

## P60: EVALUATION OF THE BIOLOGICAL DETOXIFICATION OF TABLE OLIVE PROCESSING WASTEWATERS BY UV–VIS SPECTROPHOTOMETRY AND LIQUID CHROMATOGRAPHY

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**Abstract** – The contribution of UV–Vis spectrophotometry and liquid chromatography to follow phenol degradation by *Aspergillus niger* B60 was studied in Spanish–style green olive processing wastewaters. The percent reduction of the characteristic absorption band at 270 nm in effluent UV spectra was strongly correlated with that of the total polar phenol content estimated by the Folin–Ciocalteu assay. RP–HPLC–DAD analysis of the extracts gave more information about degradation mechanism. UV spectrophotometry can be used to monitor routinely microbial degradation of phenolic compounds in table olive processing wastewaters.

**Keywords:** Spanish–style green olive wastewaters; *Aspergillus niger*; Phenolic compound biodegradation; UV–Vis spectrophotometry; Liquid chromatography

### 1. INTRODUCTION

Table olives are recognized as predominant constituents of the Mediterranean diet. Spanish–style green olives are the main type of table olives, contributing to about 50% of the total worldwide production. During the production process of this olive type, high amounts of polluting wastewaters are produced, reaching 4000 L per olive tone [1]. The generated streams constitute a serious environmental problem, due to their high organic load, and particularly due to the presence of phenolic compounds [2]. The latter are complex and simple phenolic forms derived from olive fruit, and have highly phytotoxic and antimicrobial properties [3,4]. Most of the times, the table olive processing wastewaters (TOPWs) are disposed untreated in the environment [2]. Because of their special characteristics, the detoxification of the streams becomes critical. Following the environmental enforcements, different wastewater treatment methods have been employed. Among them, biological treatment using microorganisms is widely

recognized as a green, inexpensive and effective process [1]. Due to the importance of TOPWs detoxification, it is essential to find sensitive, accurate and cheap analytical methods, among the most widely used, in order to evaluate phenol biodegradation.

The objective of this work was to study the contribution of UV–Vis spectrophotometry and liquid chromatography to follow phenol degradation by the fungus *A. niger* B60 in effluents from Spanish–style green olives (*cv.* Halkidiki) processing.

### 2. EXPERIMENTAL

#### 2.1. Samples

The lye and washing water effluents from Spanish–style green olives (*cv.* Halkidiki) processing were supplied by a private company (LADAS FOODS S.A., Halkidiki) and stored at –20 °C. Prior to biological treatment of the effluents, the pH was adjusted to 5.0 with concentrated HCl (12 N).

#### 2.2. Microorganism

The microorganisms used in this work was *A. niger* B60. The strain was subcultured on potato dextrose agar (PDA) regularly every 3 to 4 months and stored at 4 °C.

#### 2.3. Fermentation conditions

Submerged fermentation experiments were carried out using unsterile effluents under aerobic conditions in shake–flasks (an initial working volume of 50 mL in 250 mL flasks). The shaking speed was 160 rpm. The effluents were inoculated with a spore suspension containing  $2 \times 10^7$  spores/mL. Liquid cultures were incubated at 30 °C and harvested at 6–8 days followed by filtration under vacuum using Whatman No. 1 filter paper. Non–inoculated effluents were also incubated

under the same conditions and used as controls. The filtrates were used for further analysis.

## 2.4. Analytical methods

### Chemical Oxygen Demand (COD)

COD was determined by the potassium dichromate method using tube tests in an AL200 COD VARIO photometer (Aqualytic, Germany).

### Extraction of phenolic compounds

The protocol used for the extraction of phenolic compounds from the untreated and the filtrates of the fermented wastewaters is described in El-Abbassi et al. [5]. The repeatability of the extraction procedure was satisfactory (CV% = 4.4,  $n = 5$ ).

### UV spectrum analysis of effluents

The UV absorbance spectra of the effluents before and after biological treatment were recorded in the region 220–350 nm after appropriate dilution using a Shimadzu UV 1601 (Kyoto, Japan) equipped with quartz cells (CV% = 0.1 at 270 nm,  $n = 5$ ).

### Colorimetric determination of the total polar phenol (TPP) content

Total polar phenol content (TPP) of the ethyl acetate extracts was estimated by the Folin–Ciocalteu (F–C) assay [6], and results were expressed as caffeic acid equivalents (mg CA/L) at 725 nm. A calibration curve was constructed using caffeic acid solutions within the range of 50–500 mg/L ( $y = 0.011x + 0.0279$ ,  $R^2 = 0.9993$ ). The determination was performed in triplicate for lye and washing water extracts (CV% = 2.0 and 2.4, respectively,  $n = 5$ ).

