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Effect of acclimatization factors on reproducibility of biogas production in anaerobic cultures from electrochemically pre-treated or filtered olive mill wastewater

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ABSTRACT

Olive mill wastewater (OMW) is an agro industrial waste with a high organic load produced mainly in the Mediterranean region. Its inherent toxicity to anaerobic microorganisms is attributed to its high content in COD and phenols. Many treatments have been proposed for the remediation of this waste both physicochemical and biological but there is not one that is applicable in all cases. In this work, olive mill wastewater was treated anaerobically at different growth conditions. The waste introduced in the cultures was either filtered or electrochemically pretreated OMW. Two sets of experiments were conducted to evaluate the effect of temperature, HS-Co-M, fatty acids and OMW pretreatment on biogas production efficiency. The thermophilic growth exhibited extended lag phase compared to the mesophilic growth. Biogas production was strongly dependent on growth supplements. Both HS-Co-M and fatty acids enhanced biogas production in the presence of filtered and electrolyzed OMW. In a second set of experiments, the effect of a mixture of fatty acids was investigated in combination with pretreated or raw OMW.

Keywords: Anaerobic; Olive mill wastewater; Electrolytic pretreatment; Acclimatization; Biogas

1. Introduction

Olive oil production is a successful business as the worldwide demand for olive oil is increasing. This increase in demand is accompanied with an increase in the total amount of wastewater produced. Biological treatment techniques are of interest here due to environmental concerns. They are considered to be environmentally friendlier especially when biogas, its byproduct, can be further exploited. Many methods have been proposed for treating olive mill wastewater (OMW) and have been reviewed extensively [1–3]. Valorization strategies have been also reviewed for OMW and anaerobic digestion has its essential validity before final waste disposal as a bioconversion process that produces biogas [4].

Anaerobic digestion is a complex biological process. It involves the coordination of many microorganisms belonging to different groups where one's product is the substrate of the next. The major groups involved in this process are: the hydrolyzing bacteria, the acidogenic bacteria, the acetogenic bacteria and the methanogenic archaea. The latter group plays the most important role in the process as it involves the step where the production of methane occurs and where the important role of coenzyme M in the biochemistry of all methanogens has been recognized [5,6]. The interactions among the microorganisms are widely affected by the conditions prevailing in their environment such as pH, temperature and type of waste introduced. One

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of the drawbacks of anaerobic digestion is its long start up period due to lack of proper seed sludge. There is evidence that the phenolic compounds present in the OMW inhibit biogas production [7–9]. The concentration of each one of the most important phenolic constituents of OMW affects differently the methanogenesis in anaerobic experiments [7,8]. Borla et al. [8] reported that tyrosol and *p*-hydroxybenzoic acid are anaerobically degraded and even enhance methane production when concentration is up to 600 mg/l and 300 mg/l, respectively; however, the lower the concentration of tyrosol and *p*-hydroxybenzoic acid is, the shorter becomes the acclimatization period of the cultures. Polyphenols are degraded only partially in methanogenic environments but not in acidogenic [9].

Many researchers acknowledge physicochemical pretreatment as a necessity for the successful management of OMW. Mantzavinos and Kalogerakis [3] reviewed all integrated approaches that combine the main anaerobic treatment with a chemical pretreatment step. Sabbah et al. [10] reported that the efficiency of anaerobic digestion of OMW is enhanced by a pretreatment step, such as filtration through sand and activated carbon, which resulted in a reduction of the concentration of phenols from 2210 to 285 and 108 mg/l, respectively. In another study Sabbah et al. [11] found that enhanced COD removal efficiency is achieved when OMW is physicochemically pretreated. They attribute this improvement to the higher removal efficiency of polyphenols from the OMW by the pretreatment step.

Gotsi et al. [12] reported a 15% COD removal after two hours of electrochemical oxidation over a Ti/Ta/Pt/Ir anode and 100% Total Phenols removal after three hours when the initial COD concentration was 5180 mg/l. They also reported the production of organochlorides which are toxic to microorganisms while there are contradictory findings in the literature [13]. Giannis et al. [14] reported that electrooxidation of OMW with a Ti/Ta/Pt/Ir anode had best results when sodium chloride was used as an electrolyte at 3% salinity. After eight hours of reaction at 3% salinity and 0.35 A/cm^2 current density, COD was reduced by 70.8% while the specific energy consumption increased with treatment time. Kotta et al. [15] have performed electrolytic experiments with OMW and suggested that when electrolysis is used as a pretreatment step, in order to reduce the phenols, it is very important to previously separate the solid fraction from the OMW. They also reported that for high initial concentrations of phenols, their degradation increases with increasing salinity and current densities over an Ti/Pt anode.

