Cellulase Production from Olive Processing Residues

Damato G., Vivona G., Stoller M., Bubbico R., Bravi M.
Dept. of Chemical Engineering Materials Environment, Sapienza University of Roma
v. Eudossiana, 18; I-00184 Roma, Italy

One of the economic bottlenecks in the production of lignocellulosic bioethanol lies in cellulase supply. The ETOILE project (UE FP7) targets the valorisation of waste materials (pomace and vegetation water) of the olive oil production process. Here, we investigate the production of cellulase from these raw materials, targeting niche aspects favouring process integration.

1. Introduction

One of today's greatest challenges for scientists and technologists is sustainably meeting the growing energy demand to feed industry processes, transportation, and residential uses. Renewability is the leit-motiv and biological energetic resources, of which lignocellulosic biomasses are one major component, have been identified as the most promising source of biofuels. Lignocellulosic wastes are a particularly appealing resource and are being carefully considered as potential bioethanol raw materials. Olive wastes (pulp, stones, and vegetation water) are currently under close investigation under the ETOILE craft project (FP7).

One of the most (economically and technically) critical step in the production of bioethanol is the enzymatic hydrolysis of the lignocellulusic biomass; the cost of cellulase production currently accounts for a large fraction of the estimated total production costs of bioethanol. The high cost of cellulase enzymes represents a significant barrier to the commercial deployment of bioethanol production processes using lignocellulosic raw materials. In the past years significant efforts have been made to reduce the cost by focusing on optimizing the fermentation process parameters, improving the efficiency of known enzymes and microorganisms, identification of new, more active enzymes, and minimization of enzyme production costs by using, for example, waste materials as colture media and inducers of enzyme production.

In this work we present and discuss a strategy for the production of cellulase with the myceliar fungus *Trichoderma reesei* RUT-C30 using the two main by-products of the olive oil productive process: a liquid, Olive Oil Mill Wastewaters (OOMWs), and a solid, Olive Pomace (OP). OOMWs represent a serious environmental problem in the Mediterranean area: they are characterised by a high COD, a low pH, a significant suspended solids fraction and feature the presence of biorecalcitrant and inhibiting compounds, mainly polyphenols, which make traditional biological processes poorly effectives. Previous studies (D'Urso et al., 2007 and 2008) have demonstrated that mold

strains belonging to the *Trichoderma* genus are able to withstand the critical characteristics (pH and composition) of OOMWs and succesfully operate their biotreatment. Considering this, we devised a process in which cellulase production, which uses the lignocellulosic OP as inducer, is carried on by a biomass obtained from the biotreatment of OOMW.

2. Materials and methods

Microorganism. The mutant cellulase-producing strain *Trichoderma reesei* RUT-C30 (NRRL 11460) used in this work was obtained from the ARS Culture Collection. Culture was maintained on potato dextrose agar Petri plates and stored at 4 °C in refrigerator.

Culture media. Pre-culture medium: *T. reesei* RUT-C30 preculture was carried out on a propagation medium containing: glucose (10 g/l) KH_2PO4 (2 g/l); $(NH_4)_2SO_4$ (1.4 g/l), $MgSO_4•7H_2O$ (0.3 g/l); $FeSO_4•7H_2O$ (5 mg/l); $MnSO4•H2O$ (1.6 mg/l); $ZnSO_4•H_2O$ (1.4 mg/l); $CoCl_2•6H_2O$ (2 mg/l); $CaCl_2•2H_2O$ (0.4 g/l); Proteose Peptone (1 g/l); Tween80 (0.2 g/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 supplementation with 10 g/l of a specific inducer (lactose, cellulose microcristalline, OP) and, in some cases, the absence of glucose.

Cellulase production Tests. All the performed tests were carried out in 300-ml Erlenmeyer flasks with a working volume of 100 ml; the flasks were incubated at room temperature (24° C) and agitated on a rotary shaker (200 rpm). A 10% (v/v) inoculum concentration, from the pre-culture, was used to initiate the cellulase production tests. The production flasks were periodically sampled and reducing sugars (glucose or glucose plus lactose) concentration and enzymatic activity (Filter Paper Activity, FPA), were measured. All the tests were run in duplicate.

Analytical techniques. Reducing sugars were estimated by their glucose equivalents generated during the assay, as determined by the 3,5-dinitrosalicylic acid (DNS) 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); one FPU is defined as the amount of enzyme that releases 1 µmol of glucose/min under the assay conditions. Activities were reported as FPU/ml. The COD was measured with 1000-10000 mg l^{-1} O_2 Hach Lange kit.

3. Results And Discussion

3.1 Thermal-acid treatment of OOMWs

The aim of our work was to find a way to couple two very different biological processes: the "dirty" biotreatment of an agricultural liquid residue, the OOMWs, and the "clean" cellulase production process. The main difference between the two processes is that the former is usually conducted by using a consortium of uncharacterised microorganisms while the viability of the latter process relies on the manteinance of axenic conditions.

