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BIOTECHNOLOGICAL EXPLOITATION OF OLIVE MILL WASTEWATER

Coordinatore del Dottorato: Ch.mo Prof. Paolo Capretti

Tutore : Ch.mo Prof. Roberto De Philippis

Cotutore : Dr.ssa Alba Ena

Cotutore : Dr. Pietro Carlozzi

Dottorando : Cristina Pintucci

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Cristina Pintucci

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Cristina Pintucci

30 Dicembre 2009

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IMPIEGO BIOTECNOLOGICO DEI REFLUI OLEARI

Riassunto della Tesi

Scopo: Lo studio oggetto di questa tesi si pone lo scopo di trattare, sfruttare e valorizzare in modo completo le acque di vegetazione olearie (AVO), note per essere uno dei reflui a più alto tasso inquinante nel bacino mediterraneo. In questo studio viene proposto un ciclo di trattamento completo in cui le AVO vengono totalmente sfruttate da un punto di vista biotecnologico.

Metodiche e risultati: Al fine di ottenere un completo sfruttamento delle AVO, il trattamento studiato in questa ricerca ha lo scopo di depurare i reflui oleari, allo stesso tempo di recuperare importanti antiossidanti naturali, quali i polifenoli, e utilizzare le acque depurate per la produzione di "energia pulita".

Al fine di ridurre il carico inquinate del refluo, il trattamento utilizza due differenti matrici impaccate (*Azolla caroliniana* e Carbone Attivo Granulare, CAG) che sono in grado di ridurre la maggior parte delle sostanze inquinanti per adsorbimento.

Azolla e CAG vengono fortemente impaccati in un reattore plug-flow a letto impaccato dove le AVO vengono pompate contro corrente. Per ottenere la maggiore depurazione utilizzando il minor quantitativo di materiale, sono state valutate differenti concentrazioni delle due matrici. Le più efficienti sono risultate essere 100 g/L per quanto riguarda Azolla e 200 g/L per il carbone. In seguito alla filtrazione sulle matrici impaccate, il carico inquinante delle AVO risultava fortemente ridotto: il contenuto totale di sostanza organica (COD) era diminuito di oltre l'80% mentre la componente fenolica veniva rimossa in modo quantitativo.

La frazione fenolica rimasta nelle matrici durante la biofiltrazione è stata quindi recuperata utilizzando adeguati solventi e condizioni di eluizione. Le capacità adsorbenti e desorbenti assolute di entrambe le matrici sono state studiate e paragonate a quelle di due resine disponibili in commercio. Il carbone ha dimostrato la più elevata efficienza sia nell'adsorbimento che nel desorbimento. Dal desorbimento delle due matrici vegetali sono state ottenute delle polveri che sono state caratterizzate. Il profilo HPLC dei due prodotti in polvere ha rivelato una differenza nella componente fenolica da un punto di vista qualitativo: mentre l'Azolla presenta prevalentemente Acido Gallico e Tirosolo, la polvere da CAG è ricca in

Idrossitirosolo. Entrambe le polveri hanno mostrato elevate attività antiradicalica e capacità antiossidante. Queste caratteristiche rendono i prodotti recuperati da Azolla e CAG di estremo interesse a livello commerciale per future applicazioni di tipo salustico.

E' stata inoltre studiata la crescita e la produzione di bioidrogeno di *Rhodopseudomonas palustris* (*Rp. p.*) su reflui pretrattati con Azolla sia da sola che combinata con Carbone. Quando le AVO sono state trattate con la sola Azolla secca, il quantitativo totale di COD veniva ridotto in modo significativo. Nonostante questo, utilizzando mezzo contenente il refluo in concentrazione > 10% non si osservava crescita batterica. Al contrario, *Rp. p.* è risultato in grado di crescere su mezzo contenente AVO pretrattate con Azolla e Carbone. E' stata quindi studiata la foto-evoluzione di biogas (costituito all'85% da idrogeno e al restante 15% da CO_2) su AVO diluita al 25%. La produzione di biogas è stata valutata utilizzando 2 fotobioreattori a differente geometria (circolare e piatta). Gli esperimenti sono stati condotti in batch per 406 ore. Nel fotobioreattore circolare e nel piatto sono stati prodotti rispettivamente 600 e 335 mL di biogas che corrispondono, rispettivamente, a 1.12 L_{biogas}/L_{AVO} and 0.624 L_{biogas}/L_{AVO}.

Ulteriori studi sono ancora in corso per trasporre di scala il processo sopra descritto e per verificare la fattibilità del processo sviluppato in laboratorio. Un impianto pilota rappresenterebbe un punto cruciale per un approccio innovativo alla gestione dei reflui oleari.

Conclusioni: Il sistema studiato ha lo scopo di suggerire un approccio innovativo per la gestione di un refluo altamente inquinante. Il sistema di depurazione potrebbe aiutare ad alleggerire l'incalzante impatto ambientale causato dalle AVO. Inoltre nel trattamento vengono recuperati importanti antiossidanti naturali che hanno noti benefici salutistici. Il passo successivo nel ciclo di trattamento prevede la produzione di energia "pulita" che potrebbe concorrere al mantenimento energetico dei frantoi stessi. In seguito al trattamento biologico completo, l'acqua prodotta contiene ancora piccole quantità di sostanza organica e può quindi essere utilizzata come fertirrigante.

Significato dello studio e suo impatto: Considerando il consistente problema causato dallo smaltimento dei reflui oleari, è di importanza cruciale creare un trattamento

fattibile sia da un punto di vista economico che ecologico. Lo studio proposto suggerisce un trattamento di questo tipo per la gestione delle AVO. Il recupero di composti antiossidanti e la produzione di un vettore energetico "pulito" potrebbero trasformare una sostanza di scarto in una preziosa materia prima.

Lavori correlati alla Tesi

- Ena A., Pintucci C., Faraloni C., Torzillo G., 2009. An ecocompatible process for the depuration of wastewater from olive mill industry. Water Science and Technology 60(4): 1055 – 1062.

- Carlozzi, P., Pintucci, C., Piccardi, R., Buccioni, A., Minieri, S., Lambardi, M., 2010. Green energy from *Rhodopseudomonas palustris* grown at low to high irradiance values, under fed-batch operational conditions. Biotechnology Letters (DOI: 10.2007/s10529-009-0183-2).

- Ena A., Pintucci C., Scoma A., De Philippis R., Carlozzi P. Photofermentative biogas production from pretreated oil mill wastewater using two different adsorption vegetable matrices. Manoscritto sottomesso a: Current Topics Biotechnology.

BIOTECHNOLOGICAL EXPLOITATION OF OLIVE MILL WASTEWATER

Abstract

Aim: The present research aims to entirely treat, exploit and valorise the olive mill wastewater (OMW), known to be one of the most pollutant waste in the Mediterranean basin. A complete treatment cycle is here proposed, where the OMW are totally exploited from a biotechnological point of view.

Methods and Results: For a complete exploitation of OMW, the treatment studied in this research aims to depurate the olive mill wastewater and, in the meantime, to recover precious natural compounds as polyphenols and use the depurated wastewater for the production of "clean" energy.

In order to reduce the total pollutant load of the waste, the proposed OMW treatment used two different packed vegetable matrices (*Azolla caroliniana* and Granular Activated Carbon, GAC) which remove most of the pollutant substances through adsorption. Azolla and GAC were strongly packed in a packed-bed plug-flow reactor where the OMW was loaded up-flow. Different matrices concentrations have been tested according to the highest depuration efficiency with the least material utilization. The most efficient ones resulted 100 g/L OMW for Azolla and 200 g/L OMW for GAC. After filtration on the matrices, the pollutant load of the waste is highly reduced: the organic content (COD) is reduced more than 80% and the phenol compounds are completely removed.

The phenolic fraction absorbed by the matrices used in the biofiltration process, was recovered using different elution solvents and conditions. The total adsorbent and desorbent capacities of each material have been studied and compared to two commercial resin. GAC showed the highest efficiency both for adsorption and desorption. From the desorption of the vegetable matrices, two powder products have been recovered and characterized chemically. The HPLC profiles showed a qualitative difference between the two products: Azolla powder was rich mainly in Gallic Acid and Tyrosol while the GAC one presented high amounts of Hydroxytyrosol. Both the products showed antiradical activity (the Azolla and GAC IC_{50} resulted about 102 mg/mL and 200 mg/mL of powders, respectively) and

antioxidant capacity. Due to these characteristics, the two powder products resulted suitable for further commercial applications, especially in the health field.

Moreover, the growth of *Rhodopseudomonas palustris* (*Rp. p.*) and its biohydrogen production have been studied using OMW treated with Azolla alone and with GAC. When a dry-Azolla matrix was used alone for the pretreatment, the initial COD and the polyphenol content were significantly reduced. Nevertheless, no growth of bacteria was observed when using a culture broth containing >10% of pretreated OMW with only the dry-Azolla. *Rp. p.* resulted able to grow on OMW treated with Azolla and GAC. The culture broth for the biogas (constituted by hydrogen 85% and CO_2 15%) photoevolution consisted of 25% pretreated OMW. The experiments for biogas photoevolution were carried out using two different photobioreactor geometries (cylindrical and flat). The experiments were carried out under batch growth conditions and at a culture age of 406 h. 600 mL of biogas were produced by the cylindrical photobioreactor and 335 mL by the flat photobioreactor, corresponding, respectively, to 1.12 L_{biogas}/L_{OMW} and 0.624 L_{biogas}/L_{OMW}.

Further investigations to scale-up the process and eventually verify the feasibility of the laboratory treatments are still in progress. A pilot scale system would then represent a key effort in a new approach for the olive mill wastewater exploitation.

Conclusions: The studied system suggests a new approach for a complete disposal of a pollutant waste. This depuration system could help small mills lighting the increasing OMW environmental impact. Moreover, precious natural antioxidant compounds can be recovered as they have wide and important health benefits. The further step in the OMW treatment cycle is the production of “clean” energy that could help the energetic mill maintenance. After the complete biological treatment, the generated water still contains small amounts of organic compounds and could then be used as fertilizer.

Significance and Impact of the Study: Considering the environmental problem represented by OMW disposal, it is crucial to set up a feasible treatment both on an economical and ecological point of view. The present study suggests such kind of treatment for the complete exploitation of OMW. Natural antioxidants recover and clean energy vector production would turn a waste into a precious row material.

Papers related to the Thesis

- Ena A., Pintucci C., Faraloni C., Torzillo G., 2009. An ecocompatible process for the depuration of wastewater from olive mill industry. Water Science and Technology 60(4): 1055 – 1062.

- Carlozzi, P., Pintucci, C., Piccardi, R., Buccioni, A., Minieri, S., Lambardi, M., 2010. Green energy from *Rhodopseudomonas palustris* grown at low to high irradiance values, under fed-batch operational conditions. Biotechnology Letters (DOI: 10.2007/s10529-009-0183-2).

- Ena A., Pintucci C., Scoma A., De Philippis R., Carlozzi P. Photofermentative biogas production from pretreated oil mill wastewater using two different adsorption vegetable matrices. Manuscript submitted to: Current Topics Biotechnology.

CONTENT

Regular papers

- Ena A., Pintucci C., et al., 2009.
- Carlozzi, P., Pintucci, C., et al., 2010 **(**DOI: 10.2007/s10529-009-0183-2).

CHAPTER 1

INTRODUCTION

OLIVE OIL MILL WASTEWATER

Composition, production and pollutant load

Since ancient times, the olive oil has been a fundamental product of the Mediterranean regions. Already 6000 years ago, during the Copper Age, people living in the Oriental Mediterranean regions discovered the oil extraction. Later on, Romans based their economy on crops as well as olive oil. Olive oil production increased during the centuries and nowadays olive trees (*Olea europea*) are cultivated everywhere. Nevertheless, the Mediterranean area is still the most active in olive oil production (95 – 98%) (Dhouib et al., 2006; McNamara et al., 2008). The largest olive oil producers are: Spain (36% of the global production), Italy (24%) and Greece, which manufactures 17% of the world's total (FAOSTAT, 2007). Thus, if considered that worldwide olive oil production is about 2.5 million of tons per year (Dhouib et al., 2006), it seems evident which proportion the problem of olive oil waste handling has reached, especially in the Mediterranean basin.

The processes used for the olive oil extraction are the traditional and modern ones (figure 1).

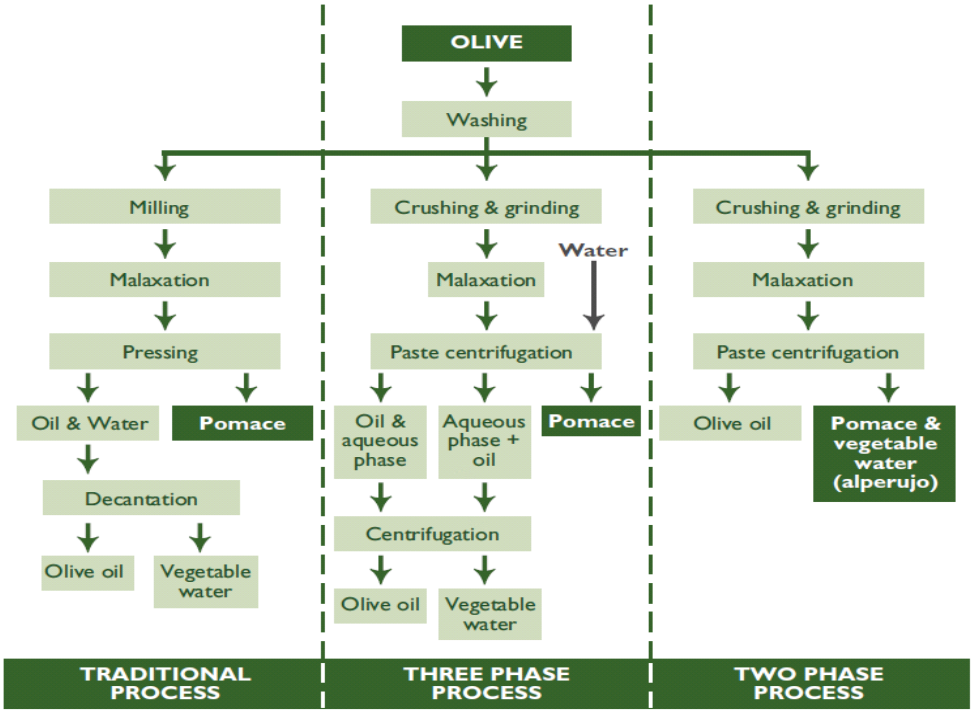

Fig. 1. Schematic processes for the olive oil extraction.

The traditional system (batch-pressure) ground the olives with millstones, the paste is then pressed by stack of fibre disks. The modern method (continuous extraction) uses an industrial decanter to separate all the phases by centrifugation. This system can be at two or three phase, depending on the addition of water and the by-products generated (oil and wet olive husk, and oil, dry olive husk and olive wastewater respectively). From olive milling different products are generated, namely (a) oil, (b) husk and (c) wastewater. Oil is obviously the most important product generated in such a process, however husk and, above all, olive mill wastewater (OMW) are the most abundant ones. Indeed, it was estimated that about 0.5-1.5 m^3 of OMW are produced from 1 ton of treated olive fruits or about 5 m^3 per ton of olive-oil produced (Gelegenis et al., 2007). Figure 2 shows the by-products deriving from the olive trees cultivation and olive oil production.

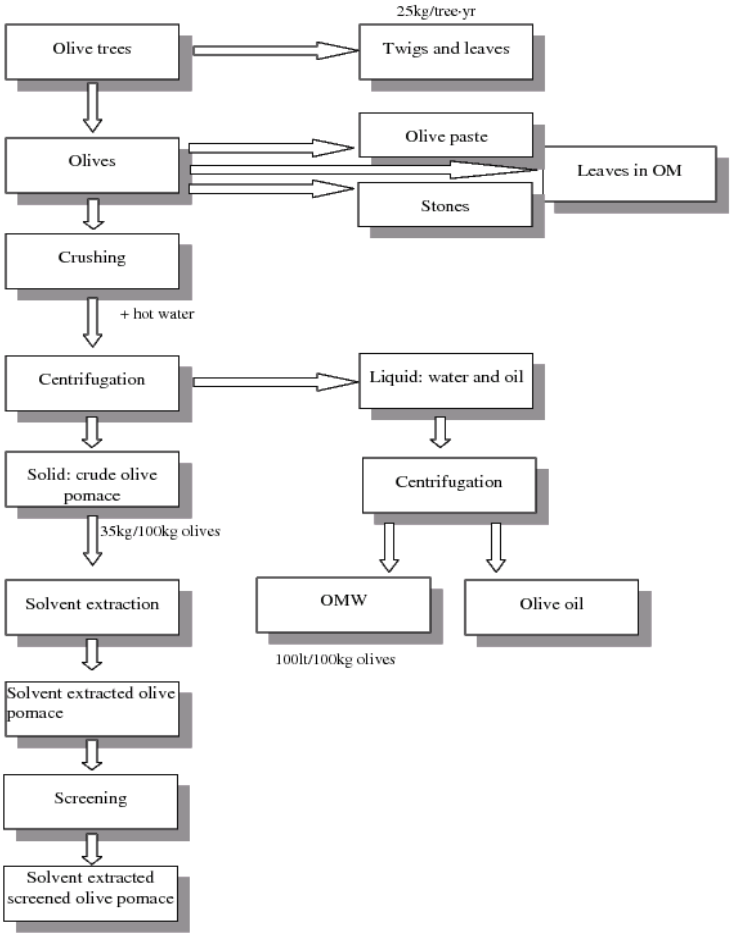

Fig. 2. By-products from the olive trees culture and olive oil industry (OM: Olive Mill), (Kapellakis et al., 2008).

Olive husks and OMW have different applications. Olive husk can undergo a chemical extraction of oil (mostly using hexane) and the dried residue can be used as fuel (pellet stove). As concerns OMW, they are known to be the most pollutant wastes in the agro-industry field.

Figure 3 shows an open pond were OMW are temporarily harvest. OMW composition, although varying according to many factors, is generally the following: water (83-94%), organic matter (measured as Chemical Oxygen Demand, COD) (4-16%) and mineral salts (0.4-2.5%) (Ramos-Cormezana et al., 1996).

Fig. 3. Collecting pool for olive oil mill wastewater in a Tuscan mill.

Table 1 shows the main chemical characteristics of OMW reported by several authors. As OMW consists of diluted juice of crushed olives, it can be safely assumed that it is completely biodegradable. Yet, some of its components (e.g. polyphenols and lipids) are decomposed at reaction rates much lower than others, like sugars and short-chain volatile fatty acids (Ena et al., 2009). The total COD is generally very high (30 - 200 kg/m^3) and the ratio between COD and BOD_5 (Biological Oxygen Demand) changes from 2.5 to 5.0, which is considered poorly degradable (Lopez, 1992). The organic compounds in the OMW, in association with its high C/N ratio and low pH, compromise the biological degradation process of soils (Marques, 2001) and can cause eutrophication when the wastewater is collected in basins with low exchange rates (closed gulfs, lakes, etc.). However, the quali-

quantitative composition of OMW widely changes depending on different parameters: olive cultivar, extraction plant, harvesting time, maturation phase of the fruit etc or simply according to the region (Niaounakis and Halvadakis, 2006).

Among all the organic compounds present in the OMW, the ones with a phenolic structure deserve a special attention because of their influence on colour and phytotoxic effect. Polyphenols (with a concentration between 0.8 and 11 g/L) (table 1) have a inhibitory effect on many bacteria and fungi, thus making the direct biological treatment of OMW quite difficult (Fedorak and Hrudey, 1984; Gonzalez et al., 1990).

Tab. 1. Main chemical characteristics of OMW given by several authors (Roig et al., 2006). (a) Vlyssides et al., 2004; (b) Filidei et al., 2003; (c) Aktas et al., 2001; (d) Piperidou et al., 2000; (e) Paredes et al., 1999; (f) Vlyssides et al., 1996; (g) Saviozzi et al., 1991; (h) Moreno et al., 1987. (EC: Electroconductivity; OM: Volatile solids; TOC: Total Organic Carbon; TN: Total Nitrogen).

	(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)
Dry matter (%)	6.35	7.1	n.d.	n.d.	7.19	6.33	n.d.	n.d.
pH	4.8	4.93	4.8	n.d.	5.17	5.00	4.2	5.0
EC (dS/m)	12.0	7.3	n.d.	10.0	5.50	n.d.	7.0	n.d.
OM (g/l)	57.4	n.d.	62.1	n.d.	46.5	57.2	n.d.	n.d.
TOC (g/l)	39.8	n.d.	n.d.	n.d.	34.2	n.d.	n.d.	n.d.
TN (g/l)	0.76	0.62	0.79	n.d.	0.63	0.86	2.1	n.d.
P_2O_5 (g/l)	0.53	n.d.	n.d.	n.d.	0.31	0.61	0.7	0.7
K_2O (g/l)	2.37	n.d.	n.d.	2.9	4.46	n.d.	3.5	10.8
Na (g/l)	0.30	n.d.	n.d.	0.2	0.11	n.d.	n.d.	0.42
Ca (g/l)	0.27	n.d.	n.d.	0.2	0.30	n.d.	n.d.	0.64
Mg (mg/l)	44	n.d.	n.d.	92	129	n.d.	n.d.	220
Fe (mg/l)	120	n.d.	n.d.	18.3	68.5	n.d.	n.d.	120
Cu (mg/l)	6	n.d.	n.d.	2.1	1.5	n.d.	n.d.	3
Mn (mg/l)	12	n.d.	n.d.	1.5	1.1	n.d.	n.d.	6
Zn (mg/l)	12	n.d.	n.d.	2.4	4.1	n.d.	n.d.	6
d (g/cm^3)	1.048	n.d.	n.d.	n.d.	1.02	1.048	n.d.	n.d.
Lipids (g/l)	1.64	8.6	12.2	n.d.	3.1	n.d.	n.d.	n.d.
Poliphenols (g/l)	10.7	0.98	3.8	n.d.	1.6	n.d.	7.8	n.d.
Carbohydrates (g/l)	16.1	4.8	4.7	n.d.	8.79	n.d.	1.4	n.d.
COD (g/l)	93	67	103	178	n.d.	130	177	n.d.
BOD_5 (g/l)	46	n.d.	n.d.	n.d.	n.d.	55	94	n.d.

Laws and restrictions concerning OMW disposal

It is worth to mention which restrictions and laws regulate OMW treatment in our community. Italy is the only olive-oil producing country in the world where legislation for disposal of olive mill wastes on soil exists. Many laws have been applied in Italy since 1976 aiming at regulating the OMW disposal on cultivated

fields. The following laws allowed the spreading of OMW on fields to help the olive oil industry that otherwise would be compromised. The present Italian Law (n. 574/96 and its following modification) allows the disposal of up to 50 and 80 m^3/ha /year olive-mill wastewater, generated by traditional and modern plants, respectively. However, the application of the law is often not practically feasible due to the hilly landscape of Italian olive-growing regions, difficulty in spreading during the winter season for lack of accessible soils, and the high energy cost required for transport and spreading of wet OMW (Altieri and Esposito, 2008).