Acclimatization of the initial seed is also considered necessary step for successful anaerobic treatment of OMW [2].

The aim of this study is primarily to investigate the acclimatization conditions of biomass which can enhance COD removal efficiency of seed biomass and contribute to the minimization of the lag phase in batch anaerobic experiments treating OMW. Additionally the aim of this study is to investigate whether electrochemically treated OMW enhances biogas production in anaerobic cultures compared to filtered OMW, whether it yields substances which are toxic to the anaerobic microorganisms; and finally, to test the reproducibility of the optimum acclimatization conditions of anaerobic cultures for OMW treatment corresponding to maximum biogas production.

2. Materials and methods

2.1. Olive mill wastewater

OMW was taken from a three phase olive mill in the region of Chania. It was centrifuged and filtered before being subjected to electrochemical or anaerobic treatment. Parameters of initial and conditioned OMW are presented in Table 3.

2.2. Biomass

The seed used in all experiments was taken from an UASB bioreactor working with olive mill wastewater. It was provided by the laboratory of Wastewater Management and Treatment Technologies at the Democritus University of Thrace. The initial biomass of the second set of experiments was also taken from the same seed.

2.3. Nutrients and chemicals

The concentration of the nutrients in the stock solution is as follows: $Na_2S \cdot 9H_2O$ (0.5 g/l), Cysteine–$HCl \cdot H_2O$ (0.5 g/l), Resazurin (0.001 g/l), $NaHCO_3$ (4 g/l), Na-formate (2 g/l), Na-acetate (1 g/l), Yeast extract (1 g/l), $FeSO_4 \cdot 7H_2O$ (0.002 g/l), $CaCl_2 \cdot 2H_2O$ (0.05 g/l), NH_4Cl (0.4 g/l), NaCl (0.4 g/l), $MgSO_4 \cdot 7H_2O$ (0.4 g/l), KH_2PO_4 (0.5 g/l).

One ml/l of trace element stock solution was added to the basal medium. The stock solution contains: HCl (2.5 g/l), $NiCl_2 \cdot 6H_2O$ (0.024 g/l), $Na_2MoO_4 \cdot 2H_2O$ (0.036 g/l), H_3BO_3 (0.006 g/l), $CuCl_2 \cdot 2H_2O$ (0.002 g/l), $ZnCl_2$ (0.07 g/l), $CoCl_2 \cdot 6H_2O$ (0.19 g/l), $FeCl_3 \cdot 6H_2O$ (2.037 g/l), $MnCl_2 \cdot 4H_2O$ (0.1 g/l).

The concentration of vitamins to the medium (stock solution) is: Biotin (2×10^{-4} w/v), Folic acid (2×10^{-4} w/v), Pyridoxine-HCl (10^{-3} w/v), Thiamine–$HCl \cdot 2H_2O$ (5×10^{-4} w/v), Riboflavin (5×10^{-4} w/v), Nicotinic acid (5×10^{-4} w/v), D–Ca–pantothenate (5×10^{-4} w/v), Vitamin B_{12} (10^{-5} w/v), *p*-Aminobenzoic acid (5×10^{-4} w/v), Lipoic acid (5×10^{-4} w/v).

Table 1
1st Experimental design—Parameters tested for optimum acclimatization conditions of initial seed: HS-Co-M, Temperature, OMW filtered and electrolyzed

Treatments	HS-Co-M	Temperature (°C)		OMW		
		T1 (36°C)	T2 (55°C)	(Control) no waste	OMW pretreated	OMW raw
α	+	–	+	+	–	–
ζ	–	–	+	+	–	–
β	+	+	–	+	–	–
Γ	–	+	–	+	–	–
δ^-	–	+	–	–	–	+
δ^+	+	+	–	–	–	+
ε^-	–	+	–	–	+	–
ε^+	+	+	–	–	+	–
Number of experiments per factor	4	6	2	4	2	2

The concentration of nutrients in the second set of experiments differs according to the experimental plan (Table 1) by dilution 4:5.

