Estimating the bioremediation of green table olive processing wastewater using a selected strain of *Aspergillus niger*

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ABSTRACT

Green and black table olive processing wastewater (TOPW) is a notoriously polluting and difficult to treat wastewater. Although in literature, there are plenty of methodologies suggested for the treatment of TOPW,, insofar no environmentally safe TOPW disposal practice has been applied systematically. Some are not applicable to green table olive processing, due to the chemical characteristics of the debittering effluents, whilst most of them do not seem appropriate for full-scale application.

This study reports on the laboratory development of a biological treatment for the wastewater arising from the debittering process of "Spanish type" green table olives, using a selected strain of *Aspergillus niger*. Two duplicated treatments from a single green table-olive producing plant were examined in order to assess the bioremediation potential of the selected strain of *A. niger*, under aerobic conditions. The wastewater arising from two different production processes was examined: a) the typical debittering protocol, using dilute NaOH solution, and, b) an alternative protocol, using dilute KOH solution.
The initial COD of the wastewater examined for the NaOH treatment ranged from about 5.0 to 21.6 g/l, corresponding to a COD removal efficiency of 60- 87%. The initial COD of the wastewater for the KOH treatment ranged from about 3.0 to 18.4 g/l, corresponding to a COD removal efficiency of 50-87%. Although a large amount of the wastewater organic load was removed, a substantial amount remains. Substituting NaOH with KOH seems a promising option, as the latter wastewater may be beneficially added to the soil after biological treatment.

Keywords: Wastewater, Table olives, Spanish type olives, biological treatment, *Aspergillus niger*.

1. INTRODUCTION

Green and black table olive processing wastewaters (TOPW) constitute a major polluting factor mainly due to their high phenolic content and organic load. Specifically in the Mediterranean region, this factor could be transformed to a real threat since high volumes of the TOPW are seasonally produced and usually disposed untreated to streams or to the sea [1]. At the best case, they are stored in evaporation ponds [2], where anaerobic conditions are quickly established leading to malodours, breading of insects and risks of surface and groundwater contamination. This does not quite reflect the trend that is noticed to the scientific literature: plenty of papers study and suggest methodologies for the treatment of TOPW [3]. Certainly, the given situation does not conform to the current and the forthcoming environmental regulations.

Referring to green table olives (so-called "Spanish type"), the preparing processing involves crop selection, cleaning, debittering using NaOH solution, washing, fermentation and canning. The largest amount of the wastewater is produced through the debittering and washing stages. The effluents of these stages also constitute the most heavily polluted fraction of the table olive processing wastewater (TOPW). Up to date, numerous treatment methods have been evolved, such as aerobic and anaerobic biological processes, advanced oxidation processes, and potential combinations of them. Biological treatment methods have been presented as overall economical and effective processes [4](, but the composition of the TOPW (i.e. polyphenols) often inhibits the biodegradation ability of microorganisms and thus, reduces the biodegradation efficiency of the treatment. An alternative approach to the minimisation of the polluting burden of TOPW, is the substitution of NaOH with compounds beneficial to soil, such as KOH, as potassium is a known fertilising nutrient, in contrast to sodium.

This study reports on the laboratory development of a biological treatment of the wastewater produced from the debittering process of "Spanish type" green table olives production, using a selected strain of *Aspergillus niger*. Treatments from two green table-olive producing plants – one located in Greece and one in Tunisia - were examined in order to assess the bioremediation potential of the selected strain of *Aspergillus niger*, under aerobic conditions. The wastewater arising from two different production processes was examined: a) the typical debittering protocol, using dilute NaOH solution, and, b) an alternative protocol, using dilute KOH solution, given that K is more beneficial to soil. Trials were carried out using flask cultures. Variations in the pH values, electrical conductivity, oxygen uptake rate, chemical oxygen demand (COD), total solids, and total phenols, for 118 hours, were reported. In order to investigate the influence of the wastewater concentration to the fungus biodegradation ability, a total of 5 dilutions (100%, 85%, 70%, 55%, 40%) were tested.

