

Production of cellulase by submerged and solid-state cultures and yeasts selection for conversion of lignocellulose to ethanol

Received for publication, October 10, 2008

Accepted, March 25, 2009

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Abstract

*Simultaneous hydrolysis and fermentation of lignocellulose to ethanol needs low-cost cellulases that work well at temperatures lower than 50°C and microorganisms able to produce ethanol at temperatures higher than 37°C. The objective of this work is to study submerged and solid-state cultures of Trichoderma for cellulase production, to compare the productivity and efficiency of the two systems of fermentation and to select yeast strains able to ferment sugars and produce ethanol at high temperatures. Submerged liquid cultures (SLC) and solid-state cultures (SSC) were carried out to compare the productivity of the two strains of Trichoderma. Comparing the productions of cellulases in the systems applied in this study, data indicate the system of solid-state culture with flushing as the most efficient (660% more efficient in *T. viride* ATCC 13.631 SSC+f than in SLC and 455% more efficient in *T. viride* CMGB1 SSC+f than in SLC). Still, *T. viride* CMGB1 show a higher production (158 FPU in SSC+f) than *T. viride* ATCC 13.631 (99 FPU in SSC+fm) in laboratory conditions. The cellulases reaches maximum activity of 3.18 FPU/ml at 50°C, pH 4,8. The activity decreases to 2.56 FPU/ml at 40°C and to 1.87 FPU/ml at 37°C. One yeast strain was found to have the same ethanol producing capacity up to 40°C. All yeast strains were inhibited at 45°C. The results recommend solid-state cultures as systems for producing cellulases at lower price than submerged cultures. The enzymes and the selected yeast strain can be applied for simultaneous hydrolysis and fermentation of lignocelluloses to ethanol.*

Keywords: cellulase, ethanol, Trichoderma, SSF, LSF, cellulose hydrolysis

Introduction

Currently, production of ethanol from starch-based corn grain is facing such challenges as limited supply and high cost of feedstock. 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 [1,2,3]. The hydrolysis of the lignocelluloses to fermentable sugars seems to be the main reason for the high producing cost of ethanol from lignocelluloses. According to preliminary evaluations of the NREL (National Renewable Energy Laboratory – USA), the cost of cellulases comprise 20% of ethanol production costs assuming them in US\$1.5/gallon. On the other hand, commercial cellulase cost (US\$16/100,000 FPU) is prohibitive for this process. In contrast, these authors indicate that the cost of producing cellulases by solid-state fermentation of corn stover could reach US\$0.15/100,000 FPU that would correspond to US\$0.118/gal EtOH, i.e. near 8% of total costs [4]. Simultaneous hydrolysis and fermentation of lignocellulose to ethanol needs low-cost cellulases, that work well at temperatures lower than 50°C and microorganisms able to produce ethanol at temperatures higher than 37°C. The objective of this work is to study submerged and solid-state cultures of *Trichoderma* for cellulase production, to compare the

productivity and efficiency of the two systems of fermentation and to select yeast strains able to ferment sugars and produce ethanol at high temperatures.

Materials and Methods

The yeasts and fungi are preserved in the collection of industrial microorganisms (CMIT) of 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:

- two strains of *Trichoderma*: *T. viride* CMIT3.4 (other name: *T. viride* ATCC13.631); and *T. viride* CMIT3.5 (other name: *T. viride* CMGB1, kindly donated by Dr. Săsărman Elena, from the University of Bucharest, Faculty of Biology).

- In addition, twenty-one yeast strains from the same collection of microorganisms (CMIT) were tested for ethanol production at high temperatures.

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%, $(\text{NH}_4)_2\text{SO}_4$ 0,14%, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 0,03%, $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ 0,04%, urea 0,03%, peptone 0,03%, tween 80 0,05%, $\text{FeSO}_4 \times 7\text{H}_2\text{O}$ sol. 5mg% 1ml, $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$ sol. 1,4mg% 1ml, $\text{MnSO}_4 \times \text{H}_2\text{O}$ sol 1,56mg% 1ml, CoCl_2 sol 2mg% 1ml, distilled 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 10% spores suspension of *Trichoderma*. The cellulose used as carbon source and substrate for cellulase in these cultures was wheat bran (containing 10% cellulose). The inoculated media were incubated in a water bath with shaker at 28°C , 180 r.p.m., for 21 days. Probes were harvested in regular basis to verify the purity of the cultures, development of fungi and cellulolytic activity.

