Effects of Bioprocess Parameters on Production of Cellulase using Miscanthus as Substrate

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Abstract

A number of fungi were isolated abroad and screened for cellulolytic potential. In this work we studied cellulase production with several fungi strains, using *Miscanthus giganteus* as substrate. An indigenous strain of *T. viride* was selected and the kinetics of cellulase production is reported. Product formation parameters of different types of cellulases indicate that the studied strain of *T. viride* is capable of producing important levels of cellulases when grown on Mandels medium with *Miscanthus* biomass as carbon source. Furthermore, it was observed that production of endoglucanase reaches its maximum during exponential phase of growth, while exoglucanase during the stationary phase. Enzyme production by solid-state fermentation was also investigated and found to be more efficient than liquid state fermentation. High production of cellulase was noted at the following parameters for liquid cultures: 4% *Miscanthus giganteus* biomass, 5% inoculum, 180 r.p.m. agitation, pH 5; and 60% humidity in the case of solid state fermentation. Comparing the productions of cellulases in the fermentations carried out in this study, data indicate *Miscanthus* as ideal substrate for cellulase biosynthesis with fungi.

Keywords: cellulase, fermentation, *Miscanthus*.

1. Introduction

Much of the biofuels produced at the moment are made from food or feed crops that are at risk of substantial ethical problems. Second generation of biofuels, produced from lignocellulosic biomass may be able to avoid these issues. Lignocellulosic biomass can be obtained by cultivating special energetic crops (e.g. *Miscanthus giganteus*). The conversion of lignocellulose to ethanol consists of three steps: in the first step the carbohydrate polymers are released from lignocellulosic complex; in the second step the carbohydrate biopolymers are hydrolyzed to fermentable sugars; and the final step consists of fermentation of sugars to ethanol [1]. In Romania, hundreds of thousands of hectares of agricultural land is not cultivated or is polluted and improper to cultivate food or feed crops. A large part of this land can be used to produce special energy crops to be used as biofuels feedstock.

Agricultural lignocellulosic biomass is more suitable to saccharification than wood, because of their soft structure, low lignin content, and low cellulose crystallinity. Glucose and xylose are the main monosaccharides released from these lignocelluloses and can be fermented to ethanol by yeast [1,2], 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].

The hydrolysis of the lignocelluloses to fermentable sugars seems to be the main reason for the high producing cost of lignocellulosic ethanol (the cost of cellulases comprises up to 20% of lignocellulosic ethanol production costs [6-8]). Therefore, major efforts are now focused on lowering enzyme-related costs in cellulosic biorefineries. The most economical way of employing the cellulolytic enzymes to hydrolyze the lignocellulosic biomass is by direct use of

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whole crude fermentation broth containing cellulolytic fungi, excluding any separation, concentration or other expensive downstream processes. Studies indicate that the cost of on-site production of cellulases is much lower and could be near 8% of total costs [4, 9, 10]. At industrial level, *Trichoderma* and *Aspergillus* are the most-widely used microorganisms to produce cellulolytic enzymes. The same energy crop used for the production of lignocellulosic ethanol, can be used as well as raw material 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 this work we evaluate the possibilities to produce on-site cellulases and to study the fermentation parameters in bioprocesses using lignocellulosic biomass from *Miscanthus giganteus* as raw material for production of cellulolytic enzyme systems.

2. Materials and methods

As substrate for cultivation of cellulolytic fungy and production of cellulases, we used lignocellulosic biomass consisting of the whole plant of *Miscanthus sinensis Giganteus*. This sterile triploid hybrid was provided by Prof. H. Barbu, from Lucian Blaga University, Sibiu (Romania). *Miscanthus* was cultivated in Copsa Mica area, (Sibiu county) a chemically polluted area, reach in lead and cadmium, as an excluder crop, accumulating reduced amounts of polluting metals in the harvestable, above the ground parts. The concentrations of polluting metals in leaves and stems of *Miscanthus* were previously reported [11]: 5 – 6.5 mg Pb x kg^{-1} DM of biomass and 2.9 - 3.7 – mg Cd x kg^{-1} DM of biomass. The concentration of cellulose and hemicellulose in the biomass of *Miscanthus* was previously reported [12]: 43.5 % cellulose and 25.8 % hemicelluloses reported to dry matter of biomass.
The biomass was air-dried and milled with a hammer mill to a mesh sieve of 2 mm.
As standard cellulose, we have used Avicel PH 101.

