

Sorghum Bagasse as Substrate for Cellulase Production by Submerged and Solid-State Cultures of *Trichoderma*

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Abstract

Sweet sorghum bagasse was used as cellulosic substrate in submerged and solid-state cultures of *Trichoderma* for cellulase production. Submerged liquid cultures (SLC) were obtained by inoculation of Mandels media containing 1% cellulose with spores suspension of *Trichoderma*. Solid-state cultures (SSC) were carried out in Erlenmayer flasks, where the substrate was distributed 1 cm layers. Comparing the yields of cellulases produced by *Trichoderma* strains in the systems applied in this study, using as substrate sorghum bagasse, we found the solid-state cultures as the system to produce the highest cellulase yields. The local strain of *T. viride* CMIT3.5. express high productivity in SSC system in laboratory conditions. The cellulolytic enzymes have maximum activity at 50°C, pH 4,8. The results recommend solid-state cultures of *Trichoderma* on sorghum bagasse as systems for producing cellulolytic products with higher activity than submerged cultures of *Trichoderma* on the same substrate.

Keywords: cellulases, sorgum bagasse, *Trichoderma*.

1. Introduction

Sweet sorghum (*Sorghum bicolor*) represents one of the most important sources of sugar, besides sugar cane and sugar beet. Due to high sugar content, *Sorghum bicolor* varieties are of great interest as raw material source for the ethanol industry. A commercial utilization of sorghum is already carried out in USA, India, Nigeria, Mexico and China. In Europe growing sweet sorghum for ethanol production is mentioned in France, Italy and Spain [1]. After the extraction of the sweet juice, results bagasse as byproduct. The spent bagasse can be further pretreated to open the lignocellulosic complex and sent to enzymatic hydrolysis of cellulose. In the enzymatic hydrolysis step, cellulolytic enzymes are used to catalyze the cellulosic and hemicellulosic compounds and obtain fermentable sugars. The sugars obtained after hydrolysis will be used as substrate to produce ethanol or other bioproducts (lactic acid, butanol etc). Glucose and xylose are

the main monosaccharides released from these lignocelluloses and can be fermented to ethanol by yeast [2], the xylose can be converted to the sweetener xylitol [3,4], while for some microorganisms these monosaccharides are ideal substrate for the production of gaseous biofuels such as hydrogen or methane [5].

Commercialization of ethanol produced from lignocellulosic biomass has difficulties mainly due to the high cost of currently available cellulolytic products [6-8]. Reduction of the costs associated with the hydrolysis phase can be achieved by direct use of on-site produced enzymes. The fermentation broth containing cellulolytic fungi, excluding any separation, concentration or other expensive downstream processes can be applied directly to hydrolyze lignocellulosic biomass. Studies indicate that the cost of on-site production of cellulases is much lower than the case of purchasing commercial cellulases from other suppliers [9, 10]. Cellulases are a group of enzymes which include endoglucanases, which hydrolyze in the interior of the cellulose polymer, exposing reducing and non-reducing ends – which are action sites for exoglucanases and

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cellobiohydrolases. Combination of these three types of enzymes will completely hydrolyze the cellulosic polymer to glucose. In lignocellulosic technology, the lignocellulosic biomass used as feed stock for the production of ethanol, can be used as well as substrate to grow cellulolytic microorganisms for production of cellulases to be used on-site in the enzymatic hydrolysis step. In this context, studying the fermentation parameters to produce maximum yields of cellulase is essential to develop an industrial process for cellulase production. In the present work we evaluate the possibilities to produce on-site cellulases and to study the fermentation parameters in bioprocesses using sorghum bagasse as substrate for production of fungi cellulases.

2. Materials and methods

2.1. Biomass feed stock and pretreatment

Sorghum (*Sorghum bicolor*) obtained from experimental sites in Timiș county (western Romania) was harvested as whole crop at the early milk stage of maturity, chopped to 2 cm theoretical length of cut using a forage harvester. Sweet juice was extracted from sorghum using a laboratory press (*5.5 Litre Ferrari Aluminium Cross Beam Press*). The sweet juice was used in other research for ethanol production, and the resulted bagasse was dried at 105°C and preserved at room temperature until use.

