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# POSTER PRESENTATIONS

## IMPACT OF DOR INCUBATED WITH SAPROBE FUNGI ON HYDROLYTIC ENZYMES ACTIVITIES AND MICROBIAL COMMUNITY STRUCTURE OF RHIZOSPHERIC SOIL OF LETTUCE

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### ABSTRACT

The dry olive residue (DOR), the solid by-product arising from the olive oil two-phase extraction system is produced in large quantities. This type of residue contains toxic components, mainly phenols, capable of inhibiting microorganisms and impact negatively the soil ecosystem. The objective of the present study was to investigate the impact of either un-treated DOR or DOR incubated with immobilized saprobe fungi *Panus tigrinus* and *Fusarium lateritium* on both enzymes activities (urease, protease, phosphatase and  $\beta$ -glucosidase) and bacterial communities of rhizosphere soil of lettuce. We observed an increase of all hydrolytic enzymes after the soil incubation with un-treated DOR for 60 days. The addition of this type of residue supposes an increase of soil organic matter and available substrates that can stimulate the soil microbial activity. We also observed an increase of all enzymes activities, except urease, in soil incubated with treated DOR. The phosphatase activity was similar in soil with all types of amendant, however the enzymes activities implicated in N cycle such protease and urease were lower in soil incubated with DOR treated with *P. tigrinus* than the soil incubated with un-treated DOR. The DGGE analysis showed that the addition of un-treated DOR and treated DOR with the fungus *F. lateritium* decreased the diversity and relative abundance of soil bacterial population. However, the transformation of this residue with the fungus *P. tigrinus* supposes an increase in the bacterial communities. It appears that this increase is related with the decrease of substrates implicated in N cycle as indicated by the decrease of the protease and urease detected activities. These results indicate that the DOR treated with *P. tigrinus* is not toxic on soil bacterial communities and this incubation process provides a residue devoid of toxicity that may be used as an organic fertilizer.

## DILUTE ACID HYDROLYSIS OF SUNFLOWER STALK AND ITS USE AS SOURCE OF XYLOSE FOR XYLITOL BIOPRODUCTION

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### ABSTRACT

Xylitol is a five carbon sugar alcohol, equivalent to sucrose in sweetness and occurs widely in nature but it is also produced in human metabolism. Unlike sucrose, it is anticariogenic, natural sweetener and can be consumed by diabetics because it is metabolized by an insulin-independent pathway. It gives a pleasant cool and fresh sensation due to its high negative heat of solution. Xylitol is used in various food products such as chewing gum, candy, soft drinks and ice cream. Despite many advantages of xylitol, the use of xylitol as sweetener is limited. Commercially, xylitol is produced from birch wood tree which is the most expensive source. The agricultural waste which is rich in lignocellulosic materials is an ideal source for the production of xylitol. Sunflower stalk is one of the most widely available waste in Turkey. It can be used as animal feed, but this use has slight economical significance, and it is usually left to rot or burned in the field after harvesting. Utilization of this material for production of xylitol does not only solve the proper disposal of these wastes, but also provides additional income for farmers and generates employment. The aim of this study was to produce xylose from sunflower stalk and conversion of xylose to xylitol production by *Candida guilliermondii*. For this purpose the effects of  $H_2SO_4$  concentration, temperature and reaction time on the production of sugars (xylose, glucose and arabinose) and on the reaction by-products (furfural and acetic acid) from sunflower stalk were investigated. Response surface methodology (RSM) was used to optimize the hydrolysis process in order to obtain high xylose yield and selectivity. The considered optimum conditions were:  $H_2SO_4$  concentration of 4%, temperature of 120 C and the reaction time of 45 min. The hydrolysates, produced under optimum conditions, was used for xylitol bioproduction by *Candida guilliermondii*.

**Keywords:** *Xylose, xylitol, sunflower stalk, wheat straw, optimization*

## EVALUATION ANTIOXIDANT ACTIVITY OF DILUTE ACID HYDROLYSATE OF WHEAT STRAW DURING XYLOSE PRODUCTION

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### ABSTRACT

Utilization of lignocellulosic wastes for industrial purposes are receiving interest due to their huge amount of carbohydrates (cellulose and hemicellulose) contents, low cost, wide availability and reduction of environmental pollution. The agricultural waste, widely found in Turkey, have a lignocellulosic nature, mainly composed of hemicellulose, cellulose and lignin and they can be used as a renewable material for production of value added products such as ethanol, glucose, xylose, xylitol and antioxidant compounds. Acid hydrolysis process not only breakdowns the hemicellulose to monosaccharides but also cleaves the β-1-4 alkyl-aryl linkages in lignin, lignin-hemicellulose linkages and forms soluble the phenolic compounds. The main phenolic components of the extracts derived from mild acid treatment of the lignocellulosic materials are *p*-hydroxybenzoic acid, ferulic acid, vanillic acid, syringic acid, and coumaric acid, syringaldehyde, *p*-hydroxybenzaldehyde, and vanillin. The aim of present investigation was to evaluate the operational conditions (temperature, time and acid) on the production of sugars (xylose, glucose, arabinose and sugar dehydration products), on the production of the phenolic and ferulic acid and on the antioxidant activity of acid hydrolysate of the agricultural waste. Hydrolysis of wheat straw was carried out under different temperature, reaction time and acid concentrations in a batch reactor. It was found that the increase in the temperature, acid and reaction time increased phenolic and antioxidant activity of the hydrolysate but decreased the sugar yield.

**Keywords:** *Xylose, wheat straw, phenolic, ferulic acid*

## RELATIONSHIP BETWEEN THE METHYL ALCOHOL CONTENT IN THE DISTILLATE FROM FERMENTED VINIFICATIONS SUB-PRODUCTS AND THEIR STORAGE CONDITIONS

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### ABSTRACT

In the traditional oenological areas grape marc and lees, after alcoholic fermentation of the residual sugars, are distilled to obtain an alcoholic beverage that has an important social and economic value. Methyl alcohol is not a direct fermentation by-product; it is formed from pectin by pectolytic enzymes. This volatile compound has no influence on the aroma but it is important to limit its content because of its high potential toxicity<sup>1</sup>. The aim of this study was to establish correlations between the methanol content in the distillate with the composition and initial characteristics of the grape pomace (humidity degree, pH, time and storage system) to evaluate which conditions have more influence in the concentration of this toxic alcohol in the final distillate. An industrial distillation unit using entrainment with steam and equipped with a rectification column was employed to distillate the raw material. GC-FID<sup>2</sup> was used to evaluate the methyl alcohol content in the twenty samples of grape marc spirits collected during each distillation process. The results obtained showed that storage time before distillation and the humidity degree in the grape marc were the two parameters with more influence in the methyl content. However, a high pH value in the raw material was not correlated with the higher content of methyl alcohol in the corresponding distillate.

**Keywords:** *grape pomace distillates, methanol, storage conditions*

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## EFFECT OF AMINO ACID SIDE CHAIN ON PREFERENTIAL CLEAVAGE OF MACROCYCLIC B ION

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### ABSTRACT

Introduction of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) into mass spectrometry has paved the way for analysis of biomolecules without degradation. Respectively, protein identification based on enzymatic digestion of proteins and tandem mass spectrometric (MS/MS) analysis of peptide fragments has become a more popular method than classical approaches like Edman degradation. In this approach, peptides are cleaved into smaller fragments by applying collision energy which mainly produce sequence-informative ions called b and y ions. However, today's protein identification tools have been constructed on a limited basis of peptide fragmentation chemistry such that peptides with acidic or basic amino acids can fragment in unusual ways that may not be predicted by the bioinformatic softwares. That's way wrong assignments can be done in protein identification that can lead to vital problems. Hence, understanding of peptide fragmentation chemistry in all aspects is considered to improve the current bioinformatic tools. In this respect, we have focussed on the study of fragmentation pathways of the model peptides AXVYI-NH<sub>2</sub> where X donates 20 common amino acids. The peptides are purchased from BL Biochem (China) and used as received. Throughout the study, a hybrid quadrupole ion trap mass spectrometer (4000 Q-TRAP Applied Biosystems) combined with electrospray ion source is used. By this approach, an informative model is aimed to be constructed in order to demonstrate the influence of amino acid side chains on the cleavage of macrocyclic b ions.

## ASSESSMENT OF PRETREATMENT METHODS FOR ENHANCING ENZYMATIC HYDROLYSIS OF KITCHEN WASTES FOR BIOETHANOL PRODUCTION

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### ABSTRACT

It is well known that utilization of low cost and abundant waste materials in microbial fermentations can reduce product costs. Kitchen wastes disposed in large amounts from cafeterias, restaurants, dining halls, food processing plants, and household kitchens contain high amounts of carbohydrate components such as glucose, starch, and cellulose. Thus, kitchen wastes have high potential to be used as a substrate in ethanol fermentation. In this study, the effect of pretreatment method and sequential use of enzymes on hydrolysis of kitchen waste was evaluated. Fermentation experiments conducted with and without fermentation nutrients were also assessed at constant conditions of pH 4.5 and 30°C for 48 h using dry baker's yeast, *Saccharomyces cerevisiae*. Un-pretreated and hot water treated samples gave close glucose concentrations. The fermentation results indicated that ethanol can be produced at similar concentrations in no fermentation nutrients added samples and fermentation nutrients added samples ( $p > 0.05$ ). Thus, it is concluded that product cost can be lowered to a large extent if i) kitchen wastes are used as a substrate and 2) no fermentation nutrient is used. The results also indicated that kitchen waste contained adequate nutrients for yeast growth and maintenance.

**Keywords:** *Bioethanol, pretreatment, enzymatic hydrolysis, kitchen waste, yield*

### INTRODUCTION

Food wastes discharged from restaurants, food production plants and household kitchens constitute a considerable proportion of municipal solid waste (MSW) all over the world. OECD [1] statistics based on seven countries; Mexico, Greece, Japan, USA, Norway, France and Belgium stated that the MSW includes 35-40% organic waste, 28% paper, and minor amounts of metal (5%), glass (7%), and plastic (10%). In Turkey, the annual generation of MSW was reported as 26 million tons. Approximately 34% of the collected solid waste consists of kitchen waste. This results in 8.84 million tons of kitchen waste per year [2]. Kitchen wastes contain 2-3% cellulose, 40-55% starch, and 55-67% total sugar [3], which can be converted to fermentable sugars. Thus, attention has been directed towards bioprocessing of kitchen wastes to produce value added products such as lactic acid [4,5] and ethanol from starchy fraction [3,6].

Bioethanol is traditionally produced from sugar and starch containing crops such as potato, rice, and sugar cane in Brazil and corn in America and China [7,8]. Starch is easily converted to glucose by commercial enzymes and subsequently fermented to ethanol by *Saccharomyces cerevisiae*. Since these materials are important food sources and abundant / low cost lignocellulosic wastes can reduce production costs, investigations have been performed to use wheat straw, crop residues, and kitchen wastes as alternative substrates [9-11].

A pretreatment method is usually needed to have effective enzymatic hydrolysis when lignocellulosic materials are used [12-14]. The purpose of various pretreatment methods are to separate or remove lignin, hemicelluloses, and cellulose, reduce the crystalline structure of cellulose, and increase the surface area, thus provide better penetration of the enzyme [15, 16]. Alkaline and acid treatments have been successfully used [15, 17, 18]. Dawson and Boopathy [15] treated postharvest sugar cane residue with acid ( $H_2SO_4$ ) and alkaline ( $H_2O_2$ ) solvents. They reported that acid hydrolysis produced higher amounts of ethanol. Yu and Zhang [18] reported high concentrations of ethanol from acid hydrolyzed cotton wastes. Alternative pretreatment methods are also available, such as hot water and steam pretreatment [19]. In studies found on kitchen waste, which mostly focused on starchy fraction, no pretreatment method has been used prior to enzymatic hydrolysis [3, 6, 20].

Kinetic models play an important role in describing performance and attributes of a process and can easily be used to control and predict these attributes. It is commonly agreed that more valuable information can be extracted from an experimental data by simple inspection, e.g. assuming first order dynamics, statistical analysis, etc.. The goal in kinetic modeling varies with the attributes of the chemical or biological process. For pretreatment prior to enzymatic hydrolysis, the optimum time and type of the pretreatment are of great value to achieve increased yields at the subsequent hydrolysis step [21]. Increasing yields of enzymatic hydrolysis would also improve the yields of ethanol. Efficient utilization of sugars is also an opportunity to reduce costs [22]. Current literature is focused on use of 1) lignocellulosic and agro-industrial wastes and 2) various microbial strains in fermentation to improve ethanol production. In addition, pretreatment methods need to be settled down for commercial use.

Therefore, the aim of this work was primarily two folds; 1) to evaluate the effect of two pretreatment methods (acid and hot water) and a control on glucose production during enzymatic hydrolysis, and 2) to study the kinetics of glucose production to select the best treatment type and period of application for improvement of enzymatic hydrolysis prior to fermentation.

## MATERIALS AND METHODS

### Raw material

The kitchen wastes were collected from food courts of Middle East Technical University (METU), Ankara, Turkey. The plastic, metal, and glass pieces were separated if present in the waste, and remaining organic fractions were combined and ground in a chopper to form the composite substrate for experiments. The composite waste was stored at 4°C until use in a day or two.

### **Enzymes, Inoculum, and Fermentation Medium**

The enzymes used in liquefaction and saccharification steps were  $\alpha$ -amylase (A6211-1MU), amyloglucosidase (AMG) (10115), cellulase (C1794-10KU), and  $\beta$ -glucosidase (49290), which were all purchased from SIGMA-Aldrich. The activity of enzymes reported by the supplier was considered in our study.

A commercial dry baker's yeast *Saccharomyces cerevisiae* was purchased from a local store and kept in a refrigerator until use. The dry yeast was dispersed in sterile water at room temperature at a concentration of 10 g/L (g dry bakers' yeast / liter of DI water) and added as an inoculum without any cultivation [23].

Fermentation medium contained pretreated and hydrolyzed waste and the yeast, *Saccharomyces cerevisiae*. The traditional fermentation nutrients were not used. Here 'traditional nutrients' meant the following commonly used liquid medium; 6 g/L yeast extract, 1.5 g/L  $\text{KH}_2\text{PO}_4$ , 1 g/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 4 g/L  $(\text{NH}_4)_2\text{SO}_4$ .

#### **Pretreatment methods**

The ground and mixed kitchen waste was subjected to pretreatment with two solutions (hot water and dilute acid) and a control (no pretreatment). For dilute acid pretreatment, sulphuric acid at two concentrations of 1 and 4 % (v/v) was added to the kitchen waste. Samples were kept at 60°C for 3 hours in all pretreatment methods [24].

#### **Enzymatic Hydrolysis**

##### **Fermentation**

Fermentation experiments were conducted in 250 ml erlenmayer flasks with a working volume of 100 ml. The yeast was added at a ratio of 10% (v/v) to the fermentation mixture under aseptic conditions. Before inoculation, the flasks and medium were sterilized by autoclaving. Sulphuric acid (0.5 M) was used to adjust the initial pH to 4.5. The temperature and agitation speed were maintained constant throughout the experiment at 30°C and 150 rpm, respectively. The fermentation period was carried out for 48 h.

##### **Analytical Methods**

The collected waste was analyzed for moisture, ash, protein, fat and total carbohydrate contents. Moisture and ash contents were analyzed according to analytical gravimetric methods [26]. Protein content was determined as 6.25 times the Kjeldahl nitrogen. Glucose was analyzed by Dinitro Salsylic Acid (DNS) method [27]. Ethanol concentration was measured by GC (SHIMADZU, Kyoto, GC-14A #124457), using ethanol with 1, 3, and 5 % (v/v) as internal standards [28].

## **RESULTS AND DISCUSSION**

### **Composition of Raw Material**

The composition of kitchen waste is summarized in Table 1. The average moisture content of the kitchen waste was about 65% (w/w), which led to 35 % (w/w) of total dry matter. Approximately 60 % of

the total dry matter was the carbohydrate fraction, which proved that the kitchen waste could be used as a valuable raw material for ethanol production.

**Table 1.** Characteristics of kitchen waste used in the experiments.

Constituent	Content (% w/w)
Moisture	64.5
Total Solids	35.6
Protein	4.5
Fat	8.8
Ash	1.8
Total CHO's	20.5

<sup>a</sup> Results belong to two replicates.

