Appl Microbiol Biotechnol (1991) 34:828–831
0175759891000762

Applied and Microbiology Biotechnology

The use of *Aspergillus niger* for the bioconversion of olive mill waste-waters

Moktar Hamdi*, Abdelkader Khadir, and Jean-Louis Garcia

Laboratoire de Microbiologie ORSTOM, Université de Provence, 3 Place Victor-Hugo, F-13331 Marseille cédex 3, France

Received 6 June 1990/Accepted 27 September 1990

Summary. Olive mill waste-water was used for protein production in small-scale experiments, using non-sterilized medium without pH control. A 14 g/l concentration of proteins, 61% chemical oxygen demand removal and a 58% reduction in total phenolic compounds were obtained using an *Aspergillus niger* strain. The removal of phenolic compounds resulted in a change in the colour of the waste-water from black to brown.

Introduction

Olive black water is a waste-water from the manufacture of olive oil. In the olive-growing Mediterranean countries, olive waste-water production is more than 30 million m^3 per year (Fiestas Ros de Ursinos 1981). Olive oil processing is mainly carried out by means of the traditional discontinuous press process or the more recent continuous solid-liquid centrifuge system. The volume of olive black water produced by the traditional press process is 0.5–0.8 m^3/ton of olives (Boari et al. 1984).

Generally, more dilute waste-waters are produced with the continuous process, but the polluting organic load in terms of the weight of processed olives amounts to 45–55 kg biological oxygen demand (BOD_5) per ton of olives, whichever processing method is used. The maximum BOD_5 and chemical oxygen demand (COD) concentrations are 100 and 220 kg/m^3 respectively (Balice et al. 1982). In olive waste-water produced by the traditional mill and press process, the average concentration of volatile solids and inorganic matter is 15% and 2%, respectively. The organic fraction includes sugars, tannins, polyphenols, polyalcohols, pectins and lipids (Fiestas Ros de Ursinos 1981).

Several studies have been carried out on single cell protein (SCP) production from olive black water (Fiestas Ros de Ursinos 1966; Giulieti et al. 1984), but the yeasts used in these processes degraded only sugars and lipids, and polluting compounds such as pectins, tannins and polyphenols were not removed. *Aspergillus niger*, which has been used for some time now in SCP production (Raimbault and Alazard 1980), possesses extracellular enzymes that hydrolyse pectins (Wood and Siddiqui 1971), polyphenols (Makasinova and Martakov 1982), and tannins (Ikeda et al. 1985). It degrades many phenolic compounds (Kieslich 1976) and can be used for olive black water treatment.

In this paper, we report preliminary results obtained on the utilization of olive mill waste-water (OMW) as a raw material for producing SCP for anti-pollution purposes, using *Aspergillus niger* in erlenmeyer flasks and a small-scale fermentor.

Materials and methods

Media. Media were based on an olive black water sample with a middle COD content, the composition of which is shown in Table 1. The liquid culture medium consisted of 1 l OMW (COD: 154 g/l), NH_4NO_3, 6 g, and $(NH_4)_2SO_4$, 4 g. Preliminary studies showed the need to add nitrogen and sulphate for *A. niger* to grow on OMW (data not reported). The OMW was inoculated with 10^9 spores/l.

The growth of *A. niger* strain A10 on several aromatic compounds was tested in erlenmeyer flasks with the following medium: NH_4NO_3, 5 g/l; $(NH_4)_2SO_4$, 5 g/l; KH_2PO_4, 4 g/l; $MgSO_4$, 0.5 g/l; NaCl, 1 g/l; aromatic compounds (p-hydroxyphenylacetate, tannin, naringin, gallate, vanillate, veratrate, protocatechuate, syringate, gentisate, caffeate – Sigma Chemical, St. Louis, Mo, USA), 5 g/l, glucose, 0.5 g/l; distilled water, 1 l; pH 5.5. The biomass was collected by filtration after 6 days, washed and dried at 105° C for 24 h.

Fungal strain. A. niger strain A10 (Alazard and Raimbault 1981) was maintained on medium containing olive black water in slanted agar at 4° C. This medium contained 50% (v/v) OMW; NH_4NO_3, 5 g/l; $(NH_4)_2SO_4$, 5 g/l; KH_2PO_4, 1 g/l, and agar (Difco, Detroit, Mich, USA), 18 g/l.