2. MATERIALS AND METHODS

2.1. Wastewater

Green table olive processing wastewaters (fresh debittering wastewater and washing effluents, as they arise in the industry) were derived from two different production lines. Two cycles of experiments were conducted: the first treating wastewater derived form a producing plant (the Agricultural Cooperation of Rovies, in Greece), during the harvesting period of 2007, and the second from processing simulated in the lab (Tunisia).

The first production line followed the typical debittering process protocol, using diluted NaOH solution, while the second was adjusted to an alternative protocol, whereby NaOH is replaced by KOH. Table olive processing wastewater (TOPW) derived from both processes, were stored independently at –20^{o}C within 24 hours after sampling to avoid TOPW denaturation due to the auto-oxidation of phenolic compounds [5].

Prior to the biological treatment, the pH value of the TOPW was adjusted to 4.6 (±0.3) adding conc. H_2SO_4, in order to retain selectivity for the proliferation of *A. niger*. The alternative option of wastewater sterilization was rejected because it would not be economically feasible for full-scale treatment.

TOPW from both large scale production lines were examined in duplicate (S1 & S2: TOPW from NaOH process; P1 & P2: TOPW from KOH process). In order to estimate the effect of the organic load and the total suspended solids concentration on the performance of the biological treatment system, five duplicated dilutions (100%, 85%, 70%, 55% and 40%) of S1, S2, P1 and P2 were biologically treated and tested. TOPW (S3: TOPW from NaOH process; P3: TOPW from KOH process) aroused by the simulated process were biologically treated undiluted. Physical and chemical properties of the S1, S2, S3, P1, P2 and P3, before pH adjustment, and the relevant dilutions used for the biological treatment are shown in Table 1.

Table 1: Physical and chemical characteristics of the wastewater solutions used, before pH adjustment.

Experiment*	COD (g/L)	pH	EC (mS/cm)	TSS (g/L)	Colour (OD 390nm)	TP (mg gallic acid/l)
$S1_{100}$	21.6	11.95	10.67	1.18		475
$S1_{85}$	17.6	12.15	9.92			252
$S1_{70}$	15.6	11.92	8.77	0.93		498
$S1_{55}$	11.2	11.50	7.42	0.73		263
$S1_{40}$	6.6	11.40	5.46	0.55		256
$S2_{100}$	14.5	12.09	9.09	0.11		248
$S2_{85}$	14.4	11.91	7.82	0.41		157
$S2_{70}$	7.9	11.73	6.53	0.38		55
$S2_{55}$	9.1	11.85	5.53	0.25		87
$S2_{40}$	4.9	11.71	4.03	0.15		75
$S3_{100}$	12.17	12.52		1.45	2.34	875
$P1_{100}$	18.4	11.32	8.75	0.30		538

$P1_{85}$	12.0	11.24	7.69	0.68		748
$P1_{70}$	10.4	11.18	6.43	0.25		200
$P1_{55}$	7.9	11.21	5.23	0.20		2
$P1_{40}$	2.9	11.16	4.10	0.38		299
$P2_{100}$	14.5	11.55	9.01	0.54		157
$P2_{85}$	10.2	11.53	7.72	0.44		223
$P2_{70}$	9.5	11.50	6.52	0.16		183
$P2_{55}$	7.1	11.26	5.34	0.15		201
$P2_{40}$	5.8	11.28	3.88	0.33		79
$P3_{100}$	8.92	12.37		1.33	1.23	638

(*) S and P denote debittering with NaOH and KOH, respectively; 1, 2,3 denote the cycle of experiment; the subscript denotes TOPW concentration.