Proposed OMW disposal solutions

As a consequence of OMW disposal problem, during the last decades many studies have been carried out to discover new feasible ways of handling them and a wide range of different processes have been proposed. These systems aim both at the reduction of the pollutant load and at its conversion into high added value products. In many of the producing countries, direct discharge on cultivated fields is the main solution applied for the wastewaters. Direct spreading of OMW on soil can be useful as the wastewaters are rich in organic matter, nitrogen, phosphorous, potassium and magnesium (Mekki et al., 2006). However OMW has also harmful effects on the soil, its structure and microbial communities. Negative effects are associated with OMW high mineral salt content, low pH and presence of phytotoxic compounds, especially polyphenols (Paredes et al., 1999). To avoid their negative effect, physico-chemical and/or biological treatments could be adopted to decrease OMW toxicity. The most applied physico-chemical treatments consist in adding chemical substances able to ease the flocculation, aggregation, condensation and/or destruction of part of the wastewater organic matter.

Biological treatments (composting, aerobic and anaerobic systems, and a combination of both) are widely studied for their eco-compatible impact on the environment. When composted, OMW is added with solid olive and olive tree residue, wheat straw, saw dust etc. During few week-month periods anaerobic and fermentative processes decrease the pollutant load.

Aerobic treatments use microorganisms able to grow on OMW, with or without previous treatments, dilution or addition of nutrients. However, aerobic bacteria seem to have a minimal effect on the more complex polyphenolic mixture responsible for

the dark coloration of OMW. Fungi were also tested for the depuration and decolourization of OMW, *Pleurotus* resulting the most effective (Tomati et al., 1991; Tsioulpas et al., 2002). One of the main drawbacks of aerobic bioreactors yet remains their configurations, which usually require high nutrient inputs to support the treatment. The process is complex to maintain and produce large quantities of waste biomass and have high capital construction costs (Gray, 1999).

Anaerobic treatments use mainly microbial consortia composed by unidentified microorganisms, recovered from municipal and other waste facilities (Dalis et al, 1996; Marques, 2001). Many papers report anaerobical co-digestions of the OMW with different effluents (Angelidaki et al., 2002; Hachicha et al., 2009; Gavalla et al., 1996). This approach is attractive as low cost nutrients are provided for digestion, inhibitory compounds present in the OMW are diluted and the digester can operate on a year-round basis (Gavalla et al., 1996; Angelidaki et al., 2002). Coupled aerobic and aerobic pre-treatment produce a usable biogas fuel, low waste biomass generation and a treated waste which is an excellent soil conditioner and fertilizer (Niaounakis and Halvadakis, 2006). Many recent studies were focused on the production and/or recover of valuable substances from the OMW such as laccase (Ergul et al. 2009), volatile fatty acids (Fezzani and Cheikh, 2009; Ntaikou et al., 2009), polyhydroxyalcanoates (Gonzalez-Lopez et al., 1996; Dionisi et al. 2004 a,b; 2005 a,b; 2006) and pectines (Cardoso et al., 2003). Moreover, OMW treatment can also lead to the production of biogas such as methane (Bertin et al., 2004 a,b) and hydrogen (Eroglu et al., 2004; 2006; 2009 a,b). The interest in recovering the OMW phenolic compounds is due to the high economical value associated to these aromatic substances. Phenols recovery treatments can be constituted by natural filters (Ena et al., 2009), resins and other non-biological substances (Gortzi et al., 2008; Visioli et al., 1999). After recovering high value compounds, an OMW with a higher compatibility for further biological treatments is produced.

PHENOLIC COMPOUNDS

Since large part of the olive phenolic compounds are hydrophilic, only a little amount is present in the olive oil (2%), the remaining part being collected in the OMW (approximately 53%) and in the olive husk (approximately 45%) (Rodis et al.,

2002). The phenolic composition of OMW has been studied by several recent groups (Della Greca, et al., 2004; Obied, et al., 2005; De Marco et al., 2007): phenols consist of monocyclic aromatic molecules, such as hydroxytyrosol, tyrosol, catechol, methylcatechol, caffeic acid and high molecular weight compounds obtained through their polymerisation (Tziotzios et al., 2007). As the latter compounds are recalcitrant to biodegradation, OMW cannot be released in the environment, but must be adequately treated before being discharged (Amaral et al., 2008). Even though phenols are considered responsible for OMW toxicity because of their high concentration, it is also well known they can have a large number of benefits for human health and food conservation, as reported by many papers (table 2).

Tab. 2. Some biological activities and applications of Hydroxytyrosol (OH-Tyrosol).

Substances	**Studies**	**Reference**
OH-Tyrosol and others	Oxidative stabilization of lard.	De Leonardis et al. (2007).
OH-Tyrosol	Preventive of protein damage induced by long-wave UV radiation in melanoma cells	D'Angelo et al. (2005).
Oleuropein, OH-Tyrosol	Small molecule HIV-1 fusion and integrase inhibitors oleuropein and OH-Tyrosol.	Lee-Huang et al. (2007).
OH-Tyrosol and others	Antimicrobial agent in food.	Soni et al. (2006).
OH-Tyrosol and others	Metabolism and health effects.	Tuck and Hayball (2002).
OH-Tyrosol	Scavenging of hydrogen peroxide produced by human neutrophilis.	O' Dowd et al. (2004).
Oleuropein, OH-Tyrosol	In vitro' antioxidant activity and biomembrane interaction.	Saija et al. (1998).
OH-Tyrosol	Antioxidant effect of OH-tyrosol and Mn^{2+} in liver of cadmium-intoxicated rats.	Casalino et al. (2002).
OH-Tyrosol	Formation of F2 isoprostanes in oxidized low density lipoprotein: inhibitory effect.	Salami M. et al. (1995).
OH-Tyrosol	Protection of human erythrocytes against oxidative damages.	Manna C. et al. (1999).

Extraction of biologically active compounds from OMW may turn a polluting residue into a source of natural antioxidants since reactive oxygen species are involved in the onset of several human diseases and in the oxidative degradation of food (De Marco et al., 2007). Polyphenols belong to the category of natural antioxidants and are the most abundant ones in our diet (Blekas et al., 2002), the suggested daily dosage of polyphenols is 100 mg. It is known that polyphenols intake is beneficial for human health, as their antioxidant activity has been associated with a lower risk of coronary heart disease (Kalogeropoulos et al., 2007; Tapiero et al., 2002), some types of cancer (Trichopoulou and Lagiou, 1997; Simopoulos, 2001), inflammation (Tapiero et al., 2002) and inhibition of platelet-activating factor activities (Simopoulos, 2001). The growing evidence that free radical-mediated events are involved in several pathological processes has stimulated interest in natural antioxidants.

Hydroxytyrosol is a product of Oleuropein hydrolysis (the major phenolic compound in leaves and fruit of *Olea oeuropaea*): as the olive fruit matures, the concentration of Oleuropein decreases and Hydroxytyrosol increases (Cimato et al., 1990; Ryan et al., 1999). Despite all its interesting properties, hydroxytyrosol is not commercially available in high amounts yet. Several methods have been proposed for Hydroxytyrosol production by means of chemical (Tuck et al., 2000) or enzymatic synthesis (Espin et al., 2001), but protocols are usually slow and expensive, resulting in a little number of commercially available products containing pure Hydroxytyrosol.

Nevertheless, several protocols under patent protection (Crea, 2002; Fernandez-Bolanos et al., 2002; Pizzichini & Russo, 2005; Villanova et al., 2006) have been developed for purification of Hydroxytyrosol and other phenolic compounds from OMW, even if different matrices have been proposed for recovering the phenolic compounds.

Activated carbon or Zeolite can be used for the absorption of the phenolic fraction, but resins are definitively the most used as they are specific for biophenols. However resins are very expensive materials.

Notwithstanding their high price, there are a few commercial products based on biophenols, mainly recovered from olive leaf. Some producers and total phenol amount expressed in Oleuropeins are shown in table 3.

Tab. 3. Companies producing commercial products based on Olive Leaf extracts.

Company Name	Country	Product Specifications
Amax NutraSource Inc.	USA	Olive Leaf 18% Oleuropeins
A.S.I. International Inc.	USA	Olive Leaf 10 - 25% Oleuropeins
Finzelberg	Germany	Olive Leaf Extract 15% Oleuropeins
Furfural	Spain	Olive Leaf Extract 6 - 20% Oleuropeins
Linnea	USA/ Swirzerland	Olive Leaf Extract 15-18% Oleuropeins
Naturex	France/USA	Olive Leaf Extract 12,5% Oleuropeins
Sabinsa Corporation	USA	Olive Leaf Extract 6% Oleuropeins

In addition to the recovery of biophenols from olive leaves, in the last few years many companies focused their attention on the recover of the antioxidant compounds from the liquid residue of OMW (table 4), which are more available

Tab. 4. Companies and products based on OMW polyphenols and Hydroxytyrosol.

Company Name	Country	Product Specifications
CreAgri Inc.	USA	Hydrox 6-10% Polyphenols
Genosa	Spain	Hytolive 99,5% Hydroxytyrosol
Seppic	USA	Prolivols Olive extract, Polyphenols 35%, Hydroxytyrosol 2%
Cohitec	Portugal	Olidrox, Hydroxytyrosol 17%
Indena	Italy	Oleaselect, 2,2% Hydroxytyrosol
Indena	Italy	Opextan, Polyphenols 10%, Verbascoside 2%

BIOHYDROGEN PRODUCTION

In the last decades, worldwide energy demand has been increasing almost exponentially, the reserves of fossil fuels have been decreasing and, especially, fossil fuels combustion has shown to have negative effects on the environment, as a consequence of massive CO_2 emissions (Rifkin, 2002). Large efforts have been made towards the exploration of new sustainable energy sources that could substitute (partially or completely) fossil fuels utilization. Hydrogen is commonly considered one of the suitable alternative fuels and "energy carrier" of the future. Hydrogen gas is a clean fuel, with no CO_2 release upon combustion**,** which owns also the smart feature of being usable in combination with new technologies as fuel cells for the generation of electricity (Goltsov and Veziroglu, 2001). Besides, hydrogen has a high energy yield (namely, 122 kJ/g), which is 2.75 times higher than hydrocarbon fuels (Kapdan and Kargi, 2006).

Up to date, conventional hydrogen gas production methods are steam reforming of methane and other hydrocarbons, non-catalytic partial oxidation of fossil fuels, autothermal reforming (which combines the methods afore cited) and electrolysis of water (Das and Verziroglu, 2001). Those methods are all energy intensive processes requiring high temperatures (more than 850°C are needed) (Kapdan and Kargi, 2006) and, above all, meaningless from an environmental point of view.

Based on the National Hydrogen Program of the United States, the contribution of Hydrogen to total energy market will be 8-10% by 2025 (Armor, 1999). In conclusion, development of cost-effective and efficient Hydrogen production technologies remain one of the key point to the wide spreading of hydrogen application (Rifkin, 2002).

Biological hydrogen production represents an alternative solution to the aforementioned methods. It is commonly believed to date that biohydrogen production would hardly represent an economical sustainable solution to the worldwide energetic crisis, although first claims for hydrogen production date back to the "Biological Energy Conversion" meeting, taking place during the first energetic crisis (Hollaender et al., 1972). Nevertheless, biohydrogen production could represent a valuable solution when applied to local energy needs. Furthermore, the capacity that biological systems show to manage the entropy factor (Schrödinger

E., 1945) remains very attracting, as no men's industry up to now was able to take advantage of low density solar energy as several kind of photosynthetic microorganisms do to grow or, for instance, produce hydrogen.

Biological production of hydrogen (figure 4) concerns a number of processes that are carried out with or without light, generally needing organic substrates to be performed.

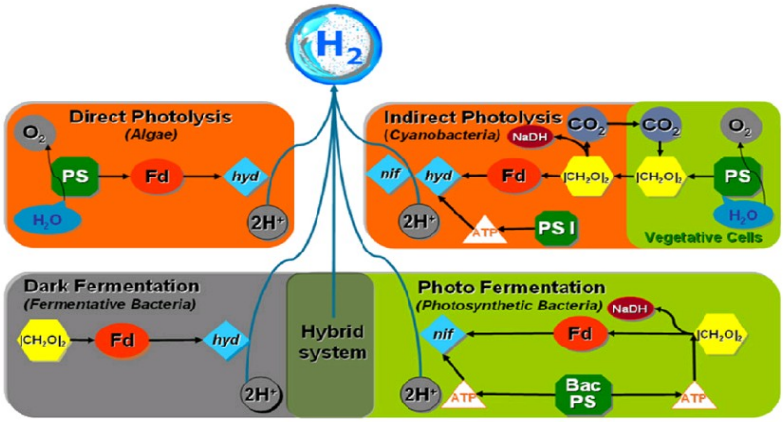

Fig. 4. Schematic systems for biological hydrogen production (Kotay and Das, 2008).

A complete list is shown hereafter (Kotai and Das, 2008):

- direct biophotolysis; hydrogen can be produced directly from water using light as the energy source (performed by some microalgae);
- indirect biophotolysis; hydrogen can be produced from water; photosynthesis is used to stock carbohydrates that are subsequently fermented to evolve hydrogen gas (performed by some microalgae and cyanobacteria);
- photofermentation; hydrogen can be produced by microrganisms able to use a wide spectral light energy and different kind of organic waste materials (performed by anoxygenic photosynthetic bacteria);
- dark fermentation; it does not need light energy and produces various valuable by-products, moreover it can use different carbon sources (performed by chemoheterotrophic bacteria);
- combined processes; a combination of both dark- and photo-fermentation to maximize substrate degradation (manly performed by mixed consortia).

All the mentioned processes take advantage of enzymatic activities carried out by a number of different hydrogenases or nitrogenases, operating in an oxygen free system.

Fermentation biotechnologies were deeply studied since the first 70's (Asada and Miyake, 1999; Hallenbeck and Beneman, 2002) and are considered much more reliable when up-scaled from the laboratory. Yet, wide applications of these biotechnologies are not going on because of operational costs (e.g. pure cultures utilization, reactor sterilization, etc) and the consequent economical concerns.

Photobiological Hydrogen production represents one of the most studied in literature (Weaver et al., 1980; Akkermann et al., 2002; Mathews and Wang, 2009), showing a good process stability (Krupp and Widmann, 2009) and, most of all, high hydrogen production rates. However, hydrogen photoproduction process still suffers of some major critical drawbacks, which negatively affects operational costs of production, such as: (i) light dependency and (ii) the relatively low conversion yields. The first point could be overcome by using solar radiation instead of artificial light, meaning that no additional energy would be spent to carry on the process. As regards the latter, an effective increase on hydrogen conversion yields could be induced by improving reactor design, optimizing culture conditions such as pH, hydraulic retention time and temperature (Carlozzi, 2009), by using suitable microbial strains and even genetic and metabolic engineering techniques to redirect metabolic pathways (Nath and Das, 2004).

For hydrogen to be renewable, it must come from renewable sources. Aiming to make hydrogen a feasible energy reality, large efforts are conducted worldwide in order to explore new sustainable energy sources that could substitute fossil fuels. Processes, producing energy from biomass are typical examples of environmental-friendly technologies as biomass is included in the global carbon cycle of the biosphere (Davila-Vazquez et al., 2008). Large amounts of biomass are available in the form of organic residues, such as solid municipal wastes, manure, forest and agricultural residues. Some of these residues can be used after minor steps of pre-treatment (usually dilution and maceration), while others may require extensive chemical transformations prior to be utilized as a raw material for biological energy production (Claassen et al. 1999). Furthermore, the production of a "clean" energy source would concomitantly lead to a recover of the treated waste that otherwise

should be depurated with high expenditure for disposal. In this respect, rice winery wastewater (Yu et al., 2002), food (Han and Shin, 2004), domestic waste (Van Ginkel et al., 2005), municipal solid waste (Lay et al., 1999), sugar refinery (Yetis et al., 2000), milk industry wastewater (Zhu et al., 1999) and vegetable residues (De Philippis et al., 2007) were all successfully tested for hydrogen gas production. OMW was already tested for hydrogen gas production (Eroglu et al., 2004, 2006; 2009 a, b) but great dilution was needed.

Among several kind of microorganisms able to carry on a photofermentation process directed towards hydrogen gas production, purple non-sulphur photosynthetic bacteria (e.g. *Rhodobacter* and *Rhodopseudomonas* strains) showed the capability to use a wide range of light intensities and a relatively high substrate conversion rate, provided that many different substrates could be supplied (Oh et al., 2004). *Rp. palustris* has been widely studied for its photobiological hydrogen production both on complete medium (Carlozzi and Sacchi, 2001; Carlozzi and Lambardi, 2009; Chen et al., 2006) and on wastewater (Vikineswary et al., 1997; De Philippis et al., 2007). The latter could be a key feature that gives these microorganisms a great relevance as they could be applied to metabolize several kinds of organic wastewaters which, as previously noted, would dramatically reduce operational costs.

CHAPTER 2

AIMS OF THE THESIS

To date, the utilization of waste as raw materials by means of biotechnological processes is fundamental to create a sustainable economy. The present research aims at entirely treating, exploiting and valorising the olive mill waste water (OMW), known to be the most pollutant waste in the Mediterranean basin. To solve the problem of its disposal, due to the high concentration of some organic compounds, it is crucial to set up a feasible treatment both on an economical and ecological point of view. Important organic compounds (e.g. polyphenols) should be recovered and sold on the market for pharmaceutical, cosmetic and food applications. Many treatments proposed and patented in the previous years aim at depurating OMW destroying its total organic load and the polyphenolic fraction as well. Since to recover is better than to destroy, the complete treatment studied in this research aims at depurating the OMW and, in the meantime, at recovering valuable natural compounds as polyphenols.

In order to achieve this goal, a first treatment was adopted to reduce OMW total pollutant load. A biofilter, consisting of two packed vegetable matrices (the aquatic fern Azolla and Granular Activated Carbon [GAC]), was designed and built. Azolla (*Azolla caroliniana*) has already been studied as fresh material for the reduction of the pollutant waste of OMW (Ena et al., 2007) and the removal of heavy metals in contaminated water (Cohen-Shoel et al., 2002; Upadhyay and Tripathi, 2007). GAC is used in many treatments of different kind of waste due to its widely known adsorption capabilities. Azolla and GAC were strongly packed in a packed-bed plug-flow reactor where the OMW was loaded up-flow., in order to avoid preferential pathways of the liquid and to ensure the whole utilization of the matrices during the biodepuration. Different matrices concentrations have been tested according to the highest depuration efficiency with the least material utilization. Azolla and GAC sorbing and desorbing capability was studied in order to evaluate their application for the recovery of OMW phenolic fraction. The phenolic fraction adsorbed by the matrices was recovered using different elution solvents depending on the matrices. The capacities of the two biofiltrating materials have been studied and compared with those of two commercial resins. From the adsorbed biofilters, two powder products were recover and characterized. The powder resulted different form a quantitative and qualitative point of views. The HPLC profiles revealed a higher amount of Hydroxytyrosol in GAC product than in the Azolla one. Both the of

products showed high antiradical activity and antioxidant capacity at the DPPH and ORAC testes. At the end, Azolla and GAC resulted suitable for the adsorption of OMW phenolic compounds and their recovery, releasing two different and interesting products.

After the biofiltration, OMW resulted clear, with no presence of polyphenols and a low COD concentration. The remaining organic content was exploited for the photobiological production of energy (i.e., hydrogen). To study its feasibility, the purple non-sulphur bacteria *Rhodoseudomonas palustris* (*Rp. p.*) has been used for the photofermentation of the residual OMW organic fraction, producing mainly composed by hydrogen (more than 85%) and CO_2 (less than 15%). It must be evidenced that the presence of a high concentration of phenolic compounds, as well as the dark colour of untreated OMW, does not permit the phototrophic growth of microorganisms, neither their hydrogen production without dilution. OMW sample dilutions were seen to work only when applied in an extremely high level (less than 5% of OMW, Eroglu et al., 2004). In the present work, 25% dilution of OMW treated with Azolla and GAC resulted suitable for both the growth of *Rp. p.* and hydrogen production.

At the end, the exhausted OMW could be supplied to cultivated soils as a fertilizer, thanks to the low concentration of the remaining organic compounds still present after such treatments. Moreover, exhausted Azolla could be used as added value feed for animal nutrition.

The final aim of the work was to scale-up the process and eventually verify the feasibility of the laboratory treatments. The pilot scale system would then represent a key effort in a new approach for the olive mill wastewater exploitation.

CHAPTER 3

AN ECO-COMPATIBLE PROCESS FOR THE DEPURATION OF WASTEWATER FROM OLIVE MILL INDUSTRY

Authors: Alba Ena, Cristina Pintucci, Cecilia Faraloni, Giuseppe Torzillo

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Abstract

Olive mill wastewater (OMW) is the by-product of olive oil industrial production. It is characterized by a dark brownish colour and a strong odour and is considered one of the most polluted agricultural wastes. In this paper we briefly describe an innovative procedure for the depuration of olive mill wastewater. With this procedure it is also possible to recover valuable substances such as phenolic compounds which have important commercial applications: they can be used in the prevention of cardiovascular disease and as antiviral, antioxidant and antitumor agents. The proposed OMW treatment uses two different packed vegetable matrices which remove most of the pollutant substances by absorption. After filtration of OMW on the matrices the pollutant load of the waste is greatly reduced: the organic content (COD) is reduced more than 80% and the phenol compounds are completely removed.

Introduction

The extraction and use of olive oil has been an integral part of Mediterranean culture for over 6000 years (Civantos, 1995; Tardàguila et al., 1996). Olive oil extraction involves a heavy consumption of water and produces large amounts of olive mill wastewater (OMW), the average volume ranging from 0.5 to 1.5 m^3 per ton of processed olives (Monteoliva-Sanchez et al., 1996; Paredes et al., 1996). In Italy, OMW is spread on cultivated fields under a strict law that strongly limits its use in agriculture (D.L. 574/96). The fresh organic matter content in oil mill wastewater causes agricultural and environmental problems in olive oil-producing countries since its effects on soil status and fertility, insect proliferation and groundwater contamination are more harmful than beneficial (Cox et al., 1996; Spandre and Dellomonaco, 1996). OMW contains a high amount of organic matter (30-200 kg COD/m^3), with a COD/BOD_5 ratio between 2.5 and 5, which is considered poorly degradable (Lopez, 1992). The organic compounds in OMW (sugars, polyphenols, tannins, polyalcohols, pectins and lipids), in association with its high C/N ratio and low pH, compromise the biological degradation process of

soils (Marques, 2001) and can cause eutrophication when the wastewater is collected in basins with low exchange rates (closed gulfs, lakes, etc.).

The toxicity of OMW is also due to its high content of phenolic compounds in a wide range of molecular weights (MW), from low-MW substituted phenols to complex high-MW phenolic compounds (Montedoro et al., 1992). During olive oil production, large quantities of phenols are released along with the wastewater, according to their partition coefficient. Phenolics are derivatives of benzene (cyclic derivatives in the case of polyphenols) with one or more hydroxyl groups associated with their ring. The dark colour of the water is caused by polyphenols (Pp) (Hamdi and Garcia, 1993) and depends on the type of olives processed, their ripening stage, the climatic conditions and the technology used. However, despite their toxicity, polyphenols are used in the food, cosmetic and pharmaceutical industries on account of their high antioxidant activity. The use of synthetic antioxidants in food processes can have negative health effects. Therefore, it is necessary to find natural substitutes that can inhibit the usual oxidization processes involved in the degradation of substances.