2. Solid-state cultures (SSC). In this case, 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 cellulolytic enzymes. Three flasks with wheat bran are prepared for each strain of *Trichoderma*. The biomass from two flasks is washed six times with double volume of liquid (100 ml) in order to remove the glucose resulted during pretreatment with steam [5]. Glucose is a inhibiting factor for cellulase synthesis. First four washings are made with sterile distillate water and the last two washings are made with specific nutrient solution used for cultivation of *Trichoderma*. The concentration of glucose in liquid collected after each washing was determined. After the last washing, the wheat bran remains saturated with nutrient solution and is inoculated with spore suspension (5 ml/flasks).

The three flasks with cellulosic biomass represent the following experimental variants:

▪ **SSC** – represent solid-state culture with biomass washed as described above, which will be incubated 21 days at 28°C ;

▪ **SSC+f** - represent solid-state culture with biomass washed as described above, which will be incubated 21 days at 28°C . Every seven days a flushing will be made (f = flushing) with two volumes of nutritive solution. This flushing will bring nutrients and will wash out cellulases and glucose;

▪ **SSC+fm** - represent solid-state culture with biomass not washed after pretreatment (we assume that the glucose is present in the culture and will inhibit the cellulase production).

Will be incubated 21 days at 28°C and every seven days a flushing will be made with two volumes of nutritive solution (fm = flushing, control).

3. The yeast cultures were carried out in 100 ml flasks closed with rubber stoppers and water traps to allow CO₂ releasing. The medium used for yeast growth consists of: yeast extract 1%, peptone 2% and glucose 5%.

Assays. Total reducing sugars were determined colorimetrically using dinitrosalicylic acid reagent, cellulase activity was determined using the modified method of Mandels [6, 7], using as substrate filter paper; the activity is expressed in FPU (filter paper units). The ethanol was determined using an enzymatic method.

Results

Activity curves for the two strains of *Trichoderma* were obtained in **submerged liquid cultures** using wheat bran as substrate. The maximum activities were 0,46 FPU/ml for *Trichoderma viride* ATCC from the 7th day until the 14th day and 0,76 FPU/ml for *Trichoderma viride* CMGB in the 10th day.

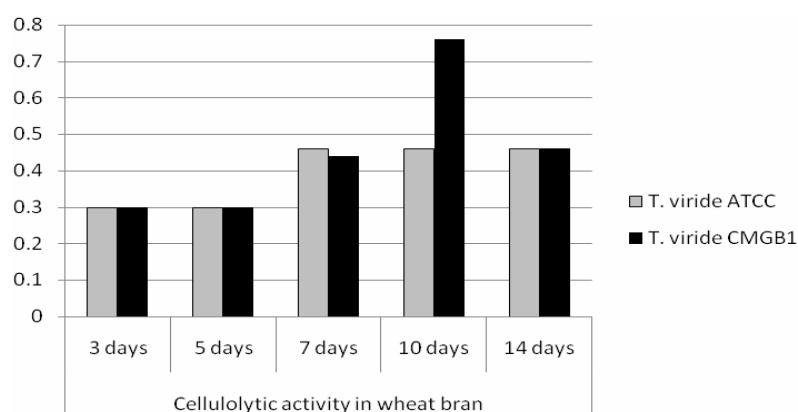


Figure 1. Cellulolytic activity of two strains of *Trichoderma* in submerged fermentation

As for reducing sugars, the results (figure 2) indicates that the fungi consume the sugars during growth, *T. viride* CMGB exhibit a faster growth of mycelia, which can be observed in a rapid consumption of sugars. The points of highest activity coincide with the point of the lowest reducing sugars content. This demonstrates that the presence of reducing sugars in medium affect negatively the production of cellulase, and vice versa. This observation leads to necessity of removing sugars from cultures of cellulolytic fungi in order to increase the cellulolytic activity.