* *Present address:* Centre de Biotechnologie, BP-W, 3038 Sfax, Tunisia

Offprint requests to: J. L. Garcia

Table 1. Analysis of olive mill waste-water from a single sample with a middle-COD content

Components	Concentration (g/l)
COD	154
BOD_5	63.5
Dry matter	105
Volatile solids	73.8
Insoluble residue	26.5
Reducing sugars	16.8
Glucose	4.7
Total Kjeldahl nitrogen	0.38
Ammoniacal nitrogen	0.12
K^+	5.2
Ca^+	0.4
Na^+	0.28
Mg^{++}	0.17
NO_3^-	0.82
pH	5.4

COD, chemical oxygen demand; BOD, biological oxygen demand

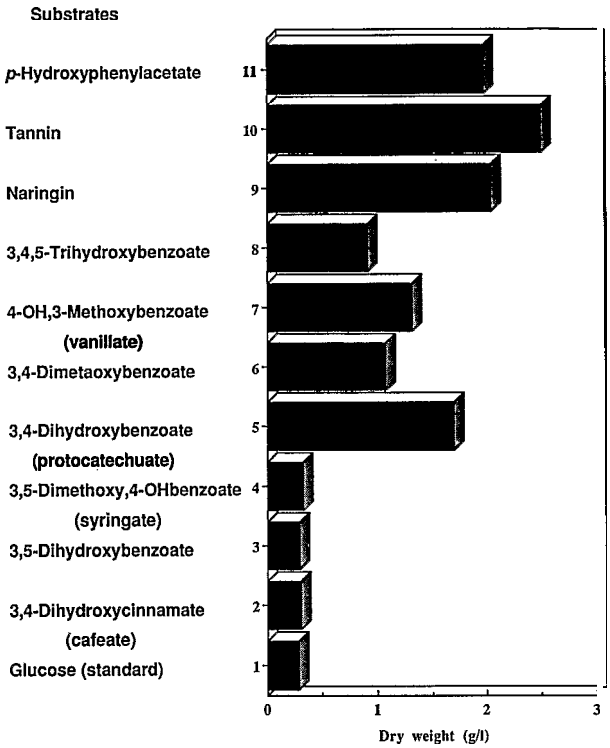

Fig. 1. Production of biomass by *Aspergillus niger* after growing on various phenolic compounds for 6 days. The standard was the yield from glucose added for growth to start without adding 5 g/l of aromatic compounds

Spore inoculation preparation. Spores were obtained from 125-ml flasks containing 15 ml medium composed of OMW, 50% (v/v); NH_4NO_3, 4 g/l; $(NH_4)_2SO_4$, 2 g/l; KH_2PO_4, 1 g/l, and agar 20 g/l. After autoclaving, the flasks were inoculated with spores from an agar slant and incubated at 35° C for 1 week. Fifty millilitres of water and a drop of Tween 80 were then added and the spores were suspended by stirring for 10 min. The spore concentration was measured by direct microscopic counts using a haematimetric cell. A sample of 1 ml spore suspension (10^8 spores/ml) was used as inoculum for 50 ml of substrate.

Fermentation experiments. Small-scale experiments were carried out in 500-ml erlenmeyer flasks containing 50 ml medium, on a rotary shaker operating at 150 rpm. Studies of batch fermentations were performed in 1.5 l of medium using a 2-l fermentor operating at an aeration rate of 1.2 vvm and an agitation speed of 300 rpm. The temperature was 35° C in all experiments. Silicone antifoam was used as required. In all experiments, non-sterilized medium was used without pH control.

Table 2. Phenolic compounds contained in olive mill waste-water (OMW) and references to their degradation by *Aspergillus niger*

Compounds	References to content	References to degradation
p-Hydroxyphenylacetate	Chichelli and Solinas 1984	Kieslich 1976
Protocatechuate	Chichelli and Solinas 1984	Kieslich 1976
Cinnamate	Balice and Cera 1984	Kieslich 1976
Quercitin	Vazequez et al. 1974	Kieslich 1976
Oleuropein	Vazequez et al. 1974	Kieslich 1976
Tannins	Balice et al. 1982	Ikeda et al. 1972
Polyphenols	Fiestas Ros de Ursinos 1981	Makasinova and Martakov 1982
Anthocianins	Tanchev et al. 1980	Blom and Thomassen 1985
Syringate	Balice and Cera 1984	