The HS-Co-M was added in a final concentration of 1 mM and 0.8 mM in the first and second set of experiments respectively.

Fatty acids mixture (final concentration in brackets): Valeric acid (0.4 g/l), Isovaleric acid (0.4 g/l), 2-Methylbutyric acid (0.4 g/l), Isobutyric acid (0.4 g/l).

OMW was added in great dilution (1:750) in the anaerobic cultures, the same dilution for pretreated and raw OMW (Table 1). The electrolyte added in the electrochemical treatment of olive mill wastewater was NaCl, at a concentration of 4% w/v.

Table 2
Second Experimental design–Parameters tested for optimum acclimatization conditions of initial seed: Mixture of fatty acids, OMW filtered and electrolyzed

Treatments	Mixture of fatty acids	OMW	
		OMW pretreated	OMW RAW
Λ (Control)	–	–	–
Δ	+	–	–
Χ	–	–	–
Φ	+	+	–
Number of experiments per factor	2×3	2×3	1×3

2.4. Equipment

The anaerobic cultures were performed in 250 ml polycarbonate bottles with adequate screw caps with septum. They were incubated in the dark under constant temperature according to the experimental plan (Table 1, Table 2). Preparation of nutrient medium, seeding and sampling all took place in laboratory anaerobic hood under anaerobic conditions with mixture of air N_2/CO_2 in a concentration 80/20 (99,999% clarity). The above concentration was also the starting atmospheric concentration in the anaerobic bottles. The electrochemical experiments were performed over a titanium–tantalum–platinum–iridium (Ti/Ta/Pt/Ir) anode. The surface of the anode area is 58 cm^2. The experimental set up of the electrochemical pretreatment step is described in details elsewhere [12].

2.5. Anaerobic batch experiments

In the first experimental set, which was comprised of 8 experiments, the effect of (i) incubation temperature, (ii) the addition of HS-Co-M, (iii) the electrochemical pretreatment step of OMW, on the performance of anaerobic digestion in terms of biogas production and COD removal efficiency was evaluated. The impact of the ratio of seed biomass volume to medium as it was 10% v/v in treatments γ, δ^-, ζ and 5% v/v in treatments α, β, δ^+, ε^-, ε^+ was also investigated. The conditions of the 1st experimental set are summarized in Table 1. All batch experiments contained all necessary nutrients as mentioned in Section 2.3.

In the second experimental set, which was comprised of 12 experiments, the effect of (i) reduced concentration of nutrients (dilution 4/5 of all nutrients), (ii) the addition of a mixture of fatty acids, (iii) the electrochemical pretreatment step of OMW, on the performance of anaerobic digestion in terms of biogas production and COD removal efficiency was investigated. The ratio of seed biomass volume to medium it was 10% v/v in all treatments. The conditions of the 2nd experimental set are summarized in Table 2.

2.6. Pretreatment of OMW

OMW was conditioned by centrifugation and filtration before being used for electrochemical treatment. Parameters of initial and conditioned OMW are presented in Table 3. Two experiments were conducted, one for each set of anaerobic experiments. The current density applied in the electrochemical experiments is 0.518 A/cm^2 and 1.034 A/cm^2. Sodium Chloride was used as electrolyte at concentration 40 g/l. Initial concentrations of COD was 32 g/l and 45 g/l with initial TPh concentration 0.98 g/l and 1.5 g/l (gallic acid equivalent) respectively (Table 3). The pretreatment step lasted 180 min.

2.7. Analytical methods

2.7.1. Chemical oxygen demand (COD)

COD concentrations were measured with a Hach spectrophotometer using the dichromate method with vials for a COD range of 0–1500 mg/l. At all cases dissolved COD was measured, thus the samples were filtered through 0.45 μm nylon filter when taken from electrochemical experiment and 0.2 μm cellulose acetate filter when taken from biological experiments. Equally treated control sample with distilled water was used. Total COD measurements were performed, without filtrating the samples through a 0.45 μm pore size filter, for determination of total COD before filtration treatment. Appropriately, diluted samples were mixed with digestion solution that contained potassium dichromate, sulfuric acid and mercuric sulfate. It was incubated for 120 min at 150 °C in a COD reactor. COD concentrations were measured colorimetrically. The absorbance of the digested solution was measured at 620 nm on a DR/2010 spectrophotometer.

