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# Anaerobic Digestion of Olive Mill Wastewaters after Detoxification by Prior Culture of *Aspergillus niger*

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*The low yields of methane production from olive mill wastewaters (OMW) which occur at high chemical oxygen demand (COD) concentration are caused by the presence of inhibitory compounds in this industrial effluent. These include tannins, phenolic compounds and oils which are toxic to methanogenic bacteria. In contrast,* Aspergillus niger *will grow on undiluted OMW, yielding reductions in COD and phenolic compounds of 61 and 58% respectively. The treated OMW is detoxified for methanogens, which will now grow easily on this treated material. Such aerobic pretreatment offers a novel approach to the degradation of OMW.*

## INTRODUCTION

The extraction of olive oil, accomplished in small, seasonally operated agro-industrial units especially in the Mediterranean region, results in the production of high-density wastewater.[1] Biological oxygen demand (BOD) and chemical oxygen demand (COD) maximum concentrations reach 100 and 220 g/litre respectively,[2] yielding a relatively toxic industrial waste. In olive wastewater produced by the traditional mill and press processes, the average concentration of volatile solids (vs) is 15% with 2% of inorganic matter. The organic fraction includes sugars, tannins, polyphenols, polyalcohols, pectins and oil. Tannin concentrations range from 8 to 16 g/litre in OMW.[3] The phenolic compound concentrations can exceed 10 g/litre.[4] The residual oil depends on the olive oil processing method and can reach 50 g/litre. The inhibition of methane production from unmodified OMW is not unexpected, as 2 g/litre of tannins,[5] 1 g/litre of phenolic compounds[6] and 5 mM of oleic acid[7] are toxic to methanogenic bacteria. Indeed, anaerobic digestion treatment of diluted OMW by anaerobic contact,[8,9] UASB[10] and anaerobic filters[11,12] show problems such as toxicity and resistance to biodegradation.

*Aspergillus niger*, used previously for SCP production,[13] possesses extracellular enzymes that hydrolyse pectins, polyphenols[14] and tannins.[15] It degrades many phenolic compounds[16] and can grow directly on OMW.[17]

The objective of this paper is the study of the effect of growth of *A. niger* on OMW and the production of a detoxified product susceptible to anaerobic digestion.

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## MATERIALS AND METHODS

### Fungal strain

*Aspergillus niger* strain *Hennebergii* isolated from a manioc (starch) fermentation[13] was maintained on medium containing OMW agar at 4°C. This medium contained 50% (v/v) OMW; $NH_4NO_3$, 5 g/litre; $(NH_4)_2SO_4$, 5 g/litre; $KH_2PO_4$, 1 g/litre and agar (Difco) 18 g/litre.

### OMW fermentation by *A. niger*

The detoxification of OMW was carried out in 1-litre Erlenmeyer flasks containing 100 ml of medium, on a rotary shaker operating at 150 rev/min at 35°C for 72 h.

The culture medium consisted of 1 litre of OMW (COD 154 g/litre), 6 g of $NH_4NO_3$ and 4 g of $(NH_4)_2SO_4$. The OMW was inoculated by $10^7$ spores/litre. Sterilized OMW was used without pH control. At the end of fermentation, the *A. niger* mycelia were separated by filtration on gauze and the filtrate was used in subsequent batch anaerobic digestion studies.

### Sludge and batch anaerobic digestion methods

The urban digestor sludge was obtained from an anaerobic mixed digester treating sludge from the aerobic treatment of urban wastewater. Ten millilitres of sludge and 0·2 ml of $Na_2S \cdot 9H_2O$ (25% w/v) were loaded under $O_2$-free nitrogen into 60-ml serum bottles stoppered with black butyl rubber closures (Bellco Glass, Vineland, NJ). These bottles were held at 4°C.