Microorganisms and propagation:
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^{o}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) *Trichoderma viride ATCC- 13.631;*
c) Trichoderma viride CMGB1 obtained from University of Bucharest, Faculty of Biology;
d) *Aspergillus niger* CMIT3.8, isolated from moldy pine saw dust;
e) *Aspergillus oryzae CMIT3.12* obtained from University of Bucharest, Faculty of Biology.
Potato dextrose agar plates were inoculated with spore suspension and incubated five days at 30^{o}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 (KH_2PO_4 0.2%, $(NH_4)_2SO_4$ 0.14%, M_gSO_4 x $7H_2O$ 0.03%, $CaCl_2$ x $2H_2O$ 0.04%, urea 0.03%, peptone 0.03%, tween 80 0.05%, $FeSO_4$ x 7 H_2O sol. 5 mg % 1 ml, $ZnSO_4$ x 7 H_2O sol. 1.4 mg % 1ml, $MnSO_4$ x 7 H_2O sol 1.56 mg % 1 ml, $CoCl_2$ sol 2 mg % 1 ml, distillated water ad. 100 ml, pH 5.5 sterilization 20 min at 121^{o}C). The suspension was appropriately diluted and used as inoculum.

Pretreatment of lignocellulosic biomass
Grinded biomass was dispensed in Erlenmeyer flasks and treated with NaOH 2% solution, and autoclaved at 1 bar for 30 minutes. The pretreated biomass was washed and neutralized.

Cellulase production by submerged liquid fermentation
The submerged cultures were obtained by inoculation with 10% spores suspension 300 ml flasks containing 50 ml Mandels media with 2% (as cellulose content) 2 mm milled miscanthus 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.

Studied parameters in liquid cultures are: carbon source concentration: 0,5 to 4 % concentration; inoculums concentration: DO_{500nm} from 0,33 to 0,96; agitation speed combined with inoculums concentration; and pH 4 to 6;

Solid state cultures (SSC).
The cellulosic substrate (pretreated *Miscanthus* biomass) 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 *Miscanthus* biomass and inoculated with spore suspension. In this case, the only studied parameter was humidity, as other previous studies have been made by our team [13], concerning other parameters.

Enzyme assays.
Two determination methods [14, 15], using as substrates: CMC for endoglucanase, and filter paper for saccharifying cellulase. The reaction was carried out at 50°C for 10 min. for CMC and 60 min. for filter paper. The amount of reducing sugar was determined by DNS method. One International Unit (IU) of enzyme was defined as the amount of enzyme that released 1 µmol of reducing sugar per minute under standard conditions.

3. Results and discussion

In the first part of our experiment we studied the capacity of the fungy to produce cellulase by submerged liquid fermentation. CMC-ase and FPU assays have been applied to analyze enzymatic activity in samples harvested from fungi fermentation. Cellulase activity was expressed depending on substrate used in the assay: if filter paper is the substrate, the enzymatic activity is expressed in FPU (filter paper units) and expresses the saccharying cellulase activity; if carboxymethylcellulose (CMC) is the substrate, the activity is expressed in endoglucanase (or CMC-ase) units. Applying CMC assay, we have found enzymatic activities in figure 1. We find that fungi strains *T. longibrachiatum*, coculture of *T. longibrachiatum* and *A. niger*, and coculture of *T. viride ATCC* and *A. niger*, expressed the highest endoglucanasic activities. As for substrate, the obtained data demonstrates that low-cost agriculture ligonecellulosic biomass, as miscanhus can serve as substrate for enzymes production at high levels.