We used a combination of physical and chemical pretreatments indicated by previous research (J. MOHAMAD & al. [11] T. VINTILĂ & al [12]) to be optimal for this kind of raw materials (steam combined with 2% NaOH). This pretreatment was done in order to release cellulose and hemicelluloses from the lignocellulosic complex and thereby increase the rate of growth for the cellulolytic fungi and subsequently increase cellulase yields. The pretreatment was made as follow: the biomass soaked in 2% NaOH was autoclaved 30 minutes at 1 bar (121°C). After this treatment, the biomass was washed with 10% H₂SO₄ until pH 6.5 and with 12 equivalent volumes of water in order to remove the inhibitors resulted during pretreatments. The main pretreatment goal is to increase the enzyme accessibility and to improve the digestibility of cellulose.

2.2. Microorganism and inoculums preparation

The fungi spores are preserved in the Collection of Industrial Microorganisms of Timisoara (CMIT), belonging to the Faculty of Animal Science and Biotechnology from Timisoara by freezing at -70°C in glycerol 16% as cryoprotective agent.

The microorganisms used in this experiment are:

a) *Trichoderma longibrachiatum* CMIT36, obtained from D.S.M.Z. Germany, where is stored as *T. longibrachiatum* DSM 769 (other name: *T. reesei* ATCC26921, initially named *T. reesei* Simons), is a mutant of *T. viride* Persoon, or QM 9123 (ATCC24449), also referred as QM 9124.

b) *Aspergillus niger* CMIT3.8, isolated from mouldy pine saw dust.

Potato dextrose agar plates were inoculated with spore suspension and incubated five days at 30°C. For inoculum preparation, fully sporulated cultures in PDA plates were used. Spore suspensions were obtained by gentle washing (using single-use plastic inoculation loop) the surface of cultures obtained above with *Mandels liquid medium* (KH₂PO₄ 0.2%, (NH₄)₂SO₄ 0.14%, MgSO₄ x 7H₂O 0.03%, CaCl₂ x 2H₂O 0.04%, urea 0.03%, peptone 0.03%, tween 80 0.05%, FeSO₄ x 7 H₂O sol. 5 mg % 1 ml, ZnSO₄ x 7 H₂O sol. 1.4 mg % 1ml, MnSO₄ x 7 H₂O sol 1.56 mg % 1 ml, CoCl₂ sol 2 mg % 1 ml, distilled water ad. 100 ml, pH 5.5 sterilization 20 min at 121°C). The suspension was appropriately diluted and used as inoculum.

2.2. Enzyme production by submerged liquid fermentation (SLF)

The submerged cultures were obtained by inoculation with 10% spores suspension 300 ml flasks containing 50 ml Mandels media with 4% milled pretreated sorghum bagasse (containing approximately 50% cellulose) as carbon source and substrate for cellulase production. As control, Mandels medium with 2% cellulose Avicel PH101 was used. The inoculated media were incubated in an incubator shaker. During fermentation, probes were harvested and FPU and CMC-ase activity were analyzed.

2.3. Enzyme production by solid state cultures (SSC).

The substrate (pretreated sorghum bagasse) was distributed in 300 ml Erlenmayer flasks in 1 cm layers (50 ml or 13 grams). The flasks were

autoclaved 30 minutes at 121°C. Mandels medium is added over the dry biomass to achieve 60% moisture. The solid, humide media are inoculated with spore suspension of the tested fungi.

2.4. Analytical methods.

Filter paper assay was used to estimate total cellulase activity in the samples harvested from the fungi cultures, cording to Ghose [13] and expressed as filter paper units (FPU). Endoglucanase activity was determined with carboxy-methyl-cellulose as substrate [13] and one unit of CMCase was defined as the amount of enzyme that released 1 µmol of reducing sugar per minute and was expressed in units per milliliter (U/ml), or in units per gram dry substrate (U/gDS).

3. Results and discussion

In the first part of our experiment we studied the capacity of the fungus to produce cellulase by **submerged liquid fermentation (SLF)**. CMCase and FPU assays have been applied to analyze enzymatic activity in samples harvested from fungi fermentation. Results in figure 1 indicate that *T. longibrachiatum* produce higher yields of cellulases than *A. niger*.

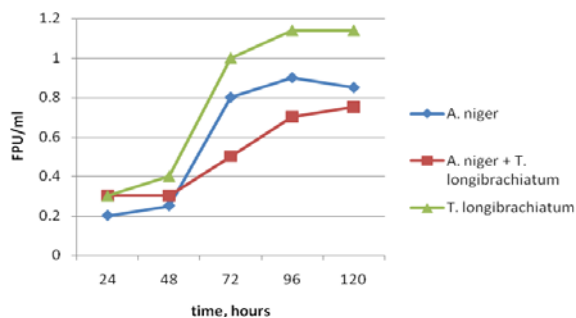


Figure 1. Cellulase activities (FPU) of *T. longibrachiatum* and *A. niger* in SLF with sorghum bagasse

Co-culturing the tested strains would not increase the cellulolytic activity of the enzymes released in the culture medium, on the contrary, the results indicate mutual inhibition of the two fungi and lower enzymatic activity.