At the time of utilization, the pH of OMW was adjusted to 7·5 with $Ca(OH)_2$ and the cultures were incubated at 35°C. The use of calcium hydroxide for pH adjustment contributes to partial detoxification of OMW because it precipitates phenolic compounds and long-chain fatty acids toxic to methanogenic bacteria, and improves the total alkalinity.[18]

### Analytical methods

The biomass was collected by filtration on a Terylene fabric sieve, and the mycelia were washed with distilled water. Chemical oxygen demand (COD) was determined according to standard methods.[19]

Gas samples were taken with a syringe and analysed by gas chromatography with a flame ionization detector (DELSI 30, Delsi-Nermag, Argenteuil) fitted with an 80-cm stainless steel column packed with 4% $H_3PO_4$ on Porapack Q (80–100 mesh). $N_2$ was used as carrier gas at 28 ml/min, with $H_2$ and air flows of 25 and 30 ml/min respectively. The oven, injector and detector temperature was 200°C. The methane concentration was calculated with an ENICA 10 integrator (Delsi-Nermag, Argenteuil).

For analysis of volatile fatty acids (VFA) and alcohols, the liquid samples were centrifuged at 300 rev/min for 10 min, acidified with 1% of $H_3PO_4$ (50%) and analysed by gas chromatography.

For the determination of phenolic acid, 2 ml of culture was centrifuged and acidified with 1% of $H_3PO_4$, and mixed with 4 ml of organic solvent (ethylacetate–acetone, 2:1). The phenolic fraction, which consisted essentially of monomeric compounds, was analysed using a Shimadzu gas chromatograph GC-9A with a flame ionization detector, fitted with a 25-m capillary column packed with OV 101. $N_2$ was used as carrier gas at 50 ml/min, with $H_2$ and air flows of 35 and 500 ml/min respectively. The oven, injector and detector temperatures were 280 and 300°C respectively.

Total phenolic compounds, including hydrolysable tannins, condensed tannins, monomeric flavoids and simple phenolic compounds, were determined as described by Balice *et al.*[4]

## RESULTS AND DISCUSSION

### Batch anaerobic digestion of crude OMW and of OMW after fermentation with *A. niger*

Batch cultures challenged with increasing amounts of OMW with COD ranging from 20 to 100 g/litre were studied. Figure 1 summarizes the cumulative methane production in these batch cultures over a 30-day incubation period. The decrease of methane production as COD concentration increased, was presumably caused by the presence of toxic compounds in the OMW; these probably included tannins, phenolic compounds and oils. Phenolic acids present in OMW are known to be very toxic at low concentrations to microbial groups carrying out methanogenesis.[20]

The cumulative methane production in batch anaerobic digestion challenged with increasing amounts of *A. niger* prefermented OMW with COD ranging from 20 to 60 g/litre (Fig. 2) showed enhanced values with greater COD. The quantities of methane produced were clearly greater than with unmodified OMW.

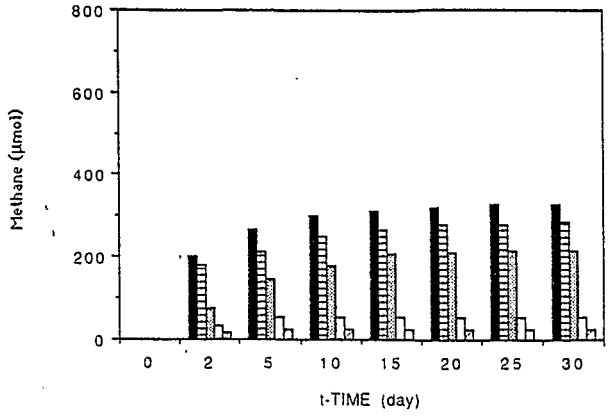


**Fig. 1.** Methane formation in batch anaerobic digestion of a crude OMW at various concentrations of COD. ■, 20 g/litre; ▤, 40 g/litre; ▩, 60 g/litre; □, 80 g/litre; ▨, 100 g/litre.

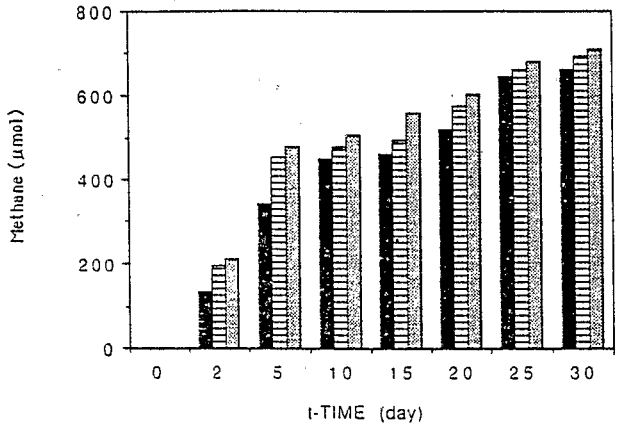


**Fig. 2.** Methane formation in batch anaerobic digestion of *A. niger* prefermented OMW at various concentrations of COD. ■, 20 g/litre; ▤, 40 g/litre; ▩, 60 g/litre.