Table 3. Degradation efficiencies of *A. niger* grown on OMW in terms of the tannin-like compounds present in the waste-water

	Total phenolic (g/l)	Hydrolysable tannins (g/l)	Condensed tannins (g/l)	Monomeric flavoids (g/l)	Simple phenolics (g/l)
OMW	18.20	7.00	2.30	1.50	6.40
Fermented OMW	7.68	1.85	1.70	1.33	2.85
Degradation efficiency (%)	57.8	73.5	26.1	22.0	55.5

Analytical methods. The biomass was collected by filtration on a gauze, and the mycelium washed with distilled water. Proteins were measured using the Kjeldahl method, because of the high content of polyphenols and tannins in the waste. Glucose and reducing sugars in the liquid were estimated by means of the glucose oxidase method (Werner et al. 1977) and the Somogyi micromethod (Nelson 1944; Somogyi 1945), respectively. Methanol was analysed as previously described (Garcia et al. 1982). The COD was estimated by means of the economical method described by Knechtel (1978). The viscosity of OMW was measured with a coaxial cylinder rotary viscometer. Total phenolic compounds, hydrolysable tannins, condensed tannins, monomeric flavoids and simple phenolic compounds were determined using a method described by Balice et al. (1988).

Results and discussion

The choice of *A. niger* was based on review of the literature indicating that the degradation of several phenolic compounds present in OMW is a common characteristic of this genus, as shown in Table 2.

The biomass obtained from *A. niger* cultured on synthetic medium with aromatic compounds is shown in Fig. 1. These results indicate that the majority of the phenolic compounds reported to be found in olive black waters were used with the exception of caffeic acid, 3,5-dihydroxybenzoic acid and syringic acid which is the most plentiful simple phenolic compound in OMW (Balice and Cera 1984).

The *A. niger* growth led to a decrease in the colour intensity of OMW, possibly due to the degradation of some phenolic compounds and adsorption of the polyphenols and tannins on the fungal mycelium. This adsorption may be due to the hydrogen bound between phenolic compounds and proteins or to the chitin of the mycelial wall which has a strongly coagulant effect (Seng 1988). The results of analysis of tannin-like compounds from OMW before and after culture of *A. niger* in erlenmeyer flasks showed that the efficiency of the degradation of simple phenolic compounds by this fungus was 55.5%; this efficiency was 73.5% for hydrolysable tannins (Table 3).

After 72 h, the COD removal rate determined on OMW filtrate fermented by *A. niger* was 61.6% in flasks and 52.5% in the fermentor. The soluble protein in filtrate obtained from flasks and the fermentor were 3.75 and 4.95 g/l, respectively.

The mycelium obtained in the fermentor adhered to the agitation paddles and inner surface of the fermentor after 15 h, giving an average biofilm thickness of 1 cm; this heterogeneity explains the low levels of COD removed in spite of the good aeration conditions. In contrast, in erlenmeyer flasks, the OMW viscosity, which was initially 70–120 cp, increased and then became non Newtonian (Blakebrough et al. 1978), due to the formation of mycelial pellets. The pellets were easily recovered by filtration. In some cases, they were decomposed into a pulpy suspension. In the fermentor, no formation of pellets was observed, and it was difficult to separate the biomass by filtration after fermentation.