2.2. Inoculum and aerobic biological treatment

S1, S2, S3, P1, P2 and P3 were treated aerobically using a specific strain (B) of the acidophilic and acid producing filamentous fungi *Aspergillus niger* (Harokopio University collection- [6]). The inoculum preparation is explicitly described by Kotsou *et al*. [1] and Kyriacou *et al.* [6]. The aerobic biological treatment of TOPW was carried out twice – in Greece and in Tunisia - at a laboratory scale, in a non-sterile system using Erlnemeyer flasks containing acidified TOPW (5:1 $V_{Erlenmeyer\ flask}$: V_{TOPW}). The inoculated flasks were continuously shaken on a rotary incubator operating at 180 rpm, at 30˚C irrespectively, for 118 hours. All cultures were performed in triplicates.

During the biological treatment samples of approximately 10ml were withdrawn from S1, S2, P1 and P2 at regular intervals (0, 6, 10, 22, 28, 34, 46, 70, 94 and 118h), to analyze the biodegradation of the reacting media. Likewise, samples of approximately 10ml were withdrawn from S3 and S4 on a daily basis.

2.3. Analytical methods and calculations performed

Electrical conductivity (E.C.), pH value, chemical oxygen demand (COD) on S1, S2, P1 and P2, and volatile suspended solids (VSS) were determined according to standard methods [7]. The efficiency of the biological treatment was assessed in terms of COD reduction (CODred %). The respiration rate, measured as oxygen consumption rate (OCR – mg O_2/L/h) was calculated from the rate of change in the dissolved oxygen concentration of the sample (YSI, probe 5718, meter 52CE). In S3 and P3 fungal biomass was determined by counting cell of Malassez and by total suspended solids (TSS) which were obtained by filtration on Whatman filter (0.45 mm). The soluble COD was measured on the centrifuged OMW at 6000

g during 30 min using a PALINTEST 5000 photometer. The results presented are means of three replicates.

2.4. Decolorization assay

Decolorization was assayed by the measurement of absorbance at 390 nm (Spectrophotometer JENWAY 63200 UV/VIS) and was calculated as defined in equation 1.

$OD_{red} = ((OD_0 - OD_f) / OD_0) \times 100$

2.5. Total phenolic compounds

Total phenolic content (TP) was measured in triplicate using the Folin-Ciocalteu's phenol reagent; involving the addition of 200µl Folin-Ciocalteu's phenol reagent to 3.6ml diluted sample. After three minutes, 800µl sodium carbonate (200g/l) were added. The mixture was let at 100°C for one minute. After cooling, the absorbance was measured at 750 nm as described previously by Catalano *et al*.[8].

2.6. Molecular mass distribution of polyphenolics

Gel filtration on Sephadex G-50 was used to analyze the polymeric aromatic fraction present in different samples of TOPW on S3 and P3. Three milliliters of sample were filtered and placed on a Sephadex coarse G-50 column (2.5 × 60 cm) previously equilibrated with $NaNO_3$ 0.05 M containing 0.02% sodium azide at a flow rate of 0.6 ml/min. The effluent was collected on the basis of 3 ml per tube. The optic density of these fractions was measured spectrometrically at 280 nm. The column was calibrated with syringic acid (MM = 198 Da), lysozym (MM = 15 kDa) and blue dextran (MM = 200 kDa).

2.7. HPLC analysis

A reversed-phase high performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds contained in the ethyl acetate extracts of TOPW'S. The HPLC chromatograph was performed on an Agilent Technologies apparatus (1200 series) composed of a VWD detector. Elutes were detected at 280 nm. The column was (4.6x250 mm) model Shimpach VP-ODS and its temperature was maintained at 40°C. The flow rate was 1ml/min. The mobile phase used was 3% acetic acid in water (A) versus 50% acetonitrile in methanol (B) for a total running time for 40 min.

2.8. Statistical analysis

To compare the growth, the decolorization and the reduction of COD of the two effluents. the ANOVA analysis were performed, and the means of the significantly different results were compared at $p < 0.05$.

3. RESULTS AND DISCUSSION

As it is pronounced by the design of this study, monitoring of the biological treatments was aiming at the estimation and thereafter the improvement of the *A. niger* biodegradation capacity, preparative to exploit it

at full-scale systems. Within this frame, factors relevant to the biological activity of *A. niger*, as well as biodegradation indices were monitored.