Treatment of OMW to recover valuable compounds like polyphenols could employ the aquatic fern Azolla as a biofilter. Dried Azolla biomass has already been used in the biosorption of a wide range of heavy metals from aqueous media (Sela and Tel-Or, 1988; Cohen-Shoel et al., 2002). Pectin is an important polysaccharide constituent of Azolla cell walls, made up of fragments of polygalacturonic acid chains that interact with Ca^{2+} and Mg^{2+} ions to form a three-dimensional polymer (Schols et al., 1989; Jauneau et al., 1997; Kamnev et al., 1998).

As OMW consists of the diluted juice of crushed olives, it can be safely assumed that it is completely biodegradable. Yet even if all the constituents of OMW are biodegradable by definition, some of them, e.g. polyphenols and lipids, are decomposed at reaction rates much lower than others, e.g. sugars and short-chain volatile acids.

In recent years several methods have been proposed for OMW bioremediation, such as physical, physico-chemical or microbiological treatment. The physical and physico-chemical methods include thermal processes (evaporation and incineration), flocculation/clarification, ultrafiltration and reverse osmosis. Some of these systems have been patented (Knobloch et al., 2002; Pizzichini and Russo, 2007). The biological

processes can be subdivided into anaerobic and aerobic ones. Nevertheless simple chemical or biological treatments cannot completely reduce OMW pollution and up to 85% of the organic substances, which could be recycled, are destroyed (Laconi et al., 2007).

The aim of this study was to test a new treatment for removing the pollution load in OMW by filtration on Azolla and granular activated carbon (GAC) to reduce the phenol, organic and inorganic matter content. This paper reports the results of laboratory experiments. The filtration system is shown in figure 1.

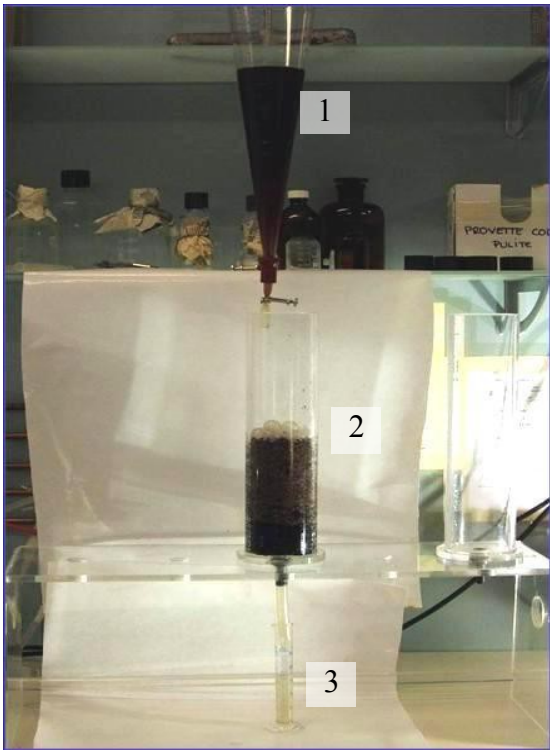


Fig. 1. Biofiltration system: (1) untreated OMW; (2) packed vegetable matrices; (3) depurated waste.

This method uses two different packed vegetable matrices (Azolla and GAC) which remove most of the pollutants by adsorption. Moreover, the phenolic compounds are almost completely recovered and the filtered water can be treated with calcium hypochlorite (ipoCa) to bring the COD to discharge values. The pretreated water can also be recovered for both hydrogen and biodegradable plastic production.

Materials and Methods

Fresh olive mill wastewater samples were supplied by a continuous olive processing plant located in Bibbona in the province of Livorno (Italy). The samples, obtained from olives collected in January 2006 and immediately processed, were stored at -18°C. Citric acid, ethanol and all other chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA). The multi-element and single element standards were supplied by CPI International (Santa Rosa CA, USA). The GAC (AFC-LS) was provided by Carboplant s.r.l. (Vigevano, PV, Italy).

Azolla cultivation

The strain of *Azolla caroliniana* (fig. 2) derives from the Botanical Institute of Naples, Italy. Since the 1980s, it has been preserved by the Institute of Ecosystem Study (ISE), Florence section, of the Italian National Research Council (CNR). The Azolla biomass was cultivated in 2 m^2 vertical tanks containing a 10 cm layer of medium (reported by Ena et al., 2007), and then harvested and dried in the sun. This vertical apparatus permits to increase the cultivated surface from 6 m^2 to 14 m^2.

Fig. 2. *Azolla caroliniana*

OMW pretreatment

Effluent (vegetation water) from a continuous olive oil extraction process was centrifuged. Table 1 shows the main characteristics of the OMW after centrifugation.

Tab. 1. Composition of the olive mill wastewater used in the present study.

Property	Unit	Value
pH	pH	3.6
COD	mg/L O_2	52500
BOD_5	mg/L O_2	16250
Polyphenols	mg/Kg	4005.00
Chloride	mg/L	593.04
Nitrate	mg/L	03.04
Sulphate	mg/L	72.05
Orthophosphate	mg/L	926.03
Total P	mg/L	428.06
Total N	mg/L	785.09
K	mg/L	16.08
Ca	mg/L	648.03
Mg	mg/L	180.03
Al	mg/L	1257
Cd	mg/L	< 0.5
Cr	mg/L	33.02
Fe	mg/L	6535
Hg	mg/L	< 0.5
Ni	mg/L	925.03
Pb	mg/L	07.08
Cu	mg/L	335.02
Zn	mg/L	4588

Each filtration experiment (in triplicate) was carried out twice and each value represents the mean of the six experiments. The pretreatment method uses two different packed vegetable matrices (Azolla and GAC) which remove most of the pollutant substances by absorption. The standard error was never higher than 5%. The results of this study demonstrate the feasibility of biofiltration on the vegetable matrices of wastewaters with an organic load of about 50-60000 ppm.

Main properties of the filtration supports.

The adsorption properties of GAC and Azolla for the organic (COD) and phenolic compounds of OMW were determined in duplicate by placing 10 g of each support (previously sterilized in an autoclave) in anaerobic 100 mL-sterile bottles containing 20 mL of a filter-sterilized defined dilution (1:1, 1:2, 1:3, 1:5 or 1:8, in distilled water) of OMW. All bottles were brought to equilibrium conditions by shaking on a rotary shaker at 25°C and 150 rpm for 2 days. The adsorption data were calculated by measuring the residual concentrations of COD and Pp in the supernatants after filtration. Freundlich sorption kinetics for the organic matter and phenolic compounds in the immobilization carriers was then studied according to Colella et al. (1998).

Analytical methods.

The analysis of BOD_5, total nitrogen, chloride, nitrate, sulphate, orthophosphate were carried out according to standard methods (Eaton et al., 2005).

- COD

The COD concentration in OMW was determined according to Ena et al. (2007).

- Polyphenols

Polyphenols (with respect to Gallic Acid) were determined spectrophotometrically according to the Folin-Ciocalteau (Ena et al., 2007) using a Beckman DU 640 as spectrophotometer.

- Total phosphorus

Total organic phosphorus was determined in unfiltered samples by acid digestion with potassium persulphate followed by colorimetric inorganic phosphate analysis with a UNICAM UV2 double ray spectrophotometer (Carmouze, 1994).

- K, Na, Mg

These ions were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) with an AGILENT 7500ce collision cell ICP-MS.

- Heavy metals (Al, Cd, Cr, Fe, Ni, Pb, Cu, Zn)

The heavy metals were determined by atomic absorption with a UNICAM 939 graphite oven (confirmation by AGILENT 7500ce ICP-MS).

- Hg

This metal was determined by atomic absorption spectroscopy at hydride development (Perkin Elmer K16; confirmation by Agilent 7500ce ICP-MS).

Results and Discussion

Data on the growth of the aquatic fern (Azolla-*Anabaena azollae* symbiosis) outdoors under the climatic conditions of Florence are reported in table 2.

Tab. 2. Yield and growth rate of *Azolla caroliniana* achieved outdoors in 2 m^2 vertical tanks.

Months	**Productivity [g (d.w.)/m^2/d^1]**	**Growth rate d^{-1}**
July	14.2 ± 0.68	0.258 ± 0.009
August	10.5 ± 0.53	0.183 ± 0.009
September	7.2 ± 0.25	0.144 ± 0.005

The highest yield [14.2 g dry weight (d.w.)/m^2/d] was obtained in July; the average productivity was 10.3 g (d.w.)/m^2/d. The average planting density was 50 g (dry weight)/m^2. A previous study had shown the ability of this aquatic fern (fresh biomass) to reduce phenol and organic matter in OMW (Ena et al., 2007). Other authors have used dried Azolla biomass in the biosorption of a wide range of heavy metals from aqueous media (Coen-Shoel et al., 2002). Physico-chemical analyses (table 1) of OMW showed that it was dark acidic waste with high levels of organic matter and polyphenols. According to Ranalli (1992), phenolic compounds are the pigments responsible for the dark colour of OMW. Moreover, OMW toxicity is mainly due to low molecular weight phenols (Della Greca et al., 2001); the toxicity of these compounds is caused by autoxidation processes (Nakai et al., 2001). This vegetation water also had high contents of chloride, orthophosphate, total P, total N, Ca and the heavy metals Ni and Fe.

As a first step, the two types of dried packed vegetable matrices were used separately: Azolla alone and GAC alone at two concentrations (50 and 100 g/L; 100 and 200 g/L respectively). The COD and Pp removal results (fig. 3) show that the lower

concentrations were most efficient (220 and 25 mg/g respectively), while the values were somehow lower (162 and 20.5 mg/g respectively) at 100 and 200 g/L. However, the higher concentrations removed greater amounts in absolute.

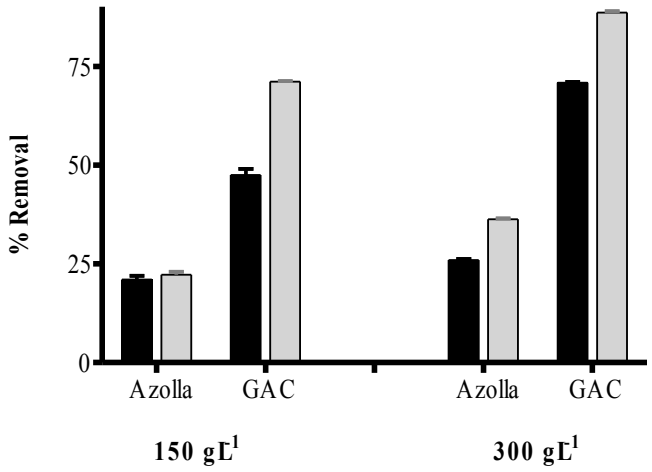


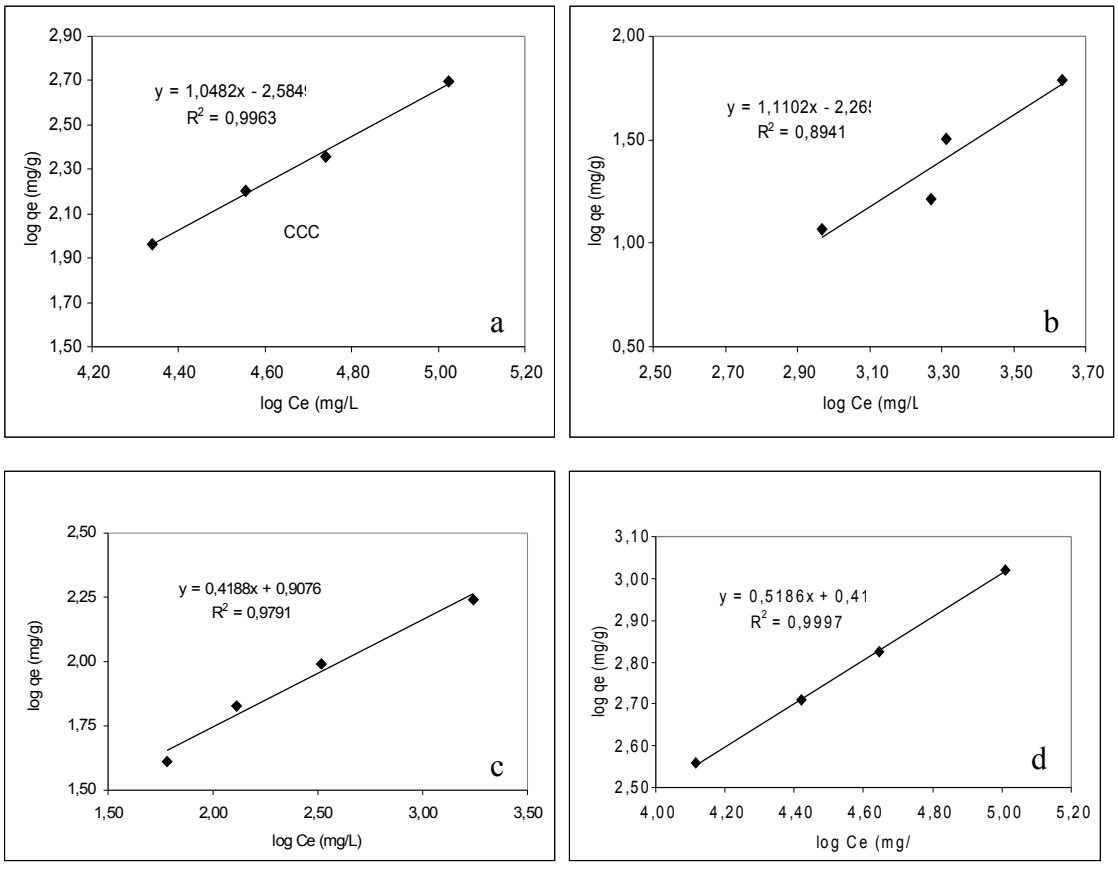
Fig. 3. Abatement percentages of COD (black bars) and Pp (grey bars) in alone (at two different concentrations) vegetable matrices.

Considering the great chemical heterogeneity of OMW, we studied the adsorption properties of GAC and Azolla in terms of removal ‘‘broad parameters’’ instead of determining adsorption isotherms for single compounds in the OMW. The Freundlich adsorption isotherm, $q_e = K*C_e^{1/n}$ (q_e = adsorbed COD or phenols per unit mass of solid material, mg/g[1], and C_e = COD or phenolic compound concentration at equilibrium, mg/L), was used to represent the COD and phenolic compound adsorption on both supports. Thus, the characteristic Freundlich parameters (k and 1/n) are best regarded here as descriptive and they were optimized by non-linear least-square regression of experimental adsorption data (table 3).

Tab. 3. Characteristic Freundlich parameters.

	k (mg/g)/[(mg/l)$^{1/n}$]	*1/n*	Correlation coefficient R^2
COD Azolla	2.60E-03	1.0482	0.9963
Pp Azolla	5.42E-03	1.1102	0.8941
COD GAC	2.63	0.5186	0.9997
Pp GAC	8.08	0.4188	0.9791

The experimental adsorption data and calculated Freundlich isotherms for COD and phenol adsorption are compared in figure 4a and 4b for *Azolla* and figure 4c and 4d for GAC.



Figs. 4. COD (a, c) and phenolic compounds (b, d) Freundlich isotherms on Azolla (a, b) and GAC (c, d).

As expected, the total organic matter (measured as COD) and phenolic fraction of OMW were both much more efficiently adsorbed by GAC (70.8% and 88.5% respectively) than by Azolla (25.95% and 36.25% respectively).

The different vegetable matrices were then tested together by packing one on top of the other in a column (concentrations: 150 g/L, 300 g/L, 360 g/L). The results indicate that the concentrations of 300 g/L and 360 g/L provided greater removal of phenols and organic matter: COD removal increased to 80.5% and 84.5 % respectively and phenol absorption to more than 99% in both conditions (figure 5).

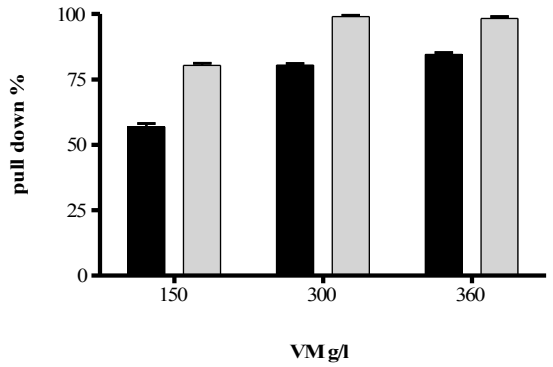


Fig. 5. Removal percentages of COD (black bars) and Pp (grey bars) at different concentrations of together packed vegetable matrices.

A higher concentration of vegetable matrices used in the biofilter (360 g/L) do not provide a significant removal of pollutant substances (Pp and COD) if compared to that obtained with the 300 g/L concentration, considering the increase of the matrices utilized. Therefore we have used the 300 g/L concentration. The progress of the treatment process is shown in figure 6: 40% of the organic load of COD was removed by centrifugation and over 80% by biofiltration; 12% of Pp was removed by centrifugation and over 99% by biofiltration.

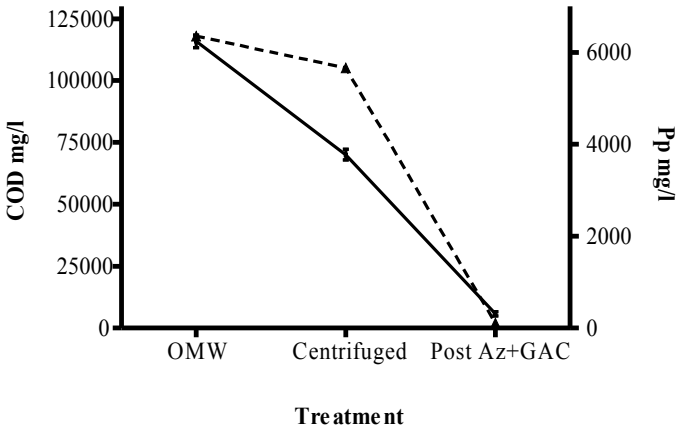


Fig. 6. Removal of COD (continuous line) and Pp (discontinuous line) in the invented treatment.

The percentage removal data for the alkaline metals (K, Ca and Mg) after biofiltration demonstrate a higher affinity of matrices for K and Mg (more than 70%) while the removal of Ca was very poor (47%). When several components are present, interference and competition for adsorption sites probably occur, leading to a lower saturation concentration of the adsorbed component. Besides, other substances such as total nitrogen and copper were almost totally absorbed by the matrices while different metallic compounds (Al and Pb excepted) were variably reduced by 50-85% (figure 7a and b).

The heavy metals with higher ionic charge probably cannot be completely adsorbed by the vegetable matrices according to the aforesaid phenomena.

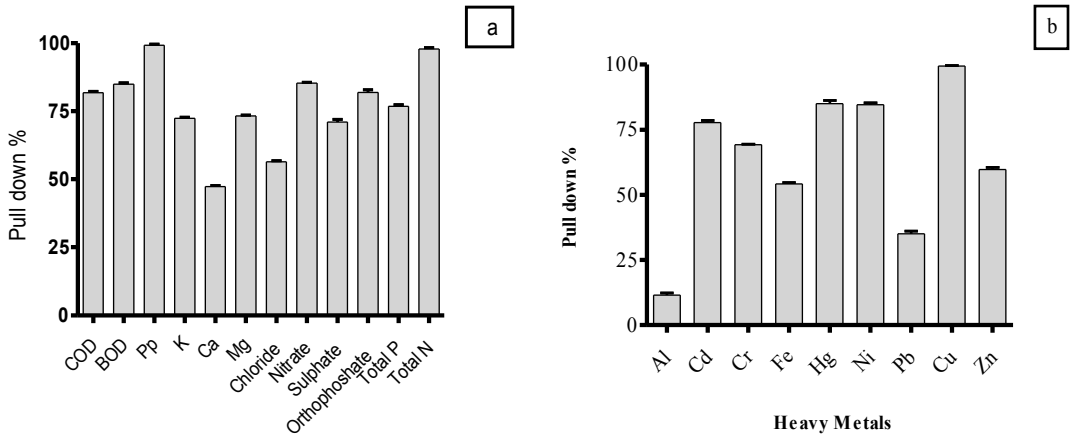


Fig. 7. Removal percentages of some chemical parameters (a) and heavy metals (b).

For complete removal of the COD, the residual waste after biofiltration was treated by oxidation with different concentrations of calcium hypochlorite (1800-15760 ppm). As shown in figure 8, the highest COD removal (366 mg/g of ipoCa) was obtained in the experiment with the highest initial concentrations of both COD and ipoCa; however this treatment was not effective because the organic load remained and there was also a large amount of chlorides.

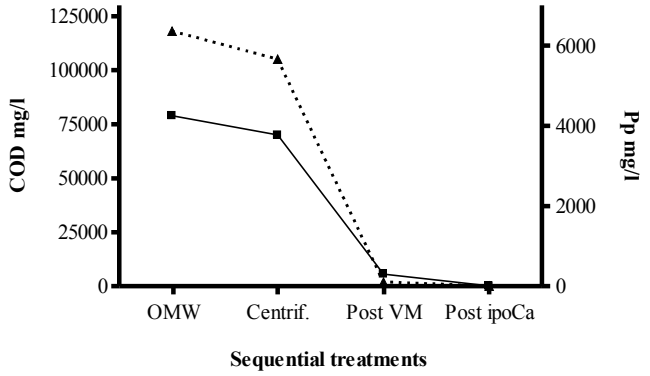


Fig 8. Complete treatment: the abatement of COD (continuous line) and Pp (discontinuous line).

The best treatment was with the lower initial contents of COD and ipoCa: the COD concentration was reduced by 92%, giving a final concentration of 150 mg/L which is

compatible with Italian law (160 mg/L COD in surface waters). Figure 9 illustrates the complete treatment.

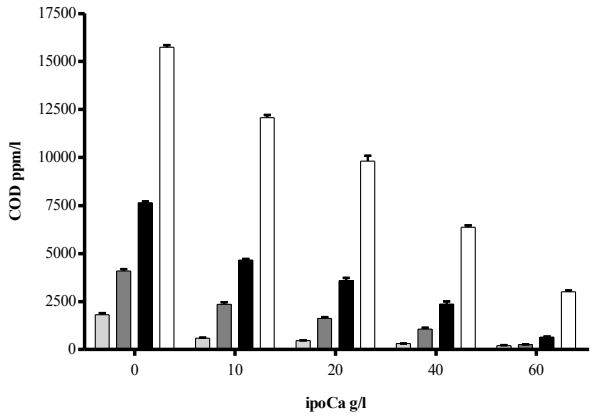


Fig. 9. Removal of COD in treated OMW with different initial COD concentrations (1800 ppm light grey bars, 4080 ppm grey bars, 7680 ppm black bars, 15760 ppm white bars) and different Ca ipoclorite concentrations.

The results obtained with this treatment are comparable to those using membrane technologies (ultrafiltration, microfiltration, nanofiltration and reverse osmosis) (Russo, 2007), although our method is much more economical.

