The Effects of Bioprocess Parameters on Cellulase Production with *Trichoderma viride* CMIT35

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Abstract

Fungal cellulases are well-studied, and have various applications in industry, health or agriculture. Species of *Trichoderma* can produce substantial amounts of endoglucanase, exoglucanase (saccharifying cellulases), and some strains are able to produce important quantities of β-glucosidase. A number of fungi were isolated abroad and screened for cellulolytic potential. In this study, the kinetics of cellulase production from an indigenous strain of *T. viride CMIT35* 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 wheat bran 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% wheat bran, 5% inoculum, 180 r.p.m. agitation, pH 5; and 60% humidity in the case of solid state fermentation.

Keywords: cellulase, culture parameters, *Trichoderma viride*

1. Introduction

Lignocelluloses represent the most abundant and lowest-cost biomass in the world and, thus, can be used as alternative raw materials for production of fuel ethanol [1]. The hydrolysis of the lignocelluloses to fermentable sugars seems to be the main reason for the high producing cost of ethanol from lignocelluloses. Hence, finding new microbial strains able to produce cellulase, and increasing the hydrolytic activity of fungal culture extracts is a way to lower the price of cellulolytic preparates used in hydrolysis of cellulose to fermentable sugars. Studying the fermentation parameters to produce maximum yields of cellulase is essential to develop an industrial process for cellulase production.

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2. Materials and methods

The fungi are preserved in the collection of industrial microorganisms (CMIT) of the Faculty of Animal Science and Biotechnology from Timisoara by freezing at -70^{o}C the spores suspension in glycerol 16% as cryoprotective agent. The strain used in this experiment is *Trichoderma viride* CMIT35 (other name: *T. viride* CMGB1, kindly donated by Dr. Săsărman Elena, from the University of Bucharest, Faculty of Biology). After thawing, the spores suspensions were inoculated on plates and tubes with agar media (MEA, PDA, Mandels) and incubated for three days at 28^{o}C. In this time the mycelia proliferated on the surface of solid media. For sporulation, the cultures were incubated at room temperature in natural light for 3-5 days. The light is the inductor for sporulation in *Trichoderma*. The tubes with cultures obtained as described above were preserved at $+4^{o}$C.

1. Submerged liquid cultures (SLC). Spores suspension of *Trichoderma* were obtained by washing 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 $7H_2O$ sol. 5mg% 1ml, $ZnSO_4$ x $7H_2O$ sol. 1,4mg% 1ml, $MnSO_4$ x H_2O sol 1,56mg% 1ml, $CoCl_2$ sol 2mg% 1ml, distillated water ad. 100ml, pH 5,5-5,6, sterilization 20 min at 121°C). The liquid cultures were obtained by inoculation 50 ml Mandels media containing 1% cellulose in 300 ml flasks with spores suspension of *Trichoderma*. The cellulose used as carbon source and substrate for cellulase in these cultures was wheat bran (containing 10% cellulose). The spore suspensions were obtained by adding 5ml of Mandels medium in each tube with *Trichoderma* sporulated cultures, gently agitate the pipette on the surface of the culture to suspend the spores (without breaking mycelia or medium), and with the same sterile pipette the spore suspension was transferred in the flasks over the fermentation medium. The inoculated media were incubated in a water bath with shaker. Probes were harvested in regular basis to verify the purity of the cultures, development of fungi and cellulolytic activity.

2. 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;

3. **Solid state cultures (SSC)**. The cellulosic substrate used as carbon source is wheat bran. The substrate was distributed in 300 ml Erlenmayer flasks in 1 cm layers (50 ml or 13 grams). The flasks with wheat bran were autoclaved 30 minutes at 121°C (1 bar). This step has two functions: first, the substrate is sterilized and second, the cellulosic biomass is pretreated using steam pressure to make it more accessible to the action of cellulase enzymes [2,3]. Mandels medium is added over the wheat bran and inoculated with spore suspension. In this case, the only studied parameter was humidity, as other previous studies have been made by our team [4], concerning other parameters.

4. **Enzyme assays**. Two determination methods [5,6], 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

Enzyme production in liquid cultures (SLC)

Enzyme production kinetics revealed that endoglucanase production starts early and the concentration of substrate have a direct influence on endoglucanase production. The highest titer of endoglucanase was obtained using the culture medium containing 4% wheat bran as carbon source and cellulosic substrate as well (figure 1). The concentration of wheat bran couldn't be increased as the viscosity of the medium will be too high at higher concentrations than 4% wheat bran. 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. viride* CMIT35, 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} of 0,5 (figure 2). In the case of higher concentration of inoculums, the production of cellulase enzymes starts earlier, but decreases during the bioprocess. 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 3 indicates 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. Using culture media with different pH values, we have found that *T. viride CMIT35* produce the highest quantity of enzymes at pH 5,5 (fig. 4).

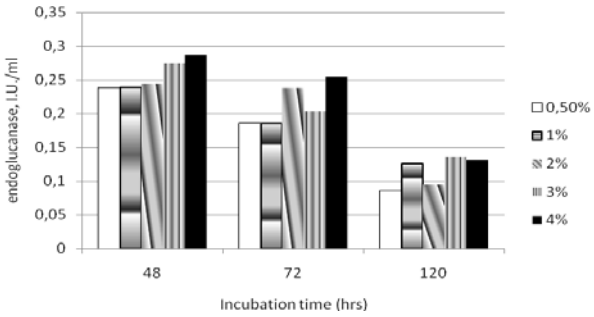

Figure 1. Enzyme production kinetics in different concentration of substrate (0.5 – 4%)