In this work, two types of effluents from the debittering and washing process of Spanish type green table olives were used to examine the effect of substituting NaOH with KOH on the biodegradability of the wastewaters by *Aspergillus niger*.

The liquid residues are characterized by an alkaline pH (11 -12.5), and a high content of organic compounds which includes sugars and phenolic compounds (Table1). The COD, the phenols content and the color were lower in the P3 compared to S3. However, in all experiments, the pH value was adjusted to 5 in order to promote the preferential growth of *Aspergillus niger* which is an acidophilic organism. Moreover, phenolic compounds are more stable and not oxidized in acid solutions [9].

The determination of biomass concentration (TSS) versus incubation time showed that mycelia grew especially during the first two days metabolizing sugars and other simple compounds then reducing COD and pH (figure 1). *Aspergillus niger* was not be affected by the toxic substances in the two effluents. However, different biomass concentrations are obtained at the end of the fermentation depending on the medium composition. An improvement of growth was observed in the waste (TOPWK) because of its lower content in phenolic compounds in comparison with TOPWNa. The final pH was decreased from 5 to 3.6 in the medium (TOPWK) and from 5 to 4 in the medium (TOPWNa). The final pH is an indicator of *Aspergillus* activity. The anova analysis of the data indicated that the difference of biomass concentration (TSS), COD and pH reductions of the two wastes were statistically significant ($p<0.05$)

The biodegradation of the two wastes was evaluated by the reduction of COD, decolorization and phenolic compounds degradation.

Aspergillus growth induced a higher rate of COD removal during the first two days. The COD reduction was 48% and 60% respectively in the medium TOPWNa and TOPWK after five days of incubation. The results of this work are relatively similar to those found by previous researchers. For example, Hamdi *et al.* [10] reported that using *Aspergillus niger* in aerobic condition reduced 52.5% the COD of olive mill wastewaters.

The COD removal was showed considerable similarity to OD removal. In fact, *Aspergillus* growth led to a decrease of color intensity and phenols content of the two wastes (figure 2). The anova analysis of the data indicated that the difference of OD removal and phenols degradation of the two wastes were statistically significant ($p<0.05$).

The analysis of the two wastewaters before and after culture of *Aspergillus niger* showed that efficiency of the degradation of total phenolic compounds was 27% in TOPWNa and 36% in the TOPWK. The coloration of the TOPW decreased markedly after cultivation with the strain of *Aspergillus niger* probably due to the degradation and adsorption of some phenolic compounds on the mycelium. These results were already shown by Hamdi *et al*. [10] when *Aspergillus niger* has been cultivated on OMW.

Strains *Aspergillus terreus* and *niger* [10, 11] have been reported to carry out degradation of phenolic compounds of OMW. Therefore, there is great interest in the use of these strains for treatment of phenolic-rich effluents.

Figure 3 showed the Sephadex G-50 chromatograms of phenolic compounds in the two wastewaters. The TOPW showed two families of phenolic compounds. The first family was eluted at the exclusion peak and responsible of dark color, while a second peak corresponds to the low molecular weight fractions.

After five days of incubation, the Sephadex G-50 chromatograms of the two TOPW'S treated with *Aspergillus niger* showed the disappearance of some phenolic compounds with high molecular weight and the appearance of others with an intermediate weight. The reduction of the polyphenols with high molecular mass was more marked in the effluent (TOPWK).

