CARVALHO, W. R.; BASTOS, F. M.; FARIA, F. P. Production of xylanolitic enzymes by the thermophilic fungus *Humicola grisea* var. *thermoidea*. In: CONGRESSO DE PESQUISA, ENSINO E EXTENSÃO DA UFG – CONPEEX, 2., 2005, Goiânia. **Anais eletrônicos do XIII Seminário de Iniciação Cientifica** [CD-ROM], Goiânia: UFG, 2005. n.p.

PRODUCTION OF XYLANOLITIC ENZYMES BY THE THERMOPHILIC FUNGUS *Humicola grisea* var. *thermoidea*

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Key words: Heterologous expression, *Humicola grisea*, *Pichia pastoris*, Xylanases – production and purification.

1. INTRODUCTION

Xylan is the major hemicellulose present in the cell wall of plants and its complete hydrolysis to monosaccharide occurs through the combined action of enzymes as exoxylanases, endoxylanases and β-D-xylosidases (Subramaniyan & Prema, 2002; Sunna & Antranikian, 1997). Interest in xylanases has increased in the last decade due to their biotechnological applications in paper, pharmaceutical, food and feed industry (Polizeli *et al*., 2005). For successful selection of xylanases suitable for specific industrial applications it is important to characterize xylan-degrading enzymes isolated from different sources. The thermophilic fungus *Humicola grisea* var. *thermoidea* isolated from Brazilian soil is described as a good producer of extracellular xylanases. Faria *et al.* (2000) demonstrated that *H. grisea* grown on sugar-cane-bagasse (SCB) secretes at least three different xylanases with 23, 25 and 35 kDa and the 23⁄25 kDa xylanase presents the main activity band in Zimogram gel. Carvalho (2003) analyzed the *H. grisea* xylanase production in different substrates and showed that the best inducer was SCB, from the growth in this substrate, the 23 kDa xylanase was isolated as an enzyme stable in pH 5.0 and 65ºC. The gene coding of the 23 kDa xylanase (*hxyn*2), isolated and expressed in *Pichia pastoris* under methanol control, was an extracellular protein active against oat-spelt xylan (Faria *et al*., 2000, 2002; Bergquist, 2002a, b). The HXYN2r xylanase produced by *P. pastoris* was used in paper industry with success, showing the biotechnological potential from *H. grisea* xylanases (Gonçalves *et al*., 2005).

In order to study the *H. grisea* xylanases and their biotechonological applications, this work has the aim of establishing the xylanases production in SCB by *H. grisea* and HXYN2r production by *P. pastoris*.

2. METHODOLOGY

2.1 – *H. grisea* xylanases production

The wild-type strain of *H. grisea* was grown on 4% (w/v) of oatmeal baby food (Quaker) medium with 1.5% (w/v) agar at 42ºC for 4 days. Conidia harvested ($0.35x10^6$ spores/mL) was inoculated in 100 mL of minimal medium ($NaNO_3$-0.6%(w/v); KCl-0.05%(w/v); KH_2PO_4-0.15%(w/v); $ZnSO_4$-0.001%(w/v) and $FeSO_4$-0.001%(w/v)) with pH 6.8, plus 1.0% SCB, 0.05% yeast extract and $(NH_4)_2SO_4$-1:1 (nitrogen source), and incubated in 250 mL Erlenmeyer flasks at 42ºC/120 rpm. Samples were taken out at each 24 h and analyzed in relation to xylanase activity by reducing sugars method (DNS) (Miller, 1959) and the proteins profile was analyzed by SDS-PAGE, Zymogram assay and two-dimensional gel**.** To optimize the xylanase

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production it was performed a 2^2 factorial design involving two concentrations for each factor (carbon - 1% and 0.5%; yeast extract and $(NH_4)_2SO_4$-1:1 (nitrogen source) – 0.25% and 0.05%) with three center points. The software Statgraphics Plus for Windows version 5.0 was used to evaluate and to compare the data.

2.2 – HXYN2r plate assays and production optimization

The clones of *P. pastoris* were chosen by activity plate assays in the mediums BMGY-U/BMMY-U (1.34% Urea, 1% yeast extract, 2% peptone), BMGY-L/BMMY-L (2.34% yeast extract, 2% peptone), BMG-U/BMM-U (1.34% urea), BMG-L/BMM-L(1.34% yeast extract) plus 100mM potassium phosphate pH 5.0, $4x10^{-5}$ % biotin, 1% glycerol or 0.5% methanol and 50 µg/mL ampicilyn for each medium. To enzymatic induction, the clones were pre-inoculated in the medium with glycerol and inoculated to a final cellular density equivalent to OD_{600} of 1 in the medium with methanol, at 30ºC for 3 days. The optimization of HXYN2r production was obtained using an experimental design 2^3, varying the nitrogen sources concentration (2.34% and 4.68%), the methanol concentration (0.5% and 1%), and the initial OD (1 and 10) in the medium BMMY-U. Samples were taken out at each 24 h and the enzymatic activity was analyzed by reducing sugars method and the proteins profile was analyzed by SDS-PAGE and Zimogram assay.