Regarding endoglucanasic activity, results in figure 2 indicate the same organism, *T. longibrachiatum* producing the highest yields of

endoglucanase and the coculture of the two strains expressing the lowest enzymes yields.

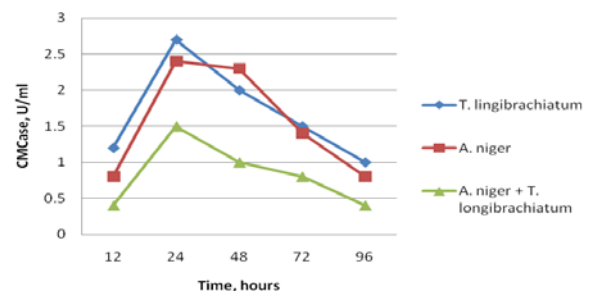


Figure 2. Endoglucanase activities of *T. longibrachiatum* and *A. niger* in SLF with sorghum bagasse

Regarding the period of growth associated with enzymes yields, the results indicate the first 24 hours as the period of mximum endoglucanasic activity, while the total cellulolytic activity reach the maximum after 96 hours of cultivation in submerged liquid system. These results are in concordance with the previous researche carried out with these microorganisms in SLF on other substrates (other types of biomass and cellulose Avicel) [14, 15].

Regarding the cellulase production in solid state cultures (SSC), literature indicates this system as technology that needs cheaper infrastructure and relatively less skilled operators, being able to use cheaper components for fermentation medium. Other advantages of SSC process compared with SLF are: saving energy, water and equipment, as well as using a more concentrated substrate. The difficulties facing SSC are: heat build up, monitoring and control of moisture, pH, temperature, aeration and agitation [16]. SSC also produces a more concentrated product, which in the case of saccharification of cellulose is very advantageous. Both strains were cultivated on sorghum bagasse moistened with liquid Mandels medium. After four days of incubation, solid state cultures were flushed with 0.1 N citrate buffer (pH 4.8). The liquid harvested from SSC and the liquid media obtained from the SLF where centrifuged to remove solid particles of substrate and fungi mycelia at 10000 rpm for 10 min. Samples from the clarified liquid were used to analyze enzymatic activity.

Table 1. Comparison of the crude enzyme preparations from *T. longibrachiatum* and *A. niger*

Organism	Type of enzyme preparation	Cellulase activity (FPU)		Endoglucanase activity (CMCase)	
		FPU/ml	FPU/gDS	U/ml	U/gDS
<i>T. longibrachiatum</i>	SLF product	1.14	28.5	2.70	67.5
	SSC product	1.20	24.0	3.42	59.8
<i>A. niger</i> CMIT3.8	SLF product	0.90	22.5	2.40	60.0
	SSC product	0.65	13.5	3.25	56.8

Data in table 1 indicate the washing liquid of substrate from solid cultures of *T. longibrachiatum* CMIT36 expressing the highest enzymatic activity. The highest titer of cellulase (1.2 F.P.U./ ml) has been obtained incubating the strain of *T. longibrachiatum* CMIT36 for 4 days in solid medium consisting of sorghum bagasse moistened with Mandels medium. A comparison among the levels of enzymes between the submerged (SLC) and solid state cultures (SSC) using sorghum bagasse as substrate, reveals that the SSC system provide higher titer of enzymes in washing liquid, but calculating the enzyme yield produced per gram of dry solid used in fermentation media, the enzymes titers are lower in SSC comparing with those found in submerged cultures. In terms of process economy, three aspects should be highlight: one is the titer of enzymes produced per gram of substrate. Data resulted in this research indicates SLF as the system producing more enzymes per gram of sorghum bagasse than SSC system. Another aspect includes investment cost and operation cost, which are known to be lower in SSC comparing with SLF [6, 16]. The third aspect is the titer of enzymes in the liquid solution harvested from the fermentation system, which is higher in SSC. If we compare the advantages and disadvantages emerging from these aspects, it is obvious that the low share of the feed stock price (sorghum bagasse) and the high share of the investment and operation cost from the economy of the enzymes production process, recommend SSC system for production of cellulolytic enzymes from sweet sorghum bagasse.