In this view, our first goal was to devise a low-cost pretreatment carrying about the microbiological stabilisation of OOMW to be performed prior to the biotreatment and the enzyme production processes. Considering that the autoclave sterilisation of huge

volumes of a waste is not economically feasible, we devised an acid-pasteurisation pretreatment process nicely fitting the needs and the features of the overall biotreatment and enzyme production process. The devised pretreatment process consists of two phases. In the first phase, OOMW, whose natural pH is usually about 5, is acidified down to pH=3, its temperature is increased to and held at 65°C for 30'. The target pH was chosen because lethal/inhibitory to most bacteria and fungi but harmless for *Trichoderma reesei* RUT-C30. Moreover, the low pH is responsible of the coagulation of some components present in the liquid waste and the formed flocs can be separated by settling in 24 hours, thereby also reducing the COD content of OOMW by about 30%. The reason of the adopted temperature value is that it can be obtained by using solar heat exchangers, thereby almost entirely offsetting the treatment costs.

The acid-pasteurization process was succesfully experimentally tested to evaluate its effectiveness in lowering the microbial load; the OOMWs that undergo the thermal-acid treatment, if plated on Petri plates, exhibit a total absence of colony-forming microorganisms growth.

3.2 Effect of gallic acid on cellulase production

OOMW is characterised, among others, by the presence of polyphenols (Proietti and Nasini, 2006). The effect of these compounds on cellulase production was previously studied in *Trichoderma viride* (Arrieta-Escobar, 1982) but their effect on the hyperproducing *T. reesei* RUT-C30 was still unknown.

To evaluate the effect of polyphenols on cellulase production, gallic acid was chosen as molecule model for this class of compounds.

Firstly, the potential inhibiting effect of gallic acid was tested in a lactose-based fermentation. In these experiments, gallic acid was added at the culture media at the concentration of 3 grams per liter, a typical concentration value of this compound found in OOMW. As shown in Figure 1A, in the considered conditions, the presence of the phenolic chemical does not influence at all cellulase production by *T. reesei* RUT-C30.

After demonstrating the absence of inhibition, gallic acid induction effect was investigated. Cellulase production was tested in a glucose-based fermentation in which gallic acid was added at the concentration of 3 g/l; the obtained results were then compared with the "basal" *T. reesei* RUT-C30 cellulase expression, that is, the amount of cellulase enzyme produced in a glucose-based fermentation in which any inducer was added. As it can be seen in Figure 1B, gallic acid has a weak but significant inductive effect on cellulase production in the studied fungus: with respect to the basal expression, the presence of gallic acid almost duplicate the maximum measured FPA (0.28 versus 0.47 FPU/ml).
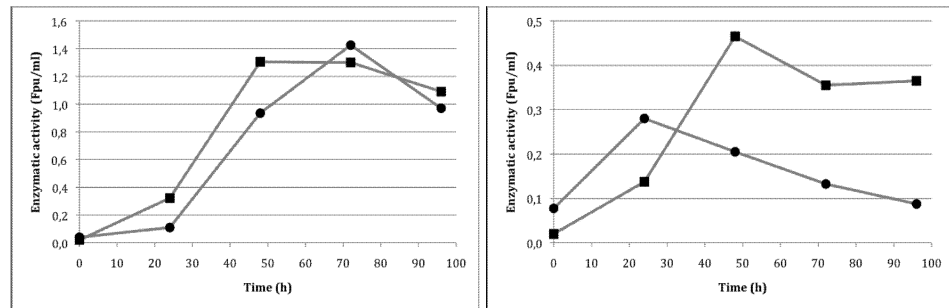
Figure 1. **Left**: *Effect of gallic acid in a cellulase fermentation induced by lactose.* **Right**: *bare gallic acid induction effect in a non otherwise induced cellulase fermentation.*

3.3 Cellulose and Olive Pomace as Inducers for Cellulase Production

One essential culture medium component in a process of enzyme production is the inducer: it is a compound, usually organic, that stimulates the production of the desired enzyme in a particular microorganism. Typical inducers of cellulase production in *Trichoderma* strains are cellulose (the enzyme target), lactose and sophorose.

Olive pomace (OP) coming from the productive process of olive oil is a lignocellulosic biomass, mainly composed by olive stones. The induction power of OP, compared with that of cellulose, was tested in two different nutritional situations: in the presence and in the absence of an additional carbon and energy source (i. e. glucose).

The esperimental results are illustrated in Figures 2A and 2B. The first notable thing is that OP is effectively usable as an inducer for cellulase production in *Trichoderma reesei* RUT-C30. As it can be seen in Figure 2A, when the fermentation is conducted in the presence of glucose, cellulase production shows an almost identical trend in OP- and cellulose- medium: after a 24 hour lag phase, cellulase production start to increase and reach a maximum of 0.8–0.9 FPU/ml at 48 hours and then it starts to slightly decrease.