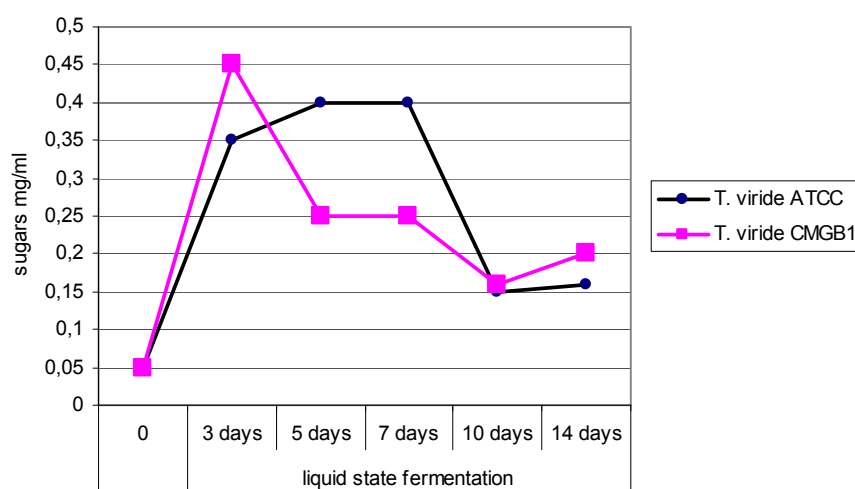


Figure 2. Evolution of reducing sugars in submerged cultures of *Trichoderma*.

The total production of cellulase in SLC (submerged liquid cultures) can be found multiplying the maximum activities of enzymes (FPU/ml) with 50 (the volume of culture). The results are:

- In *Trichoderma viride* ATCC: 23 FPU total production.
- In *Trichoderma viride* CMGB: 38 FPU total production.

In solid-state cultures, the data indicate a higher rate of enzyme synthesis and higher accumulation of enzymes in the first seven days of incubation (table 1 and figure 3). The values in the table shows the activity of the cellulases harvested in the flushing liquid, which represents double volume of the culture (100 ml liquid used to wash 50 ml solid culture). This means that each flushing harvest a quantity of enzyme much higher than the quantity of enzymes that can be excreted in submerged cultures. For example, *T. viride* CMGB has expressed 0,98 FPU/ml in 50 ml liquid culture as the maximum activity. In solid-state cultures, with the first flushing, 0,98 FPU of cellulases / ml was harvested in 100 ml washing liquid. Analyzing the values in table 1, it can be concluded that washing cellulose before inoculation leads to higher production in the first cycle of 7 days of incubation (0,61 FPU/ml in the flask with washed biomass (SSC+f), compared with 0,46 FPU/ml in the flask with unwashed biomass (SSC+fm) in *T. viride* ATCC. Still, it can be observed that the productivity in *T. viride* ATCC has increased in SSC+fm flask (unwashed biomass) after the first cycle of 7 days, probable due to glucose removal with the first flushing. The cellulolytic activity of *T. viride* CMGB is higher in the first cycle of 7 days, but decreases and is surpassed by the activity of *T. viride* ATCC until the last cycle. These data indicate that cellulolytic activity of *T. viride* ATCC is inhibited by the content in reducing sugars, while *T. viride* CMGB is less affected by the content in sugars of culture medium. This can be concluded from the activity in SSC without flushing, where *T. viride* CMGB express an activity 3 times higher than *T. viride* ATCC (0,46 FPU/ml, compared to 0,15 FPU/ml). The total production in cellulase can be found multiplying the activities of enzymes (FPU/ml) in each flushing with 100 (the volume of flushing liquid) and adding the productions of each of the three cycles. The results are:

- In SSC+f (solid-state culture with washed biomass, flushed three times, once in 7 days) of *T. viride* ATCC: 60,5 FPU total production.
- In SSC+f (solid-state culture with washed biomass, flushed three times, once in 7 days) of *T. viride* CMGB: 158,8 FPU total production.