### Effect of Pretreatment Method on Glucose Production

As stated before, the kitchen waste used in this study contained a wide variety of leftovers in raw and cooked form as well as whole edible parts and peels of fruits and vegetables. Therefore, in order to improve the yield of enzymatic hydrolysis a pretreatment method was used (Table 2). Each method was followed by enzymatic hydrolysis conducted under same conditions. Thus, the difference in final glucose concentrations was concluded to be due to the pretreatment method. This was also proved by the initial glucose concentrations after each pretreatment method (Table 2).

**Table 2.** Glucose concentrations after each pretreatment.

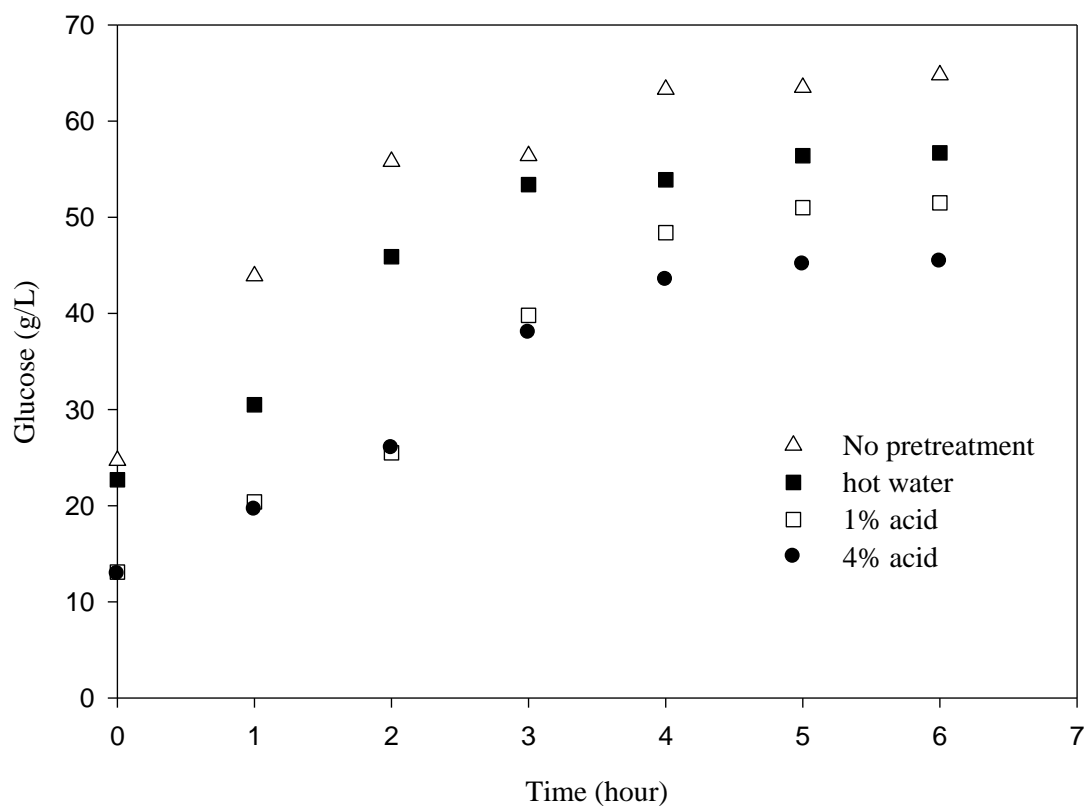
Pretreatment method	Glucose concentration (g/L)
1% acid	13.1
4% acid	12.9
Hot water	22.7
NPT	24.7

<sup>a</sup>NPT: No pretreatment method (control)

According to the tabulated values, it was found that hot water pretreated and no pretreated samples had higher glucose concentrations than the acid pretreated samples ( $p < 0.05$ ). The two acid levels (1 and 4%) had similar glucose concentrations ( $p > 0.05$ ).

The change in glucose concentration during enzymatic hydrolysis for pretreated samples over time is given in Figure 1. The concentration of glucose increased gradually with time and reached a constant value within 6 h for all pretreatment methods. The highest glucose concentration was obtained as 64.8 g/L from the unpretreated samples after 6h and followed by hot water treatment at 56.7 g/L. These values were found statistically similar ( $p > 0.05$ ) right at the edge of Tukey's confidence interval, which even can be practically taken as different. If taken similar, this result indicated that hot water pretreatment was not necessary and

the enzymatic hydrolysis process can be directly applied, being consistent with results of Tang et al. [6] and Wang et al. [3] and opposite the study of Laser et al [19]. The two acid concentrations (1% and 4%) also gave statistically similar results ( $p>0.05$ ) but still lower than no pretreatment case ( $p<0.05$ ), releasing glucose concentrations of 51.5 and 45.4 g/L, respectively. Furthermore, the glucose amount of 1% acid was the similar to the amount of hot water ( $p>0.05$ ). Thus, the acid treatment was also concluded to be an effective method for mixed kitchen wastes as reported by Dawson and Boopathy [15] who also applied acidic pretreatment on post-harvest sugarcane residue before fermentation.



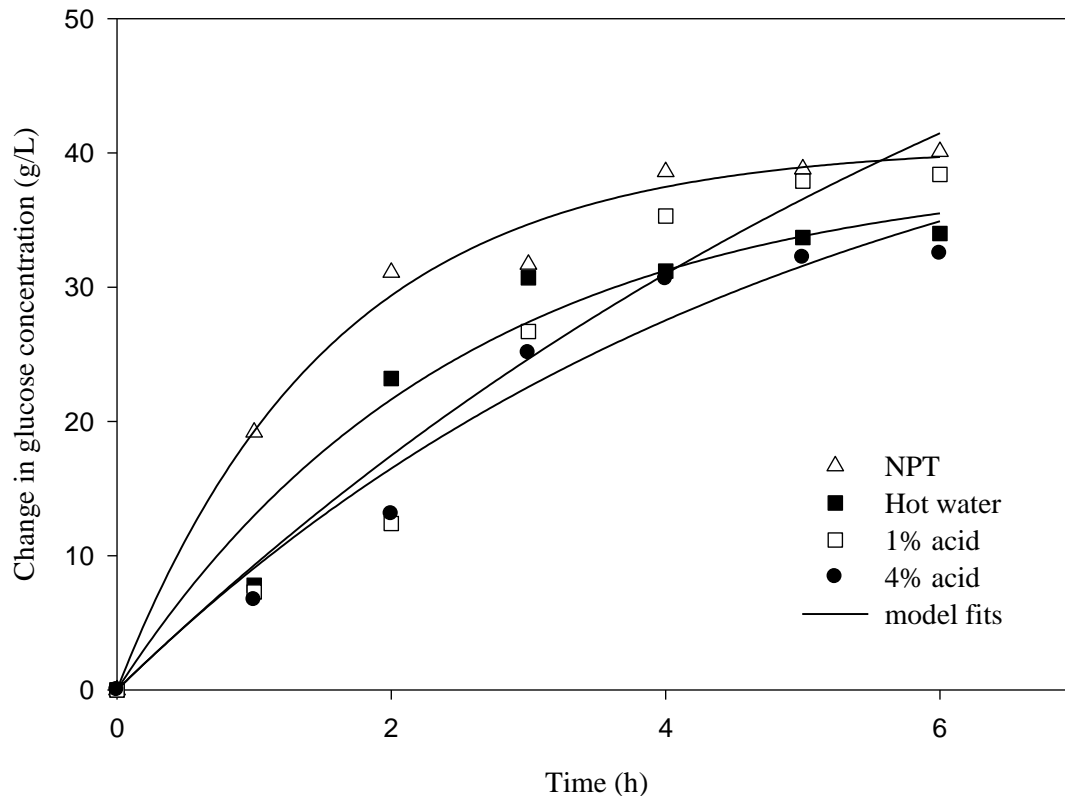
**Fig. 1.** Glucose production from hydrolyzed wastes subjected to different pretreatments

“The following equation considering first order dynamics was used to calculate the rate of glucose production during enzymatic hydrolysis after each pretreatment method:

$$C = C_m (1 - e^{-kt}) \quad (1)$$

where  $C$  is the change in glucose concentration (g/L) with respect to initial glucose concentration (i.e.,  $C(t) - C_0$ );  $C_m$  is the maximum glucose accumulated at an infinite hydrolysis time;  $k$  is the rate constant of glucose production ( $\text{h}^{-1}$ )

In order to fit the data in Fig.1 to Eqn.(1), all values were first transformed by subtracting the initial concentration from glucose produced at a given time,  $t$ , and the resulting plots with model fits are presented in Fig.2. All curves pass through the origin in the transformed form. The results of kinetic analysis for each pretreatment method are given in Table 3.



**Fig.2.** Transformed results for glucose production after each pretreatment method.

Surprisingly, the kinetic results revealed that the rate of glucose production was higher for 4% acid than the rate of 1% acid, although the final glucose concentrations for the two acid pretreatments were similar (Fig.1.). Thus, the rate constants in a descending order can be written as  $r_{NPT} > r_{HW} > r_{4\%} > r_{1\%}$  for no pretreatment (NPT), hot water, 4% acid, and 1% acid, respectively. When the time constant ( $\tau$ ), which is defined as the time required for the glucose level to reach 63.2% of the final steady level during hydrolysis process, is considered the NPT method had the smallest time constant value (1.55 h) while the 1% acid method had the highest value (7.81 h). Because the smaller the time constant is the faster hydrolysis process, the same sequence as the rate constant ( $k$ ) emerges.

**Table 3.** First order kinetics model parameters for the enzymatic hydrolysis after each pretreatment method.

Pretreatment	$C_m$ (g/L)	$R^2$	$k$ ( $h^{-1}$ )	$\tau$ (h)
NPT	40.54	0.989	0.644	1.55
Hot water	38.90	0.962	0.406	2.46
1% acid	77.52	0.958	0.128	7.81
4% acid	49.81	0.963	0.201	4.98

### Fermentation of Hydrolyzed Kitchen Wastes

Upon completion of the enzymatic hydrolysis step, the pretreated and saccharified waste was subjected to batch ethanol fermentation at pH 4.5 and 30°C for 48 h. The experimental plan and the results of fermentation experiments are shown in Table 4. The initial glucose concentration, final ethanol concentration and yield values are also given in Table 4. Statistical analysis of the results indicated that the ethanol concentrations and the yields were similar for hot water and control samples (NPT) ( $p > 0.05$ ). The ethanol concentrations were attributed to productivity values of 0.36 and 0.49 g/L.h for hot water and control samples, respectively.

**Table 4.** Fermentation results of control (NPT) and hot water pretreated kitchen wastes

Pretreatment	Glucose before fermentation (g/L)	Ethanol (g/L)	Yield (g ethanol/g glucose)
Hot water	56.7	17.2	0.30
NPT	64.8	23.3	0.36

<sup>a</sup> Results are averages of two replicates; NPT = No pretreatment method (control)

These results supported the idea that fermentation of kitchen wastes was practical without adding the traditional fermentation nutrients. Thus, it can be concluded that nutrients already present in the kitchen waste were sufficient for functioning of *S. cerevisiae* to produce ethanol. Our results are consistent with results of others. Wang et al. [3] reported fermentation of *Saccharomyces cerevisiae* with kitchen garbage at pH values of 4-6.63 and temperatures of 26.8-40°C. They obtained ethanol concentration of 22.13 g/L at 26.8°C within 48 h at pH 5, which can be taken as similar conditions of our study. Their productivity for these conditions was 0.46 g/L.h, similar to our value. It should also be noted that Wang et al. [3] used a pure yeast culture and working volume of 150 ml for fermentation experiments.

## CONCLUSIONS

The present work indicated that pretreatment prior to enzymatic hydrolysis is not strictly needed for production of high glucose levels from regular kitchen wastes. Another important result is that enzymatic hydrolysis can be as short as 6 h. Addition of fermentation nutrient is found to be not necessary for yeast to produce ethanol. The nutrients present in the original kitchen waste provide enough nutritive medium for *Saccharomyces cerevisiae* to produce high yields of ethanol. Thus, it is concluded that ethanol production costs could be lowered using kitchen wastes as substrate and by excluding the fermentation nutrients from traditional fermentation practice as well.

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## INVESTIGATION THE INTERACTIONS OF RICE HUSK WITH $Zn^{+2}$ IONS

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### ABSTRACT

The past ten years has seen a developing interest in the preparation of low-cost adsorbents as alternatives to activated carbon in water and wastewater treatment processes. Lately, the limited success of adsorbents in field applications has raised apprehension over the use of rice husk ash in the preparation of novel adsorbents as a measure to the environmental pollution control. The evolution has turned from an interesting alternative approach into a powerful standard technique by offering a numbers of advantages: better performance in terms of ulterior adsorption capacity, rate of adsorption, cost effectiveness in solving wastewater pollution problem and overcome part of the agricultural waste problem around the world. In this work, rice husk is activated and chemically reduced to provide an efficient support for zinc. The RH/Zn structures were characterized by zinc particle size. Distribution and morphology of nanoparticles have been characterized using X-ray diffraction. Scanning electron microscopy (SEM) showed that the Zn particles were distributed uniformly on the RH matrix.

**Keywords:** *active carbon, zinc, rice husk*

### INTRODUCTION

Rice is one of the major crops grown throughout the world, sharing equal importance with wheat as the principal staple food and a provider of nourishment for the world's population. Concern about environmental protection has aroused over the years from a global viewpoint. Rice husk consists of cellulose (32.24%), hemicellulose (21.34%), lignin (21.44%) and mineral ash (15.05%) (as well as high percentage of silica in its mineral ash, which is approximately 96.34%). Rice husk is insoluble in water, has good chemical stability, has high mechanical strength and possesses a granular structure, making it a good adsorbent material for treating heavy metals from wastewater. Rice husk ash, the most appropriate representative of the high ash biomass waste, is currently obtaining sufficient attraction, owing to its wide usefulness and potentiality in environmental conservation. [1]. The failure to make use of all of the RH is due, in part, to the double layer composed of silica and cuticle that resists invasion by insects and pathogenic organisms. This protective layer slows natural biodegradation, which discourages the re-utilization of RH in the agricultural and livestock industries. The ash content of RH, which is mostly silica, is approximately 20 mass%. This high ash content means that the carbon content is low, limiting the use of RH as a fuel source or as a precursor for the production of carbon based materials. However, RH do have a noteworthy advantage. The removal of heavy metals by rice husk has been extensively reviewed. Among the heavy metal ions studied include Cd, Pb, Zn, Cu, Co, Ni and Au [2].

Activated carbon is a highly porous form of solid carbon produced from carbonaceous raw materials using chemical or physical activation methods. Chemical activation has been known as an efficient method to obtain carbons with high surface area and narrow micro pore distribution. Chemical activation can be accomplished in a single step by carrying out thermal decomposition of raw material with chemical reagents. The most widely used chemicals include zinc chloride ( $ZnCl_2$ ), phosphoric acid ( $H_3PO_4$ ), and potassium hydroxide/carbonate ( $KOH/K_2CO_3$ ) [3].

Due to the outbreak of the infectious diseases caused by different pathogenic bacteria, the scientists are searching for new antibacterial agents. In the present scenario, nanoscale materials have emerged up as novel antimicrobial agents owing to their high surface area to volume ratio and the unique chemical and physical properties [16]. In recent years, the use of inorganic antimicrobial agents has been attracted interest for the control of microbes. The key advantages of inorganic antimicrobial agents are improved safety and stability, as compared with organic antimicrobial agents]. At present, most antibacterial inorganic materials are metallic nanoparticles and metal oxide nanoparticles such as zinc oxide [4].

The present work aims to provide an environmental friendly and cost-effective method for the preparation of a zinc support, prepared by activating and chemically reducing the rice husk. The RH/Zn structures were characterized by measuring zinc particle size.