HPLC analysis was used to identify the phenolic monomers that made up these polyphenols. Results revealed the presence of several phenolic compounds in ethyl acetate extracts of untreated TOPW'S as shown in Table 2. Oleuropein, tyrosol and vanilic acid were found to be the most abundant components in the effluent (TOPWNa). Besides these compounds significant concentrations of gallic acid, caffeic acid, p-coumaric acid and vanillin were also found. However, TOPWN is more charged in phenolic compounds than TOPWK. In fact, several compounds are present in this effluent and absent in the other such as: O-coumaric acid, M- coumaric acid and vanillic. Apparently, NaOH promotes more the diffusion of phenols in the medium. Treated TOPWNa by *Aspergillus niger* showed a significant removal in the concentration of total simple phenols content and the emergence of new compounds resulting from the conversion of others phenolic compounds. The decrease in the concentration of phenolic compounds has been more important in the case of the treated TOPWK.

In Table 2 the evolution of EC, SS and VSS in the experiments with undiluted TOPW debittered using NaOH ($S1_{100}$) and KOH ($P1_{100}$) are presented. The variation of pH and OCR and COD and COD reduction for the two replicate basins for each treatment are given in Figures 1 and 2 respectively. These runs were considered as typical examples of the studied wastewater media.

Table 2: Evolution with time of selected parameters in experiments $S1_{100}$ and $P1_{100}$.

Time (h)	EC (mS/cm)	SS (g/L)	VSS (g/L)	EC (mS/cm)	SS (g/L)	VSS (g/L)
		$S1_{100}$[1]			$P1_{100}$[1]	
BA[2]	10.67	1.18	0.925	8.75	0.30	0.287
0	11.40	2.86	2.300	9.30	1.93	1.319
6	11.55	1.98	1.950	9.75	2.55	1.988
10	11.61	2.64	2.013	9.52	2.08	1.488
22	11.40	3.30	2.756	9.53	2.51	2.044
28	11.52	3.64	2.744	9.63	2.36	1.963
34	11.64	4.14	3.375	9.65	2.48	3.194
46	12.17	4.16	3.569	9.90	2.64	2.188
70	12.39	4.29	3.856	10.23	3.03	2.356
94	12.07	5.18	4.175	9.63	3.27	2.663
118	12.17	3.75	3.475	9.43	2.91	2.513

1. S and P denote debittering with NaOH and KOH, respectively; the subscript denotes TOPW concentration. 2. BA: before pH adjustment

Initial pH values of TOPW were adjusted to 4.4-4.9 to improve medium selectivity for the proliferation of *A. niger*. The alternative option of wastewater sterilization was rejected because it would not be economically feasible for full-scale treatment. However, the aforementioned adjustment with conc. H_2SO_4, resulted to an increase of suspended solids and decrease of COD values (Table 2). The rise in suspended solids values has been reported in previous studies [7] and seems to feature a systematic error in TS and VS measurements.

Biological activity was followed through the pH variation, as *A. niger* is an acid producing fungi, and the respiration rate (Figure 1). Kyriacou et al. [6] have indicated the dependency of pH values on the acid production capacity of *A. niger*. In the present study, in all trials, the pH values remained almost steady for 10 to 22 h of treatment and decreased thereafter. The invariability of pH during the first 10-22 h of treatment could be attributed to the lag phase of the strain.

According to Lasaridi and Stentiford [12] the respiration rate forms a global indicator of biological activity, independently of the type of the acting microorganisms. In Figure 1b, the respiration rates (OCR) of the undiluted TOPW are illustrated. Among the four treatments, the $S1_{100}$ sample exhibited the highest OCR. It is worth mentioning that samples from the NaOH debittering processes exhibited the highest OCR value at 34h, while samples from the KOH lines exhibited peak OCR values at 22h. During the growing phase of *A. niger* (10 to 34h), OCR values were peaked. Thereafter, an exponential decrease was noticed, followed by a slight fleeting increase, which could indicate a second phase of growth on the biodegradation metabolites. However, this finding calls for further investigation. The maximum OCR value achieved for each culture flask was typical for batch cultures, demonstrating an initial phase of exponential growth, indicative of the amount of the available substrate. Considering the concentration of the examined solution, the OCR values were decreasing following the level of the dilution. Furthermore, a delay at the peak values was noted at the 40% solutions (results not shown), which could be attributed to the insufficient density of organic substances to feed the fungal population.