The observed phenomena are quite different, and more interesting, in the absence of glucose, when the microorganism can utilize either cellulose or OP as the unique carbon and energy source. In these conditions, compared to cellulose, OP seems to be a better inducer of cellulase production. As showed in Figure 2B, cellulase production starts after a 24-h lag phase in both fermentations but the maximum activity reached is higher in the OP-induced system (~1.2 FPU/ml) than in the cellulose-induced one (about 0.7 FPU/ml). Furthermore, appearently, production does not appear to have reached a plateu over the test time.

3.4 Overall Process Arrangement

Previous work by this research group has shown that *Trichoderma viride*, of which *T. reesei* RUT-C30 is a cellulase hyper-producer close relative, is able to grow in and perform the biotreatment of OOMW (D'Urso et al., 2008) as well as of other wastewaters usually deemed biorecalcitrant and toxic (D'Urso et al., 2007). A 62% DOC reduction and a 48% (C-mol base) growth yield were calculated over an HRT (Hydraulic Retention Time) and SRT (Solids Retention Time) equal to 5 days.
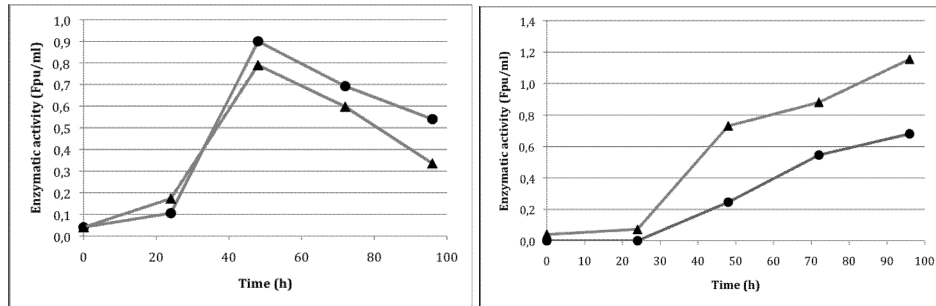
Figure 2. **Left**: *production with glucose.* **Right**: *production without glucose.* ▲: *OP;* ●: *cellulose.*

Waste biomass after the biotreatment can be used for cellulase production and, in the light of the results discussed previously, biomass concentration can be expected to affect cellulase production rate. A high biomass concentration should therefore be maintained in the cellulase production bioreactor.

As hinted by D'Urso et al. (2007), while simple polyphenols, such as gallic acid, can be degraded by *T. viride,* high molecular weight ones are not significantly degraded and are likely not degraded by our *T. reesei.* While any remaining low (and, possibly, high) molecular weight polyphenols from the biotreatment stage could even be beneficial during the enzyme production phase, and their separation before enzyme production might therefore not be necessary, their removal may be required before cellulase formulation if polyphenols-sensitive microorganisms are to be put in contact with the produced cellulase. This is the case in simultaneous saccharification and fermentation (SSF). The final cellulase activity measured in the production medium is adaequate for the hydrolysis of lignocellulosic materials, among which pomace itself, at a 10% solids weight with 15 FPU/g of solids enzyme load. After enzyme production, the fungal biomass could be recycled to the biotreatment, considering that a high biomass concentration is helpful to keeep the required HRT low. The biomass excess might be destined to anaerobic digestion.

4. Conclusions

The present work has highlighted the economic and environmental opportunity behind the integration of waste biotreatment and enzyme production in which wastewaters are used as culture media in place of traditional expensive media, thus benefitting from their negative cost, and waste solids are used as enzyme production inducers. Incidentally, residual components contained in selected wastewaters, such as polyphenols in OOMW, may even favour enzyme production. A key point in the establishment of a working operation is the adoption of a low temperature pretreatment to avoid the proliferation of obnoxious initial biological contaminants of the wastewaters.

Acknowledgements

References

Arrieta-Escobar, A. and Belin, J. M., 1982, Effects of Polyphenolic Compounds on the Growth and Cellulolytic activity of a Strain of Trichoderma Viride, Biotech Bioeng, 24, 983—989.

D'Urso, A., Galimberti, M., Bravi, M., Fungal Biomass-Based Processing of Phenolics-Rich Wastewaters, Chemical Engineering Transactions. Vol. 11 (2007) 671—676.

D'Urso, A., Gapes, D., Bravi, M., Bioremediation of Olive Oill Mill Wastewaters by Fungal (*Trichoderma viride*, strain 8/90) Sequencing Batch Reactor. Chemical Engineering Transactions. Vol. 14 (2008) 481—486.

Miller, G. L., 1959, Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. An Chem 31 (3) 426—428.

Ghose, T. K. and others, 1987. Measurement of cellulase activities. Pure Appl. Chem, 59 (2) 257—268.

Proietti, P. and L. Nasini, 2006, L'Utilizzo dei Reflui Oleari nel Nuovo Panorama Normativo (article in Italian).