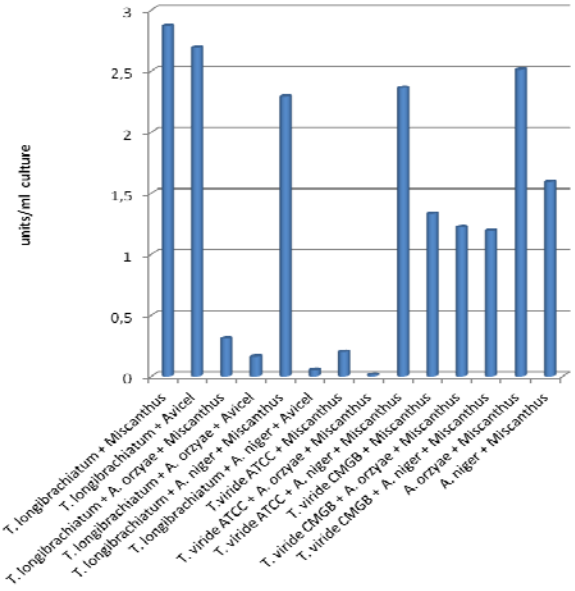

Figure 1. Endoglucanase (CMC-ase) activities of tested fungi (values represents Units/ml culture liquid)

The enzymes yields are even higher in fermentation media where *Miscanthus* was used as cellulosic substrate than fermentation media where high-purity, expensive cellulose was used to induce cellulolytic enzymes biosynthesis. Surprisingly, cocultures of *Trichoderma longibrachiatum* and *Aspergillus niger* produces endoglucanase only on media with miscanthus as substrate, and not on cellulose Avicel.
Furthermore, data in figure 2 represents the FPU activities of the tested strains. The cultures expressing the highest endoglucanasic activities, express as well the highest cellulase yields in terms of FPU activity. The batch consisting of *A. niger* grown on Miscanthus as cellulosic substrate can be added to this group, as high-yielding bioprocess.
The results presented in figures 1 and 2 recommend the strain of *Trichoderma longibrachiatum* CMIT36 to be used in the next part of our work study the fermentation parameters to produce maximum yields of cellulase. Concentration of substrate (0.5-4%), concentration of inoculums (spore suspensions with DO 0.33-0.96 and inoculation rates of 5% and10%), agitation (120-180 rpm), pH (between 4 and 6) of culture medium in submerged cultures and the humidity of substrate (60-80%) in solid culture were studied in the next phase of our work.

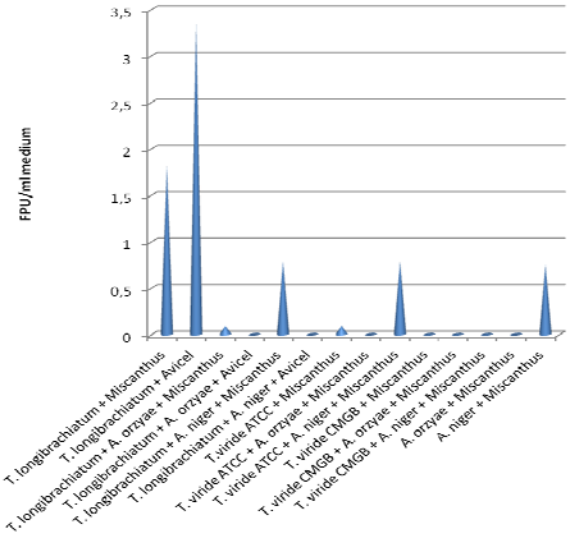

Figure 2. Saccharying cellulase (FPU) activities of tested fungi (values represents FPU/ml culture liquid)

Regarding the cellulase production in liquid cultures (SLC), enzyme production kinetics revealed that the concentration of substrate have a direct influence on cellulase production. The highest titer of saccharifying cellulase (expressed in FPU) was obtained using the culture medium containing 4% miscanthus biomass as cellulosic substrate (figure 3). The concentration of wheat bran miscanthus biomass couldn't be increased as the viscosity of the medium will be too high at higher concentrations than 4%.