2.7.2. Total phenols (TPh)

Total dissolved Phenols were estimated with the Folin Ciocalteau [16] micro method for Total Phenol in wine. Samples were taken periodically during the electrochemical experiment to follow the degradation of phenols and were filtered through 0.45 pore size filter. The sample or gallic acid standard (20 μl) was mixed with the Folin Ciocalteau reagent (100 μl), water (1580 μl) and Sodium Carbonate (300 μl). After left for two hours in the dark at room temperature, the absorbance of the solution was measured at 725 nm on a Shimadzu UV 1240 spectrophotometer. The calibration curve was prepared using 0, 50, 100, 150, 250 and 500 mg/l gallic acid standards. TPh is reported as Gallic Acid Equivalent, GAE (mg/l).

2.7.3. Biogas production

The biogas samples were taken manually, by syringe in an anaerobic hood, at predetermined dates. The hour of sampling was kept the same each time in order to readily determine daily biogas production rates.

2.7.4. Total solids (TS)

TS where measured according to the standard methods for water and wastewater [17].

3. Results and discussion

3.1. Filtration and electrochemical pretreatment: effect on COD and total phenols

The results of filtration and electrochemical treatment are shown in Table 3. As seen, a great amount of total COD is removed through filtration only. This step is

Table 3
Characteristics of raw OMW, filtered OMW and electrochemically treated OMW

	TCOD (g/l)	TS (g/l)	COD (g/l)	TPh (g/l)	TPh reduction %	pH
Raw OMW	88.3	76	52.3	2.4	–	
Filtrated OMW	–	51–53.2	32–45	1.5–1	37.5–58.3	7.4
Electrochemically treated OMW for 180 min with 40 kg/m^3 NaCl and 1.034 A/cm^2	–	52.9	37.6	1.1	26.6	7.2
Electrochemically treated OMW for 180 min with 40 kg/m^3 NaCl and 0.518 A/cm^2	–	27	28	0.52	46.6	7.34

necessary before the electrochemical treatment; otherwise in the presence of solids, electrolytic treatment results in an increase of COD due to the dissolution of the solid matter [15]. Nonetheless, the remaining COD is still high and cannot be disposed as is. Electrooxidation of undiluted and filtered OMW over Ti/Ta/Pt/Ir anode for 180 min. resulted in COD removal 15.6 % and 16.7% for initial concentration of COD 32 g/l and 45 g/l respectively. Pretreated olive mill wastewater with a final TPh concentration 0.52 g/l (GAE) and 1.1 g/l was used in the first and second set of anaerobic experiments, respectively.

3.2. Anaerobic treatment

As shown in Fig. 1, according to the cumulative biogas production at the end of the 1st set of experiments (62 d) the control culture (γ-without OMW or HS-Co-M) grown at 36°C, produced more biogas (2322 ml/l) than all the other without any lag phase. This can be justified by the volume of seed biomass to medium ratio, which was double in comparison to the other control mesophilic culture β, that exhibited 12 d of lag phase. This leads to the conclusion that the initial biomass is capable of producing biogas without any lag phase when provided with adequate amount of nutrients and seed biomass. All cultures grown with OMW exhibit less biogas production even though they contained all necessary nutrients for growth. This suggests that biogas production can be affected by OMW negatively. In the case of percentage COD removal, there is an indication that it is positively affected by the initial COD concentration, regardless of additives or treatment of OMW. This is further explored in the second experimental set.

The cultures grown at 55°C (α, ζ) had a much longer lag phase as shown in Fig. 1. In particular, culture α, incubated with HS-Co-M, had the most extended lag phase of approximately 25 d, while the other thermophilic culture (ζ) had a shorter lag phase and greater biogas production. This is probably due to the ratio of seed biomass to medium volume which was double in the thermophilic culture ζ. Thus, we can conclude that the presence of HS-Co-M, did not improve the growth of biogas producers in order to compete with double seed biomass neither at the mesophilic or the thermophilic scale.

