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# Opportunities and Issues in the Production of Cellulase from Waste Materials

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Enzymatic hydrolysis represents a key step in the production of bioethanol from lignocellulosic materials. Much research is currently focussed on the improvement of the cellulase production process, aiming at better balancing its composition and at reducing its cost. In this work we present and discuss the production of cellulase with myceliar fungi on waste materials used as culture media and inducers, making special reference to olive oil mill wastewater (OOMW) used as a culture medium and olive pomace (OP) used as an inducer. We report experimental results showing that OP is a suitable inducer of cellulase production, albeit less effective than pure cellulose and discuss their limitations and opportunities for improvement.

## 1. Introduction

Although lignocellulosic materials are widely considered to be one very promising biofuel source, there is no agreement about which is the optimal way to turn the raw materials into the target products. While chemical and thermochemical conversion paths enjoy from their simplicity and fast kinetics, biological paths offer a superior conversion efficiency at milder processing conditions.

Enzymatic hydrolysis by cellulase represent one of the key steps in the biological lignocellulosics-to-ethanol conversion process, given its (potentially) quantitative sugar yield, in the virtual absence of sugar degradation byproducts which would impair fermentation. However, enzymatic hydrolysis itself does suffer from quite a few weaknesses, namely slow reaction rate, critical pool components (endo- and exo-glucanase, beta-glucosidase, xylanase) balancing, catalyst inhibition, deactivation, and price. Currently, much research is focussed on the improvement of cellulase in these areas, which entails the improvement of the cellulase production process.

Cellulase is produced with specific fungal microorganisms secreting it, most frequently belonging to the *Trichoderma* sp., in a highly aerobic fermentation carried out in aseptic culture on a growth medium including defined salts, carbon source(s), nutrients, surfactants and inducer(s), i.e. compounds that specifically stimulate its secretion.

In this work we present and discuss the production of cellulase with myceliar fungi on waste materials used as culture media and inducers, in the light of the criticities arising from the use of the produced cellulase in the bioconversion of waste lignocellulosic materials.

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## 2. Materials and Methods

*Microorganism*. The adopted biomass was of the ascomycete *Trichoderma viride*, a cellulase high producer originally supplied by CBS, NL, maintained in purity on the following glucose-based solid medium: agar (35 g/l), $K_2HPO_4$ (2,13 g/l), $H_3PO_4$ (1 g/l), $MgSO_4 \cdot 7H_2O$ (0.67 g/l), $FeSO_4 \cdot H_2O$ (6.67 mg/l) $ZnSO_4 \cdot 7H_2O$ (6.67 mg/l) and $MnSO_4 \cdot H_2O$ (5.69 mg/l) and regularly (once per month) transplanted.

*Culture media*. Pre-culture medium: *T. viride* preculture was carried out on a propagation medium containing: glucose (7 g/l) $KH_2PO_4$ (3.5 g/l); $(NH_4)_2SO_4$ (1.2 g/l), $MgSO_4 \cdot 7H_2O$ (30 mg/l); $FeSO_4 \cdot 7H_2O$ (0.63 g/l); $MnSO_4 \cdot H_2O$ (400 mg/l); $ZnSO_4 \cdot H_2O$ (330 mg/l); $CoCl_2 \cdot 6H_2O$ (0.84 mg/l); $CaCl_2 \cdot 2H_2O$ (190 mg/l). Cellulase Production Test medium: the composition of the medium used for the cellulase production test was the same as that of the corresponding pre-culture medium, except for the concentration of the principal substrate (glucose at 15 g/l) and the supplementation with 15 g/l of a specific inducer (lactose, cellulose, OP or delignified OP).