## MATERIALS AND METHODS

### Prior carbonization of rice husk

Raw RH was milled to particles with size of about 1 mm. Activated carbon was prepared from rice husk by chemical activation with  $ZnCl_2$ . Impregnation ratio (1:1, 2:1, 3:1, 4:1) on the pore development were investigated 50 g of the air-dried rice husk and 10-100 g of  $ZnCl_2$  were well stirred in 200 cm<sup>3</sup> of distilled water using a hot plate/magnetic stirrer. Impregnation was carried out at approximately 358 K in a boiler-reflux condenser for 7 h. Several impregnation ratios, defined as the ratios of the mass of  $ZnCl_2$  to that of rice husk, were applied to prepare the impregnated samples. The impregnated sample was then filtered with a vacuum flask and dried at 105 °C for about 24 h. The rice husk powder was prepared through a carbonization treatment at 700 °C the obtained product was washed step by step: (1) with deionized water up to pH 7; (2) with 3 M HCl solution (3) with deionized water up to pH 7 again. The washed materials were dried overnight at 120 °C. Prior to surface modification, the husk were grinded and then sieved into a desired particle size of 10-40 μm. Then RH Powders were activated by stirring with a surfactant for 1 h. The activated RH powders were then immersed in 100 mL 25 wt.% aqueous ammonia to which was formed by adding  $Zn(NO_3)_2$  at room temperature. Stirring was continued under inert atmosphere at room temperature. The weight ratios of RH:  $Zn(NO_3)_2$  prepared were approximately equal to 1:0.25, 1:0.5, 1:1, 1:2, 1:5. After stirring for 1 h, dilute aqueous solution of hydrazine monohydrate was introduced to RH-Zn Composite solution in appropriate quantities using syringe. Stirring was continued under an inert atmosphere at room temperature for another 4h. Then particles were separated and washed with water, then dried. This sample was washed with deionized water several times and dried in oven at 110°C. In ion exchange study,  $Zn(NO_3)_2 \cdot 5H_2O$  was used as a cation source. Solid and solution phases were separated by centrifuging at 4000 rpm.

**Table 1.** Main characteristics of rice husk and activated carbons(RH and RHC)

Type	Rice huck	RHC <sub>1</sub>	RHC <sub>2</sub>	RHC <sub>3</sub>	RHC <sub>4</sub>
Carbonization temperature °C	-	700	700	700	700
Impregnation ratio (ZnCl <sub>2</sub> /RH)	-	1.0	2.0	3.0	4.0
Yields of activated carbon wt %	-	37.85	39.25	40.10	40.90
% Fixed Carbon	12.95	67.09	68.90	70.98	71.30
% Moisture	6.45	6.09	5.89	4.98	4.36
% ash	18.13	5.09	4.90	3.50	3.20
% Volatile Matter	62.47	21.73	20.31	20.54	21.14
% SiO <sub>2</sub>	93.5	92.35	92.28	92.08	91.75
% Al <sub>2</sub> O <sub>3</sub>	2.87	2.37	2.05	1.07	0.65
% Fe <sub>2</sub> O <sub>3</sub>	1.48	1.02	0.98	0.09	0.04
% CaO	0.2	0.0	0.0	0.0	-
Others	1.95	2.67	3.89	6.87	7.53

### Characterization

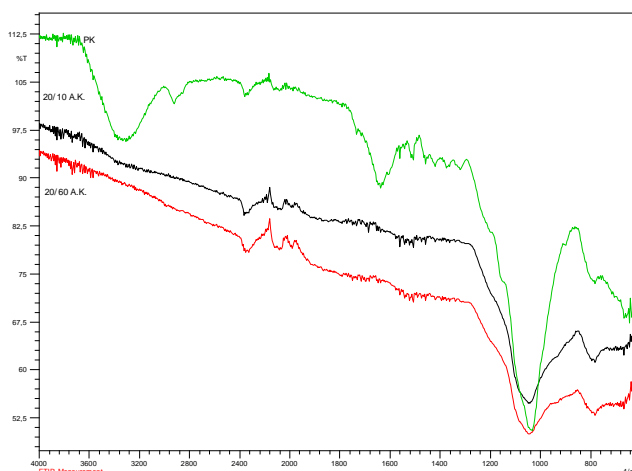
In addition, distribution and morphology of nanoparticles have been characterized using X-ray diffraction. Scanning electron microscopy (SEM) showed that the Zn particles were distributed uniformly on the RH matrix. FTIR (Fourier Transform Infrared Spectroscopy), and element analysis (EDX), studied the chemical characteristics of RH and Zn-RH char particles.

The yield of activated carbon was calculated based on the weight of rice husk on a dry basis from the following equation: Yield of activated carbon (w %)=weight of activated carbon/ weight of rice husk x100 [5]

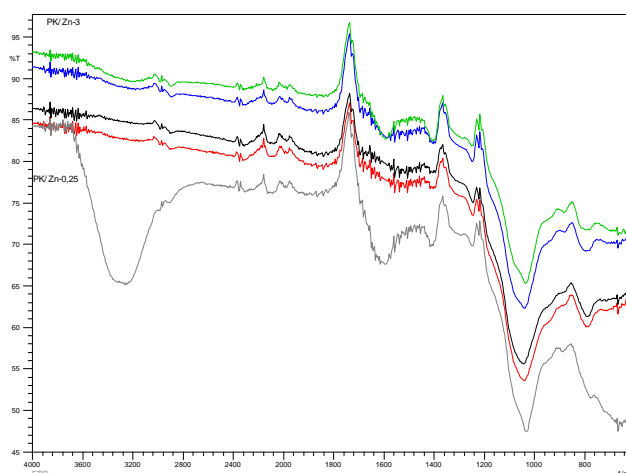
The highest yield obtained with activated carbon (RHC<sub>4</sub>) was used as starting material in the production of zinc composite.

**Table 2.** Elementary Analysis RHCZn (% w)

Type	RHCZn <sub>1</sub>	RHCZn <sub>2</sub>	RHCZn <sub>3</sub>	RHCZn <sub>4</sub>	RHCZn <sub>5</sub>
C	69.031	52.431	61.222	60.392	58.896
O	23.158	26.853	30.754	25.469	24.906
Si	7.155	18.136	3.96	8.604	8.568
Na			3.782		
Cl	0.253	0.244	0.178	0.134	0.002
Fe					
Zn	0.404	2.336	3.013	5.401	7.628

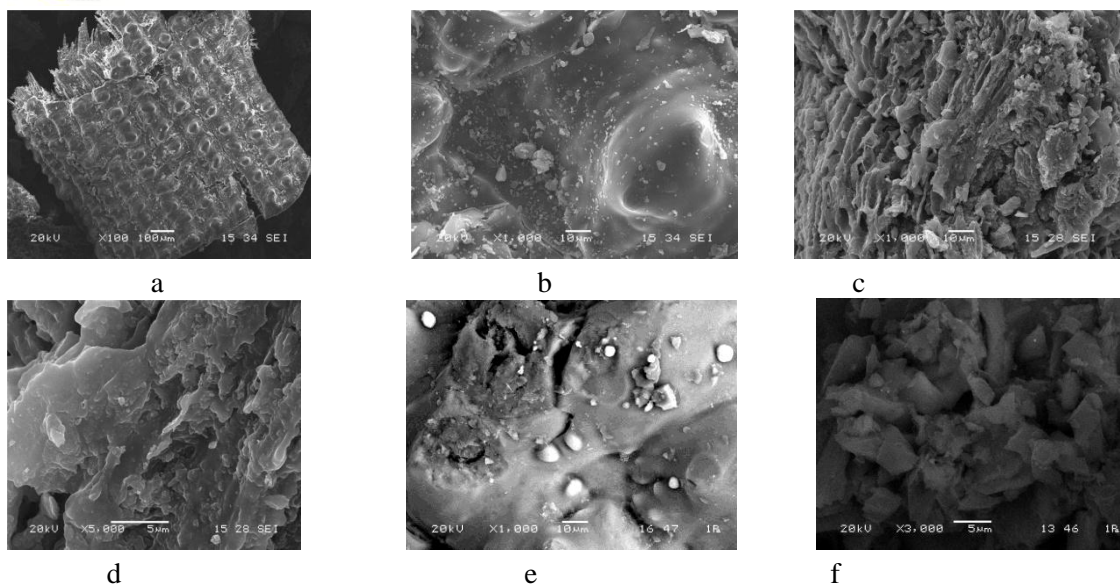


**Fig.1.** FTIR (Fourier Transform Infrared Spectroscopy), of the RH surfaces RH, RHC1, RHC2, RHC3, RHC4

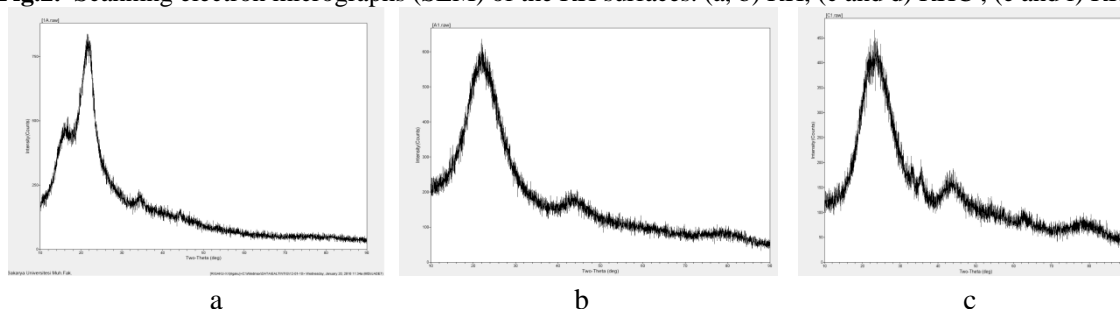


**Fig.2.** FTIR (Fourier Transform Infrared Spectroscopy), RHZn<sub>1</sub>, RHZn<sub>2</sub>, RHZn<sub>3</sub>, RHZn<sub>4</sub>, RHZn<sub>5</sub>

A band between 3200 and 3600  $\text{cm}^{-1}$  is typically ascribed to hydroxyl groups or adsorbed water. For the spectrum of cellulose, the broad band centered around 3300–3400  $\text{cm}^{-1}$  can be attributed to hydroxyl groups; the bands around 2900–2800 and 1500–1400  $\text{cm}^{-1}$  are caused by ACH<sub>2</sub>A groups; the band around 1300–1000  $\text{cm}^{-1}$  is attributed to CAO stretch. For carbons prepared from cellulose compared with the FTIR spectrum of cellulose, 1700 and 1600  $\text{cm}^{-1}$  while the bands of the spectrum of cellulose remain in the result. The band around 1700  $\text{cm}^{-1}$  is usually caused by the stretching vibration of in ketones, aldehydes, lactones, and carboxyl groups; and the band around 1600  $\text{cm}^{-1}$  is ascribed to aromatic ring stretching vibration. This indicates the formation of carbonyl-containing groups and the initial aromatization of the precursor. However, the intensities of these two bands show that the aromatization extent and the content of carbonyl-containing groups are very low at this point.



**Fig.2.** Scanning electron micrographs (SEM) of the RH surfaces. (a, b) RH, (c and d) RHC , (e and f) RHCZn



**Fig.3.** X-ray diffraction (XRD) of the RH surfaces. a) RH, b) RHC, c) RHCZn

## RESULTS AND DISCUSSION

The synthesis of the carbons with developed porosity is possible when non-carbonized RH is utilized. This method does not require a prior carbonization stage. In summary, we showed that an efficient zinc support was prepared by activation and chemical reduction of the Rice husk. The RH/Zn structures were characterized by zinc particle size. Distribution and morphology of nanoparticles have been characterized using FTIR, XRD. Scanning electron microscopy (SEM) showed that the Zn particles were distributed uniformly on the RH matrix. Rice husk carbon is thus a potential alternative to commercially available activated carbon as they have high selectivity and are efficient with low production costs.

## CONCLUSIONS

RH has appeared to be an interesting precursor for preparation of carbon because of inclusion of amorphous SiO<sub>2</sub> in significant amount in its composition, which possibly plays a role of a template during preparation of carbon materials. Regarding the experimental results, the activated carbon obtained from rice husk yield increases as the amount of ZnCl<sub>2</sub> increases. Porous rice husk particles have been successfully used as novel supports for the immobilization of zinc nanoparticles. Zn loading amount on BC increased with increasing zinc precursor concentration. RH/Zn composites are therefore believed to great potential for use as an antibacterial material.

## ACKNOWLEDGEMENT

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## UPGRADING, VIA FUNGAL FERMENTATION, OF AQUEOUS EXTRACTS FROM DRY OLIVE MILL RESIDUE

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### ABSTRACT

The present study deals with the screening of filamentous fungi able to grow on the aqueous extracts of dry olive mill residue (DOR), the solid waste derived from the olive oil two-phase extraction process, to produce enzymes of potential commercial interest, such as polyphenoloxidases. Plate tests and shaken cultures were preliminarily performed. The selection criteria were based on the capability of removing phenols, color and organic load and to produce extracellular enzymes involved in the degradation of both monomeric and polymeric aromatic compounds of the waste (*i.e.*, laccase, Mn-dependent peroxidase and mono-phenolase activities). Among the six fungal strains screened, *Phlebia* sp. DABAC 9 and *Lentinus(Panus) tigrinus* CBS 577.79 were most effective. These fungi were grown in a 3-liter bubble-column reactor and did not differ each one another with regard to the production of aromatics-degrading enzymes: they mainly released Mn-dependent peroxidase activity ( $576\pm 44$  and  $631\pm 53$  IU l<sup>-1</sup>, respectively) on aqueous DOR (25%, w/v) and, to a lesser extent, laccase and mono-phenolase activities. Phenol content and color were also significantly removed, reaching with the former fungus more than 90% removal.

**Keywords:** *dry olive mill residue, filamentous fungi, phenoloxidase enzymes; liquid culture*

### INTRODUCTION

The olive oil extraction industry worldwide has great economic and social importance especially in the Mediterranean and Middle East countries. This industry is constantly growing and generates large quantities of organic wastes and by-products with environmental problems caused by their accumulation or incorrect disposal [1-3].

In recent years a new two-phase extraction process has been introduced in modern mills. In Spain, the biggest olive oil producer in the world, the introduction of this technology was carried out in more than 90% of Spanish olive oil factories [1-3]. This process generates two fractions: a liquid phase (olive oil) and a water-rich solid organic waste ("alpeorujo"), which is dried and extracted with solvents to obtain an extra yield of oil and the dry olive-mill residue (DOR) [4]. It has been calculated that the Spain annual production of DOR, amounts to about four million tons [5].

This waste has a high concentration of organic matter which also includes toxic compounds such as polyphenols, polyalcohols and volatile fatty acids [1], capable of inhibiting microbial growth [6] and germination and vegetative growth in plants [7]. Due to its favourable C/N ratio, the application of DOR to agricultural soils as an organic fertilizer either directly or after composting has been proposed [4, 8]. However, the waste has been shown to require the addition of bulking agents in order to be effectively composted [4, 9].

Since DOR's toxicity has been mainly ascribed to phenols [10], the use of ligninolytic fungi capable of degrading such compounds [11, 12] can be an adequate upgrading approach. White rot fungi (WRF), such as, *e.g.*, *Phanerochaete chrysosporium*, *Coriolopsis rigida*, *Pleurotus pulmonarius* and *Panus tigrinus* have been shown to perform phenols removal from DOR and to detoxify the waste under solid-state conditions [13-15].

An alternative upgrading approach to the solid-state conversion of DOR implies its extraction with water leading to an aqueous extract, here termed ADOR, which, due to its content in soluble compounds (*i.e.*, simple sugars, oligosaccharides, organic acids, polyalcohols and inorganic cations), might constitute a growth medium for the microbial production of added value commodities [2, 16]. With this regard, the ADOR has been successfully used for exopolysaccharide production by *Paenibacillus jamilae* [16]. Moreover, the aqueous extraction delivers a residual solid waste characterized by a significantly reduced toxicity [17]. To date, the use of ADOR for the microbial production of enzymes has not been investigated. Besides containing compounds acting as growth substrates, DOR exhibits significant contents of phenols which can act as either stimulators or inducers of enzymes of commercial interest, such as laccase (E.C. 1.10.3.2, *para*-diphenol:oxygen oxidoreductase), Mn-peroxidase (E.C. 1.11.1.13, Mn<sup>2+</sup>:H<sub>2</sub>O<sub>2</sub> oxidoreductase, MnP) and monophenolase (E.C. 1.14.18.1, monophenol monooxygenase, MP).

Consequently, objectives of the present study were (i) to screen for filamentous fungi able to grow on the ADOR and to produce laccase, MnP and MP, (ii) to perform a preliminary assessment of process up-scaling on bench-top lab-scale bioreactors with the most promising strains and (iii) to determine the ability of the selected strains to reduce both organic load and toxicity of the ADOR.