- In SSC+fm (solid-state culture without washed biomass, flushed three times, once in 7 days) of *T. viride* ATCC: 99 FPU total production.
- In SSC+fm (solid-state culture without washed biomass, flushed three times, once in 7 days) of *T. viride* CMGB: 99 FPU total production.
- In SSC (solid-state culture with washed biomass, no flushing) of *T. viride* ATCC: 8 FPU total production.
- In SSC (solid-state culture with washed biomass, no flushing) of *T. viride* CMGB: 39 FPU total production.

Table 1. Production of cellulase of tested strains in solid-state cultures in wheat bran

	Strain	Production of cellulase (FPU / ml of washing liquid)						
		7 days		14 days		21 days		
		SSC+f	SSC+fm	SSC+f	SSC+fm	SSC+f	SSC+fm	SSC
1	<i>T. viride</i> ATCC	0,61	0,46	0,61	0,8	0,15	0,3	0,15
2	<i>T. viride</i> CMGB1	0,98	0,91	0,61	0,46	0,15	0,15	0,46

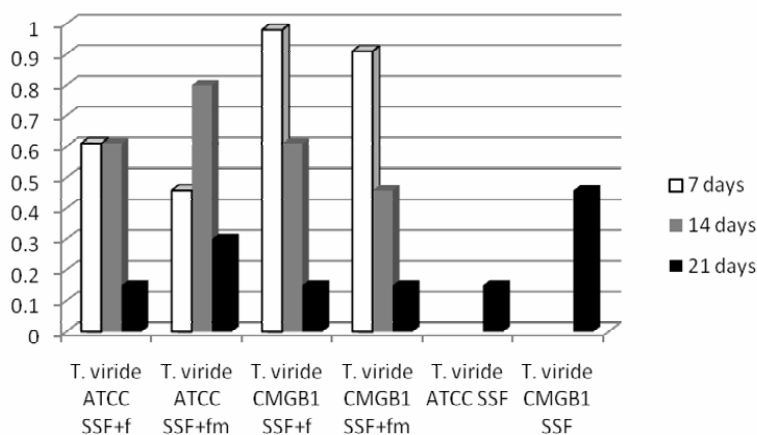


Figure 3. Production of cellulase in solid-state cultures of *T. viride* on wheat bran.

The data indicate that the most productive system is by solid-state cultures using biomass washed to remove glucose and with periodic flushings to remove reducing sugars, harvest enzymes, and add nutrients.

The cellulases produced by *T. viride* CMIT3.5 (CMGB1) were concentrated and characterized, showing maximum activity of 3.18 FPU/ml at 50°C, pH 4.8. The activity decreases to 0.6 FPU/ml at 25°C, to 0.18 FPU/ml at 90°C, or to 0 at pH 3.6 and 5.6. Regarding stability of the cellulases at high temperatures, the activity decreases in 10 minutes under 50% at 60-75°C, or to 0 in 180 minutes at the same temperatures. The results recommend this local strain to be used for cellulase production and recommend solid-state cultures as systems for producing cellulases at lower price than submerged cultures.

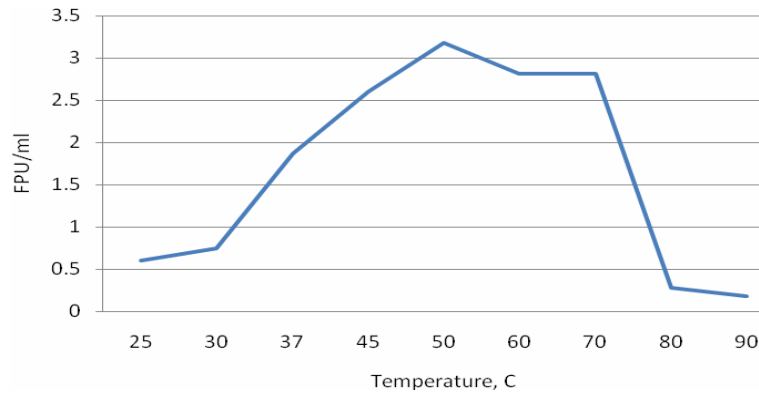


Figure 4. Activity of cellulases produced by *T. viride* CMGB1 at different temperatures

