Biological treatment of solid wastes from the tobacco industry for enzyme production

A. I. Oliveira[1], C. Curbelo[2], G. M. Alvarez[3], A. A. Vicente[1], J.A. Teixeira[1]

* Email: aiolandaoliveira@gmail.com

1 IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Campus Gualtar, 4710-057 Braga, Portugal

2 Chemical Engineering Faculty, Instituto Superior Politécnico "José Antonio Echeverría". Habana, Cuba

3 Instituto Cubano de investigación de los derivados de la caña de azúcar (ICIDCA), Habana, Cuba

Abstract

Aiming at the production of enzymes using solid wastes from the tobacco industry, the solid fermentation kinetics of *Aspergillus niger* and *Aspergillus terreus* using waste of dark tobacco and Virginia tobacco as substrate were characterized.

The efficiency of the fermentation process was evaluated by determining the enzymatic activity of the three enzymes that constitute the cellulose enzymatic system (CMCase, PFase and Xylanase).

The results obtained led to the establishment of the best initial conditions of fermentation and the selection of the most efficient microorganism for enzyme production. The best results were obtained with *Aspergillus terreus* for both tobacco residues. In the case of black tobacco, the best incubation temperature was 31 ºC for the enzymes CMCase and Xylanase and 36 ºC for the PFase and initial pH 5.5 for the three enzymes. For the Virginia tobacco, the best incubation temperature and initial pH are the same for the three enzymes, 36 ºC and 5.5 respectively.

The biological activity of the fermented tobacco residues was evaluated being the highest rate of inhibition of microbial growth – 72% - obtained with the residue of Virginia tobacco treated with *Aspergillus niger.*

Key words: Biological treatment of solid wastes, *Aspergillus niger*, *Aspergillus terreus,* dark tobacco and Virginia tobacco, solid state fermentation, enzymatic system cellulase (CMCase, PFase and Xilanase).

Resumo

De forma a atingir os objectivos propostos neste trabalho nomeadamente o tratamento biológico de resíduos da indústria tabaqueira para a produção de enzimas, foi realizado um estudo da cinética de fermentação em estado sólido dos resíduos de tabaco negro e rubio pelos microrganismos *Aspergillus niger* e *Aspergillus terreus*.

Para testar a eficiência dos processos de fermentação foi determinada a actividade enzimática através da aplicação de técnicas específicas para cada uma das três enzimas que constituem o sistema enzimático cellulase (CMCase, PFase e Xilanase).

Os resultados obtidos ao longo do estudo levaram ao estabelecimento das melhores condições iniciais de fermentação e qual o microrganismo que permite a obtenção de uma maior actividade enzimática. O microrganismo que obteve melhores resultados foi o *Aspergillus terreus* para ambos os resíduos tabaqueiros, no caso do tabaco negro com temperatura de incubação igual a 31 ºC para as enzimas CMCase e Xilanase, 36 ºC para a PFase e pH inicial de 5.5 para as três enzimas; relativamente ao tabaco rubio a temperatura de incubação e o pH inicial são o mesmo para as três enzimas sendo iguais a 36 ºC e 5.5 respectivamente.

Os resíduos tabaqueiros depois de tratados biologicamente foram submetidos a testes, no Instituto Cubano

de Investigação do Derivados de Cana-de-açúcar (ICIDCA), para verificar se possuíam actividade biológica, tendo a maior taxa de inibição de crescimento microbial (72%) sido conseguida com o resíduo de tabaco rubio tratado com *Aspergillus niger.*

Palavras-chave: Tratamento biológico de resíduos, tabaco negro e rubio, fermentação em estado sólido, *Aspergillus niger*, *Aspergillus terreus,* sistema enzimático cellulase (CMCase, PFase, Xila-nase).

Introduction

The production of enzymes is one of the most important industrial biotechnological processes. In most of the cases, enzymes are produced from a microbial source due to the high diversity that is possible to obtain from this mode of production and also due to the operational and economical difficulties that the enzyme extraction process from animal and vegetable tissues presents [1].