### RP–HPLC analysis of phenolic compounds

Aliquots of the ethyl acetate extracts were suitably diluted with MeOH and filtered through a 0.45  $\mu\text{m}$  PTFE filter (Waters, Milford, MA) before use for RP–HPLC analysis. Analysis was carried out on a Discovery<sup>®</sup> HS column C18 (250 x 4.6 mm i.d., 5  $\mu\text{m}$ ) (Supelco, Sigma–Aldrich, Germany). The elution system consisted of 0.2% aqueous phosphoric acid (solvent A) and acetonitrile (solvent B). The gradient was as follows: 0 min 10% B, 1 min 10% B, 10 min 20% B, 43 min 50% B, 48 min 95% B, 52 min 95% B, 60 min 10% B. The flow rate was 0.5 mL/min and the injection volume was 10  $\mu\text{L}$ . Phenolic compounds in the tested extracts were monitored at 240, 280 and 320 nm by a UV 6000 LP diode array detector (DAD, Thermo Separation Products, San Jose, CA). The external standard method was used for the quantification of the individual phenolic

compounds. The content of phenolic compounds at 280 nm was expressed as hydroxytyrosol equivalents (mg HTyr/L) using the equation  $y = 7864x + 138064$  ( $R^2 = 0.9993$ ). Each sample determination was carried out in triplicate (CV% = 0.6 for hydroxytyrosol,  $n = 5$ ).

## 2.5 Statistical analysis

All measurements and treatments were performed in triplicate and data obtained were reported as mean  $\pm$  standard deviation. Statistical comparisons of the mean values were carried out either by one–way ANOVA, followed by the Duncan's test or by student's  $t$ –test ( $p < 0.05$ ) using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Correlation analysis was performed by the Pearson correlation using Minitab 17 (Minitab Inc., State College, PA, USA). Significance of the Pearson correlations was evaluated using  $p$ –values.

## 3. RESULTS AND DISCUSSION

The UV absorbance spectra of the lye and washing water effluents before and after biological treatment with *A. niger* were studied to find effective wavelengths that can give information to follow phenol degradation by the fungus. The original effluent UV spectra exhibited an absorbance band with a maximum at 270 nm. The latter was attributed to the presence of phenolic compounds. After evaluation of the UV spectra of the effluents treated with the fungal cells, a strong reduction in this characteristic absorption band was recorded at the end of the incubation period. Specifically, after 6–8 days of biological treatment, the absorbance at 270 nm was reduced by 61% and 67% in lye and washing water spectra, respectively, compared with the untreated wastewaters.

A similar trend was observed for the TPP content determined by the F–C method, reflecting a phenol removal of 62% and 75% in lye and washing water effluents, respectively, after completion of the biological treatment (Table 1). The significantly lower percent decrease values observed in the lye effluent compared with the washing water stream may be related to the presence at high concentrations of toxic or non–biodegradable compounds in the former that may negatively influence the biodegradation efficiency of the fungus.

As shown in Table 1, results of TPP content measured by the RP–HPLC analysis of extracts from

treated lye and washing water effluents recorded at 280 nm indicated an efficiency of 90% and 97% phenol removal, respectively. Comparison of the experimental mean values obtained from the HPLC and the F–C methods the t–test showed significant differences (Table 1). This finding was attributed to the fact that the efficiency of this biological process is mainly corresponded to the microbial degradation of simple phenols, phenolic acids and flavonoids [7].

The non–inoculated effluents (control) showed a much lower reduction of the TPP content values determined by F–C and HPLC methods as compared to the biologically treated ones (Table 1). This result indicates that *A. niger* is mainly responsible for the degradation of the phenolic compounds.

Table 1. Percent reduction of total polar phenol (TPP) content of table olive processing wastewaters treated with *A. niger*, estimated by Folin–Ciocalteu (F–C) assay and RP–HPLC analysis.