*Cellulase Production Tests.* A small biomass amount was taken from the slant culture and inoculated in small fermenter (total volume = 1 l, working volume = 700 ml) containing 700 ml of the propagation medium. After glucose depletion (usually 48 h), 5 ml of the grown biomass suspension were sampled, centrifuged, re-suspended in water and then added to a basal medium containing glucose, nutrients and the tested inducer: glucose (15 g/l), inducer (15 g/l), $KH_2PO_4$ (7.5 g/l); $(NH_4)_2SO_4$ (2.6 g/l), $MgSO_4 \cdot 7H_2O$ (64 mg/l); $FeSO_4 \cdot H_2O$ (1.35 g/l); $MnSO_4 \cdot H_2O$ (860 mg/l); $ZnSO_4 \cdot H_2O$ (700 mg/l); $CoCl_2 \cdot 6H_2O$ (1.8 mg/l); $CaCl_2 \cdot 2H_2O$ (400 mg/l). A final test was performed using diluted OOMW (1:3) as basal medium and OP as inducer.

The production reactor was periodically sampled and reducing sugars (glucose or glucose plus lactose) concentration, ammonium concentration, enzymatic activity (Filter Paper Activity, FPA), solids content (Total Suspended Solids, TSS) and—only for OP tests—polyphenols concentration were measured. Furthermore, the reactor itself consisted in a respirometer: biomass respiration (Oxygen Uptake Rate, OUR) and $O_2$ consumption were monitored along the test timelength. Oxygen was provided by means of an external compressor at a constant flow rate of 200 l/h (5 vvm).

*Analytical techniques*. Reducing sugars were estimated by their glucose equivalents generated during the assay, as determined by the Miller method (Miller, 1959) with glucose as standard. The enzymatic activity was measured according the filter paper activity (FPA) method (Ghose et al., 1987) and expressed as international Filter Paper Units (FPUs). Polyphenols were measured by the Folin-Ciocalteau (Singleton and Rossi, 1965) method using gallic acid as standard. Biomass concentration was determined by filtering a known volume sampled from the reactor on a pre-weighed Whatman Filter Paper disk under vacuum and then drying it in an oven at 105°C for 4 h. The filter was then cooled and then weighed again. The solid concentration was calculated as the ratio of the weight difference by the sample volume. This method could not be applied to the tests performed with cellulose and OP because of the interference due to the simultaneous separation of the inducers themselves.

*Inducers*. The tested inducers were lactose, cellulose, and olive pomace.

*OUR Determination.* Dissolved Oxygen (DO) concentration in the reactor was monitored by means of a DO Probe Hach-Lange HQ10, customly modified to transfer

data to a PC. The OUR was calculated according to Badino et al. (2000), while the consumed oxygen was calculated by integration of the OUR profile.

## 3. Results and Discussion

Cellulase production was preliminarily assayed in Erlenmeyer flasks with various culture media and inducers (results not shown) and then in batch, stirred and sparged cultures, discussed in detail in the following.

### 3.1. Results of cellulase production results by *T. viride*

A preliminary test (results not shown) showed that cellulase productivity in the absence of any inducer was equal to 5 FPU.

*Lactose-induced cellulase production.* As it can be noted in Figure 1, FPA activity shows a profile resembling the OUR one. The FPA activity showed a peak (35 FPU) at the 20$^{th}$ hour. Interestingly, when reducing sugars (RS) concentration dropped to zero a significant OUR was still observed. However, this was not surprising: when lactose is hydrolized by *T. viride* enzymes, D-glucose and β-D-galactose are produced (Seiboth et al., 2007), of which only glucose is a reducing sugar detected by the Miller method (lactose is detected owing to its glucose moiety). The process is higly aerobic and reaches a oxygen-limited steady state phase after about 20 h, where the OUR is 250 $mgO_2/l/h$. In this phase, likely, all the initially available glucose (i.e., the glucose directly dosed and that arising from lactose degradation) has been removed, some D-galactose still being available in the liquor. After complete conversion of lactose into the two monomers, cellulase production is then expected to slow down/be interrupted by the fungus due to depletion of the inducer. Indeed, a decrease in FPA activity is observed, most likely caused by the vigorous stirring and aeration: cellulase deactivation by shear stress and by the presence of gas–liquid interfaces is indeed a known phenomenon (Ganesh et al., 2000). A biomass growth slow down is confirmed by the changing profile of the residual nitrogen after the 50$^{th}$ h.