The pretreated wastewater (after biofiltration) can be used for various applications:

i) Polyphenol recovery by vegetable matrix. Phenols are very interesting compounds as they can be used in the prevention of cardiovascular disease and as antiviral, antioxidant and antitumor agents. They can also be used for food, cosmetic and pharmaceutical applications. In particular, OH-tyrosol shows strong antioxidant, antinflammatory and antiviral activity (Ohno et al., 2002), higher than that of many other compounds (Manna et al., 1999; D'Angelo et al., 2005). The total desorption of polyphenols from the exhausted matrices is about 50-60%.

ii) The pretreated wastewater can be used as a substrate for the growth of a purple bacteria [*Rhodopseudomonas palustris*, (*Rp. palustris*)] able to metabolize residual organic compounds (mainly short chain organic acids) for biohydrogen production. Under the same conditions, H_2 production experiments (conducted in free nutrient

OMW) were carried out in both batch and discontinuous mode: removing and adding a fixed amount of wastewater. The results showed that in batch conditions the biogas production was ca. 600 mL per litre of culture, while in experiments carried out in discontinuous mode for more than 300 hours the yield was ca. 1200 mL per litre of culture (data not shown). These results are very good compared with those reported by Eroglù et al. (2004) because in that paper optimal condition OMW was used at 2% dilution.

iii) When growing in unbalanced conditions, *Rp. palustris* can produce biopolymers (polyhydroxyalkanoates) with potential applications in medicine and surgery as biodegradable plastic material. In these microorganisms the polyester (PHB) is used as an energy reserve compound.

At the end, the treatment proposed according to the present research not only provides for abating the pollution impact of olive mill wastewater, so that it can be directly disposed in the environment with full compliance of current legislation, but also uses this OMW as a raw material obtaining the following commercially useful products: organic fertilizers, antioxidant compounds and water that can be used for all oil press customs. In figure 10 is schemed the complete treatment suggested in this paper.

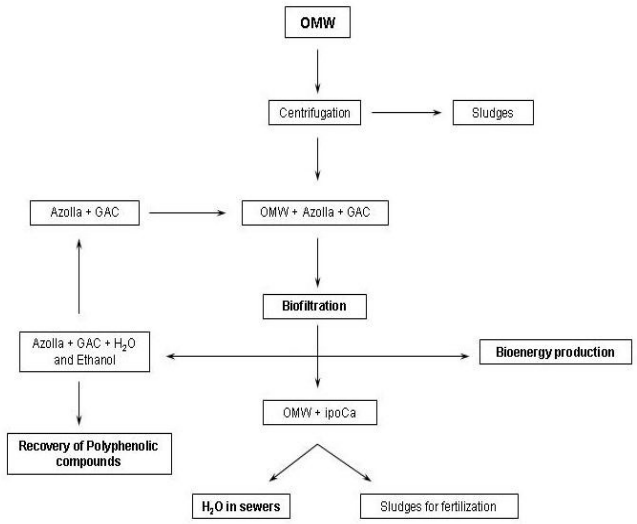


Fig. 10. Complete treatment plan of the olive oil mill wastewater.

Conclusions

This paper is the first description of a complete biodepuration treatment of olive mill wastewater carried out with a biofiltration system using vegetable matrices. The pretreated water can be utilized for various applications. For example, the OMW pretreated with the described method is a promising substrate for the biological production of valuable by-products (biohydrogen, polyhydroxyalkanoates and pigments). Consequently, the water obtained after the suggested applications can be employed for all oil press uses.

Acknowledgments

This research was supported by a contribution from the ENTE CASSA di RISPARMIO di FIRENZE. The authors thank Dr. Lisa Bonciani (General Director) and Dr. Giuseppe Sammarco (Laboratory Directory) of the Biochemie Lab and Edoardo Pinzani of the CNR – ISE for their active technical assistance. A special thank also to Francesco Peccianti, director of the Antico Frantoio Toscano (Bibbona, Li) for his economical and practical support.

CHAPTER 4

RECOVERY OF OLIVE MILL WASTEWATER POLYPHENOLIC COMPOUNDS FROM ADSORBED AZOLLA AND ACTIVATED CARBON

Abstract

OMW is considered one of the most pollutant waste in the Mediterranean basin. However, its phenolic fraction should be recovered as it shows incredible health benefits. Azolla and Granular Activated Carbon (GAC) have already been used as biofilters for the removal of OMW pollutant load. In the present study, the adsorbent and desorbent capacities of these matrices are compared with those of two commercial resins. Carbon resulted the most efficient matrix in both phenols adsorption and desorption. The total characterization of two powder products obtained by Azolla and GAC desorptions is reported, together with their antioxidant and antiradical activity. Total polyphenols in the Azolla powder product resulted more than two times higher than in the GAC product. The HPLC polyphenolic profiles of the two products resulted deeply different. GAC powder contained hydroxytyrosol in concentration 3.5 times higher than Azolla. Moreover, both the powder products showed great antiradical activities: at the DPPH test, Azolla and GAC products IC_{50} resulted 102 μg/mL and 189 μg/mL of powders, respectively. The antioxidant capacity resulted very high: 4097 μmol TE/g Azolla powder and 1277 μmol TE/g GAC products, respectively.

Introduction

Almost 97% of the world's olive oil is currently produced within the Mediterranean areas. Most frequently, olive oil mill wastewater (OMW) are pumped and discharged into evaporation ponds or directly dumped in rivers or spread on soil (Greco et al., 1999). Therefore, many countries are obliged to develop new technologies in order to reduce OMW pollutants. It is known that phenolic compounds are major contributors to the toxicity and the antibacterial activity of OMW. This limits its microbial degradability (Borja et al., 1997; Capasso et al.,1995). However, these phenolic compounds possess strong antioxidant properties (Galli and Visioli, 1999), which may turn the olive oil residues into a cheap source of natural antioxidants. An antioxidant may be defined as a substance that, when present at low concentrations compared to that of an oxidizable

substrate, significantly delays or prevents the oxidation of that substrate as carbohydrates, DNA, proteins and lipids (Halliwell, 1990).

Lipid oxidation, not only produces undesirable flavour, but also decreases the nutritional quality of oils due to the loss of essential fatty acids. The use of antioxidants to minimize the oxidation of lipids in food materials is extensively practised (Loliger, 1991; St Angelo, 1992). Antioxidants, such as BHT (Butylated hydroxytoluene), BHA (Butylated hydroxyanisole) and propyl gallate are widely used in many foods. These compounds are added at concentrations ranging from 50 to 200 ppm to fat and oils to suppress the development of peroxides during food storage (Loliger, 1991). Some recent discussion have been carried out about the undesirable use of synthetic antioxidants e. g. dietary administration of BHT to rats caused fatal haemorrhages in the pleural and peritoneal cavities and in organs such as epididymis testes and pancreas (Hirose, 1987). Also, BHT caused changes in rat livers, stimulation of DNA synthesis and induction of enzymes (Thamavit et al., 1985). Moreover, BHA has toxic and carcinogenic effects (Ito et al., 1986). However, these antioxidants are approved for food use within limits. Consequently, there is an urgent need of other types of compounds, e.g. natural compounds, that could be used as antioxidants. Olives and olive oil contain polyphenols such as Oleuropein, Hydroxytyrosol and Tyrosol (Briante et al., 2002), Rutin (Boitia et al., 2001), Quercetin (Obied et al., 2007), Caffeic Acid (Papadopoulos & Boskou, 1991), Vanillic and o- and p-Coumaric Acids (Brenes et al.1999) all with excellent antioxidant properties. These compounds were effective radical scavengers with the 2,2-diphenyl- 1-picrylhydrazyl radical (DPPH) test (Morello et al., 2005). Chimi et al., (1991) demonstrated that the antioxidant activity of biophenolic compounds, expressed in Linoleic Acid, was Hydroxytyrosol > Oleuropein > Caffeic Acid > Tyrosol. Epidemiological studies correlated the low incidence of coronary heart disease, atherosclerosis, and some types of cancer (colorectal and breast cancer) with olive oil consumption in the Mediterranean diet (Martin-Moreno, 2000). Consequently olives, and principally OMW represent an other potential source of such natural antioxidants. The recovery of polyphenols does not only reflect the need for biofunctional compounds, which may also be interesting from a technological point of view, as

valuable components of nutraceuticals in food and pharmaceutical preparations or in the cosmetics industry, but may also valorise the food production chain, respectively (Schieber et al., 2001).

Two vegetable adsorbing matrices have been studied in a previous paper to remove the organic and phenolic compounds from OMW (Ena et al., 2009). The aim of the present study was: i) to evaluate the efficiencies of these two matrices (Azolla and Granular Activated Carbon) to adsorb and desorb polyphenols from OMW, ii) to compare those efficiencies with the ones of two commercial resins (resins are widely used for OMW phenols adsorption) and iii) to recover and characterize the powder products rich in polyphenols recovered from Azolla and GAC.

Materials

Citric acid, ethanol and all other chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA). DPPH, fluorescein, AAPH, Trolox® and polyphenols HPLC standards were purchased by Sigma-Aldrich except for Hydroxytyrosol, supplied by Phytolab (Vestenbergsgreuth, Germany). Folin-Ciocateau reagent was purchased by Merck (Darmstadt, Germany).

Olive mill wastewater.

The OMW was supplied by a continuous olive processing plant located in Bibbona in the province of Livorno, Italy. It was immediately processed with addition of Citric Acid (1g/L) and stored at -18°C.

Azolla cultivation.

The strain of *Azolla caroliniana* derives from the Botanical Institute of Naples, Italy. Since the 1980s, it has been preserved by the Institute of Ecosystem Study (ISE), Florence section, of the Italian National Research Council (CNR). The Azolla biomass was cultivated in 2 m^2 vertical tanks containing a 10 cm layer of medium (Ena et al., 2007), then harvested and dried in the sun.

Granular Activated Carbon and resins.

The GAC (AFC-LS) was provided by Carboplant s.r.l. (Vigevano, PV, Italy). The resins Lewatit OC 1064 MD PH and Lewatit MP 62 WS were purchased by Sigma-Aldrich.

Methods

Polyphenol adsorption and desorption experiments

Batch adsorption experiments were carried out in 250 mL screw cap glass flasks. Fixed amount of resins, activated carbon and Azolla (12.5, 25, 50, 100, 150 and 200 g/L) were combined with OMW and shook at 150 rpm in a rotary shaker (G10 Gyrotory Shaker New Brunwick Scientific Co., New Brunswick, NJ, USA). After 24 hours, aliquots of 1 mL were withdraw for polyphenolic analyses. The amounts of total polyphenols adsorbed by matrices were calculated by difference. ‘‘Freundlich’’ adsorption kinetics for phenolic compounds on the matrices was then studied according to Bertin et al. (2004). Batch adsorption experiments were carried out in 250 mL screw cap glass flasks using ethanol for the resins and water:ethanol 50:50 (v:v) for Azolla. The eluents were corrected at pH 3 by sulphuric acid. NaOH solution was used for desorption from carbon.

Radical - DPPH· scavenging assay

DPPH· (2,2-diphenyl-1-picrylhydrazyl) is a stable radical that can be reduced by reaction with an antiradical hydrogen-donor compound. This colorimetric reaction is measured by a spectrophotometer (Beckman DU 640) at 517 nm: the radical colour shifts from violet to yellow. The extracts were dissolved in ethanol and diluted as necessary. 1 mL of diluted extract was added to 1 mL of ethanolic DPPH· solution (6.3 10^{-5} M) and mixed. The absorbance was measured every 2 minutes, from 0 to 20 minutes. The standard absorbance was given by 1 L of solution, while the zero was given by the ethanol and 1 mL of the DPPH absorbance of pure ethanol. The experiments were carried out in triplicate. The antiradical activity was calculated with the following formula:

% inhibition = $(A_s - A_e) / A_s \times 100$

where A_s is the absorbance of the standard solution (t = 0) and A_e the absorbance of the sample (t = 20 min).

ORAC Assay (Oxygen Radical Absorbance Capacity)

The method was adapted from the one described by Cao and Prior (1998) and the instrument was a fluorescence spectrophotometer (Varian Cary Eclipse) (Palo Alto, CA, USA). The sample was added to a free-radical generator (AAPH, 2,2'-azobis(2-aminopropane) dihydrochloride) and the inhibition of the free radical was measured. Fluorescein was used as target for free radical attack. Free radicals cause conformational changes in the protein structure of fluorescein, leading to a dose- and time-dependent fluorescence quenching. The following were added to 2738 μL fluorescein (25.5 mg/L solution, maintained at 4°C), 37 μL phosphate buffer solution (75 mM, pH 7.4) and 150 μL Trolox® standard (100 μM), blank (buffer solution) or sample solution. After incubation at 37°C for 30 min, the addition of 75 μL AAPH solution (86.8 mg/ml in buffer solution and kept in ice) started the reaction. The exciting λ is 490 nm and the emission λ is 512 nm. Total antioxidant capacity or ORAC unit is given by the following formula:

ORAC unit (μM of Trolox® equivalents) = $20\ k\ (S_{sample} - S_{blank}) / (S_{Trolox®} - S_{blank})$,

with *k* the dilution factor, S_{sample} the under curve area of the sample, S_{blank} the under curve area of the blank and $S_{Trolox®}$ the under curve area of the standard.

Total phenolic compounds

The total phenolic content was determined according to Ena et al., 2009.

HPLC analysis

A reversed-phase high-performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds contained in the samples. With this purpose, standard mixed solutions of phenolic compounds was analysed. The standards tested were Caffeic Acid, Gallic Acid, Vanillic Acid, Syringic Acid, Tyrosol, Hydroxytyrosol, Pirocathecol and p-Coumaric Acid.

The analyses were performed by a ProStar/Dynamax System Liquid Chromatograph (Varian Inc., Palo Alto, CA, USA) coupled with ProStar 335 PDA DAD detector. The column was Sinergy Fusion RP 80 4u (Phenomenex-Torrance CA, USA) with 250-4.6mm (5 mm), equipped with a 10-4mm pre-column of the same phase.

Each sample was solved in water:methanol (50:50 v/v) acidified with 1% Acetic Acid, the solution was washed two times with n-Hexan, centrifuged (7000 rpm, 10') and the supernatant was filtered on 0.20 nm membranes.

The method was the Ryan et al. (2001) modified. The HPLC eluents were H_2O acidified by Acetic Acid 1% v/v (A) and Methanol:Acetonitrile (95:5 v:v) acidified by Acetic Acid 1% v/v (B). The solvent gradient started from 5% B for the first 2 minutes and increased up to 99% B in 45 minutes. The flow rate was 1.0 mL/min. The DAD detector wavelength was 280 nm. All solvents used were of HPLC grade.

Results and discussion

Olive mill wastewater with its high polyphenol (Pp) content is the major environmental problem in many Mediterranean countries but olive oil extraction represents also an agro-industrial activity of vital economic importance. The profitability of an industrial process for the adsorptive purification and concentration of phenolic compounds from OMW depends mainly on the adsorption efficiency and the recovery rates during desorption. Therefore, these two independent steps were investigated and compared separately, using the two vegetable matrices [Granular Activated Carbon (GAC) and Azolla] previously employed in an OMW depurative study (Ena et al., 2009). These matrices were compared with two synthetic commercial resins (Lewatit OC 1064 MD PH and Lewatit MP 62 WS). For all matrices tested, the dosage was varied from 12.5 to 200 g/L.

As shown in figure 1, the highest values of adsorption of phenols are reached at the highest concentrations of the resins. The adsorption percentages of the resins reach the highest values of 76-78. However, the highest adsorption efficiencies were obtained at

the lowest quantity utilized (142.2 mg/g for MP 62 WS and 113.6 mg/g for OC 1064 MDPH).

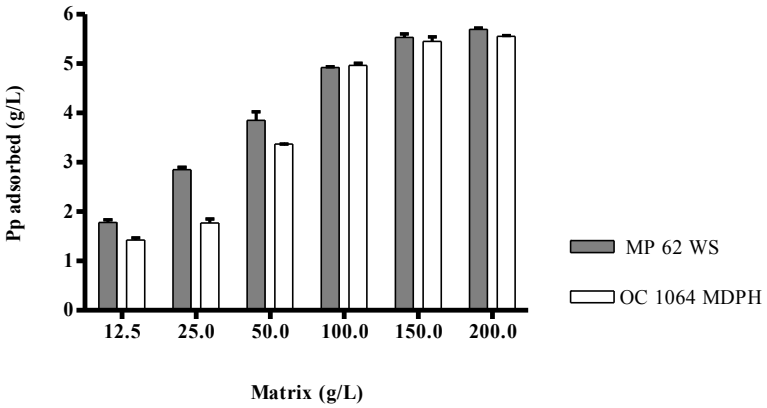


Fig. 1. Amount of phenol compounds removed by OMW as a function of resin dosage.

As shown in figure 2, the activated carbon removed the polyphenol compounds quantitatively at concentrations higher than 50 g/L, while Azolla removed the highest amount of phenol substances at the highest concentration (200 g/L). In a previous paper, it was shown that the association of Azolla with GAC in a packed column was necessary to obtain an effective biofiltration process (Ena et al., 2009). This association has the additional advantage of the much lower cost of these matrices in comparison with the synthetic resins.

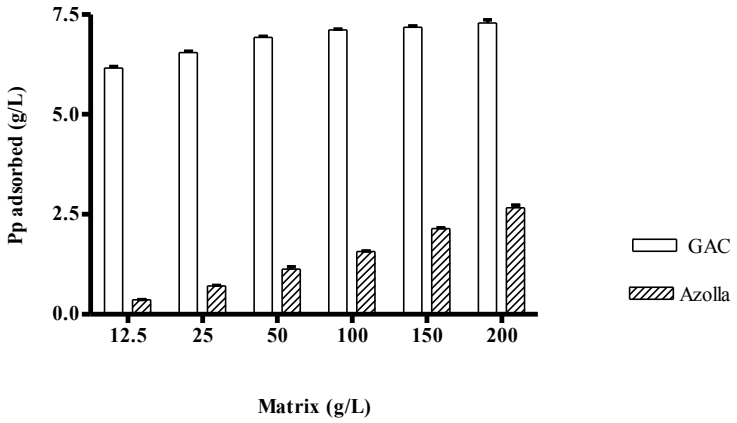


Fig. 2. Amount of phenol compounds removed by OMW as a function of Azolla and GAC dosage.

The Freundlich adsorption isotherm, $q_e = k \cdot C_e^{1/n}$ (q_e = adsorbed phenols per unit mass of solid material, mg/g; C_e = phenolic compound concentration at equilibrium, mg/L), which is the most popular adsorption model for a single solute system (Li et al., 2001), was used to represent adsorbtion of the phenolic compounds by the four tested supports.

Tab. 1. Freundlich adsorption isotherm constants for phenolic compounds.

Matrix type	Physical form	*k* (mg/g)/[(mg/l)$^{1/n}$]	*1/n*	R^2
MP 62 WS	Granular	2.84 E-03	1.261	0.9868
OC 1064 MDPH	Granular	5.32 E-02	0.862	0.8744
GAC	Granular	2.69	0.711	0.9696
Azolla	Crushed	5.42E -03	2.121	0.8921

Freundlich constants, obtained by regression analysis, are shown in table 1. The *k* value, which is an indicator of adsorption capacity, was found to be higher for the activated carbon than for the two commercial resins. Azolla showed a very little adsorption

capacity. The adsorption intensity (1/*n*) indicates the slope of the adsorption isotherm curve and its low value demonstrated that an increase in GAC or OC 1064 MDPH dose was more effective than in the case of MP 62 WS. As shown in table 1, Azolla demonstrated the higher slope of the adsorption intensity value. Relatively high R^2 values (GAC and MP62WS) indicated an acceptable correlation between adsorption isotherm data and the Freundlich equation.

Desorption studies have been carried out to elucidate the mechanism of adsorption and recovery of phenols.

Phenolic compounds have been recovered from the matrices after the adsorption experiments. Resins (figure 3) and GAC and Azolla (figure 4) have been desorbed at each tested concentration.

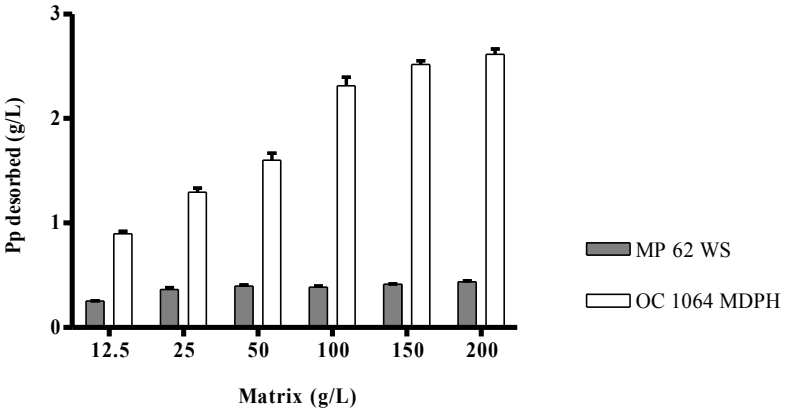


Fig. 3. Concentration (g/L) of phenolic compounds desorbed from resins at each tested concentration.

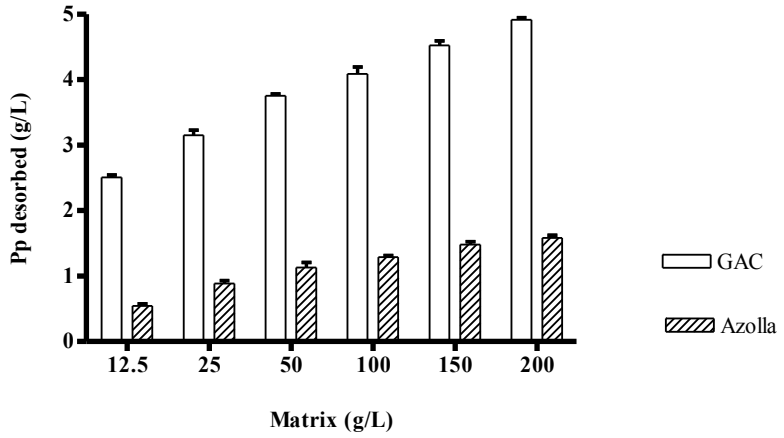


Fig. 4. Concentration (g/L) of phenolic compounds desorbed from Azolla and GAC at each tested concentration.

As shown in figures 3 and 4, the desorption of Pp from both resins carried out with NaOH resulted less effective than those obtained from activated carbon and, as a consequence, lower than the desortpion previously reported for the biofilter constituted by Azolla and GAC together (Ena et al., 2009). The GAC desorption was performed with NaOH solution, according to Ozkaya (2006). NaOH reagent was indispensable to favour the formation of sodium salt of phenols. This reaction may facilitate desorption of phenols from the carbon surfaces. Rengaraj et al. (2002) found that approximately 0.14N NaOH solution is required for quantitative desorption of phenols from activated carbon, while Ozkaya found that 0.15N NaOH solution desorbed 61% of the adsorbed phenols. In this study, 0.15N NaOH solution desorbed 68% of polyphenols from GAC surface at 200 g/L concentration. On the other hand, hydroalcoholic solution desorbed more than 82% of phenols from Azolla at 100 g/L concentration, that is the one used for the depuration treatment. As a comparison, desorption of GAC at 12.5 g/L was similar in absolute value to OC 1064 MDPH resin at 150 g/L.

Two separate powder products were obtained from desorption of Azolla and GAC and chemically characterized as shown in table 2.

Tab. 2. Chemical composition of the two powder products obtained from Azolla and GAC desorption.