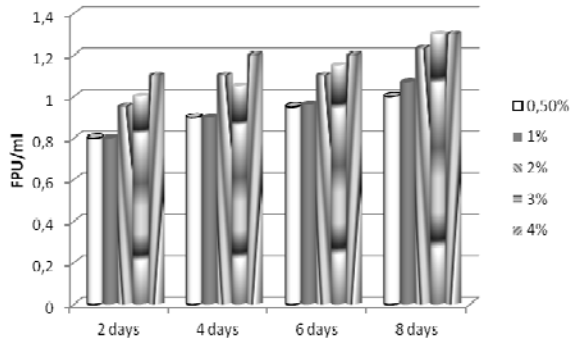

Figure 3. Enzyme production kinetics in different concentration of substrate (0.5 – 4%)

Concentration of inoculums is another factor with important influence in production of cellulase with *Trichoderma*. In the particular case of the strain used in this study, *T. longibrachiatum* CMIT36, the most appropriate concentration of inoculums to be used to start a bioprocess for production of cellulase enzymes is 5% inoculation rate of a spore suspension with DO_{500nm} 0,5 (figure 4). In the case of higher concentration of inoculums, the production of saccharifying cellulase enzymes is inhibited during the bioprocess.

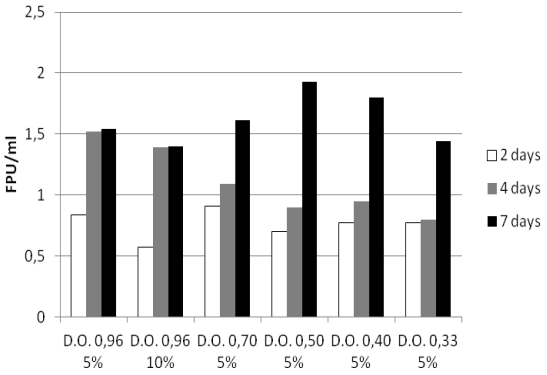

Figure 4. Enzyme production kinetics using different concentration of inoculums

Fungi like *Trichoderma* are aerobic filamentous organisms, hence the concentration of dissolved oxygen can have a high impact on enzyme production kinetics. In shake flasks, the dissolved oxygen concentration can be increased by increasing the agitation speed. But, filamentous fungi have the tendency to agglomerate and form lumps at high turbulences in liquid media, which decrease the contact surface of hypha with nutrients and oxygen, leading to lower enzyme productivity. The aeration is influenced by the concentration of mycelium in the culture as well. Taking these reasons into account, we have studied the enzyme productivity at different agitation speeds combined with two inoculums concentrations. Results obtained in this study and shown in figure 5 indicate that the optimum agitation is at 180 r.p.m. combined with the optimum inoculation rate of 0.5% spore suspension with DO_{500nm} 0.5.

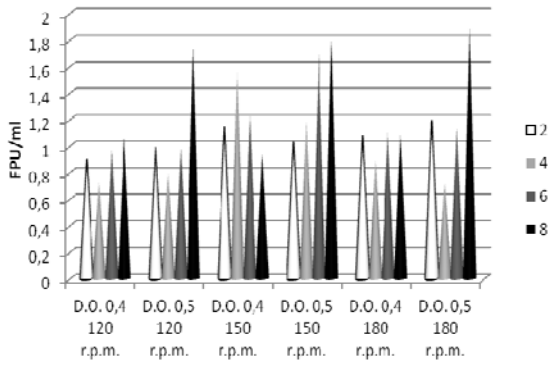

Figure 5. Effect of agitation speed and inoculation rate on enzyme production kinetics

Using culture media with different pH values, we have found that *T. longibrachiatum* CMIT36 produce the highest quantity of enzymes at pH 5,5 (fig. 6).