The application of enzymes in industries such as chemical, pharmaceutical and feed, replacing the conventional chemical catalyst, is becoming more frequent. This can be explained by the several advantages of this processes, such as higher efficiency under moderated temperature and pressure conditions, environmental pollution reduction and less secondary products formation, as result of their selectivity. About 400 companies all over the world are involved in the production of enzymes, Europe being the main producer (approximately 60 %) followed by United States of America and Japan [1].Worldwide enzyme market moves about 1.38 billions of Euros per year, with an annual increase rate of 8 % to 10%.

Nowadays, cellulase, hemicellulase and pectinase enzymes represent about 20 % of the enzymes production all over the world [2], most of them from *Trichoderma* and *Aspergillus* species [1].

The high cost and low yield of the production process of these enzymes are the major problems for their industrial application. In order to get over these difficulties several microbial strains with a high production yield are needed together with fermentation parameters optimization.

Among the several materials used as biomass, lignocellulosic biomass has been receiving a particular interest due to the reduced price and great availability. The solid state fermentation offers advantages when compared with submerged fermentation in what concerns the obtention of biomolecules from these materials.

The Cuban industries of tobacco produce annually about 4000 metric tons of solid residues rich in cellulose, hemicelluloses and lignin, that can't be used in the production of "torcidos" and "cigarrilhos". These residues can be subjected to several treatments to increase the tobacco economical value and avoid the deteriorating oh the environment [3] [4].

Material and Methods

Microorganism

The microorganisms used in this work were *Aspergillus niger* specie J-1, isolated from sugar cane bagasse, from the Collection of Chemical Engineering Faculty of the ISPJAE and *Aspergillus terreus* specie H/6.39.3, from the collection of the ICIDCA.

Aspergillus niger was maintained in sterilized malt agar and *A.terreus* in Czapck-Dox agar. The microorganisms were inoculated in erlenmeyers and incubated during 7 days at 30ºC for the obtention of the spores solution for further inoculation.

Fermentation conditions

4 black tobacco fermentations with *Aspergillus niger* were done with an initial moisture content of 70%, pH 5.5 and incubation temperature 37ºC. These conditions were selected taking in account the study carried out by Quesada C.(2004)[3].
The study by Quesada C. (2004) [3] was also used as the reference work for the fermentation experiments of both residues by *Aspergillus terreus*. Fermentations were carried out with the following initial conditions: 70 % of moisture and pH 7 or 5.5 with incubation temperatures of 31 ºC and 36 ºC.

Enzymatic activity determination
1 U of CMCase, defined as the amount of enzyme that liberates 1 µmol of reducing sugar per minute, was determined under the following conditions: 1% solution of carboxymetilcelulose in a 0.1 M sodium citrate buffer, temperature of 50 ºC, pH of 4.8 and time of reaction equal to 30 minutes.
1 U of Pfase defined as the amount of enzyme that liberates 1 µmol of reducing sugar per minute, was determined in the following conditions: 50 mg of filter paper Whatman #1 in a 0.075 M sodium citrate buffer solution, temperature of 50 ºC, pH of 4.8 and time of reaction equal to 60 minutes.
1 U of Xylanase defined as the amount of enzyme that liberates 1 µmol of reducing sugar per minute , was determined in the following conditions: 1.0 g of xylan in 500 ml of 0.1 M NaOH, temperature of 50 ºC, pH of 4.8 and time of reaction equal to 20 minutes.

Biological activity determination
The determination of the percentage of inhibition of pathogenic mold growth, in this in case *Alternaria solani*, was done as follows: the mold was incubated in a Petri plate in 15 ml of Potato Dextrose Agar PDA) that has been added 10 mL of the fermentation sample (after filtration through a 0,2 µm membrane). A control experiment, in the absence of the fermentation sample, was also made.

Results
In all the fermentations of black tobacco dust by carried *Aspergillus niger*, the obtained values for the assayed enzymatic activities were very low, even when the black tobacco dust was treated according to the procedure described by Dustet (1999) [5] .