Chemical Composition	AZOLLA	GAC
Total Polyphenols (%)	3.23	1.51
[Flavonoids (%)]	(0.34)	(0.00)
Carbon (%)	33.53	30.96
Total Carbohydrates (%)	16.80	20.14
Nitrogen (%)	2.03	0.61
True Protein (Lowry) (%)	12.20	3.82
Humidity (%)	5.80	5.90
Fatty Substance (%)	3.10	2.60
Ashes (%)	31.7	31.6

The powders resulted deeply different from both qualitative and quantitative points of view: table 2 shows that the polyphenolic content of the Azolla product is 2.13 times higher than the GAC one. Probably, it is due to the presence of pectin in Azolla leaves. The pectin is an important constituent of Azolla cell wall (Ena et al., 2007) made up of fragments of galacturonic acid chains. It reacts with ions and can create strong electrostatic interactions with positive charged molecules. This consideration on the chemical features of pectines seem to point out that both the matrices are necessary for the total adsorption of OMW phenols.

The presence and amount of phenolic compounds in the two products from Azolla and GAC were studied with reverse phase HPLC (figures 5 and 6).

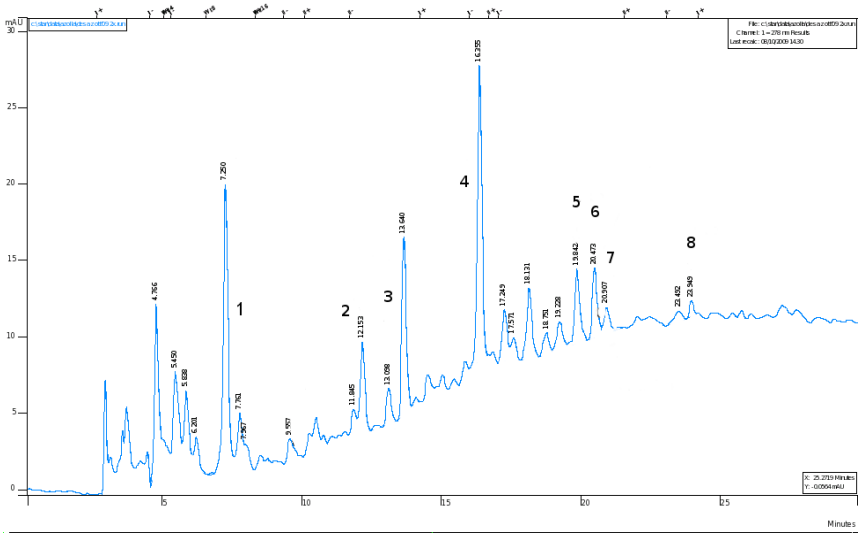


Fig. 5. HPLC profile of the Azolla product (1: Gallic Acid and derivatives; 2: Hydroxytyrosol and derivatives; 3: Pyrochatecol; 4: Tyrosol and derivatives; 5: Vanillic Acid; 6: Caffeic Acid; 7: Syringic Acid; 8: Coumaric Acid).

The HPLC profile showed in figure 5 reports several peaks corresponding to different biophenols, among which seven compounds were identified. They are part of phenyl alcohols (Hydroxytyrosol, Tyrosol) and phenyl acids (Gallic Acid, Vanillic Acid, Caffeic Acid, Siringic Acid and Coumaric Acid). As evidenced by figure 5, the most abundant components of the Azolla product were Gallic Acid and Tyrosol, whose concentration in these matrices was 14.6 mg/g and 3.86 mg/g, respectively. The concentration of Hydroxytyrosol was equal to 0.97 mg/g.

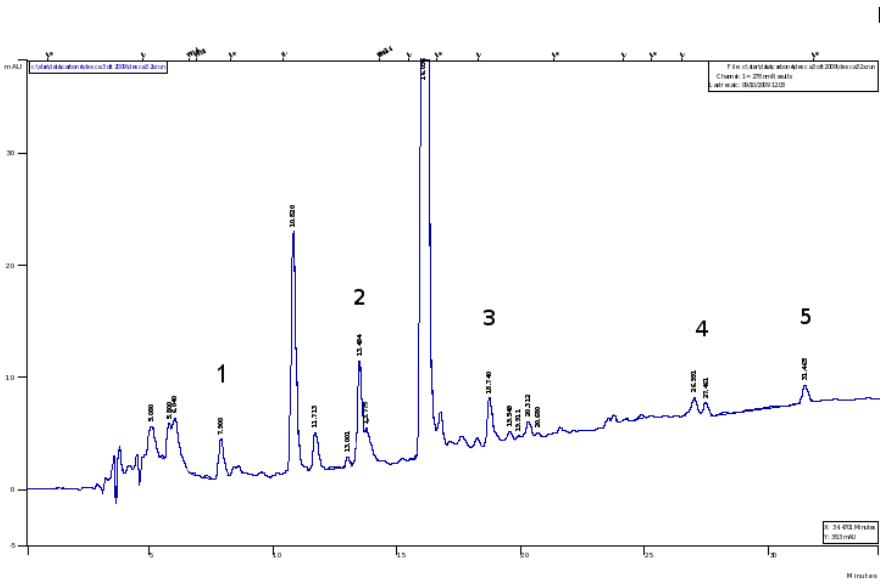


Fig. 6. HPLC profile of the GAC powder product (1: Gallic Acid; 2: Hydroxytyrosol and derivatives; 3: Tyrosol; 4: Syringic Acid; 5: Coumaric Acid).

On the other hand, biophenols identified in the GAC product were five, namely phenyl alcohols (Hydroxytyrosol, Tyrosol) and phenyl acids (Gallic Acid, Siringic Acid and Coumaric Acid). The profile of phenols recovered by GAC was dominated by two unknown peaks (figure 6) that are not present in the Azolla powder. Further studies are in progress to identify these peaks. The GAC cromatographic profile survey evidenced that Hydroxytyrosol was 3.31 mg/g, which is higher than in the Azolla product. This demonstrated that GAC possessed a high affinity towards this small molecule.

It is well known that all the quantified biophenols express antioxidant, antiatherogenic and anti-inflammatory activity. According to Ranalli (2003), polyphenolic compounds are classifiable into four groups related with their antioxidizin potencies: (i) phenols endowed with high antioxidant potency, (ii) phenols endowed with medium antioxidant potency, (iii) phenols endowed with weak antioxidant potency, and (iv) phenols having no antioxidant potency. Hydroxytyrosol is located in the first group while Tyrosol belongs to the fourth group.

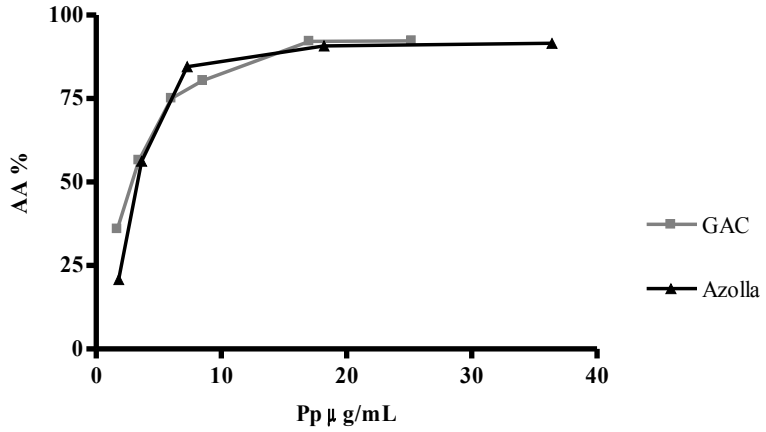


Fig. 7. Antiradical activity (AA) percentage determined at different phenol concentrations for both Azolla and GAC powder products.

Both Azolla and GAC extract products appeared to have antiradical activity determined with the DPPH test. As shown in figure 7, they showed an increasing radical inhibition over 80% at high concentrations. At concentrations higher than 10 μg/mL, the correlation between Pp concentration and antiradical activity resulted almost constant and the IC_{50} were recovered. The IC_{50} represents the tested compound concentration able to inhibit of 50% the DPPH radical activity. In table 3, the total Pp % in the powders and the IC_{50} values are shown, expressed as both mg/mL of crude powder and μg/mL of corresponding Pp content, for Azolla and GAC products.

Tab. 3. Total Pp in the two powder (%) and IC_{50} values expressed as mg/mL of crude powders and as μg/mL of corresponding Pp, in Azolla and GAC products.

Product	Total Pp %	IC_{50} (mg powder/mL)	IC_{50} (μg Pp/mL)
Azolla powder	3.23	102.22	3.27
GAC powder	1.51	198.88	2.80

As reported in table 3, the total Pp concentration is higher in the Azolla product than in the GAC one (about 2 times). This difference is confirmed by the IC_{50} expressed as mg powder/mL: a double amount of GAC product is necessary to obtain the same

antiradical activity of the Azolla powder (50%, as defined by the IC_{50}). However, the GAC IC_{50} concentration, expressed as μg Pp/mL, is not the same of Azolla as expected but rather lower. The lowest phenol concentration of GAC can be due to its qualitative Pp composition, as Hydroxytyrosol is more concentrated in the GAC product than in the Azolla one. The antioxidant capacities have been also evaluated using the ORAC test, the results are shown in figures 8 and 9.

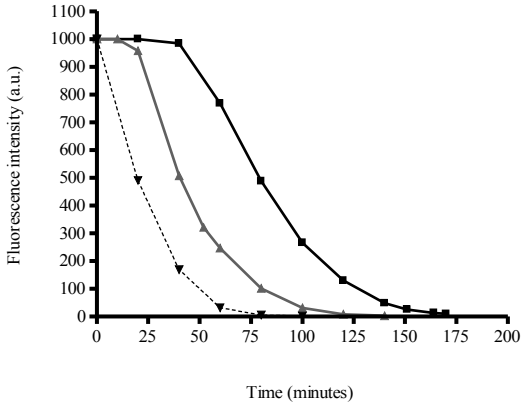


Fig. 8. Decreasing of fluorescence intensity during time of Azolla product (black continuous line), standard compound Trolox® (grey line) and blank solution (black dotted line).

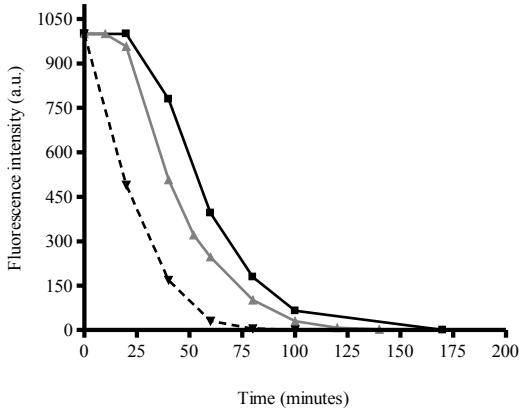


Fig. 9. Decreasing of fluorescence intensity during time of GAC product (black continuous line), standard compound Trolox® (grey line) and blank solution (black dotted line).

The Azolla product ORAC capacity was 4097 μmol TE/g powder while the GAC capacity was 1277 μmol TE/g powder. However, if considered the corresponding phenols concentration, Azolla showed ORAC capacity of 51866 μmol TE/g Pp while GAC ORAC capacity resulted 40800 μmol TE/g Pp. Even if the ORAC capacity was a little higher in Azolla than GAC, such a value was not 2 times higher than GAC one.
ORAC unit is expressed as mmol TE/100 g product and the daily antioxidant dose in human diet suggested by the National Research Council (USA) is estimated equal or more than 5000 ORAC units. Thus, less than 500 mg of both powders would be enough to cover the optimal antioxidant dose.

Conclusions

OMW represents an interesting source of biophenols with a wide range of biological activities. The recovery of important phenols is desirable, especially considering that the economical interest of these compounds could support the costs of the disposal of a highly pollutant waste such as olive mill wastewater. The biofilters Azolla and GAC resulted good matrices both for the adsorption and desorption of OMW phenolic compounds. Moreover, their desorption products showed different phenolic composition and high

CHAPTER 5

PHOTOFERMENTATIVE BIOGAS PRODUCTION FROM PRETREATED OLIVE MILL WASTEWATER USING TWO DIFFERENT ADSORPTION VEGETABLE MATRICES

Authors: Alba Ena, Cristina Pintucci, Alberto Scoma, Roberto De Philippis, Pietro Carlozzi; Paper submitted to Current Topics Biotechnology, 2009.

Abstract

In this study, we report results on some pretreatments of olive mill wastewater using one or two different vegetable matrices, such as dry-Azolla and active carbon, followed by *Rhodopseudomonas palustris* growth and biogas (composed about at 85% by hydrogen and at 15% by CO_2) photoevolution. When a dry-Azolla matrix was used alone in order to pretreat oil mill wastewater, the initial chemical oxygen demand and the polyphenol content of the oil mill wastewater were significantly reduced. Nevertheless, no growth of bacteria was observed when using a culture broth containing >10% of pretreated oil mill wastewater with only the dry-Azolla. When both vegetable matrices were used for the pretreatment of the said oil mill wastewater, 80% of the chemical oxygen demand and 99% of the polyphenol content were removed. The black-brownish colour of the oil mill wastewater faded, and the colour intensity decreased from 24.4 to 1.4. This pretreated oil mill wastewater was then used to grow *Rhodopseudomonas palustris*. The culture broth for the biohydrogen photoevolution consisted of 25% pretreated oil mill wastewater and 75% distilled water. The experiments for biohydrogen photoevolution were carried out using two different photobioreactor geometries (cylindrical and flat). The experiments were carried out under batch growth conditions and at a culture age of 406 h. 600 ml of biogas were produced by the cylindrical photobioreactor and 335 ml by the flat photobioreactor, corresponding, respectively, to 1.12 L_{biogas}/L_{OMW} and 0.624 L_{biogas}/L_{OMW}.

Introduction

Global warming and climatic change have been creating more and more apprehension at the beginning of this third millennium. Interest in renewable and clean energies such as hydrogen has increased because of the high level of polluting emissions, increasing costs associated with petroleum, and the escalating problems of global climate change (Carlozzi and Lambardi, 2009). Methods for the production, uses, and comparative analyses of different biological processes for hydrogen production have been studied (Das and Verziroglu, 2001). A recent study on the state of the art of hydrogen production by means of non-sulphur photosynthetic bacteria was carried out by (Basak and Das, 2007). Purple non-sulphur photosynthetic bacteria can photo-assimilate a wide variety of organic compounds, and their presence in a body of water is particularly

dependent upon the extent to which the said body is polluted with organic matter (Sunita and Mitra, 1993). Phototrophic bacteria have been indicated as the most promising microbial system for the biological production of hydrogen (Fascetti et al., 1998). Purple photosynthetic bacteria produce H_2 from organic compounds by means of an anaerobic, light-dependent electron transfer process in which nitrogenase functions as the terminal catalyst (Hillmer and Gest, 1977). The production of clean energy sources and the re-utilization of waste materials make photobiological hydrogen production a novel and promising approach for meeting increasing energy needs as a replacement for fossil fuels (Kargy and Kapdan, 2005). Although photobiological hydrogen production alone cannot furnish the world's requirements as regards clean renewable energy, it is desirable that photobiological hydrogen technology will in future play an important role, in view of the fact that photobioreactors for bio-hydrogen production can be positioned in fringe areas without competing with agricultural lands (Carlozzi and Lambardi, 2009). When hydrogen photo-production is achieved by means of a photo-fermentative process, such as the photo-metabolism used by purple non-sulphur bacteria, we need to add one or more organic substances to the culture medium (Carlozzi, 2009). To offset the addition of organic substances (e.g. low MW fatty acids) to the synthetic medium and to limit the high cost of bio-hydrogen production, some different waste materials have been used: distillery waste (Wolmarans and de Villiers, 2002); wheat starch (apdan et al., 2009); milk (Turkarslan et al., 1998); sugar (Vincenzini et al., 1981; Yetis et al., 2000); tofu wastewater (Zhu et al., 2002), and oil mill wastewater (OMW) (Eroglu et al., 2004). As regards this last item, a Turkish group has carried out many studies on it during this new (Eroglu et al., 2006, 2008a, 2008b, 2009). OMW coming from the manufacture of olive oil is one of the major pollutants in Mediterranean countries (Ramos-Cormenzana et al., 1995) and its antibacterial and phytotoxic effects are mainly due to phenolic compounds (Capasso et al., 1995). OMW contains high amounts of organic matter (30-200 Kg COD/m^3), which is considered insufficiently degradable (Lopez, 1992). The toxicity of OMW can also be attributed to its high content of phenolic compounds in a wide range of molecular weights (MW), from low-MW phenols to complex high-MW phenolic compounds (Montedoro et al., 1992). Moreover, the colour of OMW depends on the ratio of the two groups of polyphenols (Borja et al., 2006). The amount of

phenolic compounds contained in OMW ranges from a minimum of 200 mg/L to a maximum of 8000 mg/L (Azbar et al., 2004).

Aquatic fern Azolla biomass has already been used in the adsorption of a wide range of heavy metals (Cohen-Shoel et al., 2002) and, more recently, polyphenols (Ena et al., 2007). Moreover, activated carbon is the most popular adsorbent, and has been used with great success in treating dye wastewater, which contains large amounts of suspended solids, high chemical oxygen demand (COD) concentration, and greatly fluctuating pH (Kadirvelu et al., 2000).

The main aim of this study was to set up a system for converting by-products of pretreated OMW in hydrogen, after removing the bioactive products (polyphenols) with the use of two different vegetable matrices (dry-Azolla and activated carbon).

Materials and Methods

Assay.

To determine the colouration of OMW, three samples were collected for each wastewater source. These were centrifuged (20 min at 4000 rpm) and diluted 1:50 (v:v), and the absorbance was recorded at 395 nm (Yesilada et al., 1999). The COD concentration in the OMW was determined with a HANNA C99 Multiparameter Bench Photometer for laboratories. A Folin-Ciocalteau reagent was used to determine the polyphenols (Pp), and their total contents were estimated spectrophotometrically at 730 nm, using Gallic Acid as a standard. The adsorption process with the aquatic fern Azolla matrix has been described previously (Ena et al., 2007), while the active carbon alone or combined with dry-Azolla has been described by Ena et al. (2009). A complete adsorption process of both Pp and COD has also been reported (Ena et al., 2006).

Analytical methods.

Bacteriochlorophyll (Bchl) and dry-biomass were determined according to Carlozzi et al. (2006). The light intensity was measured using a Quantum/Radiometer/Photometer (model LI-185B, LI-COR, Lincoln, Nebraska, USA). A HPLC (Thermo Finnigan – Spectra System 6000 LP) was utilised in order to determine organic-acid concentrations in the culture broth. The HPLC was equipped with a C18 analytical column, 250 mm x 4.6 mm (I.D.). (Beckman ODS), and the column temperature was 25 °C. The mobile

phase was a solution of water + 0.1% H_3PO_4, and the flow was 1.0 mL/min. The biogas composition (trapped in a calibrated column) was determined by means of a gas chromatograph (Clarus 500 model, Perkin Elmer), using a packed column: the Carbosieve S-II Spherical, Supelco model. Hydrogen was determined by using nitrogen as a carrier gas; the other gases were determined by using helium as a carrier gas. Known amounts of pure gases were used for calibrating the instrument.

Microorganism and culture conditions.

A strain of *Rhodopseudomonas palustris* (6A) was first isolated from soil spread with OMW, and was then grown in order to investigate biogas photo-evolution by pretreated OMW. Two medium were used as controls: (i) for only the growth of bacteria (without biogas photoevolution), we used a medium containing NH_4Cl (Carlozzi et al., 2006) (ii) for both the growth of bacteria and biogas photoevolution, we used a medium containing DL-malic acid and Na-glutamate (Vincenzini et al., 1985). Before growing bacteria, the OMW samples were opportunely diluted with distilled water. The culture temperature was kept constantly at 30 ± 0.2 °C. The initial pH of the medium was 6.8. All experiments were carried out under continuous light.

Cultural systems

To test the ability of *Rhodopseudomonas palustris* for biogas photoevolution from pretreated OMW, two different cultural systems were used: (i) a cylindrical glass photobioreactor (C_GPBR) with a working volume = 1.07 L, which has previously been described by Carlozzi and Lambardi (2009), and (ii) a flat glass photobioreactor (F_GPBR) with a working volume = 1.08 L. Both photobioreactors were placed in a heat exchanger-Plexiglas water bath at a constant temperature, and the cultures were mixed using magnetic stirrers. Two needles were inserted into each silicone stopper: the first was used to withdraw culture samples periodically from the reactor, and the second acted as a gas outlet before the gas was trapped in a graduated glass column.

Oil mill wastewater properties.

Fresh OMW collected from two different olive-oil mills, a traditional system (TS) and a continuous system (CS) were investigated. The main OMW properties measured after centrifugation were the following: pH = 4.5 – 5.1; COD = 44400 - 92000 mg/L; Pp =

4367 – 7360 mg/L (Ena et al., 2007). Other details of OMW have been reported by Ena et al. (2009).

Results

A new, low-cost strategy for OMW bioremediation and polyphenol recovery was set up in 2006 (Italian patent n. FI2006A000155). Pretreated OMW, suitably diluted, were tested for the biomass production of a purple non-sulphur photosynthetic bacterial strain (*Rp. palustris*, 6A) that was previously adapted to grow in culture broth containing OMW. Figure 1 shows the growth of *Rp. palustris* 6A, express as Bchl, in four different culture broths containing 5%, 10%, 15%, and 20% OMW (pretreated with Azolla) and, respectively, 95%, 90%, 85% and 80% distilled water.

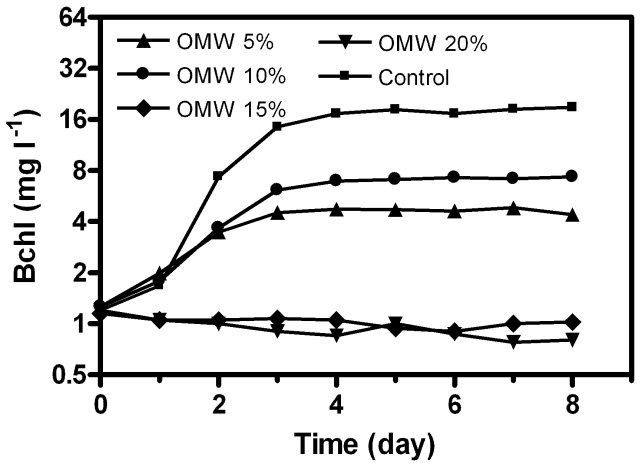


Fig. 1. Time course of Bchl content of *Rp. palustris* cultures growing in van Niel's medium (control) and in culture broths containing different percentages of OMW pretreated with a dry-Azolla matrix.

A modified van Niel's medium (Carlozzi et al., 2006) was used for the control. No bacterial growth was observed under these conditions (data not shown), but growth was noted when ammonium chloride (0.5 g/L) was added to the culture broths, with the exception of those containing more than 10% OMW (figure 1).

Table 1 shows the abatement of both COD and Polyphenol (Pp) concentrations by *Rp. palustris* grown in culture broths containing various amount of diluted OMW pretreated with Azolla matrix. No further tests for hydrogen photoproduction were carried out using the OMW pretreated with Azolla matrix as the OMW dilution ratio (10% with distilled water) adopted when supplying it to *Rp. palustris* was considered too high for a future scaling up of this technology.

Tab. 1. Abatement of both COD and Pp concentrations by *Rp. palustris* grown on diluted OMW pre-treated with Azolla matrix. NH_4Cl (0.5 g/L) was added to the culture broths.