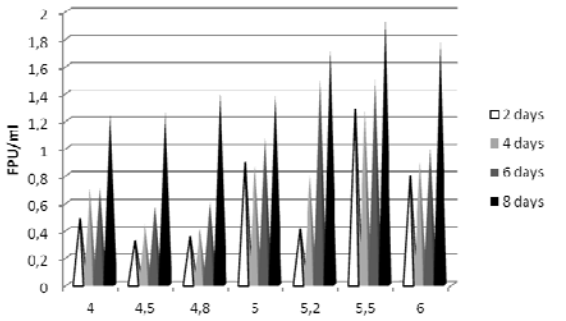

Figure 6. Effect of culture medium pH on enzyme production kinetics

Regarding the cellulase production in solid state cultures (SSC), data in figure 7 indicate the enzymatic activity found in 1 gram of substrate from solid cultures of *T. longibrachiatum* CMIT36. Enzyme production kinetics in SSC revealed that endoglucanase production is higher in a solid culture on medium consisting of miscanthus biomass moistened with spore suspension in Mandels medium, to obtain 60% humidity.

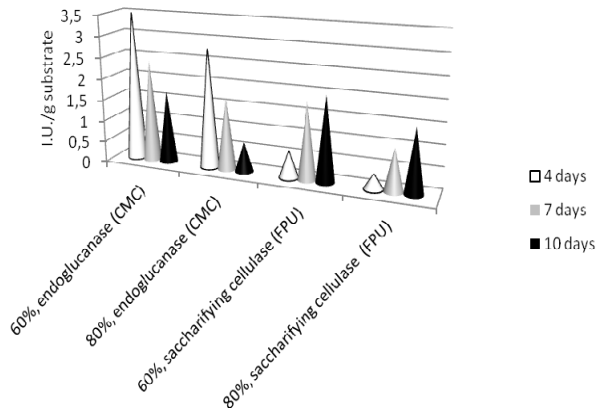

Figure 7. Effect of humidity on enzyme production in SSC of *Trichoderma longibrachiatum* CMIT36

Also, data in figure 7 indicates that the highest endoglucanase activity is achieved in the first part of incubation period. In fact, a bioprocess carried out at 60% humidity, can produce higher titer of endoglucanase than saccharifying cellulase. In each case, a humidity of 60% lead to the highest enzymatic activity. The highest titer of saccharifying cellulase (2 F.P.U./ gram substrate) has been obtained incubating the strain of *T. longibrachiatum* CMIT36 for 10 days in solid medium consisting of miscanthus biomass and Mandels medium solution at 60% humidity.

A comparison among the levels of enzymes between the submerged (SLC) and solid state cultures (SSC) using miscanthus biomass as substrate, reveals that the SSC system provide a slightly higher titer of enzymes in solid medium, but, after extraction with washing liquid, the enzymes titers are comparable with those found in submerged cultures.

4. Conclusions

Data obtained in this work allow us to conclude that lignocellulosic biomass consisting of the whole plant of *Miscanthus sinensis Giganteus* cultivated in polluted area can be used as substrate for growing cellulolytic fungy and to induce cellulase production in *Trichoderma* and *Aspergillus* species. Furthermore, a high-producing cellulolytic strain was selected (*T. longibrachiatum* CMIT36) and tested in order to set up the bioprocess parameters for high titters of cellulases in culture media. Comparing enzyme kinetics in submerged liquid cultures and solid state cultures, the optimal parameters to produce cellulase enzymes with the tested fungal strain where established (substrate concentration, concentration of inoculums and inoculation rate, agitation speed, pH of the medium, duration of the cultivation, humidity of the solid medium in SSC). Productivities of cellulases obtained in the fermentations carried out in this study, together with other considerations as low costs and availability, recommend *Miscanthus* as ideal substrate for cellulase biosynthesis with fungi.

Acknowledgements

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