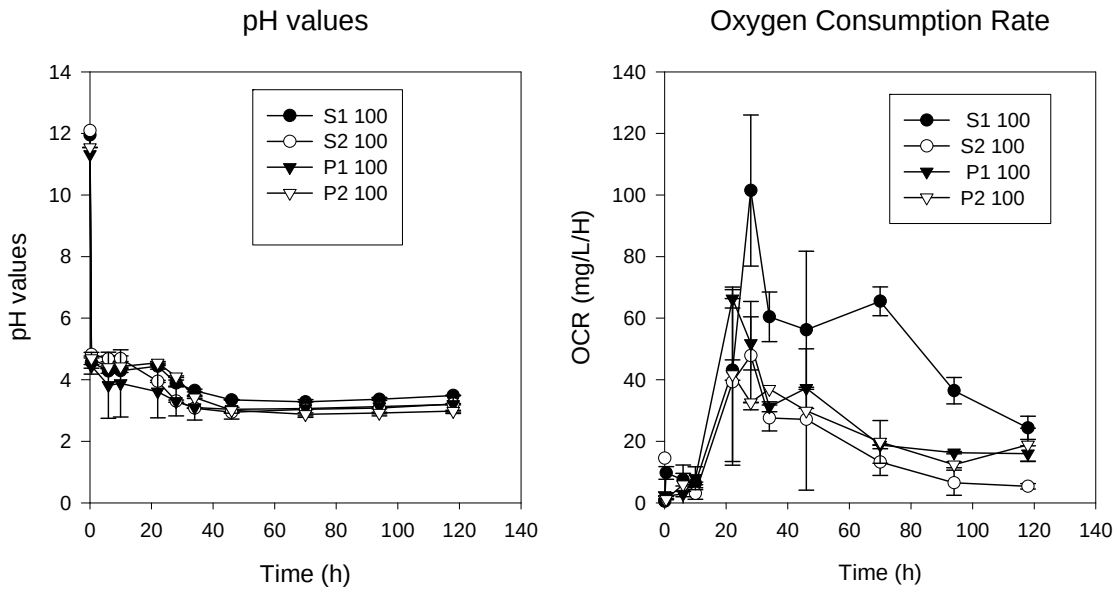

Figure 1: Variation of a) pH values, and b) Oxygen Consumption Rate, during the biological treatment of undiluted wastewater with *A. niger*. S and P denote debittering with NaOH and KOH, respectively.

Referring to the COD variation (Figure 2), it should be noted that debittering solutions even when they are derived from the same olive processing plant and the same harvest period, is characterised by heterogeneity. The COD of the effluents derived from debittering with NaOH varied among the two replicates ($S1_{100}$, 21.6g/L; $S2_{100}$, 14,5g/L). However, the COD reduction of all five dilutions (60 -87%, results not shown), indicates that even the 40% dilution showed an important organic load removal capacity. The initial COD of the wastewater for the KOH treatment ranged from about 3.0 to 18.4 g/l, with a COD removal efficiency varying from 50 to 87%. In spite of the variation in the initial COD values, the COD reduction did not differ significantly for the replicate treatments. Moreover, COD reduction was satisfactory for both debittering processes.