## MATERIALS AND METHODS

### Materials and microorganisms

DOR, withdrawn from an olive oil manufacturer (Sierra Sur S.A., Granada, Spain), was stored at – 20 °C until used. The main physico-chemical characteristics of DOR were as follows: pH 5.1, total organic carbon 58.5%, total nitrogen 1.87%, total phosphorus 0.21%, lignin 24.7%, cellulose 18%, hemicellulose 12.8%, total phenols 3.18%, total lipids 0.2%, ashes 9.2%. The most abundant elements, the concentration of which is reported in g Kg<sup>-1</sup> DOR were: potassium 30.5, calcium 13.6, magnesium 3.8, iron 1.1, sodium 0.17, copper 0.07, zinc 0.06 and manganese 0.04.

The following filamentous fungi were used: *P. chrysosporium* NRRL 6361, *L. tigrinus* CBS 577.79, *C. rigida* CECT 20449, *P. pulmonarius* CBS 664.97, *Phlebia* sp. DABAC 9 and *Botryosphaeria rhodina*

DABAC P82. The strains were maintained and routinely sub-cultured on potato dextrose agar (PDA) slants.

### **Sample preparation, inoculum preparation and culture conditions**

ADOR was obtained by extraction with water in a 1:4 (w/v) ratio for 8 h at room temperature at 170 rpm in orbital shaker and subsequent vacuum filtration through Whatman n. 41 filter paper. The recovered water extract, designated as ADOR 25%, was stored at -20 °C until use.

ADOR 25% was used as the culture medium, without any supplementation and pH correction, after sterilization (121 °C for 20 min) for both shake flask batch and bioreactor cultures.

Ten-day-old PDA slant cultures were suspended in 5 ml of sterile deionized water and used as the inoculum for pre-cultures carried out at 28°C for 7 days under orbital shaking (180 rpm) in 500-ml Erlenmeyer flasks containing 95 ml of 2-fold diluted ADOR 25%.

Shake flask cultures were carried out in 500-ml Erlenmeyer flasks containing 95 ml of ADOR 25%. Five ml of pre-culture (see above) were aseptically added to each flask and incubated in an orbital shaker (180 rpm) at 28°C for 16 days. Samples were taken daily, centrifuged (3800 x g, 10 min) and the supernatants used for all tests. All experiments were performed in triplicate.

In the case of the bench-top bioreactor cultures, experiments were conducted in a 3-liter bubble-column reactor filled with 2 l of ADOR 25%. The following probes were installed on the column top: dissolved oxygen sensor (Ingold, CH), double reference pH sensor (Phoenix, AZ) and PT 100 temperature sensor. Experiments were performed under the following conditions: inoculum amount 5% (v/v); aeration rate 0.3 vvm; silicon anti-foam, 1 ml l<sup>-1</sup>; temperature 28° C. Fermentation parameters were monitored by an adaptive/PID digital controller, ADI 1030 (Applikon Dependable Instruments, Schiedam, NL). Sampling was as above. Each condition was tested in duplicate.

### **Enzyme assays and analytical determinations**

Laccase and Mn-peroxidase were assayed according to Saparrat et al. [18] while the mono-phenolase (MP) was assayed according to the method of Espin et al. [19].

The total phenol content of ADOR as such and after fermentation was determined according to Linares et al. [7], using syringic acid as the standard. Biomass, COD and color were estimated as previously described [11].

## **RESULTS AND DISCUSSION**

### **Screening of filamentous fungi**

The six fungal strains used in shake flask experiments were selected on the basis of their capability to both grow on agar media where 25% ADOR had been incorporated and to decolorize the anthraquinone-based dye Poly-R 478 (data not reported).

It is worth noting that the liquid medium used in the present work was merely made of 25% ADOR (w/v) and consequently had high COD and phenol content (48.0 ± 5.5 and 4.2 ± 0.2 g l<sup>-1</sup>, respectively).

Table 1 reports mycelial growth and maximal laccase, MnP and MP activities produced by the fungal strains grown on ADOR. With the only exception of *P. ostreatus*, all fungi grew well on ADOR. Best laccase production was observed in *C. rigida* cultures albeit the attainment of the activity peak required 16 days of incubation. In *L. tigrinus* and *Phlebia* sp. cultures, instead, laccase activity peaks (323±8 and 469±31 IU l<sup>-1</sup>, respectively), although lower than those of *C. rigida*, were obtained within significantly shorter incubation times (8 and 4 days, respectively). *L. tigrinus* and *Phlebia* sp. were also the most effective MP producers reaching similar activity peaks values on day 4; the same fungi produced higher MnP activities than the remaining strains although the activity peaks were observed in the late phases of culture (12 and 16 days, respectively)

All the screened fungal strains showed good ability to remove both phenols and color (Table 2). Interestingly, in both *L. tigrinus* and *Phlebia* sp. cultures, the large majority of total phenols in ADOR (89.4 and 89.7%, respectively) were removed within the early 4 days of fermentation.

For this reason, the highest MDR values (39.3 mg l<sup>-1</sup> h<sup>-1</sup> for both strains), were obtained with these fungi. The least efficient strain in the removal of phenols was *P. pulmonarius* the MDR values of which were 4.2-fold lower than those of *L. tigrinus* and *Phlebia* sp.; these two strains were also the most selective phenols degraders as indicated by the high values of DS, a parameter where the amount of phenols removed is related to the mass of COD consumed.

**Table 1.** Biomass production and maximal laccase, Mn-peroxidase (MnP) and mono-phenolase activities (MP) of different fungi grown on ADOR 25% in shaken culture. Values between square brackets indicate the incubation time at which the activity peak was reached

Fungus	Laccase (IU l <sup>-1</sup> )	MnP (IU l <sup>-1</sup> )	MP (IU l <sup>-1</sup> )	s	Biomass (g l <sup>-1</sup> )*
<i>B. rhodina</i>	9±0 [4]	140±12 [4]	11.2±1.3 [8]		9.7±1.1
<i>C. rigida</i>	973±2	122±24 [4]	12.5±0.5 [8]		4.7±0.6
	[16]				
<i>L. tigrinus</i>	323±8 [8]	315±39 [16]	13.7±2.2 [4]		5.2±0.5
<i>P. chrysosporium</i>	9±0 [4]	105±5 [8]	8.9±0.3 [8]		10.3±1.
				0	
<i>P. pulmonarius</i>	9±0 [4]	77±3 [4]	3.5±0.4 [4]		1.8±0.2
<i>Phlebia</i> sp.	469±31	419±11 [12]	13.1±1.3 [4]		7.6±0.5
	[4]				

\*Mycelial biomass was determined at the end of fermentation (16 days).

**Table 2.** Maximal phenol and color removals, maximal dephenolization rate (MDR) and dephenolization selectivity (DS) observed in liquid cultures of different fungi grown on ADOR 25% in shaken flasks. Values between square brackets indicate the incubation time at which maximal removal was reached.

Fungus	Phenol removal (%)	Color removal (%)	MDR†(mg l <sup>-1</sup> h <sup>-1</sup> )	DS‡
<i>B. rhodina</i>	81.7 [8]	n.d.*	17.9	.35
<i>C. rigida</i>	91.7 [12]	60.0 [16]	13.4	.50
<i>L. tigrinus</i>	89.6 [4]	57.9 [12]	39.2	.23
<i>P. chrysosporium</i>	92.5 [12]	51.1 [12]	13.5	.31
<i>P. pulmonarius</i>	63.6 [12]	37.9 [12]	9.3	.19
<i>Phlebia sp.</i>	89.7 [4]	73.9 [16]	39.2	.42

\*n.d., not detected; †MDR, maximal dephenolization rate (mg phenols removed l<sup>-1</sup> h<sup>-1</sup>); ‡DS, dephenolization selectivity (calculated by the ratio of maximal amount of removed phenols to COD depleted at that time).

*L. tigrinus* and *Phlebia* cultures also removed chromophoric compounds from ADOR leading to decolorization efficiencies of 57.9 and 73.9%, respectively.

For all these reasons, these strains were selected for further studies at the bench-top bioreactor scale.

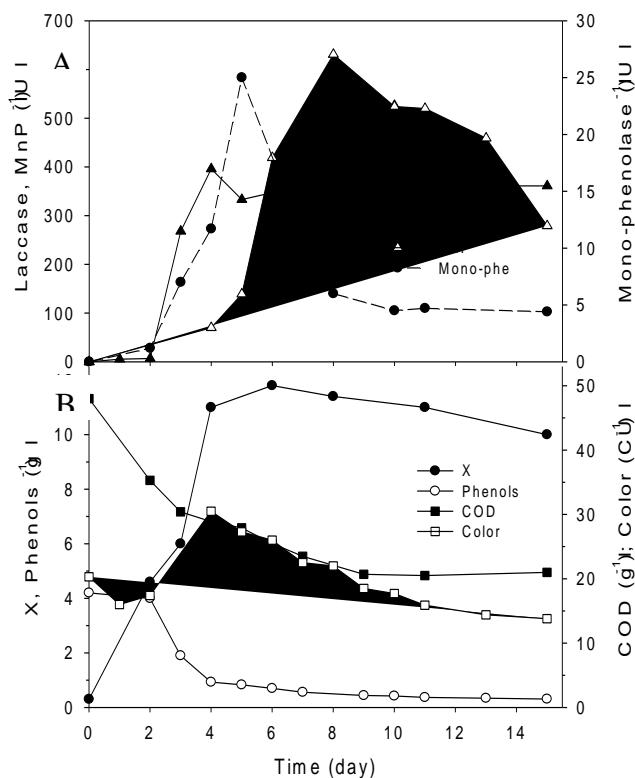
#### **Production of phenoloxidase enzyme activities in bench-top bioreactor**

To gain preliminary indications on process transfer, the two selected fungi were grown in a 3-liter bubble-column reactor. The choice of this kind of reactor with pneumatic agitation system instead of a stirred tank reactor was based on previous studies on olive-mill wastewater upgrading where *L. tigrinus* was comparatively grown in mechanical and pneumatic agitated reactors [20]: the last one resulted the most performing systems for phenoloxidase production.

Figs. 1 and 2 show the time courses of growth, enzyme productions and substrate consumption by *L. tigrinus* and *Phlebia sp.*, respectively, grown on ADOR 25% in bioreactor.

In the case of the former strain all enzyme activities increased significantly passing from shaken flask to the bioreactor scale (396±9, 631±53 and 25±1 IU l<sup>-1</sup> of laccase, MnP and MP activities, respectively) (Fig. 1A). Laccase activity reached also a second peak (526±66 IU l<sup>-1</sup>) after 10 days of fermentation. With the exception of the mono-phenolase, laccase (first peak) and MnP activity peaks were obtained with a marked reduction of the fermentation times with respect to shaken cultures. The fungus grew rapidly on the ADOR 25% up to 11.8 g l<sup>-1</sup> after only 6 days of fermentation and caused rapid COD

and phenols abatements. At the end of fermentation COD, total phenols and color were reduced by 57.1, 92.4 and 32.0%, respectively (Fig. 1B).



**Fig. 1 A, B.** Time course of mycelial growth (X, 1B) and laccase, Mn-peroxidase (MnP) and mono-phenolase activity productions (1A) by *L. tigrinus* CBS 577.79 grown on ADOR 25% in bubble-column bioreactor. Evolution of COD, phenol content and color is also reported (1B).

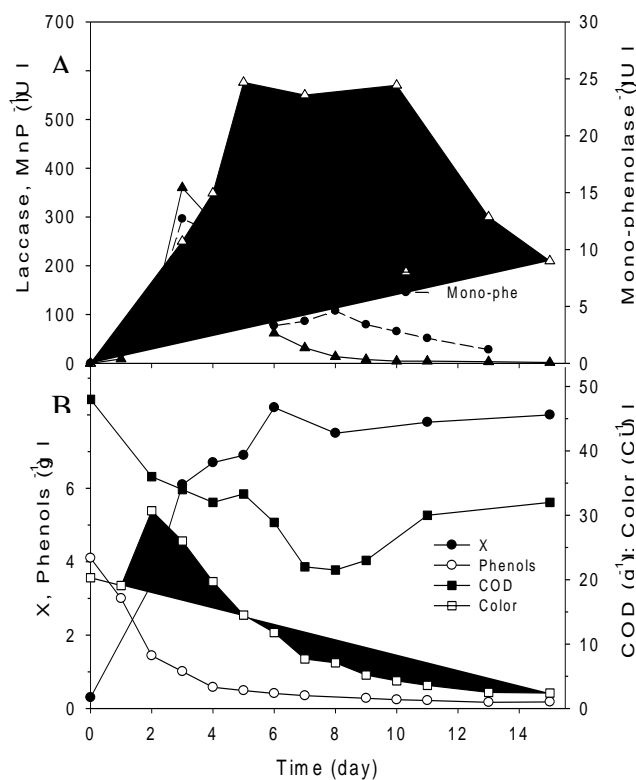
Similarly, the production of enzyme activities by *Phlebia* sp. peaked earlier than shaken cultures but only MnP activity increased significantly ( $576.5 \pm 74 \text{ IU l}^{-1}$ ) (Fig. 2A). Laccase activity was markedly lower ( $360 \pm 41$  vs.  $469 \pm 31 \text{ IU l}^{-1}$  shaken flask culture), while MP one did not significantly differ ( $12.7 \pm 1.7$  vs.  $13.1 \pm 1.3 \text{ IU l}^{-1}$ ). Fungal growth was rapid and reached the maximum ( $8.3 \text{ g l}^{-1}$  after only 6 days) to maintain this biomass level thereafter. COD removal reached the minimum ( $21.6 \text{ g l}^{-1}$ , 55.1% of removal) after 8 days and then increased most likely due to a partial lysis of the mycelium. On the other hand, *Phlebia* sp. showed a good efficiency in total phenols and color removal (95.7 and 88.0%, respectively, at the end of fermentation).

Although the maximal productions of phenoloxidase activities by *L. tigrinus* were higher than by *Phlebia* sp., the enzyme volumetric productivities of the latter were better with the exception of mono-phenolase activity (Table 3).

## CONCLUSIONS

The upgrading of ADOR aimed to fungal production of phenoloxidase enzymes of potential industrial interest is technically possible. In particular, *L. tigrinus* CBS 577.79 and *Phlebia* sp. DABAC 9 were able to grow on this waste mainly producing MnP activity and, to a lesser extent, laccase and mono-phenolase activities. Also the scale transfer to bench-top bioreactor of the fermentation process was positive since a shortening of the enzyme production kinetics and/or an increase of enzyme activity levels occurred. Phenol content, color and COD were also significantly removed at the end of the fermentation processes.

Although, further optimization and scale-up experiments are still needed the technical feasibility of a fermentation process for fungal production of phenoloxidase, mainly MnP-dependent peroxidase, activities on ADOR is realistic.



**Fig. 2 A, B.** Time course of mycelial growth (X, 2B) and laccase, Mn-peroxidase (MnP) and mono-phenolase activity productions (2A) by *Phlebia* sp. DABAC 9 grown on ADOR 25% in bubble-column bioreactor. Evolution of COD, phenol content and color is also reported (2B).

**Table 3.** Laccase, Mn-peroxidase (MnP) and mono-phenolase (MP) volumetric productivities when *L. tigrinus* CBS 577.79 and *Phlebia* sp. DABAC 9 were grown on ADOR 25% in bubble-column reactor. In bracket is reported the corresponding fermentation time.