Culture broths	COD		Pp	
	COD_0 (mg/L)	COD_f (mg/L)	Pp_0 (mg/L)	Pp_f (mg/L)
5 %	2805	1150	210	180
10 %	5610	3300	420	365
15 %	8415	8400	630	nd
20 %	11280	11300	845	nd

COD_0 and Pp_0 indicate the initial concentrations

COD_f and Pp_f indicate the final concentrations

nd, not determined

On the other hand, a dilution rate lower than 10% was possible by using two adsorbent matrices (dry-Azolla and activated carbon) for the pretreatment of OMW. The adsorption effects of the pre-treatment processes are shown in table 2.

Tab. 2. The adsorption effect of both vegetable matrices, on the pre-centrifuged OMW

Adsorption matters	COD_0 (mg/L)	COD_f (mg/L)	COD Adsorption efficiency (%)	Pp_0 (mg/L)	Pp_f (mg/L)	Pp Adsorption efficiency (%)
Centrifugation	-	52,500	39	4,490	4,005	10.8
Dry-Azolla[a]	52,500	41,475	21	4,005	3,116	22.2
Dry-Azolla[b]	52,500	38,850	26	4,005	2,555	36.2
Active carbon[1]	52,500	27,300	48	4,005	1,157	71.1
Active carbon[2]	52,500	15,225	71	4,005	461	88.5
Dry-Azolla[a] + Active carbon[1]	52,500	22,575	57	4,005	785	80.4
Dry-Azolla[b] + Active carbon[2]	52,500	10,237	80.5	4,005	40	99.0

Dry-Azolla: [(a)] 50 g/L; [(b)] 100 g/L

Active carbon: [(1)] 100 g/L; [(2)] 200 g/L

The removal efficiency of the COD was high, especially when both dry-Azolla and activated carbon were used. The adsorption efficiency was, respectively, 80% for the COD and 99% for the Pp. This OMW was tested for biogas photoproduction with *Rp. palustris* 6A, using two different photobioreactor-geometries (C_GPBR and F_GPBR).

Figure 2 shows the results of both Bchl synthesis and cumulative biogas achieved with an OMW:water ratio of 1:3 (v:v), which corresponded to a 25% OMW dilution. The experiment was carried out under batch growth conditions. The Bchl concentration increased rapidly in both reactors during the first 48 h, then it increased more in the C_GPBR than in the F_GPBR (figure 2a). At a culture age of 406 h, 600 mL of biogas were produced from the C_GPBR and 335 mL in the F_GPBR. These values corresponded, respectively, to 1.12 L_{biogas}/L undiluted OMW and 0.624 L_{biogas}/L undiluted OMW (figure 2b)

Figure 3 shows the biogas production rate [µL/mg(Bchl)/h] as a function of the culture age. The highest rates were achieved in both photobioreactors at a culture age of 44 h,

then the rates progressively decreased. At first, the biogas production rate was higher in the F_GPBR than in the C_GPBR; but when the culture age increased (≥ 70 h), the rate decreased more in the F_GPBR than in the C_GPBR.

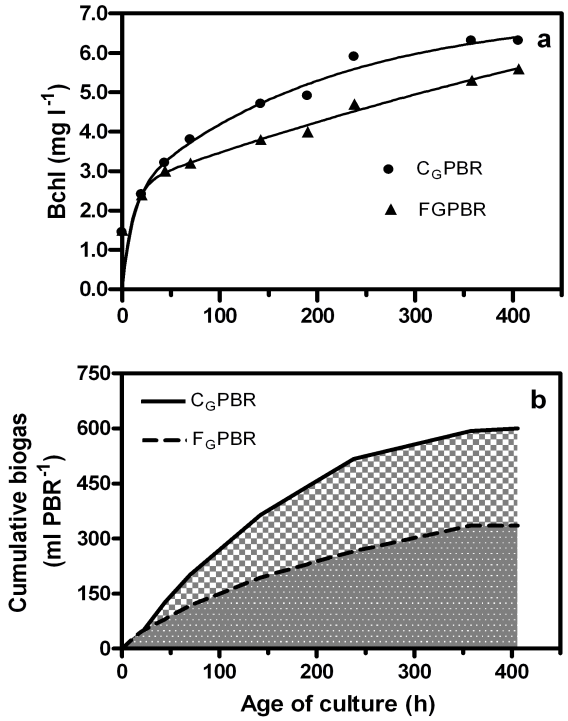


Fig. 2. Time course of (a) Bchl concentration and (b) cumulative biogas production versus the culture age of *Rp. palustris*. The investigation was carried out in two different photobioreactors (C_GPBR and F_GPBR). For these experiments, we used 25% water diluted OMW, pretreated with two adsorbent matrices (dry-Azolla and active carbon).

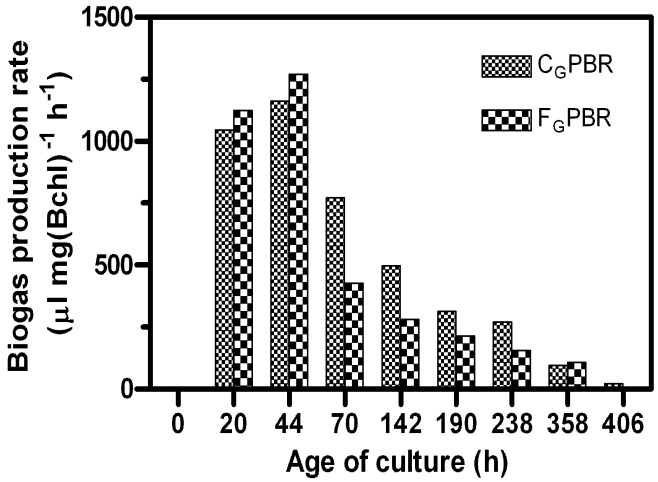


Fig. 3. Biogas production rate versus the culture age of *Rp. palustris*. The investigation was carried out in two different photobioreactors (C_GPBR and F_GPBR). The experimental conditions were the same as those reported in figure 2.

Figure 4 shows the time course of organic acids consumption observed in the OMW culture broths used for both the bacterial growth and the biogas photoevolution. All acids were consumed progressively; however, in both photobioreactors bacterial cells consumed succinic and malic acids at a higher rate than acetic and tartaric ones. At the end of the biogas photoevolution, tartaric acid was the only acid still found in the culture broth. The dry biomass yield at the end of bacterial growth was slightly higher in the C_GPBR (0.65 g/L) than in the F_GPBR (0.61 g/L).

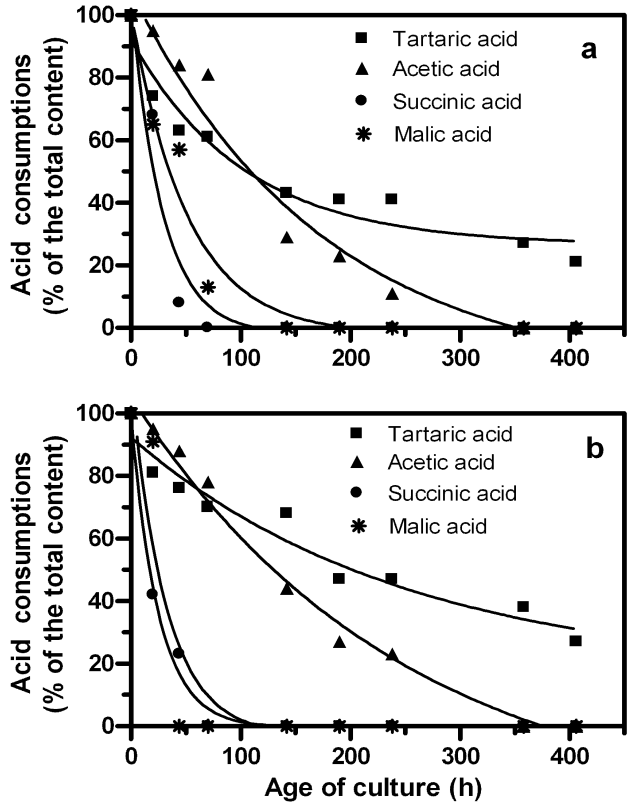


Fig. 4. Acid consumption versus the culture age in *Rp. Palustris* growing in: (a) C_GPBR; (b) F_GPBR. The experimental conditions were the same as those reported in figure 2.

Table 3 summarizes the results obtained using two different photobioreactors, characterized by cylindrical (C_GPBR) and flat geometry (F_GPBR). Both of them were operated under the same light conditions (200 μE/m/s).

Tab. 3. Photofermentation of pretreated OMW by *Rp. palustris* grown in two different photobioreactors (C_GPBR and F_GPBR). The results are compared with those obtained using a synthetic medium (runs 3 and 4). R_E = removal efficiency (%).

Run	Reactors	Time (h)	Productivities		COD_0 (mg/L)	COD_f (mg/L)	R_E (%)
			Biogas (mL/PBR)	Dry-biomass (g/PBR)			
1	F_GPBR	406	335	0.47	2,740	1,148	58.1
2	C_GPBR	406	600	0.49	2,740	1,162	57.6
3	F_GPBR	188	904	1.18	5,065	1,065	79.0
4	C_GPBR	188	230	2.12	5,065	635	87.5

Biogas produced by *Rp. palustris* and stored in the traps contained 88.7 ± 4.1 % of H_2 and 11.3 ± 4.2 % of CO_2 (data expressed as mean ± SE).

To evaluate the efficiency of the system using a culture broth containing 25% pretreated OMW and 75% distilled water, a comparative study was carried out using a synthetic medium containing DL-malic acid and Na-glutamate (Vincenzini et al., 1985). The experiments were performed using both reactors (C_GPBR and F_GPBR), and the results are shown in figure 5. The Bchl concentration was always higher in the C_GPBR than in the F_GPBR, and at the end of the experiment reached 26 mg/L and 21 mg/L.

The initial chemical oxygen demand (COD_0) was 5065 mg/L. At a culture age of 188 h, the biogas photoproduction came to a halt, and the final chemical oxygen demand (COD_f) was 635 mg/L in the C_GPBR and 1065 mg/L in the F_GPBR. The total amount of biogas produced was very low (230 mL/PBR) in the C_GPBR, and increased to 904.3 mL/PBR in the F_GPBR. Since the lag phase before the biogas photoevolution was 45 h, the average biogas photoevolution rate was 5.98 mL_{biogas}/L/h in the F_GPBR, and decreased to 1.5 mL_{biogas}/L/h in the C_GPBR. On the contrary, the average dry-biomass productivity was 5.91 mg(dw)/L/h in the F_GPBR, and increased to 9.94 mg(dw)/L/h in the C_GPBR.

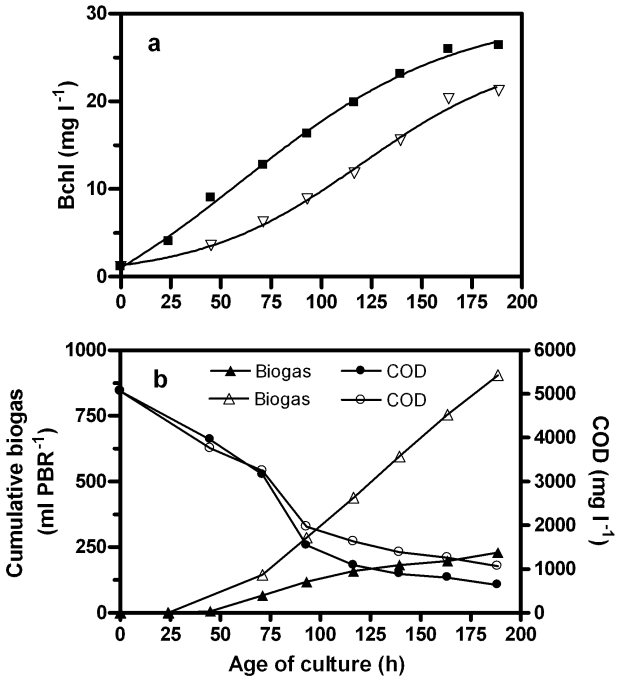


Fig. 5. (a) Bchl synthesis versus the culture age of *R. palustris*; (b) cumulative biogas. Cultures were grown in the synthetic medium (Vincenzini et al., 1985). The investigation was carried out in two different photobioreactors: C_GPBR (filled symbols) and F_GPBR (empty symbols).

Discussion.

The main characteristics of the OMW collected from the traditional and continuous extraction systems (TS and CS, respectively) have been reported by Ena et al., 2007. The high adsorption efficiency in both COD_0 and Pp_0 using two vegetable matrices provided us with the possibility of diluting the pretreated OMW with water (25%), which corresponded to the OMW-to-water ratio of 1:3 (v:v) for both the growth of bacteria and the biogas photoevolution. In 2004, using diluted OMW (4%, v:v), Eroglu et al. (2004) carried out an interesting study growing *R. sphaeroides* for the photoproduction of biohydrogen. In our study, *Rp. palustris* was grown in a broth containing OMW 6.2 times more concentrated than the findings in Eroglu et al., (2004). After 406 h, 600 ml and 335 mL of biogas were produced by *Rp. palustris* grown in the C_GPBR and in the F_GPBR, respectively. These results corresponded, respectively, to 1.12

L_{biogas} /L untreated OMW and 0.624 L_{biogas} /L untreated OMW (figure 2b). Although the biogas productivities obtained in this study seem to be very low if compared to those reported by Eroglu et al. (2004) (10.1 L/L_{biogas} /L untreated OMW, at OMW dilution of 4%), it has to be stressed that in the present study the dilution ratio was much lower in comparison with the one used by Eroglu et al., (2004). The technological significance of this aspect is relevant, particularly considering future outdoor biotechnological applications: e.g. for treating 1,000 L of OMW for biogas photoproduction, a volume of 4,000 L of culture broth containing 25% OMW inside the photobioreactor is needed; on the other side, to treat the same volume (1,000 L of OMW) by using Eroğlu's dilution (4%, v:v), the overall culture broth to be used inside the photobioreactor would be 25,000 L. When OMW has a relatively low initial organic load (e.g. COD_0 = 52,500 mg/L and Pp_0 = 4,005 mg/L), both COD_0 and Pp_0 decrease, respectively, to 10,237 mg/L and 80.1 mg/L, after the adsorption process (Ena et al., 2009). As the Pp_f content is very low, for biogas photoproduction it should be possible to use the pretreated OMW (Ena et al., 2009) with an OMW-to-water ratio of 1:1 v:v, which corresponds to a dilution of 50%. Consequently, the starting culture broth containing 50% of pretreated OMW should have COD_0 = 5,118 mg/L and Pp_0 = 40.05 mg/L.

The characteristic black-brownish colour of untreated OMW is due to slowly biodegradable compounds, such as polyphenols, which are difficult to remove. Hence, an important step in the decolourization of the olive-oil wastewater is the breakdown of coloured polymeric phenolics into monomers (Eroglu et al., 2009). The acidification (pH 3.5) of OMW used before the pre-treatment process caused the breakdown of Oleuropein into a monomer such as OH-tyrosol (Capasso et al., 1997), which is more easily adsorbed by both vegetable matrices during the filtration process (data not shown).

When a synthetic medium (containing DL-Malic Acid and Na-Glutamate) was used to produce biogas, the removal efficiency of COD_0 was 87.5% in the C_GPBR, and decreased to 79% in the F_GPBR. The total biogas produced during 188 h of cultivation was about four times higher in the F_GPBR than in the C_GPBR. On the contrary, the dry-biomass yield obtained after 188 h of growth was lower (1.32 g/L) in the F_GPBR respect to the C_GPBR. This was in agreement with results of our previous studies (Carlozzi et

al., 2008; Carlozzi and Lambardi, 2009). Hydrogen photo-evolution rate was significantly affected by the diameter of the photobioreactors: the larger the diameter of the photobioreactor the lower the hydrogen production rate (Carlozzi et al., 2008). The lower amount of hydrogen produced in the C_GPBR can be attributed to a very high photolimitation, due to an average culture thickness about two times larger than the F_GPBR's one. In support of this hypothesis, El-Shishtawy and co-workers obtained the highest hydrogen production rate at the lowest culture thickness, but the rate decreased when the thickness of the culture increased (El-Shishtawy et al., 1997).

When *Rp. palustris* 6A was grown using a culture broth containing 25% pretreated OMW, in both photobioreactors very low Bchl concentrations were attained (5.6 mg/L in F_GPBR and 6.3 mg/L in C_GPBR) after a 406 h period. On the contrary, when supplying a synthetic medium, a 4 times higher Bchl concentration was achieved in a shorter period of time (188 h). It is not clear why Bchl did not reach a concentration higher than 6.3 mg/L in the photobioreactors containing pretreated OMW. Most likely, growth was limited by a lack of nitrogen since nitrate concentration in the original untreated OMW was between 3.4 and 10.9 mg/L (Borja et al., 2006; Ena et al., 2009). Further experiments are in progress in order to better understand this unclear behaviour.

In conclusion, more efforts are necessary to set up a system for hydrogen production using a 50% (v:v) pretreated OMW-to-water ratio. Since the annual production of OMW worldwide is estimated to be more than 30 million m^3/year (Ntaikou et al., 2009), finding the best OMW-to-water ratio (e.g. 1:1, v:v) for biogas production could enormously reduce the total volume that has to be treated in order to recover bioenergy from OMW. In addition, the production costs would thus be cut down. Moreover, since the decolourization of OMW is easy to attain, as shown in table 4, a proposal for the management of pretreated OMW diluted more than 25% by using the process reported by Ena et al., (2009), could possibly be a system for the production of biogas by means of the photofermentative process.

Tab. 4. Colour intensity of different oil mill wastewater first, and then the adsorption process with only dry-Azolla and also dry-Azolla + active carbon.

OMW	Colour intensity					
	Centrifugated OMW (-)	Dry-Azolla		Centrifugated OMW (-)	Dry-Azolla + Active carbon	
		After adsorption (-)	B_E (%)		After adsorption (-)	B_E (%)
TS [a]	21.4	11.77	45	24.4	1.39	94
CS [b]	15.7*	7.54*	52*	13.6	1.70	87

(TS [a]) - fresh OMW collected from a traditional system

(CS [b]) - fresh OMW collected from a continuous system

(B_E) - Bleaching efficiency

(*) - Ena et al. 2007

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CHAPTER 6

WORK IN PROGRESS

Olive mill wastewater biofiltration

A 500 litres pilot plant have been designed and built for the semi-continuous depuration of OMW (fig. 1). Such a plant is able to process one tonn OMW each day as it can operate two cycles of depuration per day.

The pilot plant was first placed at the Institute of the Ecosystem Study (ISE) of the National Research Council (CNR), unit of Florence, on an outside area, and then moved at the mill factory Antico Frantoio Toscano (Bibbona, Livorno, Italy) which collaborates with our research group.

The plant system consisted in three stainless steel operating units: a main structure where the depuration takes place and two tanks where the OMW is placed before and after the treatment. The packing pressure of the matrices, the pH and COD of the treated OMW are controlled by an electronic panel, while OMW flow through the filtration tank is regulated by two adjustable pumps.

Fig. 1. 500 litres pilot plant for OMW semi-continuous process: OMW collecting tank (1); biofiltrating main structure (2); treated OMW collecting tank.

The pressure needed to pack GAC and Azolla is a relevant step of the process. As the biofiltration is conducted in reversed current, the matrices should be pressed neither excessively nor weakly: in the former case, the incoming OMW can end up lifting the

biomass or block the process itself, while in the latter the absorbent capacity would be obviously reduced. Concerning this, the optimal pressure to be applied resulted to be 250 kg/cm^2. In order to easily handle the matrices, Azolla and GAC were placed in separated and removable structures inside the main filtrating unit and a movable electric hoist was used to handle them. Picture 2 shows the OMW before and after the biofiltration process.

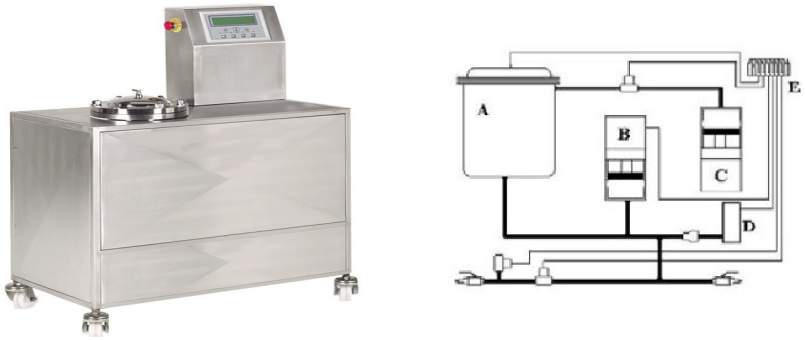
Fig. 2. Picture of OMW before and after the biofiltration.

The 500 litres pilot plant here shown represents a first fundamental step towards a further upscale to industrial applications. Indeed, considering the feasible application of the proposed biofiltration system, it was necessary to test whether lab-scale results were obtainable also upscaling the system to higher volumes (namely, 1000 times greater). In this respect, it was surprising to note that, while obtaining comparable results, the problems usually arising when these kind of systems are up-scaled (e.g., the contact time between the OMW and the matrices, the pressure of Azolla and other parameters depending by the pollutant load of the waste) were solved.

Recovery of the phenolic fraction from olive mill wastewater

The matrices used in the pilot plant experiments were collected and desorbed. A pilot system had been adjusted for an exhaustive exploitation and recovery of the phenolic fraction. This system couples the extraction of the substances from the matrices with their concentration. A solid-liquid extractor (Timatic Mini 5, by Tecnolab, Spello, PG,

Italy) was used for a semi-automatic extraction of the active compounds from absorbed GAC (figure 3).



1 2

Fig. 3. Semi-automatic extraction device (1) and general scheme of its principle of function (2): A, extraction chamber; B, active pressure piston; C, secondary pressure piston; D, pump; E, pneumatic valve.

The Timatic is a solid-liquid extractor of the kind used for the industrial production of herbal spirits. According to the manufacturer, the device simulates the effects of a percolation. This is achieved by a dynamic phase within which a pre-set pressure is being generated.

The yields obtained from the Timatic extraction were higher than those achieved within laboratory experiments: thanks to the automation of the extraction, only a little handle was necessary for the treatment of the matrix. This provided a major protection by air contact and the eventual oxidation of the phenolic compounds present in the extract. As observed in laboratory experiments, the extraction eluent was ethanol acidified by sulphuric acid. Afterwards, the ethanolic extract was concentrated by evaporation of the solvent. A solvent reclaimer (type IST N-DIGIT, Italia Sistemi Tecnologici, MO, Italy) was used for the recover of the solvent and the concentration of the extract. The extracts resulting from the complete automatic process (extraction and concentration) had a total phenolic content that was more than two times higher than the manual one. Moreover, while the laboratory extraction needed at least 3 days to take place, the automatic system took about 8 hours.

Comparing the automatic extraction process with the laboratory one, some relevant differences were obtained. In particular, the concentration of Hydroxytyrosol in the automatic process resulted more than 5 times higher than in the laboratory extract. Thus, such result showed that an optimization of phenols recovery was effectively reached and, interestingly, they concerned one of phenol compounds that arises a wide commercial interest.