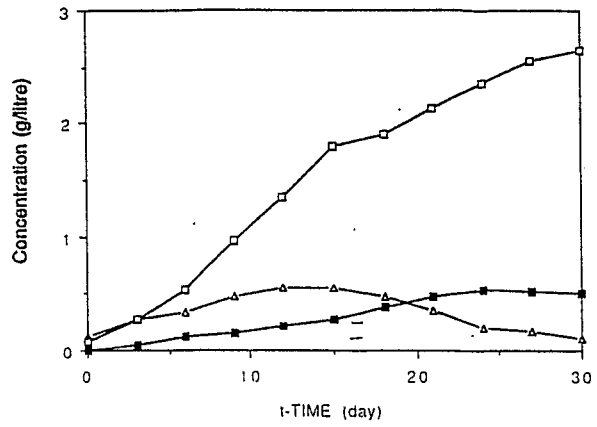


**Fig. 3.** Time course of acetate (□), propionate (■) and ethanol (△) in batch anaerobic digestion of crude OMW.

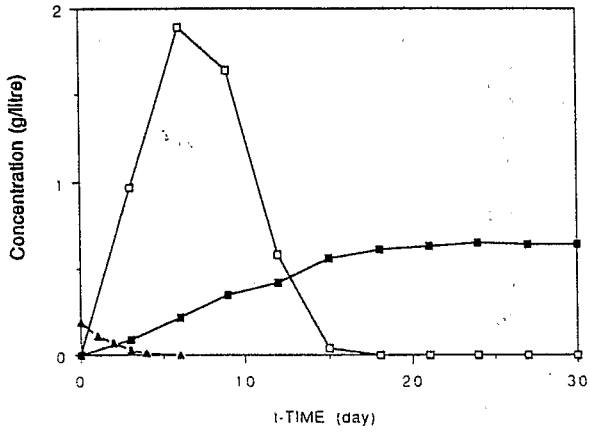


**Fig. 4.** Time course of acetate (□), propionate (■) and methanol (▲) in batch anaerobic digestion of *A. niger* prefermented OMW.

Using the *A. niger* prefermented OMW, the production of methane per gram of introduced COD was greater than that obtained with unmodified OMW (Fig. 3). The analysis of VFA and alcohols showed that there was no accumulated acetate when OMW was prefermented by *A. niger* (Fig. 4). Moreover, the transitory acetate production in batch anaerobic digestion of prefermented OMW was greater than with unmodified OMW because of the lack of the inhibition of fermentative bacteria and the hydrolysis of tannins and other polyphenols contained in OMW. Ethanol accumulated only in batch anaerobic digestion of crude OMW, whereas propionate accumulated in batch anaerobic digestion of OMW and *A. niger* prefermented OMW. The values for removal of soluble COD after batch anaerobic digestion of OMW and prefermented OMW (20 g COD/litre) were 55% and 23% respectively.

## The degradation of phenolic compounds by *A. niger*

The *A. niger* grew well on undiluted OMW without any inhibition. At the end of the fermentation, the COD removal determined on the filtrate of OMW detoxified by *A. niger* reached 61·6%. The accumulation of methanol up to 10 mM is probably the result of pectin degradation. The growth of *A. niger* on OMW increased greatly its ease of filtration, partly as a result of the degradation of pectin and the entrapment of olive pulp particles in the fungal biomass.[21]

The growth of *A. niger* concomitantly decreased the colour intensity of OMW. This could be due to the degradation of some phenolic compounds, and the adsorption of the polyphenols and tannins on the fungal mycelia. Such adsorption phenomena can be obtained by hydro-