Fungus	Laccase productivity (IU l <sup>-1</sup> h <sup>-1</sup> )	MnP productivity (IU l <sup>-1</sup> h <sup>-1</sup> )	MP productivity (IU l <sup>-1</sup> h <sup>-1</sup> )
<i>L. tigrinus</i>	4.12 [4] 2.19 [10]	3.28 [8]	0.21 [5]
<i>Phlebia</i> sp.	5.00 [3]	4.80 [5]	0.17 [3]

### ACKNOWLEDGEMENT

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## FATE AND BIODEGRADABILITY OF OLIVE MILL WASTEWATER IN A VETISOL-TYPE SOIL

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### ABSTRACT

Controlled land spreading of untreated olive mill wastewater (OMW) has been widely practiced as a mean of their disposal. The Israel Ministry of Environmental Protection permits land spreading of 40-50 m<sup>3</sup>-OMW ha<sup>-1</sup> yr<sup>-1</sup>, every alternate year in the same area. However, judicious selection of sites that are safe for spreading is still hampered by the limited knowledge about potential transport and biodegradation rates under field conditions. In the present study the fate and biodegradability of OMW after application is being evaluated in a Vertisol-type (clayey) soil from Jezreel Valley, northern Israel, representing a region of potential OMW application in Israel. Biodegradation rates are assessed from the dynamics of dissolved organic carbon (DOC) and total phenols (TP) in soil extracts. Other effects on soil properties are evaluated from the pH, EC, microbial counts (total and fungal) and phytotoxicity to cress (*Lepidium sativum* L.). The soil was collected from an organic peach orchard. It was mixed with OMW (at doses equivalent to 80 m<sup>3</sup> ha<sup>-1</sup>) or with the same amount of tap water (control) and further wetted to 70% field capacity. Replicate 500 g soil portions were transferred to pots and incubated for three months under cool/warm (12 or 25°C) and moisture-non-compensated (dry) or moisture-compensated (wet) conditions (20-24% or 28% moisture contents), in four different combinations: cool-dry, warm-dry, cool-wet and warm-wet. Biodegradation rates were found to be generally greater under wet conditions. After 90 days of incubation under warm-wet conditions, the concentrations of DOC and TP decreased by 83 and 88%, respectively. Under warm-dry conditions, the DOC decreased by 69% during the first two weeks but then increased substantially, presumably due to release of organic matter from desiccated microbial cells. The TP decreased by 79% under these conditions. The phytotoxicity of OMW application (expressed by roots lengths as compared to water control) was completely disappeared after two weeks of incubation. It is evident from these results that weather conditions (ambient temperature and rainfall) would affect the fate of OMW after application. Relatively warm temperatures and high moisture can maximize biodegradation rates and minimize leaching of OMW constituents. An on-going study focuses on Loess-type soil (sandy loam) which represents parts of the Negev region, southern Israel. This is another potential region of OMW application. After completion, this study will provide information that is necessary to better assess potential accumulation and leaching of OMW organic constituents and the potential phytotoxicity associated with permitted doses of application in Israel.

**Keywords:** olive mill wastewater, land spreading, biodegradation, phytotoxicity, total phenol

## **MAKING ORGANIC FERTILIZERS BY COMPOSTING TWO-PHASE OLIVE MILL WASTE (“ALPERUJO”) AND POULTRY MANURE**

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### **ABSTRACT**

A 500-ton windrow was composted in a factory to manufacture marketable organic fertilizers by mixing a two-phase olive-mill waste ("alperujo"), stored in open basins for 5 years, with 25% of fresh poultry manure as a bulking and amending agent. After previous mixing and homogenization during 1 week, an industrial by-product mainly consisting of iron sulphate was added to a separate 50-ton portion of the windrow in order to decrease N losses during composting and also increase available Fe, then aeration by mechanical turning was extended for 19 more weeks until the thermophilic phase of composting was finished. For marketing purposes, both resulting end-products were catalogued as NPK organic fertilizers according to the Spanish guidelines, due to their substantial (N+P<sub>2</sub>O<sub>5</sub>+K<sub>2</sub>O) content, close to 9%, and C/N ratio

### **INTRODUCTION**

The wide use of the two-phase continuous-centrifugation system for olive oil extraction in Spain has led to the production of a solid material known as olive husk or “alperujo” (AL), which is the main by-product generated by this industrial sector (approximately four million tons annually). AL is composed of olive pulp, residual oil, vegetation water and fragments of the stone, together with a small amount of water occasionally added during the extraction process. It is an acidic and very humid material, rich in organic matter, potassium and nitrogen, but also containing fats and water-soluble carbohydrates and phenols (Alburquerque et al., 2004), the later being mainly responsible for its phytotoxicity.

Composting, as a method for preparing organic fertilizers and amendments, is economically and ecologically sound and may well represent a suitable option for adding value to AL. The compost obtained shows advantages mainly due to a high content of humic-like substances (HS), which play a major role in soil fertilization, but also due to the abundance of nutrients such as potassium and nitrogen. The latter, however, is easily lost during composting mainly by ammonia volatilisation due to the rapid pH increase. The N-losses diminish economical benefits (fertilizer value) and contribute to air pollution. Several strategies have been assayed to reduce N-losses during composting ( Mahimairaja et al., 1994; Hao and Benke, 2008), one of them has been the use of acidifying agents such as elemental sulphur, calcium sulphate, iron pyrite and sulphuric acid; iron sulphate was employed in our research.

The role of organic matter has changed in modern agriculture, where chemical fertilizers have become the major source of crop nutrients. The use of intensive agricultural methods generally leads to fertility loss, soil

erosion, water contamination, soil compaction and organic matter content decline. Nowadays, there is considerable evidence about the pollutant impact of mineral fertilizers on the environment, due to their huge application and the fact that crops use them inefficiently; thus, increasing attention has been paid to organic fertilizers that might provide plant nutrients more properly and help growers to maximise crop yields. Further, simultaneously applying irrigation water, nutrients and HS to soil in the same operation, the so-called fertigation, satisfies the requirements of many plant species at their different development stages and avoids nutrients losses, thus enhancing crop yield and reducing evaporation of water and irrigation frequency (Parrado et al. 2008).

On the basis of the above information and from previous studies of our research group into AL composting (Cegarra et al., 2006; Alburquerque et al., 2007; Alburquerque et al., 2009), our technical assistance was required by TETMA S.A., a Spanish company which collects a great variety of agricultural wastes and produces organic fertilizers, operating in a modern composting plant located in Eastern Spain.

The objective of this research project was to produce marketable solid and liquid organic fertilizers, rich in HS and nutrients, by composting AL with poultry manure (PM), the latter being very abundant in the area near the composting facility. The first step has been reached already, making pellets from the composts for selling. The second is now in progress: the manufacture of liquid organic fertilizers by stirring the composts with alkalis according to different extraction conditions already defined but not yet all tested.

## **MATERIALS AND METHODS**

### **Description of the raw materials**

Due to the large scale of the composting operation and likely heterogeneity of the starting materials (AL and PM), the analytical data provided should be considered as merely indicative. Thus, the AL exhibited a clear alkaline pH value, low fat content and a TOC/TN ratio around 14 (Table 1). It also had considerable contents of P, K, Ca and TN, a short fraction of it being ammonium, as well as an abundance of micronutrients, mainly Fe and Zn. All these parameters were out of the typical ranges found by Alburquerque et al. (2004) for AL composition. These results must be related mostly to the prolonged storage period (up to five years) of the AL employed, which induced compositional changes in AL due to spontaneous anaerobic fermentations.

The PM showed lower pH, but higher electrical conductivity (EC) values and ammonium content than AL. In addition, the organic matter content was similar in both materials, but those of total organic carbon and lignin were much lower in manure, revealing the more complex chemical structure and recalcitrant characteristics of the AL substrate. The by-product rich in iron sulphate (FeB), added to the composting mixture as an acid mineral amendment, was an industrial waste from TiO<sub>2</sub> production.

### **Composting performance**

A 500-ton windrow was composted by mixing the AL with 25% (on a fresh weight basis) of PM as a bulking and amending agent. After previous mixing and homogenisation during one week, the FeB was added to a separate 50-ton portion of the windrow. Frequent mechanical turnings, at least one per week,

were applied during the active phase of composting in order to homogenise, aerate and avoid high temperatures in the composting substrate.

Due to the vast size derived from the industrial composting scale, two areas per windrow were limited for sampling. Sub-samples were taken from randomised sites in the mentioned areas and composite, representative samples of approximately 5 kg were obtained after mixing and homogenising them; later, they were sub-divided into three new sub-samples in the laboratory. One of them was frozen (-20°C) and kept for the determination of  $\text{NH}_4^+$ -N and  $\text{NO}_3^-$ -N, the second was dried in an oven at 105°C for 24 hours to determine the moisture content and the third was air-dried and ground to less than 0.5 mm prior to analysis.

**Table1.** Main characteristics of the raw materials: “alperujo” (AL) and poultry manure (PM) (dry weight basis).

Parameters	AL	PM
pH	8.53	6.84
EC ( $\text{dS m}^{-1}$ )	5.11	7.92
Organic matter (%)	80.71	82.41
Lignin (%)	34.10	16.25
Fats (%)	3.95	1.40
TOC (%)	40.66	36.96
TN (%)	2.95	3.80
$\text{NH}_4^+$ -N ( $\text{mg kg}^{-1}$ )	1706	7324
$\text{NO}_3^-$ -N ( $\text{mg kg}^{-1}$ )	62	7
TOC/TN ratio	13.8	9.8
P (%)	1.21	1.36
K (%)	3.59	3.44
Ca (%)	3.40	2.36
Mg (%)	0.56	0.64
Na (%)	0.19	0.48
S (%)	0.50	0.48
Fe ( $\text{mg kg}^{-1}$ )	2470	686
Cu ( $\text{mg kg}^{-1}$ )	251	68
Mn ( $\text{mg kg}^{-1}$ )	225	470
Zn ( $\text{mg kg}^{-1}$ )	625	329

#### **Extraction and fractionation of potential liquid organic fertilizers and analytical methods for solid and liquid samples.**

Based on previous research (González, 2005), the potential use of the end-composts to obtain liquid organic fertilizers containing HS and nutrients was evaluated by treating them with 0.1 M NaOH (1:20 w/v) and constantly stirring in sealed bottles for 24 h at 20°C; later they were centrifuged at 23,500g for 20 min and the supernatant (alkali-extractable fraction, AEF) carefully removed. Fractionation of AEF was carried out by carefully adjusting the pH to 2.0 with concentrated HCl. The

precipitate obtained (humic-like acids, HA) was allowed to coagulate for 24 h at 4 °C and was then separated by centrifugation as before from a new supernatant corresponding to the fulvic-like fraction (FF). Both AEF and FF were analysed as described before and analytical results of FF were subtracted from those of the initial supernatant, thus allowing characterisation of the HA. The FF in turn was fractionated into its organic fraction (OFF) and mineral fraction (MFF) by treating it with active charcoal. The treatment was performed by stirring in sealed bottles for 1 h at room temperature an aliquot of FF with the charcoal (1:50 w/v), later centrifuging as above and separating the colourless supernatant (MFF) for elemental analysis. The elemental content of OFF was calculated by subtracting analytical results of MFF from those obtained for FF. It is to be noted that NaOH was used in this preliminary research in order to ascertain the K extractability, even if Na is known to affect negatively both soil and plant growth, but use of KOH instead of NaOH is planned in further experiments since K is well recognised as an essential plant nutrient: the similar alkalinity of both extractants means that they will be similarly able to release soluble elements from compost.

The nutrient and organic carbon contents in raw materials, composts and the resulting extracts (AEF, FF and MFF) as well as other analytical parameters were determined as follows: pH and EC in a water suspension of 1:5 (w/v) extract; ash content after ignition at 550 °C for 24 h; total nitrogen (TN) and organic carbon (TOC) in solid samples by automatic microanalysis (Navarro et al., 1991); P, K, S, Na, Ca, Mg, Fe, Cu, Mn and Zn in both solid and liquid samples by inductively coupled plasma-optical emission spectrometry after H<sub>2</sub>O<sub>2</sub>/HNO<sub>3</sub> digestion in an ultraclave-microwave; fats by Soxhlet extraction with petroleum ether followed by further weighing, and lignin according to the American National Standards Institute and American Society for Testing and Materials (ANSI and ASTM, 1977). In addition, organic carbon and nitrogen in liquid samples were determined by using an automatic analyzer, NH<sub>4</sub><sup>+</sup>-N by a colorimetric method based on Berthelot's reaction (Sommer et al., 1999), after extraction with 2 M KCl, and NO<sub>3</sub><sup>-</sup>-N by ionic chromatography.

### **Statistical analyses**

Statistical analysis of data (ANOVA) was performed using the SPSS 18.0 program for Windows.

## **RESULTS AND DISCUSSION**

### **Characteristics of the solid organic fertilizers.**

The resulting composts (Table 2), without (C) and with FeB addition (FeC), showed the same organic matter content (75%, dry weight basis) with being lignin its major component (41% with respect to the total organic matter content). However, the total organic carbon represented 43% and 48% of the total organic matter in C and FeC composts, respectively, suggesting a relatively more complex organic structure in FeC. The fat content and the TOC/TN ratio of both composts were quite similar, while the addition of FeB led to significantly lower pH and higher EC values also in FeC.

With respect to the nutrient content, K and Ca showed the highest concentrations in both composts (> 3%) whereas those of TN and P were lower, the latter being slightly lower in FeC. On the contrary, both total- and

ammonium-N concentrations were significantly higher in FeC: these findings should be related to a certain decrease of N-losses during composting due to the above mentioned acidifying effect of the FeB. Finally, S, Fe and Mn contents were also clearly higher in FeC since all of them were added in the FeB.

For marketing purposes, both resulting composts were catalogued as NPK organic fertilizers according to the Spanish agricultural guidelines (BOE, 2005), due to their substantial N+P<sub>2</sub>O<sub>5</sub>+K<sub>2</sub>O content (higher than 9%) and TOC/TN ratio <15. Thus, the fertilizers obtained exceed largely the official requirements, which stipulate 4% as the minimum content of the sum of the three nutrients and 1%, at least, of each one.

**Table 2.** Characteristics of the AL composts (dry weight basis).

Parameters	C	FeC	ANOVA
pH	8.97	8.13	***
EC (dS m <sup>-1</sup> )	5.40	7.18	**
Organic matter (%)	74.65	74.63	NS
Lignin (%)	30.61	30.92	NS
Fats (%)	0.85	0.81	NS
TOC (%)	31.96	35.90	***
TN (%)	2.48	2.83	**
NH <sub>4</sub> <sup>+</sup> -N (mg kg <sup>-1</sup> )	1811	2610	***
NO <sub>3</sub> <sup>-</sup> -N (mg kg <sup>-1</sup> )	146	110	NS
TOC/TN ratio	12.9	12.7	NS
P (%)	1.28	1.18	*
K (%)	3.06	3.01	NS
Ca (%)	3.67	3.79	NS
Mg (%)	0.74	0.74	NS
Na (%)	0.32	0.32	NS
S (%)	0.61	0.94	***
Fe (mg kg <sup>-1</sup> )	3409	6413	***
Cu (mg kg <sup>-1</sup> )	135	131	NS
Mn (mg kg <sup>-1</sup> )	409	564	***
Zn (mg kg <sup>-1</sup> )	465	448	NS

NS: not significant, \*\*\*, \*\* and \*: significant at the probability level P < 0.001, 0.01 and P < 0.05, respectively.

### Characteristics of the potential liquid organic fertilizers

The highest extraction rate (ER), calculated as “(alkali-extractable element/total element)×100”, was > 80% for K (Table 3), followed by S (close to 70%) and N (40-50%); whereas the lowest ER value was shown by Mn (close to 10%). Consequently, the alkali-extracts obtained from both composts were mainly rich in K (roughly 1.2 g/l) and N (0.5 g/l), but also in TOC (around 4.5 g/l) as a typical component of the organic matter (data not shown). They contained much less of the micronutrients, only Fe, Mn and Zn being detected.

When the extracts from both composts were fractionated into HA, OFF and MFF, more than 70% of the TOC and 60% of the TN were included in the HA as both are typical organic matter constituents, while more than 50% of P, K and Mn contributed to the less polymerised chemical structure of the OFF and more than 30% of K, Mn and Zn contributed to the MFF fraction.

The comparison of the two composts revealed that all macronutrients, except S, had relatively lower ER values in FeC, but the opposite was found for the micronutrients. All nutrients were mostly included in both organic fractions, but the FeB addition caused generally greater retention of them in the OFF fraction; this effect was mainly evident for the two elements added in FeB (Fe and S). The influence of the FeB addition on the nutrient content of the mineral fraction was generally scarce, except for the mentioned added elements whose content in mineral form increased appreciably as a result of this addition.

**Table 3.** Elements extractable with 0.1 M NaOH from the AL composts (dry weight basis).

Element	Compost	ER (%)	HA (%)	OFF (%)	MFF (%)	HA/OFF
TOC	C	31.87	75.89	23.21	0.90	3.27
	FeC	26.34	71.20	27.52	1.28	2.59
	ANOVA	***	***	***	***	***
TN	C	51.77	65.66	23.47	10.87	2.81
	FeC	41.81	61.69	26.74	11.57	2.31
	ANOVA	***	**	*	NS	**
P	C	34.32	6.73	69.96	23.31	0.10
	FeC	29.43	5.95	73.69	20.36	0.08
	ANOVA	***	NS	**	**	NS
K	C	88.94	6.94	56.28	36.78	0.12
	FeC	84.14	4.41	57.83	37.76	0.08
	ANOVA	*	NS	NS	NS	NS
S	C	68.84	50.10	36.10	13.80	1.39
	FeC	77.16	25.86	48.82	25.32	0.53
	ANOVA	**	***	***	***	***
Fe	C	17.30	50.88	40.98	8.14	1.24
	FeC	21.51	36.45	50.05	13.49	0.73
	ANOVA	***	***	**	*	**
Mn	C	11.17	9.18	57.70	33.12	0.16
	FeC	13.20	6.54	59.37	34.09	0.11
	ANOVA	**	**	**	NS	**
Zn	C	19.76	23.21	43.94	32.85	0.53
	FeC	24.56	17.69	46.73	35.58	0.38
	ANOVA	**	NS	NS	*	NS

ER (%) = (alkali-extractable element/total element) × 100.