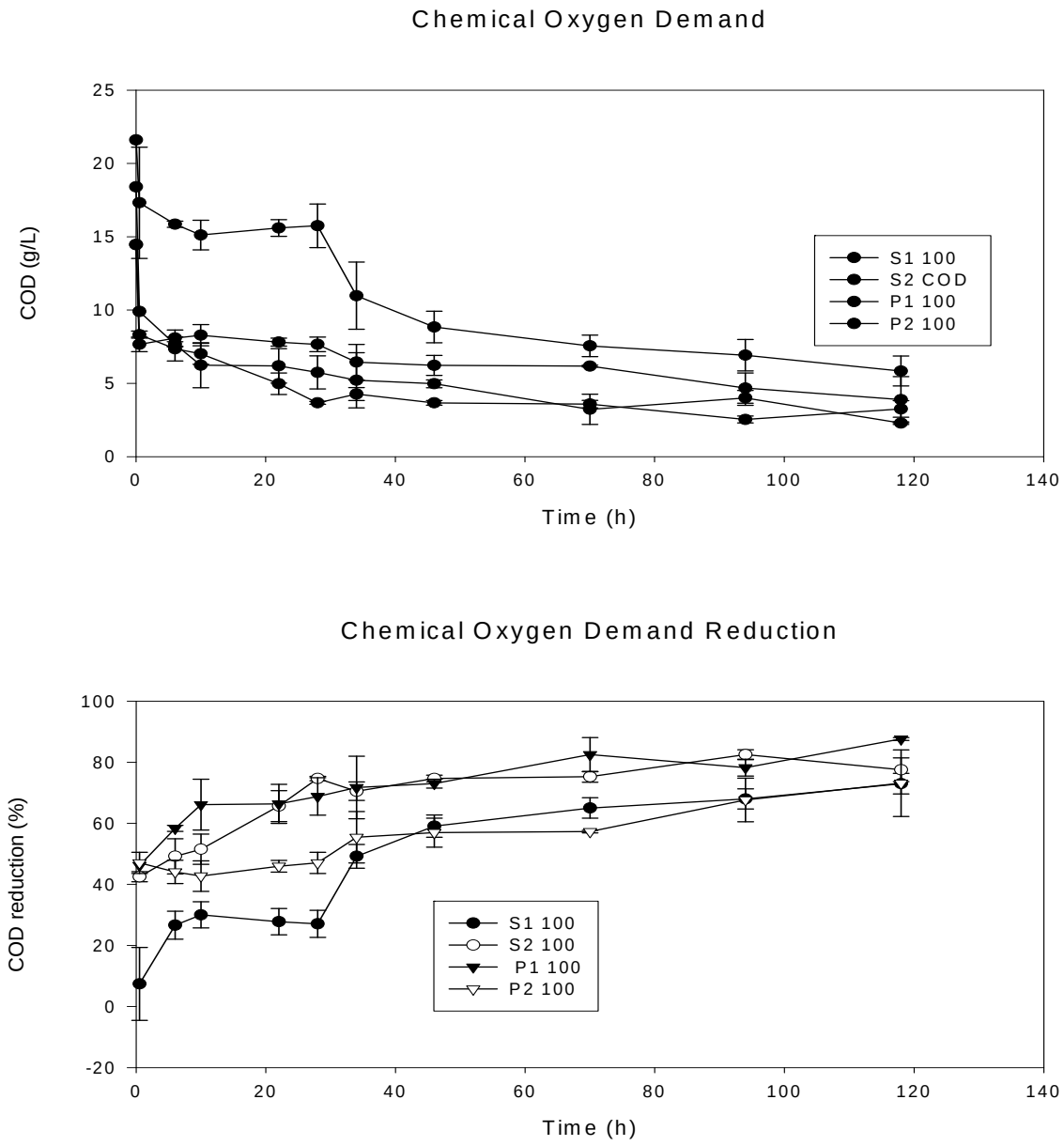

Figure 2: Variation of a) COD, and b) COD Reduction, during the biological treatment of undiluted wastewater with *A. niger*. S and P denote debittering with NaOH and KOH, respectively.

Total phenolics (results not shown) increased slightly after acidification, which is in accordance with the findings of Lasaridi et al. [5]. During the biological treatment, they were decreased. The same pattern was noticed by Kotsou et al.[7].

4. CONCLUSIONS

A large part of the organic load was removed from TOPW through the biological treatment with *A. niger*. Although this amount reached 87%, the use of further treatment should be taken under consideration.

Substituting NaOH in the debittering process of olives with KOH was shown to be a promising and feasible approach. The biodegradation of polyphenolics and their potential contribution to the inhibition of the wastewater biodegradation requires further investigation.

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