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Use of olive mill wastewater by lipolytic yeasts

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The olive mill industry is a traditional agricultural industry in Mediterranean countries. These countries produce almost all the olive oil sold worldwide. In Portugal is predicted, for 2007, a production of 35×10^3 m^3 of olive oil (INE, Agricultural Statistics). During the olive oil production (short periods of time) a large amount of liquid waste is generated. This liquid waste, also called olive mill wastewater (OMW), constitutes a major environmental problem. The quality and quantity of the constituents in OMW depends on many factors, such as, type of olives, type of soil, cultivation system and production process. The large variety of components found in OMW (carbohydrates, polysaccharides, sugars, lipids and phenolic compounds) difficults their treatment, and their disposal presents a critical environmental problem (Niaounakis and Halvadakis, 2004).

The inhibitory effects of phenolic compounds on cellular activity limit the OMW treatment, in traditional biological plants. Many proposals have been reported to reduce this problem, including the modification of oil extraction process (ecological olive mill) to decrease the OMW production, re-utilization of OMW in olive cultivation and OMW treatment by physical–chemical and biological process. Several disposal methods have been proposed ad mainly include physical–chemical treatments (decantation with lime and/or chemical oxidation, concetration, drying and incineration, ultrafiltration and reverse osmosis) (D'Annibale et al., 2006). The anaerobic biological degradation of OMW can lead to methane production although large periods of biomass adaptation have been reported as a disadvantage of the process. Biological treatment by aerobic microrganisms isolated in OMW (fungi and yeasts) has also been proposed but no valorisation of the OMW is made (Eusébio et al., 2002). Yeast species such as *Yarrowia lipolytica*, *Candida rugosa* and *Candida cylindracea* can grow well in OMW media, consume the organic material and, at the same time, produce biomass and other valuable products, like enzymes (such as lipases) and organic acids (such as citric acid).

The aim of the present work was to study the best conditions to use olive mill wastewater to produce high-values compounds by lipolytic yeasts such as *Yarrowia* and *Candida* strains.

OMW from different regions of Portugal were used and characterized chemical and biochemically. OMW with COD ranging from 20 to 200 g/L were supplemented with yeast extract and ammonium chloride proportionally to its organic composition. Preliminary studies of OMW consumption were carried out in bacth yeast cultures of *Y. lipolytica* and *C. rugosa*.

The strains were able to grow in the OMW used without dilution, to consume almost all of the sugars present in the media and to significantly reduce COD. In spite of the low degradation of phenolic compounds, no cell growth inhibition was notice. Moreover, lipase production was observed in yeast cultures of OMW based medium with common profiles usually found in synthetic media.

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doi:10.1016/j.jbiotec.2007.07.916

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Optimization of the conditions for the expression of an anti-transthyretin scFv in *Pichia pastoris*

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Familial amyloidotic polyneuropathy type I (FAP) is an autosomal dominant hereditary disease with high prevalence in the northern part of Portugal and is caused by the systemic deposition of a variant of transthyretin (TTR V30M) (Costa et al., 1978). Specific removal of TTR from plasma in an extracorporeal immunoapheresis procedure (Regnault et al., 1992) has been under investigation as a possible treatment for FAP. The high costs related to the production and purification of the anti-TTR monoclonal antibodies has prompted us to develop immunoadsorbents based on single-chain antibody fragments (scFvs).

Using phage display technology we isolated 4 anti-TTR scFvs from the human combinatorial $V_H + V_L$ phagemid library "Griffin.1." (Griffiths et al., 1994) which were expressed in *E. coli* suppressor strain ER2537 up to 10 mg/ml. Since the construction of an immunoadsorbent requires several grams of purified anti-TTR scFv, an attempt was made to express these scFvs in high yields using *Pichia pastoris* (Cereginho and Cregg, 2000).

Anti-TTR scFv-D was subcloned from the phagemid pHEN2 into the vectors pPICZαB and pGAPZαB for the expression under the control of the methanol-regulated AOX1 promotor, and the constitutive glyceraldehyde-3-phosphate dehydrogenase promoter, respectively. Presence of scFv insert was confirmed by PCR, and the recombinant plasmids were propagated in *E. coli* TOP10F′. *P. pastoris* X33 (Mut^+) was transformed with the linearized plasmids by electroporation, and transformants were selected on YPD plates + 100 μg/ml zeocin. Several clones were tested for the highest scFv production under standard conditions (30 °C, 6 days, unbuffered medium) and the clones with the highest level of anti-TTR scFv expression were used for fur-