in medium with methionine (1.506 IU/ml) and alanine (1.251 IU/ml).

As it results from the graph, ß-glucosidase activity was stimulated by nearly all amino acids, and it had a high activity in the control sample (1.178 IU/ml). Looking at the dynamic of this enzyme, we see an increase in the first period of cultivation, a slight decrease in the next period, followed by a considerable increase at 9 days. At 12 days the values almost doubled compared to the previous records.

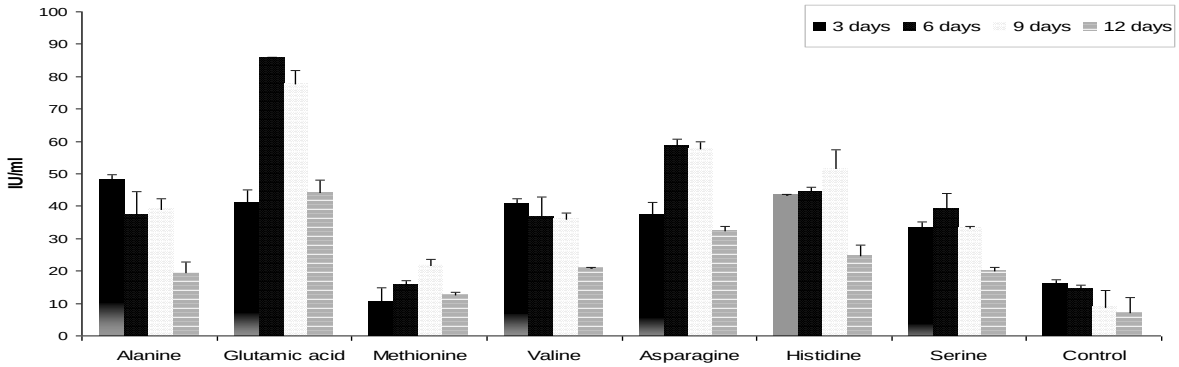

Figure 4. **ß-xylanase activity of *Trichoderma reesei* QM-9414**

Figure 4 illustrates ß-xylanase activity. Enzyme activity was stimulated by the use as nitrogen source of glutamic acid (85.598 IU/ml) and asparagine (58.456 IU/ml). Similar values were recorded in media with alanine (48.211 IU/ml), serine (39.262 IU / ml) and valine (40.629 IU/ml). The lowest values were recorded in medium with methionine (15.697 IU/ml) and in control sample (15.915 IU/ml).

The graph shows that ß-xylanase activity fluctuated during the 12 days of cultivation. In media with glutamic acid, serine and asparagine, enzyme activity peaked at 6 days after inoculation. ß-xylanase activity increased in the first period in the cultivation medium with alanine and serine. The activity decreased progressively until the fourth period.

CONCLUSIONS

The addition in the culture medium of glutamic acid and asparagine has a stimulating effect on cellulase and ß-xylanase activity of the filamentous fungi *Trichoderma reesei* QM-9414.

In contrast, the presence of methionine in the culture medium causes a decrease in enzyme activity. Low activity was also recorded in the control sample.

The low values in endoglucanase activity, ß-xylanase and exoglucanase of the control sample may indicate the need for the presence in the culture medium of at least one nitrogen source.

Enzyme activity varied during the 12 days of cultivation, depending on the amino acid present in the culture medium.

ACKWLEDGEMENTS

This work was supported by the the European Social Fund in Romania, under the responsibility of the Managing Authority for the Sectoral Operational Programme for Human Resources Development 2007-2013 [grant POSDRU/107/1.5/S/78342

REFERENCES

Antongiovanni, M., Sargentini, C., 1991- *Variability in chemical composition of straw*, Opt. Meditterr., Ser. Semin., Num. 16, 49-53.

Bailey, M.J., Baily, P., Poutanen, R., 1992 – *Interlaboratory testing of methods fro assay of xylanase activity*. J. Biotechnol., 23: 257-270

El-Hawary, F.I., Mostafa, Y.S., Laszlo, E., 2003 – *Cellulase production and conversion of rice straws to lactic acid by simultaneous saccharification and fermentation*, Acta Aliment. Hung., 30:281-95.

Fereirra, S.M.P, Duarte A.P, Queiroz, J.A, Domingues, F.C., 2009 – *Influence of buffer system on Trichoderma reesei RUT C-30 morphology and cellulase production***.** Electronic Journal of Biotechnology. 12(3):1-9

Graham, H., and P. Aman, 1984 –*A comparison between degradation of in vitro and in sacco of constituents of untreated and ammonia treated barley straw*. Anim. Feed Sci. Technol. 10:199.

Ghose, T.K., 1987 – *Measurement of cellulase a*ctivity. Pure and Appl. Chem., 59: 257-268

Kubicek, C.P., Messner, R., Gruber, F., Mach, R.L., Kubicek-Pranz, E.M., 1993 – *The Trichoderma cellulase regulatory puzzle: from the interior life of a secretory fungus*, Enzyme Microb. Technol., 15(2):90-99.

Kubicek, C.P., Penttila, 1998 – *Trichoderma and Gliocladium, Enzymes, biological control and commercial applications,* vol. 2, Bristol, p. 49-67.

Lawford, H.G., Rousseau, J.D., 2003 – *Cellulosic fuel ethanol-alternative fermentation process designs with wild-type and recombinant Zymomonas mobilis*, Appl. Biochem. Biotechnol. 105:457-69.

Lee, J., 1997 – *Biological conversion of lignocellulosic biomass to ethanol*, J. Biotechnol., 56 (1): 1-24

Miller, G.L., 1959 – *Use of dinitrosalicilyc acid reagent for determination of reducing sugars*, Ann. Chem., 31: 426-428.

Saha, M., Cotta, A., 2006 - *Ethanol Production from Alkaline peroxide pretreated enzymaticallz saccharified wheat straw,* Biotechnol. Prog., 22, 449-453.

Taguchi, F., Yamada, K., TakiSaito, T., Hara, K., 1996 – *Continuous hydrogen production by Clostridium sp. strain no 2 from cellulose hydrosalysate in an aqueous two-phase system*, J. Ferm. Bioeng., 82:80-3.