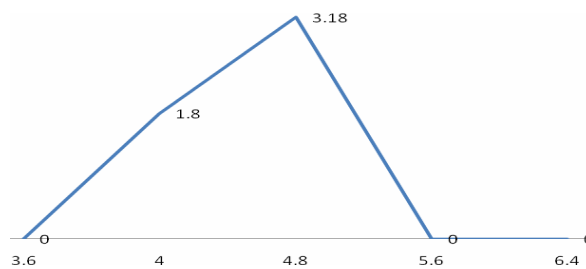


Figure 5. Activity of cellulases produced by *T. viride* CMGB1 at different pH values

Regarding the capacity of ethanol production in yeasts, seven yeast strains with the highest capacity to produce ethanol are presented in figure 6. One yeast strain (*S. cerevisiae* CMIT2.18) was found to have the same ethanol producing capacity up to 40°C. All yeast strains were inhibited at 45°C. Comparing the activity of cellulases and the capacity for ethanol production of yeasts, the temperature of 40°C can be used for simultaneous hydrolysis and fermentation of lignocellulose, if the enzymes produced by *T. viride* CMIT 3.5 (CMGB1) are used for hydrolysis and the yeast strain *S. cerevisiae* CMIT2.18 is used for fermentation of resulted glucose to ethanol.

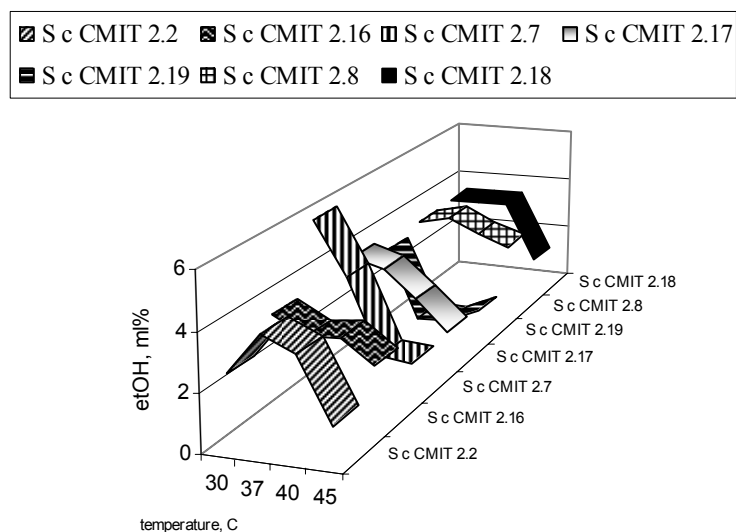


Figure 6. Capacity to produce ethanol of several yeast strains at different temperatures.

Conclusions

Comparing the productions of cellulases in the systems applied in this study, data indicate the system of solid-state culture with flushing as the most efficient (660% more efficient in *T. viride* ATCC 13.631 SSC+f than in SLC and 455% more efficient in *T. viride* CMGB1 SSC+f than in SLC). Still, *T. viride* CMGB1 show a higher production than *T. viride* ATCC 13.631 in laboratory conditions, which recommend this local strain to be used in industrial applications for cellulase production. These results obtained in yeast cultures indicates the strain *S. cerevisiae* CMIT2.18 to be used in simultaneous hydrolysis and fermentation of lignocellulose together with the cellulases produced by *T. viride* CMIT3.5 at the temperature of 40°C. This system can lower the price of ethanol produced from lignocellulosic biomass.

Acknowledgements: This research was funded by the Romanian Ministry of Education and Research – PNII - IDEI grant no. 427/2007, cod CNCSIS 1236.

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