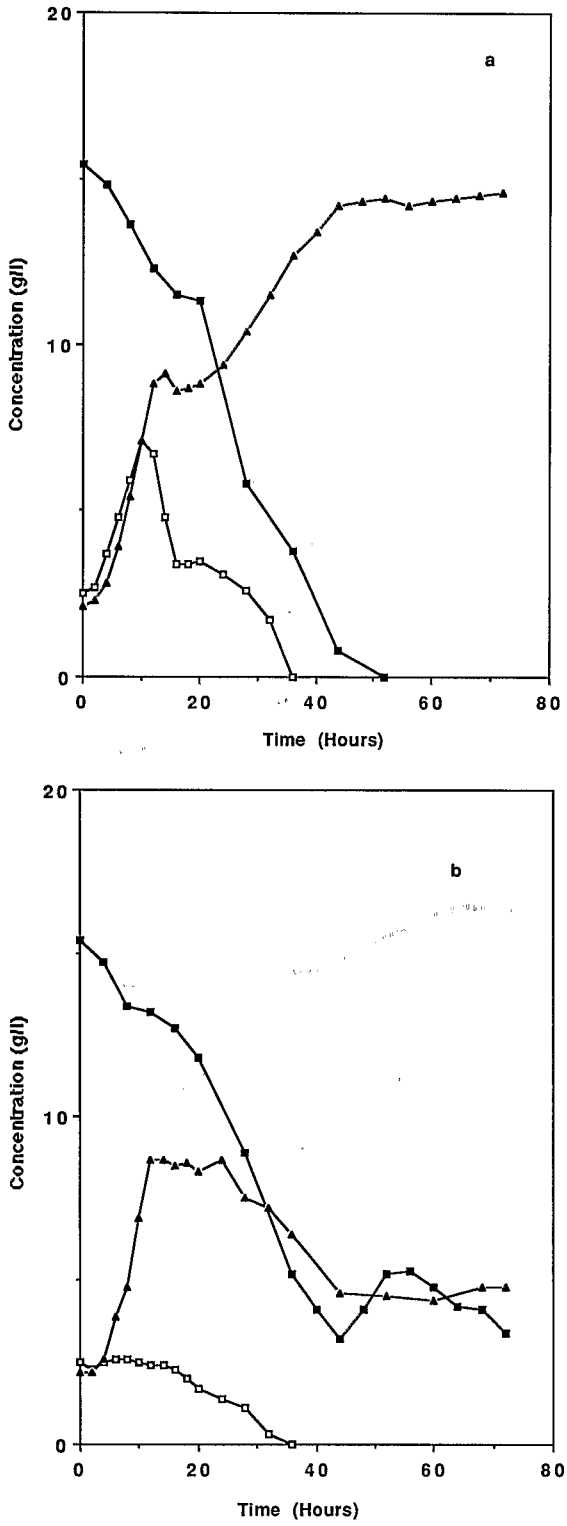

Fig. 2. Consumption of glucose (□), reducing sugars (■), and protein production (▲) of *A. niger* grown on olive mill waste-water (chemical oxygen demand: 154 g/l) in erlenmeyer flasks (a) and in a 2-l fermentor (b)

By the end of fermentation, the biomass had reached 55 g/l. This biomass also contained olive pulp particles (50–90% cellulose and lignin) (Fernandez-Diez 1983). The biomass protein content was between 11.5% and 23.0% and depended on the OMW composition and the incubation time. The mycelium yield resulted from the bioconversion of sugars, lipids, pectins, tannins, anthocyanins, and aromatic compounds associated with the decrease in COD. The protein content of the mycelium produced in flasks and the fermentor, are given in Fig. 2. The proteins given for the fermentor culture take into account only the mycelium in suspension in the medium and do not include the whole synthesized mycelium.

The kinetic degradation of glucose and reducing sugars differed between the flasks and the fermentor (Fig. 2). The initial increase in the glucose level observed in the flasks may have been due to solubilisation of the olive pulp and the enzymatic hydrolysis of several compounds such as cellulose, pectin and tannins. With fungal growth in the fermentor, the initial consumption of glucose was faster than in the flasks, but towards the end of the fermentation process, reducing sugars accumulated, either because of damage to the mycelium or the heterogeneity of the medium.

The degradation of pectin from OMW by *A. niger* has been measured from the accumulation of methanol (Wood and Siddiqui 1971). The methanol concentrations at the end of the fermentation were 0.47 and 0.55 g/l in the fermentor and flasks, respectively. The carboxylic acids and polyols produced by the metabolism of *A. niger* were not quantified.

Our results show that *A. niger* can be used as an efficient means of OMW bioconversion. Since the present experiments were carried out under non optimized conditions, optimization of the medium composition and adaptation of fermentor technology to mycelium production are now being attempted. Attempts are also being made to find fungi which are able to reduce more COD and decrease the colour of OMW, while maintained a good level of protein production.

Acknowledgements. Many thanks to Dr. M. Raimbault for providing strain A10 of *A. niger*, and for his critical review of this manuscript, and to J. Blanc for revising the English.