4. Conclusions

Sorghum bagasse can be used as substrate for growing cellulolytic fungi and to induce cellulase production in *Trichoderma* and *Aspergillus*

species. *T. longibrachiatum* CMIT36 proved to be a high-producing cellulolytic strain both in SLF and SSC using sorghum bagasse as substrate. Comparing enzyme titer in liquid products harvested in the two fermentation systems, we recommend SSC system to produce liquid product with higher enzymatic activity. Productivities of cellulases obtained in the fermentations carried out in this study, together with other considerations as low costs and availability, recommend sorghum bagasse as suitable substrate for cellulase biosynthesis with fungi.

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References

1. Deb, U. K., Bantilan, M.C.S., Roy, A.D., Parthasarathy Rao, P., Global Sorghum Production Scenario, Brochure, <http://oar.icrisat.org>.
2. Lynd, L.R., Overview and evaluation of fuel ethanol from cellulosic biomass: technology, economics, the environment, and policy, *Ann. Rev. Energy Environ.*, 1996, 21 403–465.
3. Vintila, T., Dragomirescu M., Vintila D., Croitoriu V., Hydrolysis of three types of lignocelluloses from agriculture using commercial enzymes and culture filtrate of *Trichoderma viride*, *Journal of Biotechnology*, 2010, vol. 150S.
4. Sanchez, O.J., Cardona C.A., Trends in biotechnological production of fuel ethanol from different feedstocks, *Bioresource Technology*, 2007, xxx 1 – 26.
5. Diguța, C., Jurcoane, Ș. Israel– Roming, F., Brule, M., Mukengele, M., Lemmer, A., Oechsner, H., Studies concerning enzymatic hydrolysis of energy crops, *Roumanian Biotechnological Letters*, 12 (2), 3203, 3207 (2007).

6. Sukumaran, R. K., Singhanian, R. R., Mathew, G. M., Pandey, A., Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production, *Renewable Energy* 34 (2009) 421-424.
7. Wingren, A., Galbe M., Zacchi G., Techno-economic evaluation of production ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks, *Biotechnology Progress*, 2003, vol. 19, no.4, 1109-1117.
8. Sassner, P., Zacchi G., Integration option for high energy efficiency and improved economics in a wood-to-ethanol process, *Biotechnology for Biofuels*, 2008, vol. 1, article 4.
9. Barta, Z., Kovacs K., Reczey K., Zacchi G., Process design and economics of on-site cellulase production on various carbon sources in a softwood-based ethanol plant, *Enzyme Research*, 2010, article ID 734182.
10. Merino, S.T., Cherry J., Progress and challenges in enzyme development for biomass utilization, in *Biofuels*, vol. 108 of *Advances in Biochemical Engineering/Biotechnology*, 2007, pp. 95-120.
11. Mohammad, J. T., Keikhosro K., Pretreatment of lignocellulosic Wastes to Improve Ethanol and Biogas Production. *International Journal of Molecular Sciences*, 2008, 9(9) 1621,1651,
12. Vintila T., Dragomirescu M., Croitoriu V., Vintila C., Barbu H., Sand C., Saccharification of lignocellulose - with reference to Miscanthus - using different cellulases, *Romanian Biotechnological Letters.*, 15(4), 5498, 5504 (2010).
13. Ghose, T.K. - International union of pure and applied chemistry – Applied chemistry division – Commission on biotechnology, Measurement of cellulase activities, in *Pure and Applied Chem.*, 1987, vol. 59, No.2, 257-268.
14. Vintila, T., Croitoriu, V., Dragomirescu, M., The Effects of Bioprocess Parameters on Cellulase Production with *Trichoderma viride* CMIT35, *Scientific Papers Animal Sciences and Biotechnologies*, 2010, Vol. 43 (1),.
15. Trulea, A., Vintila T., Pop G., Dragomirescu M., Effects of Bioprocess Parameters on Production of Cellulase using Miscanthus as Substrate, *Scientific Papers Animal Sciences and Biotechnologies*, 2013, 46 (1).
16. El-Nawawy, A.S., Microbial biomass production from organic solid substrates. In: *Solid Substrate Cultivation*, H.W. Doelle, D.A. Mitchell, E.R. Carlos, Ed. Elsevier, 1992, pp. 247-265.