3. RESULTS AND DISCUSSION

3.1 – Xylanases production by *H. grisea* in SCB

Experiments with a 2^2 factorial design was carried out and the results showed, after 96 h of cultive, the maximum peak of xylanase production, using concentrations of 1% SCB and 0.25% nitrogen source (12.8 U/mL). Pareto chart was used to compare the amount of xylanase produced in all media and clearly shows that only the nitrogen source affected the xylanase production while SCB did not have significant effect. This result is according to that for other microorganisms, where the main impact on growth is generally the nitrogen source. The secreted proteins were analyzed by Zymogram assay and showed three different xylanase activity bands with 35, 25/23 (main activity band) and 14 kDa. The two-dimensional gel to activity was performed and was observed at least one halo of xylanase activity with 23 kDa and pI 6.1.

3.2 – HXYN2r plate assays and production optimization

The clones with the best xylanolytic activities in plate were 12.3, 43.1, and 43.4 and the best cultivation medium in plate was BMM-U, followed by BMM-L and BMMY-U. On the kinetic of xylanase production in flakes, the growth in the medium BMM-U showed an insignificant xylanolytic activity. The results with BMMY-U medium showed bigger xylanolytic activity than with BMM-L medium and, the highest xylanolytic activity was 409.2 U/mL, obtained by 12.3 transformant after 120 h of cultivation. These results were much higher than the ones obtained with the same clones in YNB medium (Banhe *et al.*, 2004).

The best result obtained by optimization with the BMMY-U medium was 478.2 U/mL using 2.34% of nitrogen sources, 1% of methanol, and OD of 10 and, only the nitrogen source was significant at a confidence level of 95%. The 23 kDa protein was active against oat-spelt xylan in Zymogram assay.

4. CONCLUSION

The xylanolytic enzymes were produced by *H. grisea* and, the maximum peak of xylanase production was detected after 96 h of cultivation, using 1% BCA and

0.25% yeast extract-ammonium sulphate (1:1); it was also observed three different xylanase activity bands in Zimogram assay.

The results showed that the best conditions for HXYN2r production were growing the 12.3 transformant in the BMMY-U medium using an initial OD_{600} of 10, 2.34% of nitrogen sources and 1.0% methanol and that HXYN2r was the predominant enzyme in the culture supernatant.

5. REFERENCES

BANHE, A. A.; Moraes, L. P. & Faria, F. P. Expressão do Gene HXYN2 em *Pichia Pastoris*: Análise da estabilidade genética dos transformantes. Trabalho apresentado na XXIV Reunião de Genética de Microrganismos, Gramado – RS, 2004.

BERGQUIST, P. L.; Te'o, V.S.J.; Gibbs, M. D.; Cziferszky, A. C. E.; Faria, F. P.; Azevedo, M. O. & Nevalainen, K. M. *H.* Expression of xylanase enzymes from thermophilic microorganisms in fungal hosts. Extremophiles, 6:3, 2002a.

BERGQUIST, P. L.; Te'o, V.S.J.; Gibbs, M. D.; Cziferszky, A. C. E.; Faria, F. P.; Azevedo, M. O. & Nevalainen, K. M. *H.* Production of recombinant bleaching enzymes from thermophilic microorganisms in fungal hosts. Applied Biochemistry and Biotechnology, 98-100:165-176, 2002b.

CARVALHO, W. R. Produção e Purificação da Xilanase de 23 kDa do Fungo Termofílico *H. grisea* var. *thermoidea*. Dissertação de mestrado em Biotecnologia Industrial, FAENQUIL/DEBIQ, 2003.

FARIA, F. P.; Téo, V. S. J.; Azevedo, M. O. & Nevalainen, H. Expression and processing of a major xylanase (XYN2) from the thermophilic fungus *Humicola grisea var*. thermoidea in *Trichoderma reesei*. Lett. in Appl. Microbiol., 34:119-123, 2002.

FARIA, F. P. Clonagem, Caracterização e Expressão do gene de xilanase *Hxyn*2 do fungo *Humicola grisea* var. *thermoidea*. Tese de doutorado em Biologia Molecular, UnB, 2000.

GONÇALVES, A. R., Moriya, R. Y. and Faria, F. P. Enzymatic bleaching of the organosolv sugarcane bagasse pulps with recombinant xylanase of the fungus *Humicola grisea* and with commercial Cartazyme HS xylanase. Applied Biochemistry and Biotechnology, 121-124:195-204, 2005.

MILLER, G. L. Use of dinitrossalicylic acid reagent for the determination of reducing sugars. Analytical Chem. 31:426-428, 1959.

POLIZELI, M. L.; Rizzatti, A.C.; Monti, R.; Terenzi, H. F.; Jorge J. A. & Amorim, D. S. Xylanases from fungi: properties and industrial applications. Appl Microbiol Biotechnol, 67:577-591, 2005.

Subramaniyan, S. & Prema, P. Biotechnology of Microbial Xylanases: Enzymology, Molecular Biology, and Application. Critical Reviews in Biotechnol., 22(1):33-64, 2002.

SUNNA, A. & Antranikian, G. Xylanolytic enzymes from fungi and bacteria. Critical Reviews in Biotechnology,17:39-67, 1997.

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