CONCLUSIONS

The huge amount of studies for innovative OMW disposal face the increasing problem of its pollutant effects. The present research aimed at entirely treating, exploiting and valorising the olive mill wastewater, known to be one of the most pollutant waste in the Mediterranean basin. A complete treatment cycle is here proposed, where the OMW are totally exploited from a biotechnological point of view. The purpose of the treatment studied in this research was to depurate the olive mill wastewater and, in the meantime, to recover economically valuable natural compounds as polyphenols and use the depurated wastewater for the production of “clean” energy. The resulting products could be used in different fields. These current studies with their scaled systems represent a key effort for a concrete approach towards a complete olive mill wastewater disposal, from laboratory to industrial scale.

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CHAPTER 1 AND CHAPTER 2

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An eco-compatible process for the depuration of wastewater from olive mill industry

A. Ena, C. Pintucci, C. Faraloni and G. Torzillo

ABSTRACT

Olive mill wastewater (OMW) is the by-product of olive oil industrial production. It is characterized by a dark brownish color and a strong odor and is considered one of the most polluted agricultural wastes. In this paper we briefly describe an innovative procedure for the depuration of olive mill wastewater. With this procedure it is also possible to recover valuable substances such as phenolic compounds which have important commercial applications: they can be used in the prevention of cardiovascular disease and as antiviral, antioxidant and antitumor agents. The proposed OMW treatment uses two different packed vegetable matrices which remove most of the pollutant substances by absorption. After filtration of OMW on the matrices the pollutant load of the waste is greatly reduced: the organic content (COD) is reduced more than 80% and the phenol compounds are completely removed.

Key words | biofiltration treatment, olive mill wastewater, phenol recovery, phenol removal

A. Ena (corresponding author)
C. Pintucci
C. Faraloni
G. Torzillo
National Research Council (CNR)—Institute of Ecosystem Study (ISE),
Florence section Via Madonna del Piano 10, 50019 Sesto Fiorentino (FI),
Italy
E-mail: *ena@ise.cnr.it*

INTRODUCTION

The extraction and use of olive oil has been an integral part of Mediterranean culture for over 6000 years (Civantos 1995; Tardàguila *et al.* 1996). Olive oil extraction involves a heavy consumption of water and produces large amounts of olive mill wastewater (OMW), the average volume ranging from 0.5 to 1.5 m^3 per ton of processed olives (Monteoliva-Sanchez *et al.* 1996; Paredes *et al.* 1996). In Italy, OMW is spread on cultivated fields under a strict law that strongly limits its use in agriculture (D.L. 574/96).

The fresh organic matter content in oil mill wastewater causes agricultural and environmental problems in olive oil-producing countries since its effects on soil status and fertility, insect proliferation and groundwater contamination are more harmful than beneficial (Cox *et al.* 1996; Spandre & Dellomonaco 1996). OMW contains a high amount of organic matter (30–200 kg COD m^{-3}), with a COD/BOD_5 ratio between 2.5 and 5, which is considered poorly degradable (Lopez 1992). The organic compounds in OMW (sugars, polyphenols, tannins, polyalcohols, pectins and lipids), in association with its high C/N ratio and low pH, compromise the biological degradation process of soils (Marques 2000) and can cause eutrophication when the wastewater is collected in basins with low exchange rates (closed gulfs, lakes, etc.).

The toxicity of OMW is also due to its high content of phenolic compounds in a wide range of molecular weights (MW), from low-MW substituted phenols to complex high-MW phenolic compounds (Montedoro *et al.* 1992). During olive oil production, large quantities of phenols are released along with the wastewater, according to their partition coefficient. Phenolics are derivatives of benzene (cyclic derivatives in the case of polyphenols) with one or more hydroxyl groups associated with their ring. The dark color of the water is caused by polyphenols (Pp) (Hamdi & Garcia 1993) and depends on the type of olives processed, their ripening stage, the climatic conditions and the technology used. However, despite their toxicity, polyphenols are used in the food, cosmetic and

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pharmaceutical industries on account of their high antioxidant activity.

The use of synthetic antioxidants in food processes can have negative health effects. Therefore, it is necessary to find natural substitutes that can inhibit the usual oxidization processes involved in the degradation of substances.

Treatment of OMW to recover valuable compounds like polyphenols could employ the aquatic fern *Azolla* as a biofilter. Dried *Azolla* biomass has already been used in the biosorption of a wide range of heavy metals from aqueous media (Sela & Tel-Or 1988; Cohen-Shoel *et al.* 2002). Pectin is an important polysaccharide constituent of *Azolla* cell walls, made up of fragments of polygalacturonic acid chains that interact with Ca^{2+} and Mg^{2+} ions to form a three-dimensional polymer (Schols *et al.* 1989; Jauneau *et al.* 1997; Kamnev *et al.* 1998).

As OMW consists of the diluted juice of crushed olives, it can be safely assumed that it is completely biodegradable. Yet even if all the constituents of OMW are biodegradable by definition, some of them, e.g. polyphenols and lipids, are decomposed at reaction rates much lower than others, e.g. sugars and short-chain volatile acids.

In recent years several methods have been proposed for OMW bioremediation, such as physical, physico-chemical or microbiological treatment. The physical and physico-chemical methods include thermal processes (evaporation and incineration), flocculation/clarification, ultrafiltration and reverse osmosis. Some of these systems have been patented (Knobloch *et al.* 2002; Pizzichini & Russo 2007).

The biological processes can be subdivided into anaerobic and aerobic ones. Nevertheless simple chemical or biological treatments cannot completely reduce OMW pollution and up to 85% of the organic substances, which could be recycled, are destroyed (Laconi *et al.* 2007).

The aim of this study was to test a new treatment for removing the pollution load in OMW by filtration on *Azolla* and granular activated carbon (GAC) to reduce the phenol, organic and inorganic matter content. This paper reports the results of laboratory experiments.

The filtration system is shown in Figure 1.

This method uses two different packed vegetable matrices (*Azolla* and GAC) which remove most of the pollutants by adsorption. Moreover, the phenolic compounds are almost completely recovered and the filtered water can be treated with calcium hypochlorite (ipoCa) to bring the COD to discharge values. The pretreated water can also be recovered for both hydrogen and biodegradable plastic production.

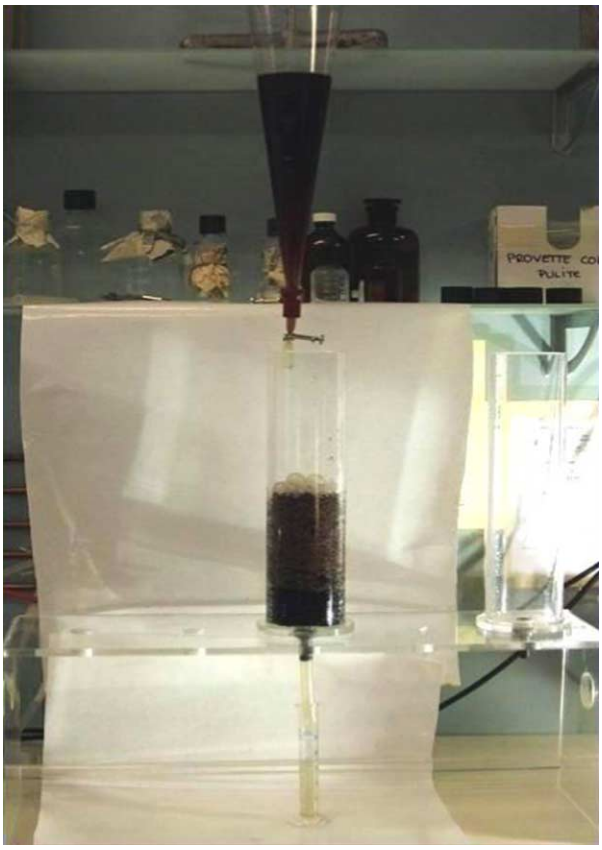

Figure 1 | Biofiltration system.

METHODS

Materials

Fresh olive mill wastewater samples were supplied by a continuous olive processing plant located in Bibbona in the province of Livorno (Italy). The samples, obtained from olives collected in January 2006 and immediately processed, were stored at −18°C. Citric acid, ethanol and all other chemicals were purchased from Sigma-Aldrich (St. Louis MO, USA). The multi-element and single element standards were supplied by CPI International (Santa Rosa CA, USA). The GAC (AFC-LS) was provided by Carboplant s.r.l. (Vigevano, PV, Italy).

Azolla cultivation

The strain of *Azolla caroliniana* derives from the Botanical Institute of Naples, Italy. Since the 1980s, it has been preserved by the Institute of Ecosystem Study (ISE), Florence section, of the Italian National Research Council (CNR). The *Azolla* biomass was cultivated in 2 m^2 vertical tanks containing a 10 cm layer of medium (reported by Ena *et al.* 2007), and then harvested and dried in the sun. This vertical apparatus permits to increase the cultivated surface from 6 m^2 to 14 m^2.

OMW pretreatment

Effluent (vegetation water) from a continuous olive oil extraction process was centrifuged. Table 1 shows the main characteristics of the OMW after centrifugation.

Each filtration experiment (in triplicate) was carried out twice and each value represents the mean of the six experiments. The pretreatment method uses two different packed vegetable matrices (*Azolla* and GAC) which remove most of the pollutant substances by absorption. The standard error was never higher than 5%. The results of this study demonstrate the feasibility of biofiltration on the vegetable matrices of wastewaters with an organic load of about 50–60,000 ppm.

Table 1 | Composition of the olive mill wastewater used in the present study

Property	Unit	Value
pH	pH	3.6
COD	mg/l O_2	52,500 ± 2,700
BOD_5	mg/l O_2	16,250 ± 800
Polyphenols	mg/Kg	4,005.0 ± 185
Chloride	mg/l	593.4 ± 27.2
Nitrate	mg/l	3.4 ± 0.18
Sulphate	mg/l	72.5 ± 3.5
Orthophosphate	mg/l	926.3 ± 46.2
Total P	mg/l	428.6 ± 18.6
Total N	mg/l	785.9 ± 35.8
K	mg/l	16.8 ± 0.77
Ca	mg/l	648.3 ± 32.0
Mg	mg/l	180.3 ± 9.1
Al	mg/l	1,257 ± 50.1
Cd	mg/l	< 0.5
Cr	mg/l	33.2 ± 1.2
Fe	mg/l	6,535 ± 280
Hg	mg/l	< 0.5
Ni	mg/l	925.3 ± 36.0
Pb	mg/l	7.8 ± 0.2
Cu	mg/l	335.2 ± 11.2
Zn	mg/l	4,588 ± 181

Main properties of the filtration supports

The adsorption properties of GAC and *Azolla* for the organic (COD) and phenolic compounds of OMW were determined in duplicate by placing 10 g of each support (previously sterilized in an autoclave) in anaerobic 100 ml-sterile bottles containing 20 ml of a filter-sterilized defined dilution (1:1, 1:2, 1:3, 1:5 or 1:8, in distilled water) of OMW. All bottles were brought to equilibrium conditions by shaking on a rotary shaker at 25°C and 150 rpm for 2 days. The adsorption data were calculated by measuring the residual concentrations of COD and Pp in the supernatants after filtration. Freundlich sorption kinetics for the organic matter and phenolic compounds in the immobilization carriers was then studied according to Colella *et al.* (1998).

Analytical methods

The analysis of BOD_5, total nitrogen, chloride, nitrate, sulfate, orthophosphate were carried out according to standard methods (Eaton & Greenberg 2005).

COD

The COD concentration in OMW was determined according to Ena *et al.* (2007).

Polyphenols

Polyphenols (with respect to gallic acid) were determined spectrophotometrically according to the Folin-Ciocalteau (Ena *et al.* 2007) using a Beckman DU 640 as spectrophotometer.

Total phosphorus

Total organic phosphorus was determined in unfiltered samples by acid digestion with potassium persulfate

followed by colorimetric inorganic phosphate analysis with a UNICAM UV2 double ray spectrophotometer (Carmouze 1994).

K, Na, Mg

These ions were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS) with an AGILENT 7500ce collision cell ICP-MS.

Heavy metals (Al, Cd, Cr, Fe, Ni, Pb, Cu, Zn)

The heavy metals were determined by atomic absorption with a UNICAM 939 graphite oven (confirmation by AGILENT 7500ce ICP-MS).

Hg

This metal was determined by atomic absorption spectroscopy at hydride development (PERKIN ELMER K16; confirmation by AGILENT 7500ce ICP-MS).

RESULTS AND DISCUSSION

Data on the growth of the aquatic fern (*Azolla-Anabaena azollae* symbiosis) outdoors under the climatic conditions of Florence are reported in Table 2.

The highest yield (14.2 g dry weight (d.w.) $m^{-2} d^{-1}$) was obtained in July; the average productivity was 10.3 g (d.w.) $m^{-2} d^{-1}$. The average planting density was 50 g (dry weight) m^{-2}.

A previous study had shown the ability of this aquatic fern (fresh biomass) to reduce phenol and organic matter in OMW (Ena *et al.* 2007). Other authors have used dried *Azolla* biomass in the biosorption of a wide range of heavy metals from aqueous media (Cohen-Shoel *et al.* 2002).

Table 2 | Yield and growth rate of *Azolla filiculoides* achieved outdoors in 2 m^2 vertical tanks

Months	Productivity (g (d.w.) $m^{-2} d^{-1}$)	Growth rate d^{-1}
July	14.2 ± 0.68	0.258 ± 0.009
August	10.5 ± 0.53	0.183 ± 0.009
September	7.2 ± 0.25	0.144 ± 0.005

Physico-chemical analyses (Table 1) of OMW showed that it was dark acidic waste with high levels of organic matter and polyphenols.

According to Ranalli (1992), phenolic compounds are the pigments responsible for the dark color of OMW. Moreover, OMW toxicity is mainly due to low molecular weight phenols (Della Greca *et al.* 2001); the toxicity of these compounds is caused by autoxidation processes (Nakai *et al.* 2001). This vegetation water also had high contents of chloride, orthophosphate, total P, total N, Ca and the heavy metals Ni and Fe.

As a first step, the two types of dried packed vegetable matrices were used separately: *Azolla* alone and GAC alone at two concentrations (50 and 100 g l^{-1}; 100 and 200 g l^{-1} respectively). The COD and Pp removal results (Figure 2) show that the lower concentrations were most efficient (220 and 25 mg g^{-1} respectively), while the values were somewhat lower (162 and 20.5 mg g^{-1} respectively) at 100 and 200 g l^{-1}. However, the higher concentrations removed greater amounts in absolute.

Considering the great chemical heterogeneity of OMW, we studied the adsorption properties of GAC and *Azolla* in terms of removal "broad parameters" instead of determining adsorption isotherms for single compounds in the OMW. The Freundlich adsorption isotherm, $q_e = k.Ce^{1/n}$ (q_e = adsorbed COD or phenols per unit mass of solid material, mg g^{-1}, and Ce = COD or phenolic compound concentration at equilibrium, mg l^{-1}), was used to represent the COD and phenolic compound adsorption on both supports. Thus, the characteristic Freundlich parameters (k and $1/n$) are best regarded here as descriptive and they

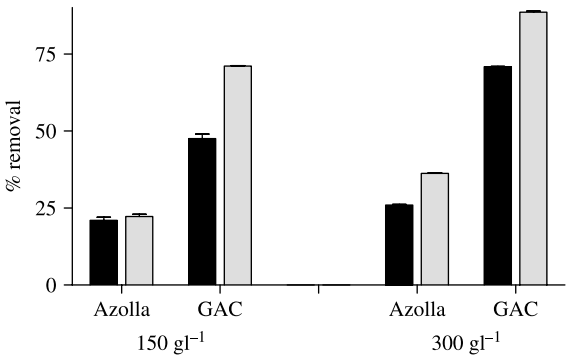

Figure 2 | Abatement percentages of COD (black bars) and Pp (grey bars) in alone (at two different concentrations) vegetable matrices.

Table 3 | Characteristic Freundlich parameters

	k(mg/g)/[(mg/l)$^{1/n}$]	1/n	Correlation coefficient
COD *Azolla*	2.60E-03	1.0482	0.9963
Pp *Azolla*	5.42E-03	1.1102	0.8941
COD GAC	2.63	0.5186	0.9997
Pp GAC	8.08	0.4188	0.9791

were optimized by non-linear least-square regression of experimental adsorption data (Table 3).

The experimental adsorption data and calculated Freundlich isotherms for COD and phenol adsorption are compared in Figure 3a and b for *Azolla* and Figure 3c and d for GAC.

As expected, the total organic matter (measured as COD) and phenolic fraction of OMW were both much more efficiently adsorbed by GAC (70.8% and 88.5% respectively) than by *Azolla* (25.95% and 36.25% respectively).

The different vegetable matrices were then tested together by packing one on top of the other in a column (concentrations: 150 g l^{-1}, 300 g l^{-1}, 360 g l^{-1}). The results indicate that the concentrations of 300 g l^{-1} and 360 g l^{-1} provided greater removal of phenols and organic matter: COD removal increased to 80.5% and 84.5% respectively and phenol absorption to more than 99% in both conditions (Figure 4).

A higher concentration of vegetable matrices used in the biofilter (360 g l^{-1}) do not provide a significant removal of pollutant substances (Pp and COD) if compared to that obtained with the 300 g l^{-1} concentration, considering the increase of the matrices utilized. Therefore we have used the 300 g l^{-1} concentration. The progress of the treatment process is shown in Figure 5: 40% of the organic load of COD was removed by centrifugation and over 80% by biofiltration; 12% of Pp was removed by centrifugation and over 99% by biofiltration.

The percentage removal data for the alkaline metals (K, Ca and Mg) after biofiltration demonstrate a higher affinity of matrices for K and Mg (more than 70%) while the removal of Ca was very poor (47%). When several components are present, interference and competition for adsorption sites probably occur, leading to a lower saturation concentration of the adsorbed component. Moreover, other substances such as total nitrogen and

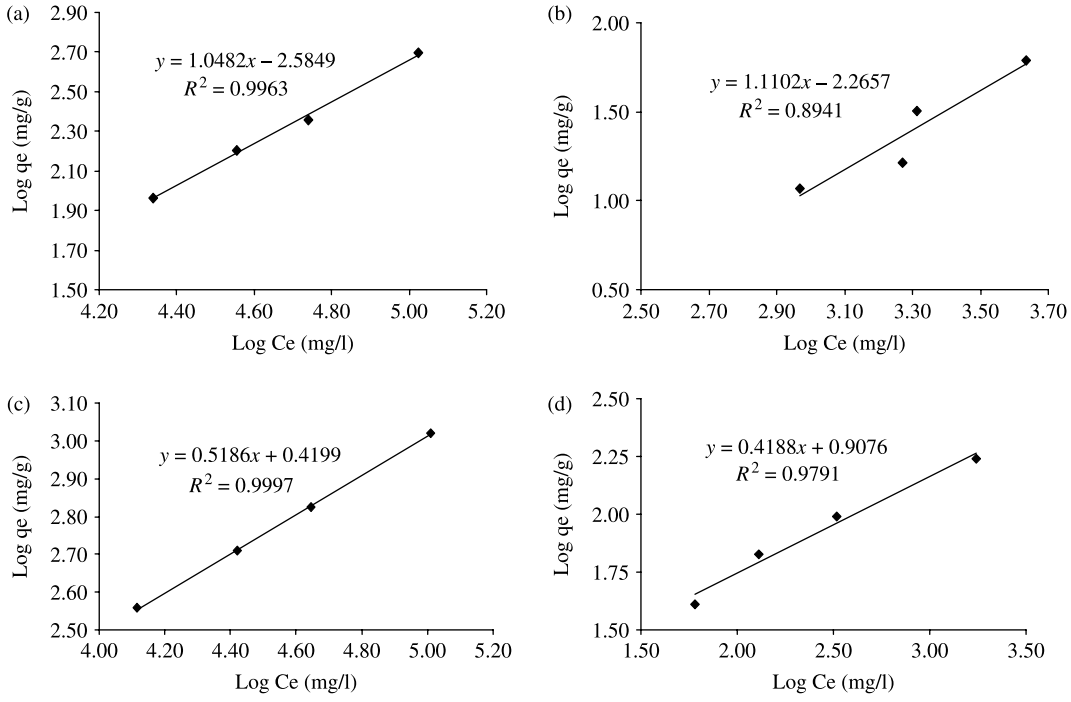

Figure 3 | COD (a, c) and phenolic compounds (b, d) Freundlich isotherms on *Azolla* (a, b) and GAC (c, d).

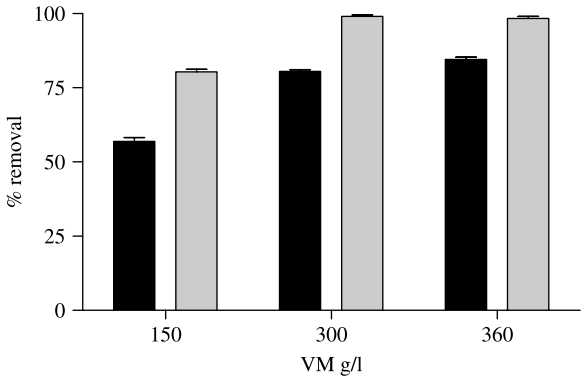

Figure 4 | Removal percentages of COD (black bars) and Pp (grey bars) at different concentrations of together packed vegetable matrices.

copper were almost totally absorbed by the matrices while different metallic compounds (Al and Pb excepted) were variably reduced by 50–85% (Figure 6a and b).

The heavy metals with higher ionic charge probably cannot be completely adsorbed by the vegetable matrices according to the aforesaid phenomena.

For complete removal of the COD, the residual waste after biofiltration was treated by oxidation with different concentrations of calcium hypochlorite (1,800–15,760 ppm). As shown in Figure 7, the highest COD removal (366 mg g^{-1} of ipoCa) was obtained in the experiment with the highest initial concentrations of both COD and ipoCa; however this treatment was not effective because the organic load remained and there was also a large amount of chlorides.

The best treatment was with the lower initial contents of COD and ipoCa: the COD concentration was reduced by 92%, giving a final concentration of 150 mg l^{-1} which is

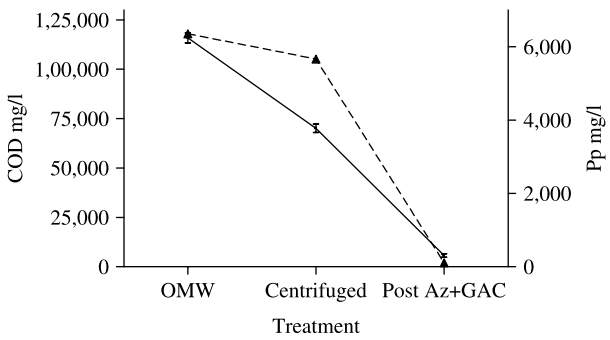

Figure 5 | Removal of COD (continuous line) and Pp (discontinuous line) in the invented treatment.