Both cultures with pretreated and filtered OMW produced more biogas in the presence of HS-Co-M (δ^{+}, ε^{+}) when compared to the ones without coenzyme M (δ^{-}, ε^{-}). HS-Co-M had a more positive effect on the culture with filtered OMW (138% more cumulative biogas production) even though the initial seed biomass of δ^{+} was half of δ^{-}. The effect of coenzyme M is positive also for % COD removal and for biogas produced per COD removed as shown in Fig. 2. The effect of the electrolytic pretreatment was not apparent in cumulative biogas production since in the presence of HS-Co-M it was negative ($815_{\varepsilon+}$ ml/l < $1028_{\delta+}$ ml/l), whereas in the absence of the cofactor it was positive ($771_{\varepsilon-}$ ml/l > $431_{\delta-}$ ml/l).

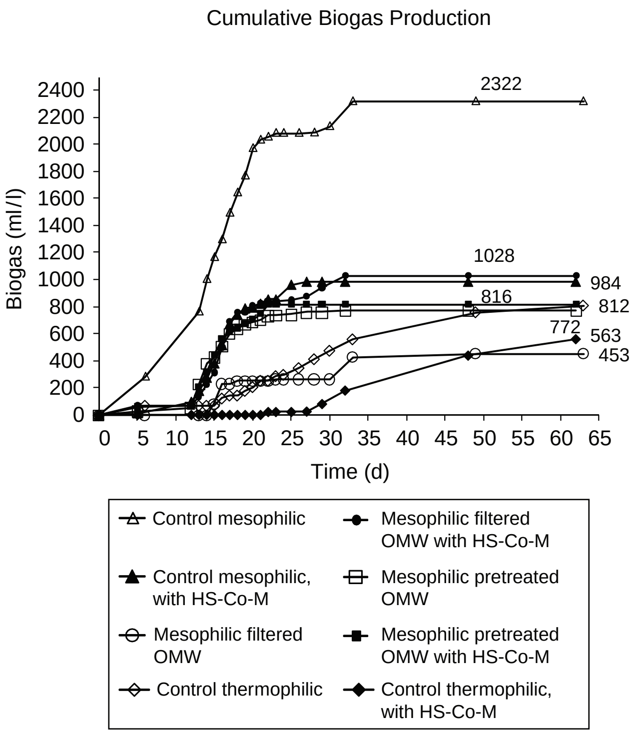

Fig. 1. Kinetics of biogas production (ml/l) of 1st set of batch anaerobic experiments.

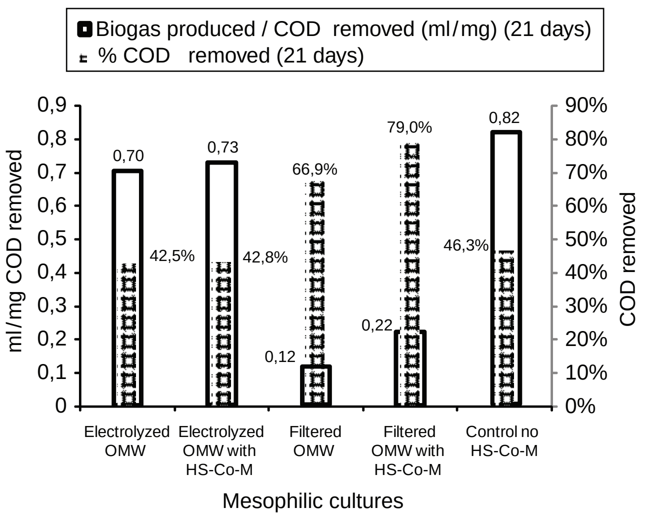

Fig. 2. Effect of HS-Co-M factor and electrochemical pretreatment on % COD removal and Biogas produced/COD removed of the 1st anaerobic set.