Wastewater effluent	TPP content	
	F–C (mg CA/L)	RP–HPLC (mg HTyr/L)
Lye treated with <i>A. niger</i> for 8 days	62% <sup>a,A</sup> ±2	90% <sup>a,B</sup> ±1
Lye (control*)	14% <sup>b,A</sup> ±2	26% <sup>b,B</sup> ±1
Washing waters treated with <i>A. niger</i> for 6 days	78% <sup>c,A</sup> ±1	98% <sup>c,B</sup> ±0
Washing waters (control*)	12% <sup>b,A</sup> ±1	20% <sup>d,B</sup> ±1

\*Non–inoculated effluents incubated under the same conditions as those for the biologically treated ones. Data are the mean ± standard deviation ( $n = 3$ ). Different letters in the same column (a, b, c, d) and row (A, B) represent significant differences in percent reduction ( $p < 0.050$ ).

The overall percent reduction in TPP content of treated washing waters was strongly correlated with that in the absorption band at 270 nm ( $R^2 = 0.893$ ,  $p = 0.000$ ) (Fig. 1). The respective correlation for the treated lye was slightly poorer ( $R^2 = 0.771$ ,  $p = 0.002$ ), but significant (Fig. 2).

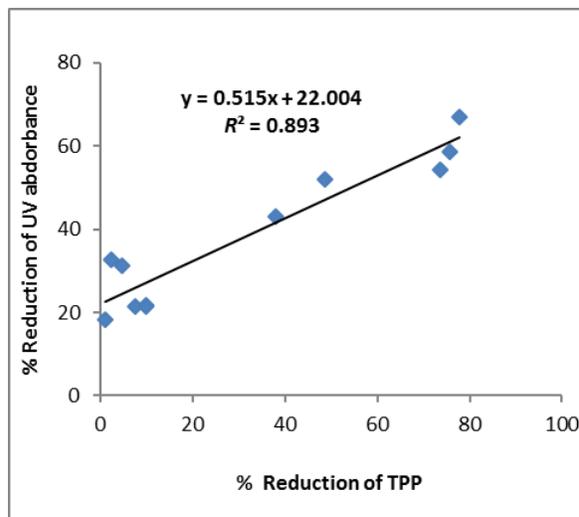


Figure 1. Relation of the percent reduction of the UV absorbance at 270 nm and total polar phenol (TPP) content (Folin–Ciocalteu assay) in washing water effluents treated with *A. niger*.

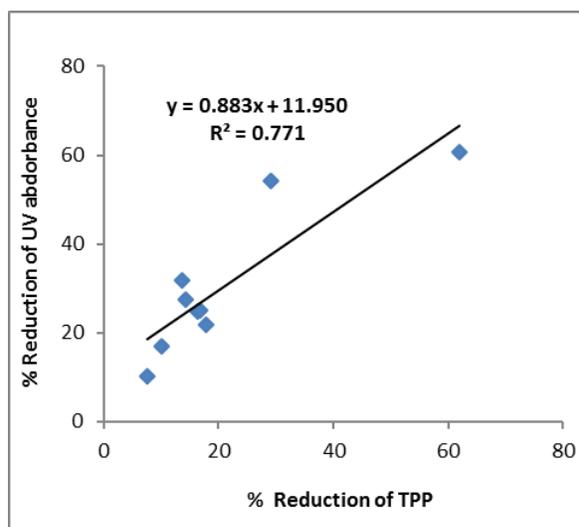


Figure 2. Relation of the percent reduction of the UV absorbance at 270 nm and total polar phenol (TPP) content (Folin–Ciocalteu assay) in lye effluents treated with *A. niger*.

After 6–8 days of the biological treatment, the degree of total organic pollutant matter removal, as measured by the COD values in the lye and washing water effluents, were 58% and 74%, respectively, in line with previous information. This finding indicates that the fraction of COD that is removed is mainly attributed to the biodegradable phenolic compounds. Based on all the above, UV–Vis spectrophotometry can be proposed as a simple

cost-effective methodology to monitor routinely the efficiency of this detoxification biological process using *A. niger*.

#### 4. CONCLUSIONS

The use of UV-Vis spectrophotometry allows the effective control of the detoxification efficiency of Spanish-style green olive processing wastewaters by *A. niger* B60. The percent reduction of the absorption band at 270 nm in effluent UV spectra was strongly correlated with the respective one of TPP content determined by the F-C assay. RP-HPLC analysis gave more information about degradation mechanism, demonstrating high fungal selectivity in degradation of simple phenolic classes. As a result, the simple, rapid and low-cost UV spectrophotometry could be a useful tool for monitoring phenol removal from the streams and, thus, the detoxification efficiency of the biological treatment.

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