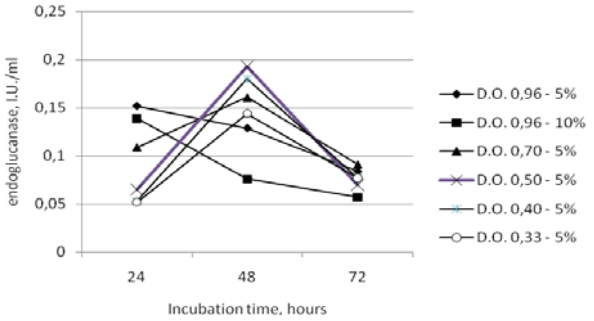

Figure 2. Enzyme production kinetics using different concentration of inoculum

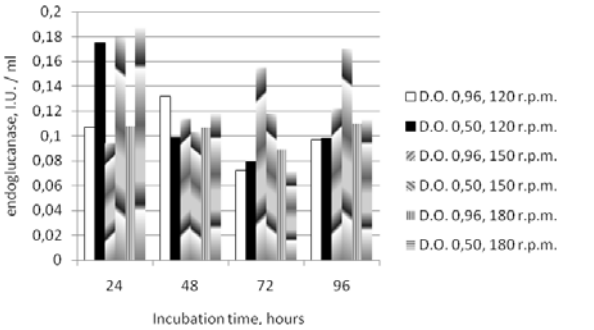

Figure 3. Effect of agitation speed and inoculation rate on enzyme production kinetics

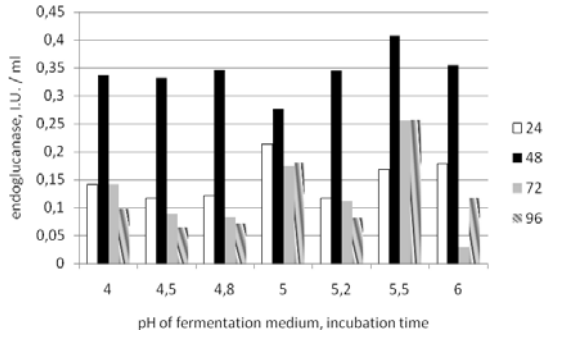

Figure 4. Effect of culture medium pH on enzyme production kinetics

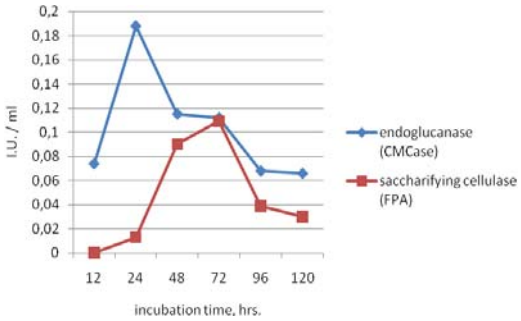

Figure 5. Endoglucanase and saccharifying cellulase production kinetics of *T. viride CMIT35*

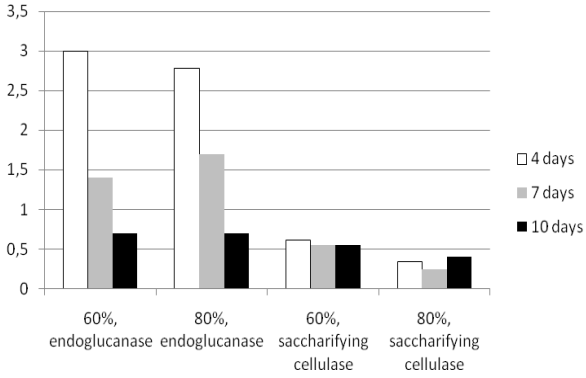

Figure 6. Effect of humidity on enzyme production in SSC of *T. viride CMIT35*

Enzyme production kinetics revealed that in this study, in the presence of wheat bran as carbon source and cellulosic substrate as well, the endoglucanase production (figure 5) started earlier, (during the lag phase), but its titer slightly decreases during the log phase. As for Filter Paper Activity (FPA) kinetics, data in figure 5 indicates that saccharifying cellulase follows the growth kinetics of fungi – it increases during the log phase (first 72 hours) and drastically decreases during stationary phase.

Enzyme production in solid state cultures (SSC)

Data in figure 6 indicate the enzymatic activity in 1 gram of substrate from solid cultures of *T. viride CMIT35*. Enzyme production kinetics in SSC revealed that endoglucanase production (figure 6) is higher in a solid culture medium consisting of wheat bran moistened with spore suspension in Mandels medium, to obtain 60% humidity. Also, data in figure 6 indicates that the highest endoglucanase activity is achieved after four days of incubation. 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 (0,62 E.U./ gram substrate) has been obtained incubating the strain of *T. viride* for 96 hours in a 60% humidity solid medium consisting of wheat bran and Mandels medium solution.

A comparison among the levels of enzymes between the submerged (SLC) and solid state cultures (SSC) using wheat bran as substrate, reveals that the SSC system provide a 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

1. Comparing enzyme kinetics in submerged liquid cultures the following parameters appears to be optimal to produce cellulase enzymes with fungal strain *T. viride CMIT35*: substrate concentration (wheat bran): 4%; concentration of inoculums: 5 %, $D.O._{500nm}$ = 0,5; agitation speed: 180 r.p.m.; pH: 5,5; cultivation time: for endoglucanase 24 hours, for saccharifying cellulase 72 hours.

2. A more concentrate cellulosic preparate can be obtained by solid state cultures at 60% humidity of solid medium. Still, in solution obtained by washing the solid medium, the enzymes titers are comparable with those obtained in submerged cultures. It is important to note that endoglucanase production has increased after 96 hours of solid state cultures.

3. The optimal parameters to be applied in the bioprocess for cellulase production with fungal strain *T. viride CMIT35* have been settled, and the researches can be further conducted to bioreactor conditions for scaling up.

Acknowledgements

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