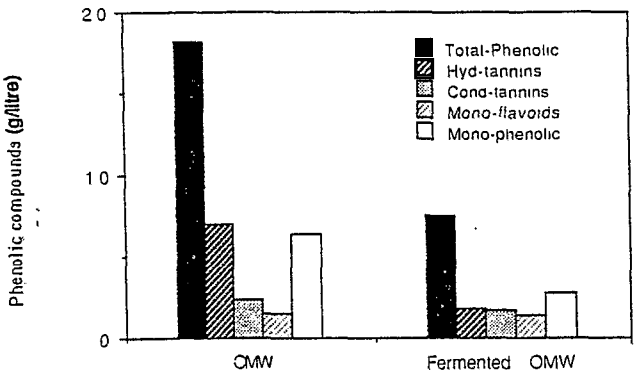


**Fig. 5.** Phenolic compounds present in crude OMW and *A. niger* perfermented OMW.

gen bonding between phenolic compounds and proteins or by the mycelial chitin which can promote coagulation.[22] On the other hand, the results of analysis of tannin-like compounds from OMW before and after culture of *A. niger* showed that the efficiency of the degradation of simple phenolic compounds by this fungus was 55·4%, and 77·5% for hydrolysable tannins (Fig. 5). However, the black colour of OMW was due principally to polyphenols that remained. The main phenolic acids in OMW include syringic acid, *p*-hydroxyphenylacetic acid, vanillic acid, veratric acid, caffeic acid, protocathechuic acid, *p*-coumaric acid and cinnamic acid.[23,24] There are also polyphenols,[3] tannins[4] and anthocyanins.[25] The monomer phenols of OMW were changed after fermentation (data unreported). All these compounds found in raw OMW had decreased after the growth of *A. niger* but there was also the appearance of new peaks. Overall, *A. niger* grew on the majority of the simple phenolic compounds identified in OMW.[17]

**Comparison between OMW pretreatments**

The dilution of OMW can reduce its toxicity to methanogenic bacteria but also decreases the volumetric capacity of digestors. Thus pretreatment to facilitate the anaerobic digestion of OMW and removal of such toxicity have been evaluated.

For the anaerobic digestion of OMW without dilution and with minimal inhibition of methanogenic bacteria, Medici *et al.*[26] proposed a differential distillation to concentrate the organic matter. Three fractions were drawn from this pretreatment step. Two fractions represent feed for subsequent anaerobic digestion, and the other is for aerobic biodegradation.

Pretreatments included the culture of yeast, an acidogenesis approach and finally aerobic fermentation by *A. niger* (this work).

The culture of *Saccharomyces cerevisiae* and *S. uvarum* on OMW removed 85% of oil which inhibited methanogenic bacteria and 42% of the COD in 3 days. After the subsequent removal of the biomass, OMW treated by anaerobic digestion gave 54% removal of COD.[27]

Acidogenesis of OMW has been used to facilitate the anaerobic digestion of OMW. High gas production rates (up to 30 $m^3$ of gas, 78·5% $CH_4$/$m^3$/d) were obtained with a hybrid anaerobic upflow sludge blanket–upflow fixed filter (Hausbuff) treating acidified OMW in the open holding basin, where it was stored for 5–7 months.[1] The acidified OMW obtained by this acidogenesis step was slightly less toxic than unmodified OMW. The acidogenesis step can potentially be carried out in a stirred tank reactor, as the transient VFA production can be improved by agitation.[18]

As toxicity of OMW is caused especially by tannins and simple phenolic compounds, biological pretreatment is a potential detoxification approach. Furthermore, *A. niger* efficiently removed tannins.[15] Pretreatment by *A. niger* reduced the toxicity of OMW towards methanogenic bacteria and improved its biodegradability. as the production of methane per gram of introduced COD was higher than that obtained with crude OMW (Figs 1 and 2). Moreover, the methanol probably derived from hydrolysis of pectin by *A. niger* can stimulate the activity of methanogenic bacteria and thus facilitate rapid start-up of the anaerobic digestion process.

## CONCLUSIONS

Growth of *A. niger* on OMW produces mycelia that aid subsequent filtration and remove compounds inhibitory to fermentative and methanogenic bacteria. Thus this pretreatment of OMW by *A. niger* improves indirectly its biodegradability. The detoxification of OMW is especially significant in that it can result in a decrease in the amount of dilution water routinely used in anaerobic digestion. These trials were carried out under non-optimized conditions. Efforts are now concentrated on screening for better fungi and optimization of the current process.

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