HA: humic acid fraction, OFF: organic fulvic fraction and MFF: mineral fulvic fraction. Data expressed as % with respect to the total amount of element extracted with alkali.

NS: not significant, \*\*\*, \*\* and \*: significant at the probability level  $P < 0.001$ ,  $0.01$  and  $P < 0.05$ , respectively.

The ratio between the two humic-like organic fractions HA and OFF (called the polymerisation index in the case of TOC) exhibited values higher than 1 for both extracts, indicating a predominance of organic carbon in the HA. This ratio was lower in FeC, which suggests a less complex molecular structure in the dissolved organic matter extracted from this compost. When the same ratio was calculated for the rest of the elements, its values in the extract from FeC were equally lower, as before, than in the extract from C, but the predominance of HA was only observed for TN and, only in this extract, also for S and Fe.

Experiments in course are addressed to produce a marketable liquid organic-mineral PK fertilizer from AL compost with the following minimum requirements: 6% ( $P_2O_5+K_2O$ ), 2%  $P_2O_5$ , 2%  $K_2O$  and 4% organic carbon contents as established by the Spanish legislation for fertilizers (BOE, 2005), which should be attained by using heat and KOH as alkaline extractant.

## CONCLUSIONS

The co-composting of AL and PM yielded end-products rich in organic matter and nutrients that were catalogued as NPK organic fertilizers. The addition of FeB diminished pH and increased EC value and the contents of Fe, S and Mn.

The alkali-extracts from both composts were mainly rich in K and N, but also in TOC. The FeB addition reduced the solubility of most macronutrients and increased that of micronutrients and S. All the elements were found mainly in both organic fractions, HA and OFF, from the extracts, but the mentioned addition generally enhanced retention in the latter. This effect was mainly evident for the two added elements Fe and S. Based on these results, further research is now planned to produce a marketable liquid organic-mineral PK fertilizer.

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## BIOTECHNOLOGICAL RECYCLE OF OLIVE MILLS WASHING WATER BY MICROALGAE

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### ABSTRACT

The olive fruit washing is the first treatment in oil extraction process, which eliminates the impurities collected during harvesting and temporary storage. During this process an enormous amount of fresh water is consumed in Mediterranean countries, which exhibit serious shortage of water. Furthermore, the disposal of the water used to wash olives may pollute the environment. The EU funded project ALGATEC (FP7-SME-2008-/232331) aims to propose a cost-efficient system for the on-site treatment and reuse of olive washing water generated in olive oil mills, with high pollutant content, by means of an affordable and compact photobioreactor using microalgae followed by a membrane technology module, capable to recover and recycle the majority of the drinkable water used in the process of olives washing. Furthermore, the problem of the disposal of wastewater from olive oil mills will be reduced because the reutilisation of the washing water will diminish the overall volume of wastewater, with both economical and environmental benefits. A group of Small Medium Enterprises (SME), end-users and a multi-national research team with experience in the field of water characterisation and treatment will closely cooperate. The project will create working prototypes to be tested at olive mill conditions. The ALGATEC system will provide a decentralised, safe and cost-efficient wastewater treatment and water reuse system, especially applicable for small and medium sized olive oil producers and will reduce water consumption of the process by 80 % contributing in the protection of the environment.

## INHIBITORY ACTIVITY OF OLIVE MILL WASTE AND BIOCONTROL BACTERIA AGAINST SOILBORNE PLANT PATHOGENS

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### ABSTRACT

Soilborne diseases, mainly caused by fungal pathogens, determine severe loss in yield of several horticultural and fruit crops world-wide. In the last decades they have been occurring with an increasing frequency and severity in nursery as well as in open field cultivations. By far, their control has been relied on the use of synthetic pesticides with consequent increasing of environmental concerns. It is therefore necessary to develop and test alternative methods, more environmentally friendly. Preventive methods, adopted even in the nursery (i.e. pathogen free soil and planting material, suppressive substrates and biocontrol agents), are considered key factors for an efficient disease control. Recently, alternative plant growing substrates containing natural organic amendments have been studied not only for agronomic properties, but also for their suppressiveness against soilborne pathogens. This study aimed at evaluating the suppressive activity against fungal infections of a selected natural amendment made up of destoned olive mill wet husk mixed with dry hygroscopic organic additives (waste wool, straw and sawdust), used after a short period of aerobic storage. In our assays the amendment was used alone or enriched with selected bacteria (*Bacillus amyloliquefaciens* and *Burkholderia cepacia*) as biocontrol agent against fungal pathogens (*Fusarium oxysporum* f.sp. *lycopersici*, *Phytophthora* spp. and *Verticillium dahliae*). In order to preliminary assess possible antifungal activity of amendment water extracts (AWE), bioassays were carried out *in vitro* in multiwell plates, using unsterilized or sterilized AWEs. Higher reduction of fungal biomass production was exerted by the unsterilized AWE thus evidencing the repressiveness exerted by the natural antagonist microflora contained in the amendment. The amendment was also tested for its repressiveness against soilborne fungi in pot experiments, in mixture (15% v/v) with a standard artificially contaminated substrate. The density of fungal pathogen propagules was periodically monitored by semi-selective media. The results showed a significant reduction of fungal pathogen propagules in the amended rhizosphere, particularly when the substrate was enriched with biocontrol bacteria. Further studies are in progress to optimize the suppressive activity of these innovative substrates for a more eco-compatible plant protection strategy.



## LIGHT CONTROLLED HEAVY METAL CARRYING E. COLI MACHINES

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### ABSTRACT

Heavy metals are natural part of environment that we live in. However they have toxic effects when they are above certain concentration. Since they are not degradable and accumulates in living organisms, soil change researches predict that heavy metal contamination may become major problem in close future. Currently bacteria with heavy metal binding abilities have been designed as an efficient and low cost solution for heavy metal contamination. Certain surface proteins have been already reported to tolerate heterologous metal binding peptide insertions to design heavy metal binding bacteria. Given that metals adsorbed to peptides are desorbed under acidic condition, we showed that bacterial metal binding and release can be controlled by light. In this work we designed a system with which can we control metal adsorption and desorption using light only. However one problem is still to be solved. The adsorption and desorption should be decoupled. It was already reported that E.coli can be engineered to exhibit stable phototactic response. Hence decoupling can also be done by light again, but using specific wavelengths. Moreover, MBMs can also be used to construct ion exchange matrix to uncouple adsorption and desorption.

## CHEMICAL AND STRUCTURAL CHARACTERIZATION OF ORGANIC MATTER FROM URBAN WASTEWATERS IN A PILOT PLANT TO PRODUCE METHANE

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### ABSTRACT

Anaerobic transformation of organic matter in acetate seems to be the most advantageous way to process urban sludge and wastewaters from both the environmental and economical standpoints. An anaerobic fermentation aimed at producing gases (e.g. methane) is usually performed in two main steps: (acetogenesis and methanogenesis) that generally occur at the same time. Two types of bacteria are involved in anaerobic fermentation, acetogens and methanogens. The former belong to rather diverse, 19 bacterial genera and produce acetates, whereas methanogens are able to extract methane from the previously generated acetates. Acetogens can inhabit very diverse ecosystems with a wide range of temperature, pH, and salinity. It has been estimated that acetogenesis globally yields billions of tons of acetate per year and, therefore, acetogens play an important role in the global carbon cycle. Acetogenesis is a process that, in many instances, organizes the organic matter through a chemical reduction into molecules with increased energy and stability. In the present study, a separation of acetogenesis from methanogenesis was obtained, and different chemical parameters were used to describe both acetogenesis and methanogenesis. The entire methanation process took place in two series of digesters fed with the same raw sludge and working in steady-state conditions; the first digester was used for hydrolysis, acidification and acetogenesis, whereas the second one was for methanogenesis. The sludge samples were taken daily from both digesters for analyses. Special focus was placed on characterization of organic matter using diffuse reflectance infrared transform spectroscopy (DRIFT), carbon stable isotopic ratio, thermogravimetric (TG) and differential thermal (DTA) analyses. Moreover, the composition of volatile (VFA) and non volatile free fatty acids (NV-FFA) was analyzed and discussed. Valuable information about the phase of degradation or stabilization of sludges was obtained by means of these analytical techniques, suggesting the possibility of a more efficient recycling of urban wastewaters organic matter.

## INTRODUCTION

Anaerobic digestion of the wastewater sludge can contribute efficiently to solid waste and greenhouse gas emission reduction by means of methane production [1]. Due to the great importance of anaerobic digestion, different models have been suggested to optimize the process. Most of the proposed models refer to a single-stage process, where hydrolysis, acidogenesis, acetogenesis and methanogenesis take place in the same reactor. In these conditions, the understanding of chemical and biological processes that occur at the same time is extremely difficult and it has actually been approached by working on single bacterial strains or under artificial conditions [2]. Two-stage anaerobic processes have been proposed for splitting volatile fatty acids (VFA) and methane forming stages, so as to optimize each step [3-7], which would allow a better analysis of the chemical process involved. Every decomposition stage of sludges is characterized by significant chemical variations of organic matter. These changes can be directly identified by using a multiple analytical approach similar to that applied to chemical modifications occurring in the composting process [7].

In the present work, the chemical features of sludges from two series of digesters (the first for acetogenesis and the second one for methanogenesis) were studied by using chemical analysis,  $\delta^{13}\text{C}$  isotopic ratio, HS-SPME-GC-MS, DTA analysis, and DRIFT spectroscopy, in order to monitor chemical modifications during acetogenic and methanogenic digestions.

## MATERIALS AND METHODS

### Experimental design

The entire process took place in two series of digesters, the first for acetogenesis and the second one for methanogenesis; both digesters were fed by the same raw sludge and worked in steady state conditions. To uniform distribution throughout the digesters, the sludges and the gas were injected by means of a distribution system. The influent was obtained from a sequence of stabilization processes, which were carried out under aerobic-anaerobic conditions at an urban wastewater treatment plant in Bologna (Italy).

The acetogenic sludge inoculum (about 1.8-liter corresponding to 1.8 kg with a pH value of 4.5) was placed in each of ten 2-liter total volume digesters for acetogenesis. Since the first stage was operated at an hydraulic retention time (HRT) of 6 days,  $300 \text{ mL d}^{-1}$  corresponding to about  $300 \text{ g d}^{-1}$  total weight of influent were added in each acetogenic digester. The acetogenic cultures were incubated in a thermostatic chamber at a constant temperature of  $25^\circ\text{C} \pm 1.0$ . All acetogenic digesters were continually flushed with 1.5 liter  $\text{d}^{-1}$  of  $\text{CO}_2$ , resulting in a light mixing of the anaerobic culture without damage and a good  $\text{CO}_2$  dissolution rate in the liquid phase.

The methanogenic sludge inoculum (about 1.8-liter corresponding to 1.8 kg with a pH value of 7.0) was placed in each of ten 2-liter total volume digesters for methanogenesis. Since the second stage was also operated at a HRT of 6 days,  $300 \text{ mL d}^{-1}$  corresponding to about  $300 \text{ g d}^{-1}$  total weight of acetogenic sludge was passed from the acetogenic to the corresponding methanogenic digesters. The methanogenic cultures were incubated in a temperature-controlled water bath at  $42^\circ\text{C}$ . Agitation in methanogenic digesters derived

from the mixing caused by acetogenic sludges inflow and biogas produced. During the experimental period, biogas production measurements ( $\text{CH}_4$  and  $\text{CO}_2$ ) were taken every 24 h; biogas collection and measurement was performed by water displacement method from both acetogenic and methanogenic digesters. The biogas composition was determined by using Geotech GA 2000 gas analysers (Keison Products, Chelmsford, UK). The gas output was more or less constant throughout the whole experiment and similar among replicates. At the 6<sup>th</sup> day, the experiment was stopped and the sludges from each digester were freeze-dried to stop the biological activity and were subsequently analyzed.

### **Chemical analyses**

Total C and N contents were measured with an elemental analyzer (Thermo Finnigan mod. EA 1110). The C isotopic ratio was analyzed by continuous flow-isotope ratio mass spectrometry (CF-IRMS mod. Delta Plus, Thermo Electron, Bremen, Germany). The determination of soluble chemical oxygen demand (SCOD), total suspended solids (TSS), volatile suspended solids (VSS), volatile fatty acids (VFA), total alkalinity (TA) and pH were carried out according to APHA [8].

Lipid fraction was extracted using the Folch's procedure. Identification of volatile compounds was performed by using HS-SPME-GC-MS [9]. The retention time and mass spectra of each substance were compared with those of the corresponding standards, as well as data reported in the literature [9].

DRIFT spectra were recorded using a Bruker TENSOR series FT-IR Spectrophotometer (Ettlingen, Germany) equipped with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT). Thermogravimetric analysis (TG) and differential thermal analysis (DTA) were simultaneously performed, using a TG-DTA92 instrument (SETARAM, France).

## **RESULTS AND DISCUSSION**

The main chemical and physical parameters of the sludges here studied are shown in Table 1. The pH values in both acetogenic and methanogenic digesters were similar to those found at the beginning of the study, without any pH adjustments. The progressive decrease in VSS gave an idea of the organic matter degradation in both digesters. The SCOD values significantly differed in both digesters, being highest in acetogenic digesters. The considerable increase in SCOD is probably related to the transformation of organic matter into VFA (Table 1). In contrast, the low SCOD and VFA values found in methanogenic digesters, suggested that a part of organic matter was converted into methane.

**Table 1.** Physico-chemical characteristics and biogas production in raw sludge, acetogenetic and methanogenetic digesters. Numbers within the brackets correspond to standard error (n= 9)

	<i>Raw sludge</i>	<i>Acetogenesis</i>	<i>Methanogenesis</i>
T °C	rt	25(1.0)	42(2.0)
pH	6.0(0.1)	4.5(0.2)	7.1(0.1)
TSS gL <sup>-1</sup>	35(1.2)	31(0.9)	26(0.9)
VSS gL <sup>-1</sup>	24(1.1)	22(0.8)	16 (0.9)
SCOD gL <sup>-1</sup>	4.4(1.0)	20(4.3)	2.4(0.9)
N-NH <sub>4</sub> <sup>+</sup> gL <sup>-1</sup>	0.2(0.03)	0.3(0.02)	0.7(0.02)
VFA gL <sup>-1</sup>	0.5(0.01)	7.6(0.04)	0.3(0.01)
TA gL <sup>-1</sup>	1.5(0.3)	2.5(0.1)	2.9(0.1)
VFA/TA	0.3(0.1)	2.9(0.1)	0.1(0.09)
CO <sub>2</sub> Ld <sup>-1</sup>	nd	0.9(0.07)	0.6(0.05)
CH <sub>4</sub> Ld <sup>-1</sup>	nd	nd	1.0(0.05)

rt = room temperature; nd =not detected

The difference between the VFA/TA ratio in acetogenesis with respect to the methanogenesis one, indicates an efficient phase separation process during the study. Furthermore, a VFA/TA ratio smaller than 0.3 is considered a good stability index in methanogenesis [10]. The highest N-NH<sub>4</sub><sup>+</sup> amount was detected in the methanogenesis digesters; however, at this concentration, the CH<sub>4</sub> production did not seem to be influenced.

The daily biogas production in the acetogenic digesters showed a significant difference between CO<sub>2</sub> input (1.5 Ld<sup>-1</sup>) and output (0.9 Ld<sup>-1</sup>). Under this condition, the carbon dioxide dissolved in the sludge did not cause a variation of total alkalinity. However, the lower amount of CO<sub>2</sub> output and the considerable amount of VFA found in acetogenesis suggested that a part of CO<sub>2</sub> is transformed into fatty acids according to Wood-Ljungdahl pathway [11]. These results confirm that the digestion regularly took place

The biogas production in the methanogenic digesters was composed by about 60% of methane and 40% of carbon dioxide, while VFA were present as trace. No biogas production was estimated in raw sludge.