References

Alazard D, Raimbault M (1981) Comparative study of amylolytic enzymes production by *Aspergillus niger* in liquid and solid-state fermentation. Appl Microbiol Biotechnol 12:113–117

Balice V, Cera O (1984) Acidic phenolic fraction of the juice of olives determined by gas chromatographic method. Grasas Aceites 25:178–180

Balice V, Boari G, Cera O, Abbaticchio P (1982) Indagine analitica sulle acque di vegetazione. Nota 1. Inquinamento 7:49–53

Balice V, Carrieri C, Cera O, Rindone B (1988) The rate of tannin-like compounds from olive mill effluents in biological treatments. In: Hall ER, Hobson PN (eds) Proceedings of the Fifth International Symposium on Anaerobic Digestion, Bologna, Italy. Pergamon Press, Oxford, pp 275–280

Blakebrough N, McManamey W, Tart KR (1978) Rheological measurements on *Aspergillus niger* fermentation systems. J Appl Chem Biotechnol 28:453–461

Blom H, Thomassen MS (1985) Kinetic studies on strawberry anthocyanin hydrolysis by a thermostable anthocyanin-β-glucosidase from *Aspergillus niger*. Food Chem 17:157–168

Boari G, Brunetti A, Passino R, Rozi A (1984) Anaerobic digestion of olive oil mill wastewaters. Agric Wastes 10:161–175

Cichelli A, Solinas M (1984) I composti fenolici delle olive e dell'oloi di oliva. Riv Merceol 23:55–69

Fernandez Diez MJ (1983) Olives. In: Rehm HJ, Reed G (eds) Biotechnology, vol. 5. Verlag Chemie, Weinheim, pp 379–397

Fiesta Ros de Ursinos JA (1966) Installacion comercial para la obtencion de levura duras-pienso. Grasas Aceites 17:41–47

Fiestas Ros de Ursinos JA (1981) Différentes utilisations des margines. In: F. A. O. (ed) Proc of Séminaire International sur la Valorisation des Sous-Produits de l'Olivier, Monastir, Tunisie, décembre 1981, pp 93–110

Garcia JL, Guyot LP, Ollivier B, Trad M, Paycheng C (1982) Ecologie microbienne de la digestion anaérobie: technique de numération et d'isolement. Cah ORSTOM Sér Biol 45:3–15

Giulietti AM, Ercoli E, Ertola R (1984) Purification and utilization of olive black water and distillery slops by microbial treatment. Acta Cient Venez 35:76–86

Ikeda Y, Takahashi E, Yokogama K, Yoshimora Y (1972) Screening for microorganisms producing gallic acid from chinese and tara tannins. J Ferment Technol 50:361–370

Kieslich K (1976) Microbiol transformations of non-steroid cyclic compounds. Wiley, New York

Knechtel RJ (1978) A more economical method for the determination of chemical oxygen demand. Water Pollut Control May/June:25–29

Makasinova SY, Martakov AA (1982) Assimilation of phenolic compounds by fungi of the genus *Aspergillus*. Tr In-st Mikrobiol Virusol Acad Nauk Kaz ssr 27:30–36

Nelson N (1944) Photometric adaptation of Somogyi method for determination of glucose. J Biol Chem 153:375–380

Raimbault M, Alazard D (1980) Culture method to study fungal growth in solid fermentation. Eur J Appl Microbiol Biotechnol 9:199–209

Seng JM (1988) Chitine, chitosane et dérivés: de nouvelles perspectives pour l'industrie. Biofutur 71:40–44

Somogyi M (1945) Determination of blood sugar. J Biol Chem 160:61–68

Tanchev S, Joncheva N, Genov N, Codounis M (1980) Identification of anthocyanins contained in olives. Georgike Eurena 4:5–13

Vazequez RA, Maestro DR, Graciani CE (1974) Componentes fenolicos de la aceituna. II. Poliphenoles del alpechin. Grasas Aceites 25:341–345

Werner W, Rey HG, Wielinger RH (1977) Über die Eigenschaften eines neuen Chromogens für die Blutzucker Bestimmung nach der GOD/POD Methode. Z Anal Biochem 252:224–228

Wood PJ, Siddiqui IR (1971) Determination of methanol and its application to measurements of pectin ester contents and pectin methyl esterase activity. Anal Biochem 39:418–428