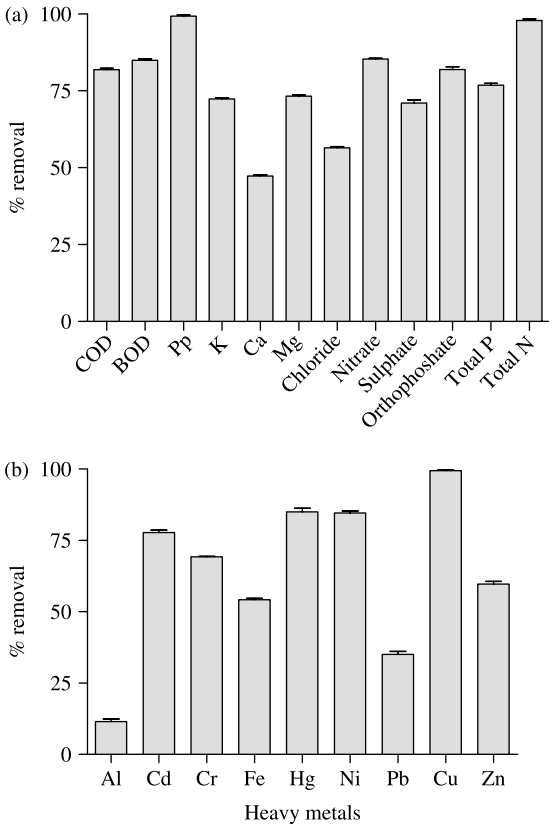

Figure 6 | Removal percentages of some chemical parameters (a) and heavy metals (b).

compatible with Italian law (160 mg l^{-1} COD in surface waters). Figure 8 illustrates the complete treatment.

The results obtained with this treatment are comparable to those using membrane technologies (ultrafiltration, microfiltration, nanofiltration and reverse osmosis) (Russo 2007), although our method is much more economical.

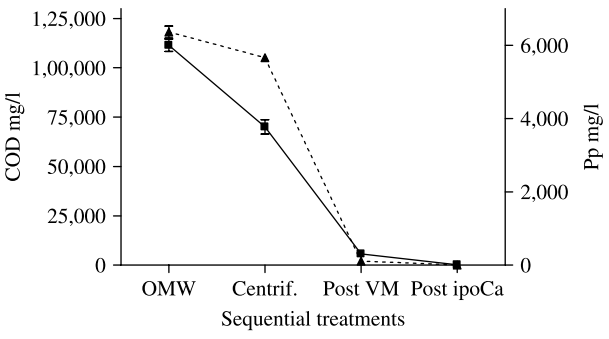

Figure 7 | Complete treatment: the abatement of COD (continuous line) and Pp (discontinuous line).

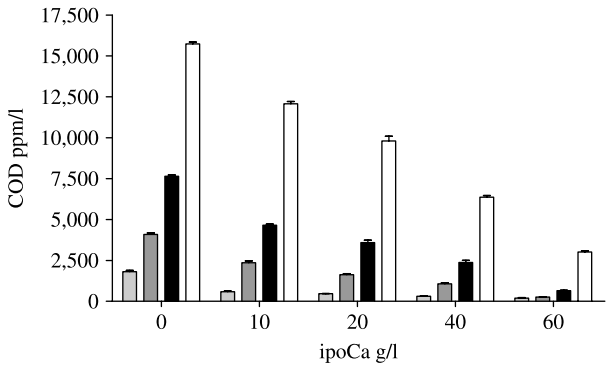

Figure 8 | Removal of COD in treated OMW with different initial COD concentrations (1,800 ppm light grey bars, 4,080 ppm grey bars, 7,680 ppm black bars, 15,760 ppm white bars) and different Ca ipoclorite concentrations.

The pretreated wastewater (after biofiltration) can be used for various applications.

(i) Polyphenol recovery by vegetable matrix. Phenols are very interesting compounds as they can be used in the prevention of cardiovascular disease and as antiviral, antioxidant and antitumor agents. They can also be used for food, cosmetic and pharmaceutical applications. In particular, OH-tyrosol shows strong antioxidant, antinflammatory and antiviral activity (Ohno *et al.* 2002), higher than that of many other compounds (Manna *et al.* 1999; D'Angelo *et al.* 2005). The total desorption of polyphenols from the exhausted matrices is about 50–60%.

(ii) The pretreated wastewater can be used as a substrate for the growth of a purple bacteria (*Rhodopseudomonas palustris*) able to metabolize residual organic compounds (mainly short-chain organic acids) for biohydrogen production. Under the same conditions, H_2 production experiments (conducted in free nutrient OMW) were carried out in both batch and discontinuous mode: removing and adding a fixed amount of wastewater. The results showed that in batch conditions the biogas production was ca. 600 ml per liter of culture, while in experiments carried out in discontinuous mode for more than 300 hours the yield was ca. 1,200 ml per litre of culture (data not shown). These results are very good compared with those reported by Eroglù *et al.* (2004) because in that paper optimal condition OMW was used at 2% dilution.

(iii) When growing in unbalanced conditions, *R. palustris* can produce biopolymers (polyhydroxyalkanoates) with potential applications in medicine and surgery as biodegradable plastic material. In these microorganisms the polyester (PHB) is used as an energy reserve compound.

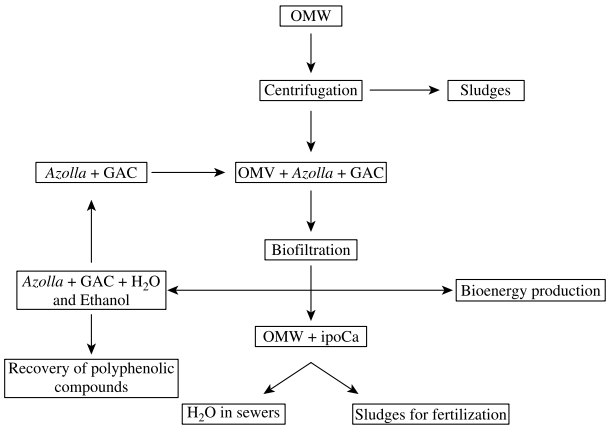

Figure 9 | Complete treatment plan of the olive oil mill wastewater.

In the end the treatment proposed according to the present research not only provides for abating the pollution impact of olive mill wastewater, so that it can be directly disposed in the environment with full compliance of current legislation, but also uses this OMW as a raw material obtaining the following commercially useful products: organic fertilizers, antioxidant compounds and water that can be used for all oil press customs. Figure 9 sets out schematically the complete treatment suggested in this paper.

CONCLUSIONS

This paper is the first description of a complete biodepuration treatment of olive mill wastewater carried out with a biofiltration system using vegetable matrices. The pretreated water can be utilized for various applications. For example, the OMW pretreated with the described method is a promising substrate for the biological production of valuable by-products (biohydrogen, polyhydroxyalkanoates and pigments). Consequently, the water obtained after the suggested applications can be utilized for all oil press uses.

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ORIGINAL RESEARCH PAPER

Green energy from *Rhodopseudomonas palustris* grown at low to high irradiance values, under fed-batch operational conditions

Pietro Carlozzi · Cristina Pintucci · Raffaella Piccardi · Arianna Buccioni · Sara Minieri · Maurizio Lambardi

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Abstract *Rhodopseudomonas palustris* was grown under continuous irradiances of 36, 56, 75, 151, 320, 500, and 803 W m^{-2}, for a co-production of both bio-H_2 and biodiesel (lipids) using fed-batch conditions. The highest overall bio-H_2 produced [4.2 l(H_2) $l_{culture}^{-1}$] was achieved at 320 W m^{-2}, while the highest dry biomass (3.18 g l^{-1}) was attained at 500 W m^{-2}. Dry biomass contained between 22 and 39% lipid. The total energy conversion efficiency was at its highest (6.9%) at 36 W m^{-2}.

Keywords Green energy · Bio-H_2 · Biodiesel · Hydrogen production rate · *Rhodopseudomonas palustris*

P. Carlozzi (✉) · C. Pintucci · R. Piccardi
Istituto per lo Studio degli Ecosistemi, Sede di Firenze, Consiglio Nazionale delle Ricerche, Polo Scientifico, Via Madonna del Piano n. 10, 50019 Sesto Fiorentino, Firenze, Italy
e-mail: p.carlozzi@ise.cnr.it

A. Buccioni · S. Minieri
Dipartimento di Scienze Zootecniche, Università degli Studi di Firenze, Via delle Cascine n. 5, 50144 Florence, Italy

M. Lambardi
Istituto per la Valorizzazione del Legno e delle Specie Arboree, Consiglio Nazionale delle Ricerche, Polo Scientifico, Via Madonna del Piano n. 10, 50019 Sesto Fiorentino, Firenze, Italy

Introduction

Purple non-sulfur photosynthetic bacteria can decompose organic acids by using light energy and nitrogenase in a photofermentation process (Basak and Das 2007). Green energies (bio-H_2 and biodiesel) need cultural systems to be developed. Bioreactor design, hydrodynamic aspects and light distribution are key points to improve H_2 yield. Improvements could also be achieved through the genetic modification (Nath et al. 2004). Chisti (2007) reported: "Biodiesel derived from oil crops is a potential renewable and carbon neutral alternative to petroleum fuels. Unfortunately, biodiesel from oil crops, waste cooking oil and animal fat cannot realistically satisfy even a small fraction of the exiting demand for transport fuels". A review of biodiesel production from oleaginous microorganisms was recently carried out by Ratledge and Cohen (2008). Purple, non-sulfur photosynthetic bacteria, such as *Rhodopseudomonas palustris*, is able to use irradiance more efficiently than do crop plants, and oil production from bacteria could be improved under adequate or stressed culture conditions.

The purpose of this study is to investigate a co-production of bio-H_2 and rich biomass content of lipids for biodiesel production by means of *Rhodopseudomonas palustris* (strain 42OL) grown at low irradiance (LI) (36, 56 and 76 W m^{-2}), at both medium (MI) (151 and 320 W m^{-2}) and high irradiance (HI) (500 and 805 W m^{-2}). The relationship between light intensity and both H_2 production rate

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and the total energy conversion efficiency has also been investigated.

Materials and methods

Description of the cultural system

A cylindrical glass photobioreactor [internal diam = 9.6 cm; working volume (V) = 1.07 l] was held at 30 ± 0.2°C. All irradiance was measured at the surface of the photobioreactor using a flat glass surface (10 × 16 cm) an averaged from nine different location points. The heat exchanger-Plexiglas basin was not filled of water during the irradiance measurements. The average radial depth of water between the Plexiglas (inside wall) and the photobioreactor (outside wall) was 7.3 cm. The gas produced by bacteria was trapped in a graduated glass column (Carlozzi and Lambardi 2009).

Organism and culture conditions

Rhodopseudomonas palustris (strain 42OL) was taken from the culture collection of the former Centro di Studio dei Microrganismi Autotrofi of Florence, now the Institute of Ecosystem Study, CNR, Florence, Italy. It was grown at 30°C on a medium previously described (Carlozzi and Sacchi 2001) but with glutamic acid at 0.865 g l^{-1} and malic acid at 3.26 g l^{-1}. The initial pH of the medium was 6.8. All experiments were carried out under continuous light at irradiances of 36, 56, 75, 151, 320, 500, and 803 W m^{-2}. The photobioreactor irradiated from one side was operated in a fed-batch mode (Carlozzi and Lambardi 2009). This feeding strategy was used for long-term investigations (max = 408 h); otherwise, the yields produced (biomass and H_2) would have ceased for lack of macronutrients.

Analytical methods

Dry biomass was determined by using the empirical equation of Carlozzi and Sacchi (2001). Growth was determined from the bacteriochlorophyll (Bchl) concentration (Carlozzi et al. 2006). Cultures were irradiated with a 250 W OSRAM Power-star HQI-TS lamp, and the irradiance was measured using a Quantum/Radiometer/Photometer (model LI-185B, LI-COR, Lincoln, Nebraska, USA). To determine organic acid concentrations in the bacteria cultures, a HPLC was used. Disposable syringe filter units (MFS-13 mm, 0.45 μm pore size) were used to remove the cells and the supernatant was tested for malic acid. The HPLC used a C18 analytical column (250 mm × 4.6 mm) and was run at 25°C. The mobile phase was 0.1% H_3PO_4 at 1 ml min^{-1}.

Gas and lipid evaluation

The gas mixture produced (CO_2 and H_2) was first made to flow into a basin containing a saline solution absorber of NaOH, which stripped CO_2. H_2 was then trapped in a calibrated column, where it was collected and the volume measured to determine its production. No CO_2 was found inside the calibrated column. This was checked by sampling 0.1 ml from the calibrate column and injecting it into a gas chromatograph equipped with a thermal conductivity detector and a silica gel 60/80 grade 12 column (Alltech, Derfield). The carrier gas was He; pure H_2 was used as the standard. Total lipid extraction and the fatty acid profile were determined in accordance with Pushparaj et al. (2008).

Light conversion efficiency

The total energy conversion efficiency (η') was determined using the following equation:

$$\eta'(\%) = \frac{33.61\rho H_2 V H_2 + (P_B(-\Delta P_B))}{I A\, tH_2 + (MA_c(-\Delta MA)) + (GA_c(-\Delta GA))} \times 100 \quad (1)$$

where ρH_2 is the concentration of H_2 (g l^{-1}); VH_2 is the volume of H_2 produced (l); P_B is the total biomass produced (g) (ash = 4.5% of dry biomass; $(-\Delta P_B)$ is the heat of combustion of ash-free biomass (dw) (kcal g^{-1}); I is the irradiance (W m^{-2}); A is the irradiated area of the photobioreactor (m^{-2}), which was calculated as being ½ of the cylindrical reactor surface ($2\pi r_i h$), with r_i and h indicating, respectively, the internal radius and the height of the cylindrical reactor; tH_2 is the evolution time (h); MA_c is the malic acid consumed (g); $(-\Delta MA)$ is the heat of combustion of MA (kcal g^{-1}); GA_c is the glutamic acid consumed (g), and $(-\Delta GA)$ is the heat of combustion of GA (kcal g^{-1}).

Results and discussion

Growth of *R. palustris* and H_2 production are shown in Fig. 1. Both bio-H_2 and dry-biomass productivities and the total energy conversion efficiency versus the irradiance are shown in Fig. 2. The total H_2 produced in the photobioreactor (working volume: 1.07 l) increased in accordance with the irradiance up to 4.2 l(H_2) $l^{-1}_{culture}$ at 320 W m^{-2}. By increasing the irradiance further, the H_2 yield decreased by about

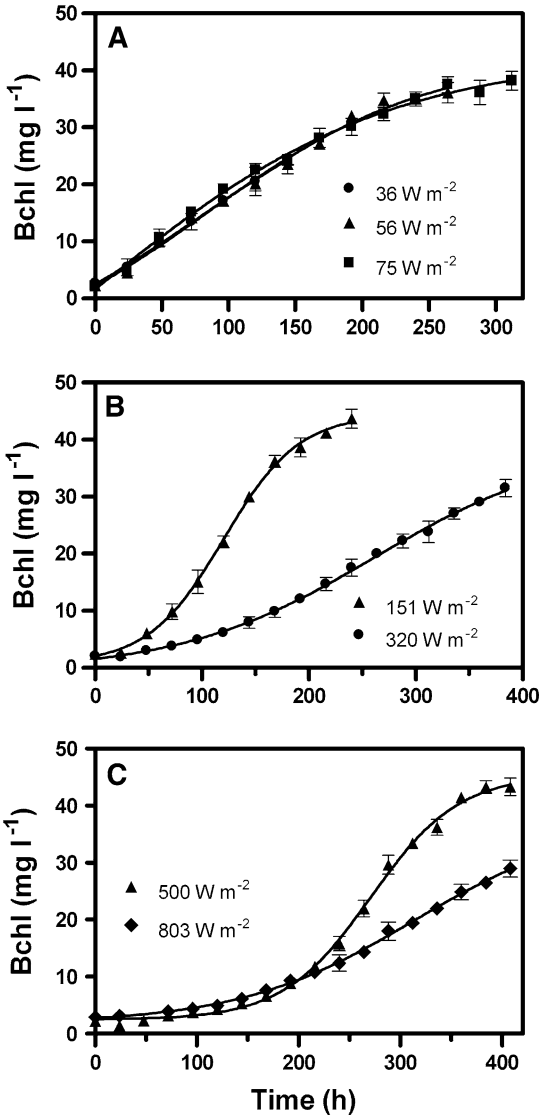

Fig. 1 Trends of bacteriochlorophyll (Bchl) synthesis versus time attained with *R. palustris* grown at low (**a**), middle (**b**) and high irradiance. Values are means of three replications ± standard deviation

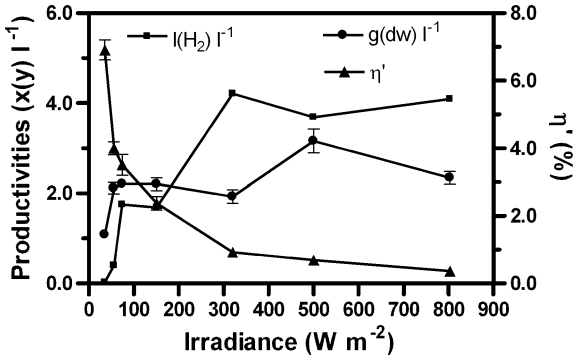

Fig. 2 Bio-H_2 and dry-biomass productivities and the total energy conversion efficiency achieved at seven different irradiances (36, 56, 75, 151, 320, 500 and 803 W m^{-2}). Bars ± standard deviation

7.5%. The average H_2 production rate (HPR_{av}) was 12.3 ml l^{-1} h^{-1} 320 W m^{-2}. Chen et al. (2008) predicted an overall H_2 photoevolution rate of 12.6 ml l^{-1} h^{-1} by using acetate and butyrate as dual carbon substrates. The yield of H_2 achieved in this study was about 33% higher than that reported by Carlozzi and Lambardi (2009), and the H_2 photoevolution rate was within the range of those cited in the literature for use of the same microorganism species (Chen et al. 2006; Eroğlu et al. 2008). The highest cumulative H_2 was achieved at 320 W m^{-2}, while the highest dry biomass was attained at 500 W m^{-2}. This was because the biomass production competed with the H_2 photoevolution.

The total energy conversion efficiency was at its highest (6.9%) at 36 W m^{-2}, and went progressively down as the irradiance increased. In 2009, Obeid et al. wrote: "The conversion efficiency of light energy to hydrogen is the key factor in the development of a biological process devoted to hydrogen production". The η' of 6.9% is among the highest values cited in the literature (Kondo et al. 2006; Basak and Das 2009).

Biomasses of *R. palustris* harvested at the end of each fed-batch cycle, during bio-H_2 photoevolution, were analysed in order to determine the total lipid content. The results are shown in Fig. 3. The lipid content 36–39% of the dry biomass when the irradiance was within the 56–151 W m^{-2} range. A further increase in the irradiance caused a relevant and progressive drop in the lipid content in the *R. palustris* biomasses: the minimum content (22%) was reached when the irradiance was at its highest (803 W m^{-2}).

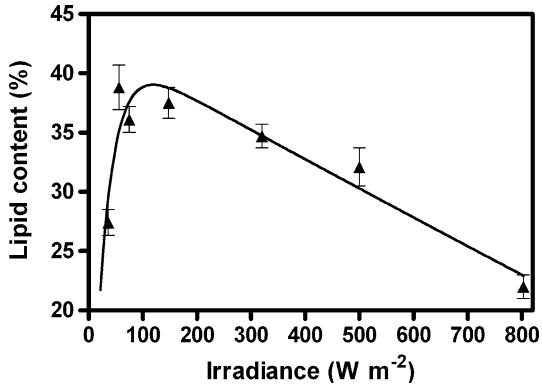

Fig. 3 Lipid content in dry biomass of *R. palustris* grown from 36 to 803 W m^{-2} during hydrogen photo-evolution. Values are means of three replications ± standard deviation

With an irradiance of less than 56 W m^{-2}, the lipid content of the biomass dry weight also decreased. The major fatty acid of *R. palustris* grown inside a tubular underwater photobioreactor (UwTP) (Carlozzi and Sacchi 2001) were 16:0 (17%); 16:1 (7%); 18:1 (73%); 18:0 (3%). Our data are similar to the four *R. palustris* strains cited by Imhoff and Bias-Imhoff (1995). Co-production of both bio-H_2 and biodiesel, as biofuels, is proposed in this study. For biodiesel production, triacylglycerols (TAG) are transesterified with methanol. The reaction occurs stepwise: TAGes are first converted to diacylglycerols, then to monoacylglycerols, and lastly to glycerol + methyl fatty esters (biodiesel) (Chisti 2007). The author listed 14 microalgal species with their respective oil contents. The oil content we found in *R. palustris* biomasses can be included in the group of the five microalgal species, with the highest oil content, reported by Chisti (2007).

Bacteria accumulate fewer lipids than do microalgae, and the average oil content is about 20–40% of the dry biomass (Meng et al. 2009). Microorganisms that can accumulate lipids at more than 20% of their biomass are defined as oleaginous species (Ratledge and Wynn 2002). Since the lipid content of the *R. palustris* that we investigated was within the range of 22–39% of the dry biomass, we could define our bacterial strain as being oleaginous. Carlozzi and Sacchi (2001) reported outdoor production of *R. palustris* dry biomass of 64 g m^{-2} day^{-1}. On the basis of Carlozzi's investigations, a projection of the monthly

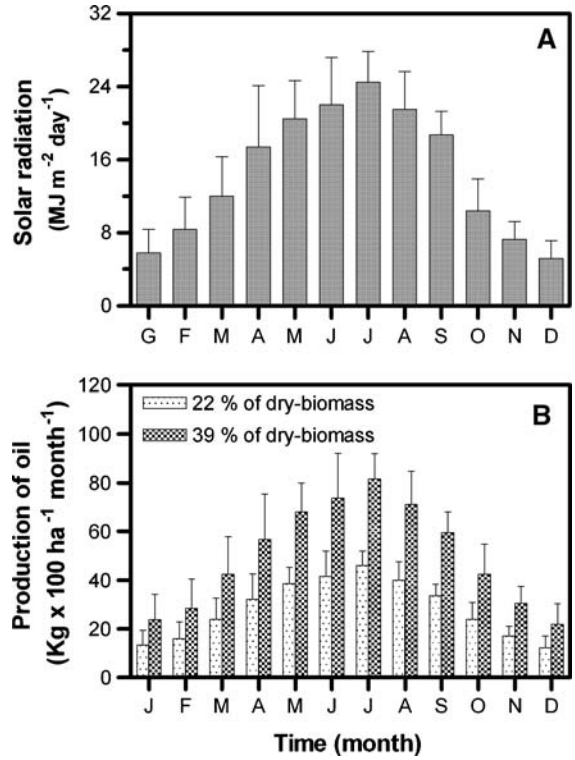

Fig. 4 **a** Solar radiation and **b** predicted results of the monthly oil production from *R. palustris* grown outdoors in the climatic conditions of Florence, Italy (latitude: 43°46′N; longitude: 11°15′E). Bars ± standard deviation

oil production over the year can be made from *R. palustris* grown outdoors in the climatic conditions of Florence, Italy (latitude 43°46′N, longitude 11°15′E) (Fig. 4). Projections on bio-H_2 production, outdoors, were not carried out, because we did not have enough experimental results, even though a first, very short investigation has recently been carried out (Carlozzi et al. 2008). To reduce the greenhouse gas effect, oil production (biodiesel) from plants, crops, microalgae, yeast and, recently, bacteria has already been suggested (Ratledge and Wynn 2002; Chisti 2007; Rittmann 2008). Nevertheless, we would remark that this was the first time, which a non-sulfur photosynthetic bacteria (*R. palustris*) has been proposed as a potential oil source for biodiesel production.

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