*Cellulose-induced cellulase production.* Oxygen uptake rate again showed an exponential profile in the first part of the test, followed by an oxygen limited steady state. Interestigly, the FPA activity showed a maximum (~30 FPU) when glucose in the medium was depleted. The rapid FPA decrease can be explained by the fact that a significant fraction of the secreted enzyme is actually absorbed on the cellulose matrix surface and therefore not available in the liquor. Likely, cellulose hydrolysis was made slower by the crystalline nature of the cellulose used. This hypothesis is supported by the fact that the OUR was still high and that an exogenous substrate was available. Reducing sugars analysis was likely unable to detect significant amounts of carbohydrates as they were taken up by the biomass itself too quickly.

*OP-induced cellulase production.* Biomass respiratory activity was lower and irregular, a result reflected in a lower cellulase production. However, the enzyme activity profile was similar to that of the other inducers. Cellulase may have partially bound to the lignin contained therein, which makes up 19.3% of the dry weight of pomace, while cellulose accounts for 17.5% (Roig et al., 2006). OP also contains polyphenols, which might have exerted some promotion action of their own on cellulase excretion, as pointed out by Arrieta-Escobar and Belin (1982).

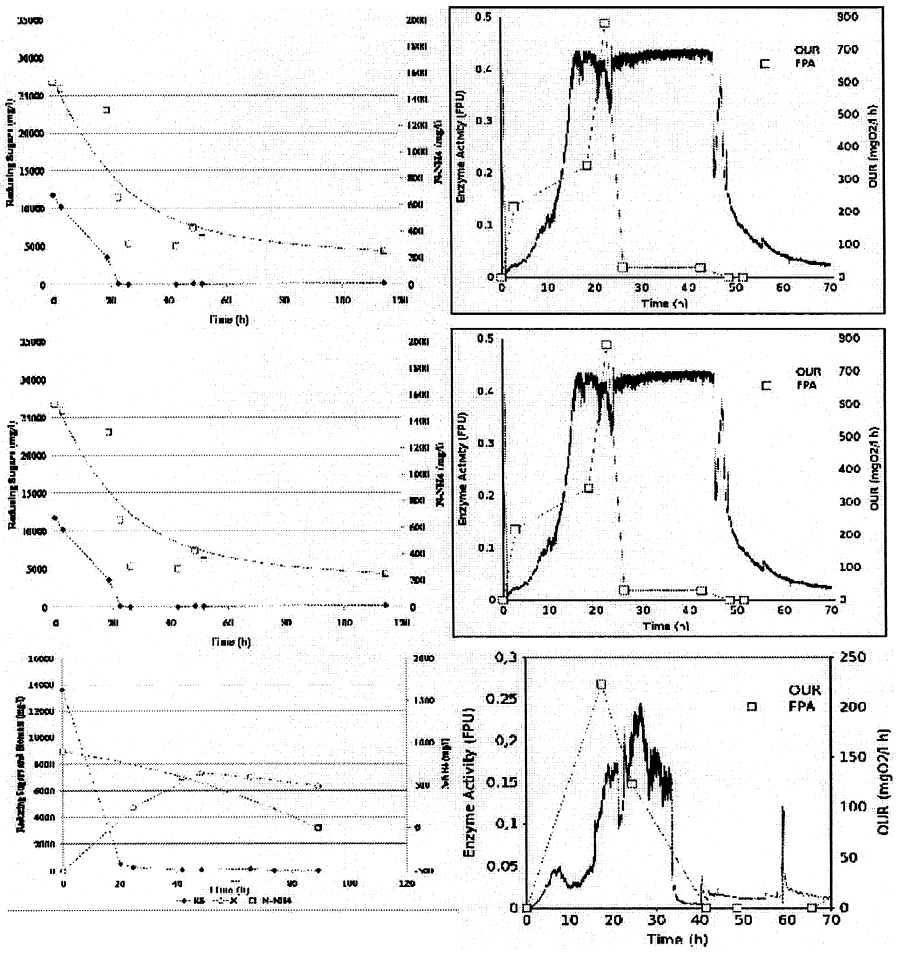


*Figure 1. Process parameters during cellulase production culture using lactose (upper series), cellulose (middle) and olive pomace (lower series). Time profile of enzyme activity and oxygen uptake rate (left) and concentrations in the culture (right).*