Table 4
Characteristics of 1st set of batch cultures

Culture	COD (mg/l) removed (12–33 d)	Biogas produced/ COD removed (ml/mg) (12–33 d)	COD % removed
ε^-	1020	0.70	42.5
ε^+	1027	0.73	42.8
δ^-	3640	0.12	66.9
δ^+	4297	0.22	79.0
γ	1890	0.82	46.3

ε^- : 36°C pretreated OMWW without HS-Co-M
ε^+ : 36°C pretreated OMWW with HS-Co-M
δ^- : 36°C filtered OMWW without HS-Co-M
δ^+ : 36°C filtered OMWW with HS-Co-M
γ: Control 36°C, without HS-Co-M

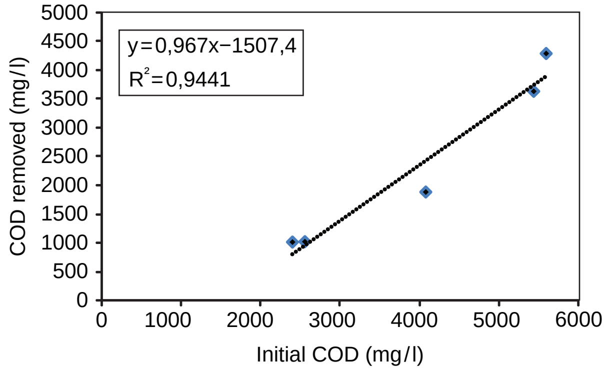

Fig. 3. Effect of Initial COD (mg/l) on COD removed of 1st anaerobic set.

In Fig. 2 the positive effect of electrolysis on biogas production per mg of COD removed is also shown which was calculated over the period that biogas was produced. There is a difference in the utilization of substrate by microorganisms reflecting different values in ratios; biogas production per COD removal (ml/mg). Thus, cultures grown with pretreated OMW, ε^- and ε^+, show 0.7 and 0.73 ml biogas production per mg COD removed (Table 4), suggesting that the constitution of substrate favors biogas production and *hence* the microorganisms that produce it. Additionally, in Fig. 2 the superiority of cultures grown with filtered OMW (δ^+, δ^-) is depicted on the percentage of COD removed, however, this is attributed to the higher initial COD of these cultures as also shown in Fig. 3.

In the second set of experiments the conditions that were proved beneficiary were kept the same with some minor modifications. That is the nutrient medium and the cofactor were diluted by 4:5. The dilution of the OMW and the ratio of seed biomass volume to medium were kept constant, however a mixture of fatty acids was introduced in two treatments. The cultures were grown at 36 °C in triplicates and the results shown in Table 5 represent average values of the three replications. The standard deviation values of the parameters is also shown.

In this set of anaerobic cultures there was not as much lag phase as in the previous set as seen in Fig. 4 and this is due to the higher initial seed biomass ratio (10%). Cultures grown with fatty acid mixture show higher cumulative biogas production (ml/l), Δ-1672 ml/l and Φ-1051 ml/l, and greater ml biogas produced per mg COD removed Δ-0.257ml/mg and Φ-0.176 ml/mg (Table 5). The effect of initial COD on % COD removed matches the result of the previous set of experiments. In Fig. 6, the high positive correlation of initial COD on % COD removal of anaerobic cultures in the area of initial COD tested (1740–6940 mg/l) is shown.

The effect of electrochemical pretreatment prior to anaerobic digestion did not appear to be superior to the filtered OMW as shown in Table 5. This is because the dilution

Table 5
Characteristics of 2nd set of batch cultures

Culture	COD* (mg/l) initial	COD* (mg/l) final 21st day	COD* (mg/l) removed (±sd)	Biogas produced/ COD removed* (ml/mg) (21 d) (±sd)	COD%* removed (±sd)	Biogas produced* ml/l (21 d) (±sd)
Λ	1740	604	1136 ± 91	0.258 ± 0.308	65% ± 5%	275 ± 321
Δ	6940	418	6522 ± 58	0.257 ± 0.050	94% ± 1%	1672 ± 309
Φ	6560	566	5994 ± 63	0.176 ± 0.149	91% ± 1%	1051 ± 883
X	3160	481	2679 ± 42	0.148 ± 0.140	85% ± 1%	400 ± 380

*Average values of triplicate cultures with standard deviation (sd)
Λ: Control
Δ: filtered OMWW with fatty acid mixture
Φ: pretreated OMWW with fatty acid mixture
X: pretreated OMWW without fatty acid mixture