Table 2 reports the C, N, δ<sup>13</sup>C values and percentage of total lipids in the lyophilized sludges. As expected, a slight difference in δ<sup>13</sup>C values in acetogenic and methanogenic digesters was observed, which could be ascribed to the different biochemical pathways followed by acetogenic and methanogenic bacteria. In general, the fermentation processes led to a greater fractionation of <sup>12</sup>C over the <sup>13</sup>C isotope because of the lower energy required to break a <sup>12</sup>C-<sup>12</sup>C bond as compared to a <sup>13</sup>C-<sup>12</sup>C one. This fact can result in a <sup>13</sup>C- enriched organic residue. In the present experiment, a δ<sup>13</sup>C value similar to that of acetate (-24 ‰), was found in acetogenesis. This result might be supported by the considerable VFA amount produced in acetogenesis (Table 1). A slight shift of δ<sup>13</sup>C to less negative values in methanogenesis suggests that the organic carbon residual was enriched in heavier isotopes as a possible consequence of VFA conversion into

methane. As an alternative explanation, the difference in the total lipid content (acetogenesis>methanogenesis) might explain the shift of the  $\delta^{13}\text{C}$  value in both processes. On the contrary, no reasonable explanation can be provided for the  $\delta^{13}\text{C}$  value in the raw sludge, because no anaerobic fermentation is supposed to take place.

**Table 2.** C and N contents, isotopic ratio ( $\delta^{13}\text{C}$ ) and percentage of total lipids in lyophilized sludges. Numbers within the brackets correspond to standard error (n= 3)

	<i>Raw sludge d.m.</i>	<i>Acetogenesis d.m.</i>	<i>Methanogenesis d.m.</i>
C (%)	35(0.7)	33(0.4)	28(1.1)
N (%)	3.5(0.03)	4.1(0.2)	3.5(0.07)
$\delta^{13}\text{C}$ (‰)	-25.8(0.1)	-24.2(0.2)	-23.7(0.2)
Total fat (%)	5.6(0.9)	12.3(1.1)	8.2(0.9)

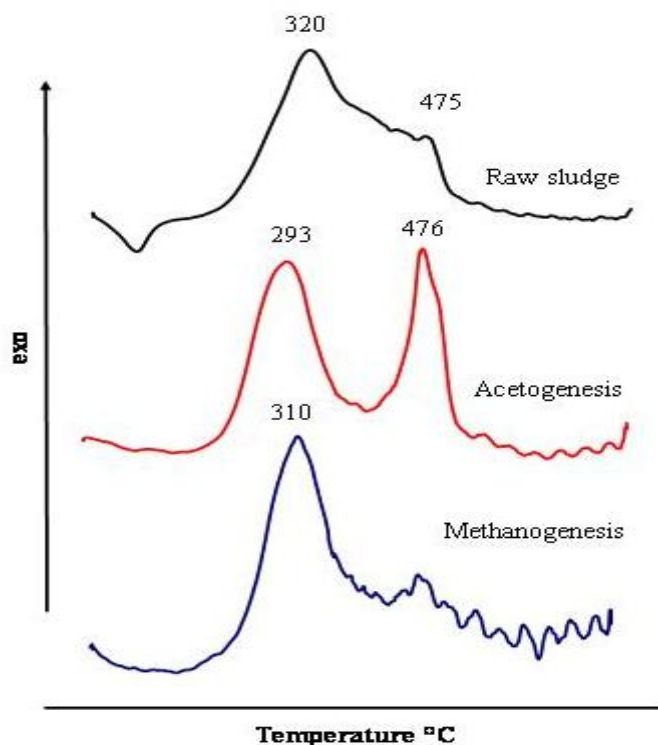
d.m.=dry matter

A different chromatographic pattern of VFA in both digesters was observed. VFAs mainly consisted of acetic (C2), propionic (C3), iso-butyric, (C4), butyric (C4), iso-valeric (C5), valeric (C5) and caproic (C6) acids and they were dominant in acetogenesis. This implies that homoacetogenic fermentation and acetate/butyrate fermentation were prevalent in this process. The presence of propionate, instead, indicated that hydrogen and formate are not low enough to activate the acetogenic bacteria involved in the decomposition of propionate [12]. In methanogenesis, acetate and *n*-butyrate were completely degraded, as supported by Gallert and Winter [13], while the propionate degradation seems to be a time-requiring process [13]. Other VFA were present in traces, such as isovaleric and caproic acids. In raw sludge, the absence of the VFA GC peaks, might be due to their low concentration (Table 1).

In the present study, the DTA curves of the acetogenic and methanogenic digester sludges showed different, well-defined thermal events (Fig 1). In fact, the DTA curve of the acetogenic sludge exhibited two strong exothermic reactions at 293°C (weight loss of 34.46%) and 476°C (weight loss of 16.61%), attributed to the organic matter decomposition. The bimodal thermal profile in acetogenesis is related to the presence of a complex mixture of organic components involved in the heating phase; the first peak at 293°C was mainly produced by the decomposition of carboxyl groups (probably fatty acids), whereas the exothermic reaction at higher temperatures (~500°C) was originated by decomposition of refractory C.

Due to the high amount of lipids, it might be possible that the double bonds of unsaturated fatty acids were broken during heating and that some molecules are modified into saturated structures characterized by a higher thermal stability [14].

A unique strong thermal reaction at 310°C (weight loss of 49.12%) characterizes the methanogenesis DTA curve, which is evidently different from that of acetogenesis.



**Fig. 1.** DTA curves of raw sludge, acetogenic and methanogenic freeze-dried sludges.

It is of particular relevance the variation of combustion heat released during thermal decomposition of sludges. The total energy per unit mass released during combustion is directly proportional to the total area of the DTA peaks. On this basis, the combustion heat of the acetogenesis sludge ( $-5802 \mu\text{V sec/mg}$ ) was greater than that of methanogenesis ( $-3553 \mu\text{V sec/mg}$ ), but it was not so different from that of the raw sludge ( $-6453 \mu\text{V sec/mg}$ ). This variation in both fermentation processes seems to be related to the higher amount of lipids found in acetogenesis (Table 2). The DTA curve of raw sludge displayed a broad and unique exothermic reaction at  $320^\circ\text{C}$  (weight loss of 50.38%) and a shoulder at  $475^\circ\text{C}$  that may be related to organic matter with complex structures.

The structural changes observed using TG-DTA analysis are supported by DRIFT spectra (Fig. 2). Some quantitative and qualitative differences can be observed between spectra. The relative intensity of the band corresponding to carbonyl/carboxyl groups was lower in methanogenesis and it shifted from  $1561$  to  $1540 \text{ cm}^{-1}$ , indicating a different content in acidic groups as also supported by VFA concentration and DTA analysis. The carbonyl frequency in fatty acids usually increases and decreases alternatively for odd and even carbon number [41]. The lack of a shift of the CH band frequency suggested that the aliphatic component in both sludges was similar and mainly characterized by methylenic chains [15]. The strong bands between  $1170$  and  $1000 \text{ cm}^{-1}$  were mainly attributed to O-H stretching in mineral components.

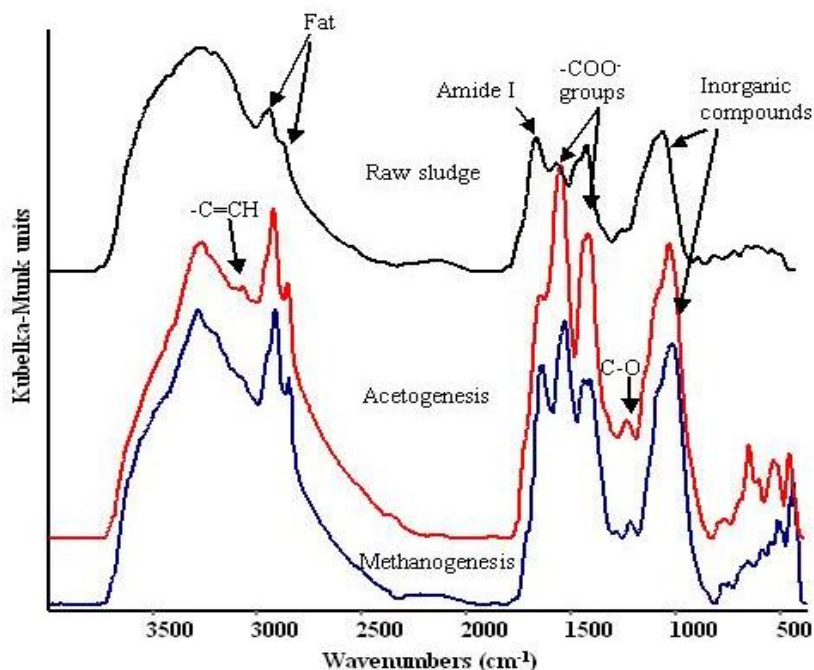


Fig. 2. DRIFT spectra of lyophilized sludges: upper, raw sludge; middle, acetogenesis; bottom, methanogenesis.

## CONCLUSIONS

The utilization of two separate phase digesters, one for acetogenesis and the other one for methanogenesis, led to a better understanding of various metabolic aspects of each phase of the process. The acetogenesis role is, in general, fundamental for lipid hydrolysis and the consequent VFA production, molecules with high energetic potential. Moreover, the data obtained proved that VFA production in acetogenesis can be increased, if it is kept separated from methanogenesis. The decrease of the lipid content in methanogenic sludges reinforces the importance of the role of these molecules in methanogenesis. A more detailed study will surely provide further information about the role of lipids and single molecular species involved in the biogas production, a basic issue in renewable energy.

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## **INFLUENCE OF INDUSTRIAL LUMP SULPHUR BY-PRODUCT ON SOIL CHEMICAL AND BIOCHEMICAL PROPERTIES AND ON YIELD AND QUALITY OF SUGAR BEET (BETA VULGARIS L).**

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### **ABSTRACT**

A field experiment was carried out to evaluate modifications on the chemical and biochemical parameters of an agricultural alkaline soil (pH=8.31) under sugar beet (*Beta vulgaris* L.) by applying different amounts of sulphur by-product coming from oil desulphurization process. Soil chemical properties such as sulphate formation, pH, soil electrical conductivity (EC), total and extractable organic carbon (TOC and TEC), and biochemical parameters such as microbial biomass carbon content (MBC) and total hydrolytic capacity (FDA-hydrolysis), arylsulphatase (ArS-ase) and o-diphenoloxidase (o-DPO), were monitored. Commercial parameters of yield and quality production of sugar beet (yield of roots, % of polarization, extractable saccharose content and purity of thick juice) were also determined. All soil chemical and biochemical properties and commercial parameters of sugar beet were monitored in plots treated with 1(S1), 5(S5) and 10(S10) t ha<sup>-1</sup> of sulphur by-product and compared with untreated plots (S0). In S1, 60 days after amendment the highest values of sulphate formed (310 mg kg<sup>-1</sup>) and the EC (1.596 mS cm<sup>-1</sup>), were observed. In S5, the sulphate formed was 2583 mg kg<sup>-1</sup> and soil EC was 4.138 mS cm<sup>-1</sup> after 210 days, while in S10 the amount of sulphur added probably exceeded soil oxidation capacity since it continued more slowly till the end of the experiment. After 210 days, soil pH reached the lowest value in all the treated plots and it was 7.75, 7.52 and 7.26 in S1, S5 and S10, respectively. No significant modifications were made on soil biochemical parameters tested by the different amounts of sulphur added. A decrease in the production yield of sugar beet (27.2%) was found only when the highest amount of sulphur was applied. In contrast, no worsening of main commercial parameters was observed.

## USING OF DIFFERENT BULKING AGENTS FOR AEROBIC STABILISATION PROCESS OF IZMIR-ÇIĞLI WASTEWATER TREATMENT PLANT SLUDGE

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### ABSTRACT

Sludge treatment is an indispensable component of waste water treatment plants. Depending upon the climate, economical conditions and compliance with the environmental regulations the country, various methods are in use for the purpose of sludge treatment. Municipal waste water treatment plant in Izmir city establishment in 2000 and currently 600 tons of dry sludge on the average is produced on daily basis. The current sludge stabilization method is to condition the excess sludge with lime, which has many disadvantages. Therefore, searching economically acceptable alternative disposal methods are crucial and became a driving force for this study. In the first part of the study, natural zeolite was used to adjust the moisture content of the piles and thereby the dry matter which was volumetrically around 15-16% was increased up to 30-34%. Following the successful adjustment of moisture with zeolite, various filling materials on compost quality were investigated. The best organic material reduction was achieved by raw material combination of beer pomace, grass, sludge, zeolite (8/8/4/1) (v/v/v/v). The best results were obtained with an initial dry matter content of 32-38%. The temperature to a maximum of 62<sup>0</sup>C in 4 days and temperature was maintained at 55<sup>0</sup>C for at least 10 days. At the end of 21 days of experimental period, an organic material reduction of 38% was achieved. The compost was observed to remove heavy metals (zinc, copper, arsenic, total chrome, nickel and lead) and to reduce some indicator and pathogenic bacteria (total and fecal coliform bacteria, *Clostridium* sp., *Escherichia coli* and *Salmonella* sp.) successfully. As a result, it was concluded that sludge combined with natural zeolite, beer pomace and grass is a suitable alternative for composting.

**Acknowledgements:** The authors are especially grateful to the staff of Izmir Municipal Wastewater Treatment Plant for the given permission.

**Keywords:** *Composting, aerobic stabilisation, natural zeolite, beer pomace, grass*

## **THERMAL AND STATIC COMPOSTING PROCESSES FOR BIOREMEDIATION AND MANAGEMENT OF OLIVE MILL SOLID WASTE**

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### **ABSTRACT**

Olive production is an economically significant horticultural industry in several countries around the world. Olive oil extraction process gives rise to a recalcitrant waste material known as olive husk (OH). The OH is generated in large quantities in short periods of time and, is characterized by its phytotoxicity, hydrophobicity, salinity, low pH and polyphenols (1). The presence of phenols as well as short and long chain fatty acids is considered to be responsible for its phytotoxicity (2). The common method of disposal of raw OH is to spread it on farm lands. Italy is the only country that has regulated this practice. Although it is feasible to use untreated OH as soil amendment, this will leave significant amounts of non-humified carbon in the soil for considerably long time (3, 4). Such amendments also reduce the rate and degree of humification. There is at present a significant shift from the practice of spreading untreated OH in the field to the use of OH that has undergone a bioremediation process. Composting is one such bioremediation process. We have successfully used thermal and static composting processes to convert raw OH to a humified organic medium. Thermal composting, as the name suggests, requires heat to be generated mainly by the activities of resident populations of bacteria, fungi and actinomycetes under aerobic conditions. Static composting, on the other hand, is a decomposition process conducted under partial anaerobic condition, especially at the earlier stages during the composting process. It is important to identify markers for determining process efficiency of methods used in bioremediation of OH. We propose that these markers are levels of phenols and humification and absence of phytotoxicity in the end product. Using these markers, we have been able to amend olive orchard soils with humified compost produced by thermal and static composting of untreated OH. In addition, substrate for growing the cultivated mushroom *Agaricus bisporus* was prepared using OH as an ingredient. The thermal and static composting methods used in our work will be outlined in this paper. Some of the benefits derived from using the humified compost for horticultural purposes will be mentioned.

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## EVALUATION OF VARIOUS AGRO-INDUSTRIAL CO-PRODUCTS FOR THE PRODUCTION OF TRICHODERMA MICROPROPAGULES AS A BIOCONTROL AGENT UNDER SOLID STATE FERMENTATION CONDITIONS

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### ABSTRACT

Biological control is an environment friendly approach which promotes the use of specific microorganisms to protect plants against plant pathogens and pests instead of chemical treatments. As a biocontrol agent, *Trichoderma* type fungi, can be used to control a wide spectrum of plant pathogen fungi. When compared with similar agents, *Trichoderma* based biocontrol agents are commercially preferred due to their plant growth stimulation characteristic and high activity on soil bioremediation. Also by holding a 50% share of the fungal biological control agent market, *Trichoderma* species, became a popular research material for the scientists. Besides of all the favorable aspects, low efficiency, low spore yield, expensive raw materials of culture medium are some of the significant constraints of *Trichoderma* species. In this study, *Trichoderma harzianum* EGE-K38 was used for micropropagules production in solid state fermentation (SSF). Various inexpensive agricultural co-products including wheat bran, sawdust, rice straw, hazelnut shell, grape marc, cotton seed cake were used as organic substrates and various organic nitrogen sources (malt sprouts, peptone, wine lees, urea, soy flour, yeast extract) were tested for micropropagules production. In all tested SSF media the effect of initial moisture content, initial pH, incubation temperature, light and inoculation ratios were determined in erlenmayer flasks and different laboratory scale bioreactor configurations were investigated for effective micropropagules production. Under optimum conditions up to 10<sup>10</sup> micropropagules (cfu/g substrate) was achieved in erlenmayer flask productions.