In what concerns the fermentation experiments of the black tobacco and Virginia tobacco dust by *Aspergillus terreus*, residual values for enzymatic activity were obtained for both substrates at pH 7 and T 31 ºC. It is interesting to notice that a CMCase value of 0.2128 U/mL was obtained at the third day of fermentation, when using Virginia tobacco dust.
When the initial conditions were pH 5.5 and T 31 ºC, the obtained PFase enzyme activity values are very low for both wastes, even if there is a significant improvement in the case of black tobacco. In what concerns CMCase and xylanase activities a significant increase also occurs. The obtained maximum values of enzymatic activity using the black tobacco dust were 0.0154 U/mL at 72 hours of fermentation for the PFase, 0.1488 U/mL at 72 hours of fermentation for xylanase and 0.4163 U/mL at 96 hours of fermentation for CMCase. For the Virginia tobacco waste, the obtained maximum values of enzymatic activity were 0.3729 U/mL and 0.1148 U/mL for CMCase and xylanase, respectively, at 120 hours of fermentation and 0.0281 U/mL at 72 hours for the PFase.
Using as initial conditions pH 5.5 and T 36 ºC, the maximum values of enzymatic activity for the black tobacco dust are 0.0304 U/mL at 144 hours of fermentation for PFase, 0.1464 U/mL at 120 fermentation hours for xylanase and 0.3829 U/mL at 72 hours of fermentation for the CMCase. For the Virginia tobacco dust the maximum values of enzymatic activity registered are 0.5019 U/mL and 0.1647 U/mL, in the case of the CMCase and xylanase enzymes, respectively, at a fermentation time of 72 hours and 0.0344

U/mL for the PFase with a fermentation time of 96 hours.
The antimicrobial activity of the fermented tobacco wastes was evaluated and the results are presented in Table 1.

Table 1. Percentage of microbial growth inhibition by the treated tobacco wastes with *Aspergillus terreus* and *Aspergillus niger*.

	% of inhibition
Virginia tobacco – *Aspergillus terreus*	5.8
Black tobacco – *Apergillus terreus*	2.4
Tobacco enzymatic solution	8.6
Tobacco enzymatic solution	8.6
Black tobacco – *Aspergillus terreus*	25
Virginia tobacco – *Aspergillus niger*	71
Virginia tobacco – *Aspergillus niger*	72
Virginia tobacco – *Aspergillus niger*	8.6

All samples display antimicrobial activity, being the Virginia tobacco sample treated with the microorganism *Aspergillus niger* the one that presents a bigger percentage of microbial growth inhibition. These results indicate that bioactive metabolites are formed during the fermentation process.

Conclusions

The results obtained demonstrate that both tobacco wastes, black and rubio tobacco dust, can be biological degraded. The best results for enzymatic activity were obtained when fermentations were carried with *Aspergillus terreus* and Virginia tobacco dust was used as substrate.
The tests for biological activity evidence the formation of bioactive metabolites during fermentation.

Acknowledgements

Authors thank the financial support of VALNATURA Project.

References

[1] M. K. Bhat CELLULASES AND RELATED ENZYMES IN BIOTECHNOLOGY. Biotechnology Advances 18 (2000) 355–383
[2] Mantyla A, Paloheimo M, Suominen P. INDUSTRIAL MUTANTS AND RECOMBINANT STRAINS OF *TRICHODERMA REESEI*. In: Harman GF, Kubicek CP, editors. *Trichoderma* & *Gliocladium*—Enzymes, biological control and commercial applications, Vol. 2. London: Taylor & Francis, 1998. pp. 291–309.
[3] Quesada C. TRANSFORMACIÓN BIOLÓGICA DEL RESIDUAL TABACALERO TIPO RAPÉ MEDIANTE UN PROCESO DE FERMENTACIÓN EN ESTADO SÓLIDO. Instituto Superior Politécnico "José Antonio Echeverría", 2004.
[4] Curbelo C. PRODUCCIÓN DE AROMATIZANTE NATURAL A PARTIR DE RESIDUOS DE LA INDUSTRIA TABACALERA. Instituto Superior Politécnico "José Antonio Echeverría", 2002
[5] Dustet, J. CONTRIBUCIÓN AL ESTUDIO Y AL DESARROLLO DE LA FERMENTACIÓN EN ESTADO SÓLIDO DE MATERIALES LIGNOCELULÓSICOS. Trabajo presentado en opción al grado científico de Doctor en Ciencias Técnicas, Instituto Superior Politécnico "José Antonio Echeverría",1999.