In all the reported results, the peak time of the production culture medium activity profile is close to the exhaustion time of the exogenous substrate. Its shape, which defines the useful harvest time window, most likely is the effect of two overlapping phenomena: enzyme release and enzyme deactivation. Release is expected to show a burst during the relief of catabolyte repression, and to decay shortly after the shortage in the supply of a readily available carbon source (glucose) shows its effects. Activity loss occurs continuously, due to stirring and to the massive presence of gas-liquid interfaces and, for the case of cellulose- and pomace-induced tests, to the binding onto the solid surface of the inducer (expecially toward lignin, which is nonoproductive and, therefore, often irreversible: Xu et al., 2008).

### 3.2. Opportunities, Limitations, and Possible Developments

Cellulase is harvested some some time after the production culture is started. In general, a development phase of the biomass ahead of the production culture will be required, in turn requiring a suitable growth medium and time for the biomass to grow, both at a cost. Strategies for reducing it may include using negative-cost culture media, such as wastewaters, and saving culture time by sharing this biomass growth time with a biotreatment. Residual open questions include whether a biotreatment can be maintained in monocultural state, which is essential for the production of the desired metabolytes, and whether cellulase excretion by a biomass developed on a specific wastewater is not repressed (e.g., due to interplay with lipid accumulation), of which *Trichoderma viride* has been demonstrated by D'Urso et al. (2008a) to be capable both on glucose and on OOMW. The results (not reported) of the initial qualitative screening investigation suggest that this may be feasible.

Cellulase is a mixture of enzymes synergically operating the breakdown of cellulose and its activity is defined through the amount of released reducing sugars, independent of their actual identity. The hydrolysis of cellobiose, a reducing sugar itself, may be the controlling step of the overall production rate of glucose—which is normally the case with the *Trichoderma* genus, due to an imbalance between β-glucosidase and the rest of the cellulase pool. Other than rebalancing it by supplementing *Trichoderma* cellulase with an appropriate amount of β-glucosidase produced separately, other opportunities include running the bioprocess with a mixed culture comprising a suitable β-glucosidase producer (such as *A. niger*), or using a suitably genetically modified *Trichoderma.* Successful co-culturing of *T. viride* and *A. niger* has been recently demonstrated by ul-Haq et al. (2005), who found, however, that strain compatibility (lack of antagonistic effects) is a key issue; the supernatant from compatible cultures had significant more saccharifying ability than that from either culture alone. Ahamed and Vermette (2008) observe that substrate cost is still one key cost factor of cellulase production, and grow their *Trichoderma-Aspergillus* co-culture on a mixture of cellulose and lactose. Whether the co-culture may be grown on biorecalcitrant wastewaters, thus minimising the culture cost, has only partial answers in the literature. *T. viride* monocultures have been successfully grown on biorecalcitrant model systems in batch and continuous reactors and real-life biorecalcitrant wastewaters such as distillery wastewaters, cork processing wastewaters and OOMW in batch and continuous cultures by D'Urso et al. (2007, 2008b and 2008c). An indigenous *A. niger* strain was successfully used in OOMW treatment by Aissam et al. (2007). However, co-culturing of *Trichoderma* and *Aspergillus* in biorecalcitrant wastewaters, appearently, has never been attempted. Application of a continuous co-culture to the treatment of OOMW aiming at the simultaneous withdrawal of the excess biomass for further uses may present with unexpected difficulties, such as the washout of the slower-growing species.

## 4. Conclusions

Despite recent advances, the availability of low-cost cellulase is still recognized as a hindrance to the deployment of lignocellulosic bioethanol. Production of cellulase at a low cost is an outcome of process optimization, including an appropriate deployment of the time profile of the enzyme activity during the production fermentation, and the

choice of suitable inducers. The present experimental work has discussed the relationship between the culture parameters, including the oxygen uptake rate, and the measured activity of the enzyme in solution; this relationship may be worked into a readily measured indicator of the optimal stopping time for a production fermentation. While OOMW is able to support *Trichoderma viride* growth with negligible inhibition from contained phenolics and variable organic load removal efficiencies, olive pomace may be used a suitable inducer of cellulase production, albeit less effective than pure cellulose.

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