**Keywords:** *Biocontrol agent, Micropropagule Production, Trichoderma, Solid state fermentation*

## OLIVE MILL WASTEWATER BIOREMEDIATION BY *Bjerkandera paranensis*: A SUSTAINABILITY AND TECHNOLOGICAL EVALUATION

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### ABSTRACT

Remediation of olive mill wastewater (OMW) is an important issue associated with olive-oil manufacturing, a widespread activity in the Mediterranean area. This high organic loading effluent contains water, organic acids, high-molecular-weight polyphenols such as tannins, antocyanins and catechins, which are considered to be responsible for its brownish black colour and toxic properties. The composition of OMWs is highly variable with respect to each individual component, depending on the process conditions and on the agricultural specificities. In this work, the ability of a “white-rot” fungus, *Bjerkandera paranensis*, to use undiluted OMW from a two phase process mill (COD = 11.1 gL<sup>-1</sup>; Phenol Content = 3.9 gL<sup>-1</sup>; ColourAbs395nm = 7.8) as a substrate was studied. The biodegradation potential of *B. paranensis* was assessed monitoring several physico-chemical parameters. A chronic ecotoxicity test (*Vibrio fischeri* growth inhibition test) was carried out to follow the detoxification ability of this fungus. In work, the results demonstrate that OMW was a suitable medium for cultivation of *B. paranensis*, with corresponding changes in the physico-chemical properties of the OMW. The results showed that *B. paranensis* removed 93% phenols and 54% COD from the culture medium within 21 days of treatment. In addition, the IC50s values obtained for the different treated samples showed a significant decrease in the effluent chronic toxicity to *V. fischeri* when the OMW pH was adjusted to 6.0 prior to the treatment (71.8 %), highlighting the OMW detoxification capacity of *B. paranensis*.

**Keywords:** *OMW; biological treatment; B. paranensis; ecotoxicity evaluation; detoxification.*

### INTRODUCTION

The olive oil industry represents one of the most important economic agro-food sectors in the bordering Mediterranean countries that produce more than 98% of the world’s olive oil, estimated at over 2.5 million metric tons per year of which about 75% is produced in the European Union (EU). The largest European olive oil producers are Spain, with 36%, Italy, with 24%, and Greece, with 17%, of the world’s total production. The next largest producer is Portugal, with a production of one order of magnitude lower than the three leading countries, followed by France, Cyprus and Croatia (1, 2).

During olive oil extraction a process that is conducted by mechanical procedures in olive mills, large amount of liquid effluents and solid residues are produced, with a high organic load, the nature of which

depends on the technology of the extraction system employed. Three systems are used worldwide for industrial-scale extraction of oil from olives, the traditional press-cake system, the three phase decanter system and the modern two-phase centrifugation system. Nowadays, in European countries, two-phase and three-phase centrifugation systems (continuous processes) are the ones most commonly used.

The quality and quantity of the constituents of olive mill wastewater (OMW) are dependent on many factors: type of olives, type of soil, cultivation system and production process. The OMW contains a majority of the water-soluble chemical species present in the olive fruit, a very high organic load (chemical oxygen demand, COD) typically ranges from 50-150  $\text{g l}^{-1}$ , about two orders of magnitude higher than municipal wastewater and has an acidic pH (4-6). Phenolic compounds that are present in olive stones and pulp tend to be more soluble in the water phase than oil, resulting in concentrations ranging from 0.5-25.0  $\text{g l}^{-1}$  (McNamara *et al.*, 2008). These phenolic compounds are the main determinants of antimicrobial and phytotoxic olive-mill wastes actions and are responsible for its characteristic black colour (3).

A common way of dealing with the OMW in many Mediterranean countries was to discharge directly into sewer network an option that is unacceptable without a previous complex and expensive pretreatment; alternatively and when no sewage network is available the favored option it is to store it on artificial lagoons beside the mills where it is left to evaporate until the next season. These ponds are often leaking causing ground water pollution and mal odor problems. The use of this water for irrigation is possible but under stringent regulations, in many countries. Since the setting up of more stringent regulations concerning public waste disposal, there is a growing interest in the development of new technologies and procedures for the purification of this wastewater (4).

Due to the seasonality of olive oil production the OMW treatment process should be flexible enough to operate in a non-continuous mode. Besides, the olive mills are small enterprises, scattered around the olive production areas, making individual on-site treatment options unaffordable (5, 6, 7). The treatment of liquid wastes (OMW) produced from olive oil production is still a major challenge facing this industry and still unsolved in the olive-oil-producing countries. The high recalcitrant organic load and the associated toxicity make the treatment of OMW a challenge.

Many physical, thermal, physico-chemical and biological management strategies or combined and miscellaneous processes have been proposed for the treatment and valorisation of OMWs but a solution both environmentally friendly and economically viable is not yet widely available.

The physico-chemical treatments (coagulation, precipitation or flocculation of OMW organic compounds) are very expensive and/or do not completely solve the problem of the need to dispose the sludge or the by-products that derive from the process (8). The composition of OMW is highly variable with respect to each individual component, mainly because OMW is a natural product, processed from a raw material and subject to varied conditions that are difficult to control, and the traditional biological methods used to treat industrial wastewaters cannot be applied to this type of effluent (9).

Several studies have reported the biological disposal of this wastewater by anaerobic digestion (10), being the main interest the production of energy (biogas) and the potential re-use of the effluent in

irrigation (11). The major limitation of this type of treatment is the inhibition of metanogenic bacteria by the phenolic compounds and the organic acids present in the OMW (12), showing that a pre-treatment is necessary to remove undesirable compounds. In this context, a large range of aerobic biological processes, technologies and microorganisms have been tested for OMWs treatment, aiming to reduce organic load, dark colour and toxicity of these effluents. Several treatments focused on the degradation of phenolic compounds showed that fungi (13, 14, 15) are more effective than bacteria in OMW detoxification. These fungi appear quite effective achieving removal rates as 40 – 88 % for COD, 60 – 100 % for phenolics, and 45 – 80 % for colouration (7). The reason for this lies in the structure of the aromatic compounds present in OMWs that is analogous to that of many lignin monomers and only a few microorganisms, and among this mainly white-rot fungi, which produce a variety of lygninolytic enzymes, are capable of completely oxidize phenols (16). The main fungal genera described in the available scientific information for OMW dephenolization are: *Aspergillus*, *Coriolus*, *Phanerochaete*, *Lentinula*, *Penicillium* and *Pleurotus*.

The purpose of the present work was to investigate the ability of a “white-rot” fungus, *Bjerkandera paranensis*, to use undiluted OMW (COD = 11.1 mg/l; Phenol Content = 3.9 g/l ; Colour<sub>Abs395nm</sub> = 7.8) as a substrate. The results obtained proved that OMW is a suitable media for cultivating *Bjerkandera* B33/3, and its growth on OMW cause drastic changes in physical and chemical properties. OMW decolourization was observed during mycelium growth, with a colour reduction since the 7<sup>th</sup> day (50 %). The results showed that *Bjerkandera* B33/3 removed phenols (90 %) and COD (75 %) from the culture medium after 21 days of treatment.

In addition, the IC<sub>50s</sub> values obtained during the treatment show a significant decrease in the OMW chronic toxicity to *V. fischeri* (60.8 %), changing its classification from toxic to lightly toxic.

## MATERIALS AND METHODS

### OMW Sampling and Characteristics

Sampling of olive mill wastewater (OMW) was carried out from a two phase Portuguese mill farm (Trás-os-Montes, Portugal). Prior to any assay performed with the OMW, this effluent was centrifuged (45 min at 10000 x g) to remove residual solids, and autoclaved at 121 °C during 20 min. The main properties of this OMW (mean values SD; n=3) were: pH 4.8 ± 0.2; chemical oxygen demand (COD) 11.1 ± 0.4 gl<sup>-1</sup>; total phenolic content 3.9 ± 0.1 gl<sup>-1</sup>, determined as caffeic acid equivalents; colorimetric value (A<sub>395</sub>) 7.8 ± 0.2.

### Microorganism and Culture Conditions

A white-rot basidiomycetes *Bjerkandera paranensis*, a novel fungal strain (17, 18), isolated and identified in our laboratory, exhibiting high decolourisation activities in different dyes (17) was used for the biological treatment of the OMW. This fungus was grown on potato dextrose agar (PDA, Difco, France) slants at 28 °C and stored at 4°C. *B. paranensis* was maintained through periodic subculture every 3 weeks on PDA plates.

Liquid cultures were conducted to monitor several parameters, such as phenol concentration, colour and chemical oxygen demand (COD), during the growth of *B. paranensis*. OMW liquid medium was prepared using undiluted OMW set to pH 6.0. The medium was autoclaved at 121 °C during 15 min. Volumes of 150 ml of the OMW media were used in 500 ml Erlenmeyer flasks and then inoculated with 15 mycelium plugs (7 mm) cut from the front of an actively growing *B. paranensis* fungus in a PDA plate. Incubations were carried out on an orbital shaker at 130 rpm and 28 °C. The assays were carried out during 21 days. In addition, abiotic controls using no inoculated OMW medium were performed at the same incubation conditions.

### **Ecotoxicological Evaluation**

The ecotoxicological evaluation of OMW samples collected from the liquid culture assays, before and after treatment by *B. paranensis* (T21 days), was carried out using a miniaturized chronic toxicity test: the growth inhibition test using a bioluminescent bacterium culture, *Vibrio fischeri*. This test was performed in 96-well microplates (NUNC<sup>TM</sup>, Denmark), based in DIN 38412-L37 -1999 (19). The OMW samples were tested without further pH adjustment and after filtration (membrane filter of pore size: 0.45  $\mu$ m). Toxicity results were expressed in IC<sub>50</sub>, the concentration responsible for the growth inhibition in 50% of the tested population. IC<sub>50</sub>-6h values were estimated from the sigmoidal concentration - inhibition curves fitted by the maximum likelihood - logit method using the ToxCalc V5.0.23F (Tidepool Scientific Software, McKinleyville, CA, USA).

### **Analytical procedures**

Total phenolic content (with respect to caffeic acid) was determined according to a modification of the Folin-Ciocalteu method. According to this method, 500  $\mu$ l Folin-Ciocalteu (4-fold-diluted) phenol reagent was added to 100  $\mu$ l of 20-fold-diluted samples. After 5 min, 500  $\mu$ l sodium carbonate (200  $\text{g l}^{-1}$ ) was added and the absorbance was measured at 725 nm against a blank after being kept at room temperature for 30 min. OMW colour was determined spectrophotometrically in diluted samples (1:20) by measurement of absorbance at 395 nm (9). Analysis of chemical oxygen demand (COD) was carried out following the APHA (2005) (20).

## **RESULTS AND DISCUSSION**

### **Treatment of undiluted OMW by *B. paranensis***

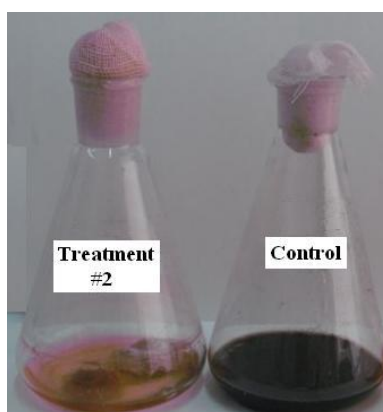
Biological treatment of undiluted OMW (pH 6.0) was conducted in liquid cultures (batch process in shake-flask on an orbital shaker 120 rpm) with *B. paranensis*. This treatment by *B. paranensis* was monitored following the parameters: phenol concentration, colour, COD and pH.

The results from the treatment are presented in Table 1 as % reduction of the different physico-chemical parameters assessed. To evaluate the inherent degradation, abiotic remediation of OMW was assessed by 21-day incubation of an OMW sample (pH 6) in the absence of *B. paranensis* (Table 1 - Abiotic Control). In this control, COD, phenol content, and colour decreased by 10.3 %, 8.1 %, and 6.3 %, respectively.

**Table 1.** Reduction (%) of several parameters (COD, phenols content, colour and toxicity) within 21 days of undiluted OMW treatment by *B. paranensis*.

OMW SAMPLES	% REDUCTION			
	COD	Phenol	Colour	Toxicity
Abiotic Control	10.3	8.1	6.3	---
Treated OMW	57.5	93.0	74.0	71.8

The results obtained showed with *B. paranensis* was able to remove a significant part of phenolic content from the OMW - culture medium, under the conditions tested, reaching a maximum of 93% reduction in undiluted OMW medium, without any addition of nutrients but with pH adjustment to 6.0, in contrast with other fungi previous studied that need a prior dilution of OMW to dilute its initial phenol content values to  $\leq 3 \text{ gl}^{-1}$ , with or without additional nutrient (9, 21, 22, 23).



**Fig. 1.** Decolourization of undiluted OMW (pH 6.0) after 21 days of treatment by *B. paranensis*.

At the end of the treatment of undiluted OMW (21<sup>st</sup> day), *B. paranensis* was also able to a significant reduction of the pollutant load, 57.5 % in COD, and an extensive decolourization (74%), as can be seen in Fig 1. These results seem greatly satisfactory as real OMW with no dilution was treated. In addition, the results of the ecotoxicological evaluation of the OMW samples, before (untreated) and after treatment by *B. paranensis* (T21 days), using the *V. fischeri* chronic toxicity test (growth inhibition test) were:  $\text{IC}_{50-6\text{h}} (\%) = 3.4\%$  and  $\text{IC}_{50-6\text{h}} (\%) = 12.1\%$ , respectively, corresponding to an OMW detoxification of 71.8% (Table 1). This significant decrease in the chronic toxicity of the treated OMW to *V. fischeri*, is probably related to a reduction of 93% in phenol content, from  $3.9 \text{ gl}^{-1}$  to  $0.3 \text{ gl}^{-1}$ , that is considered the main factor responsible by OMW toxicity.

Aggelis *et al.* (2003) (24) carried out a chronic toxicity test using *Heterocypris incongruens* a freshwater ostracoda (growth inhibition test), to evaluate the detoxification ability of *Pleurotus ostreatus* in OMW treatment. *P. ostreatus* have reduced the OMW phenol content from  $4.18 \text{ gl}^{-1}$  to  $1.13 \text{ gl}^{-1}$  (73%

reduction) during its treatment, however this reduction did not corresponded to a significant detoxification. The inhibitory effect of their OMW on the growth of *H. incongruens* was maintained after the treatment, being the IC<sub>50</sub>-6days values for both untreated and treated OMW identical (IC<sub>50s</sub> = 3% OMW). This was probably due to the fact that the remaining phenolics or oxidation products in OMW were more toxic for *H. incongruens* than the initial phenolics. In contrast, in the current study, *V. fisheri* showed a decrease in OMW toxicity when treated by *B. paranensis* (71.8 %). *V. fisheri* and *H. incongruens* presented a similar sensitivity for the initial untreated OMW samples (with an equivalent phenol content): IC<sub>50</sub>-6h = 3.13 % and IC<sub>50</sub>-6 days = 3.0%, respectively.

## CONCLUSIONS

The OMW treatment results obtained by a white-rot fungus *Bjerkandera paranensis*, highlight the potential of this novel strain to be used as a good alternative strain for OMW bioremediation in comparison with the fungal strains already described in studies of OMW treatments.

In the undiluted OMW treatment by *B. paranensis* it was achieved a reduction of 57.5 % in COD, 93% in phenol content and 74% in colour, when the pH was adjusted to 6.0. In addition, an OMW detoxification of 71.8% was attained by this strain.

These are promising results for further research combining an eco-efficient aerobic-anaerobic technology for the bioremediation of this agro-industrial effluent, using *B. paranensis* as the microorganism responsible by the aerobic step, due to its potential to remove OMW polluting load (COD, phenol content and colour).

Moreover, studies based on the production of bioproducts (enzymes and biopolymers) by *B. paranensis* can be a useful tool to a possible OMW valorisation and further scale-up of OMW bioremediation technology, which together will form a pillar for future developments within this field.

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