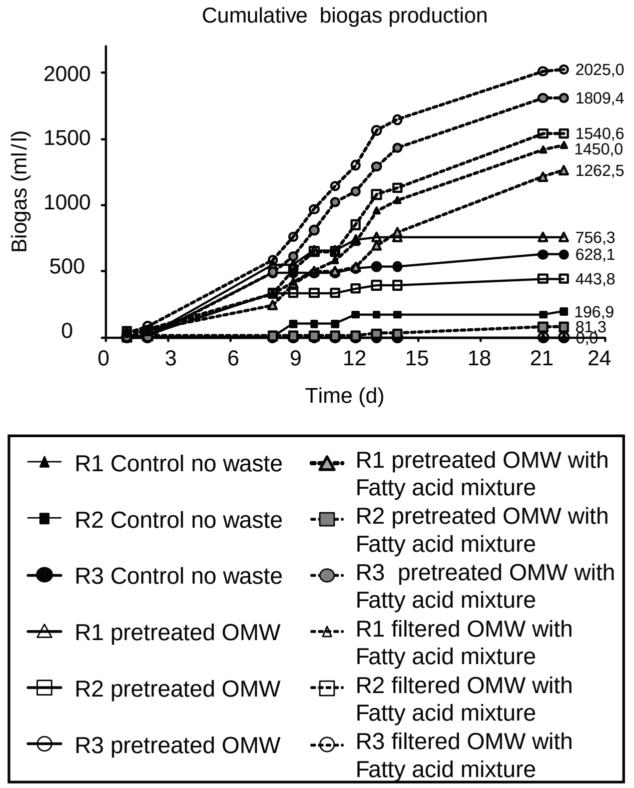

Fig. 4. Kinetics of biogas production (ml/l) of 2nd set of batch anaerobic experiments with incubation time.

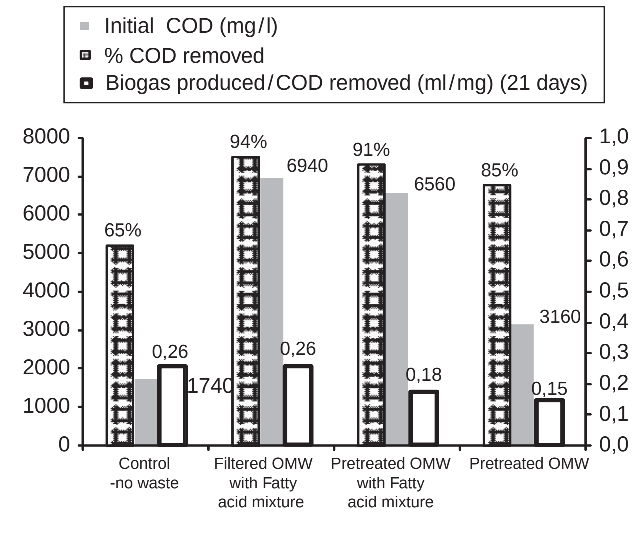

Fig. 5. Effect of electrochemical pretreatment on % COD removal and Biogas produced/COD removed of the 2nd anaerobic set.

of the waste added in the cultures was such that the concentration of the phenolics did not affect negatively the production of biogas. Although the difference of % COD removed between cultures Δ and Φ (with filtered and pretreated

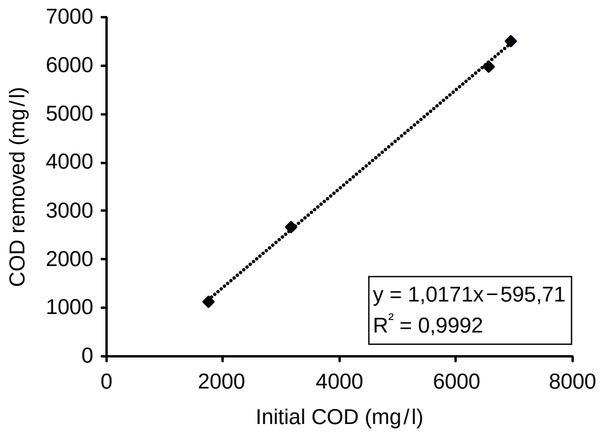

Fig. 6. Effect of Initial COD (mg/l) on COD removed of 2nd anaerobic set.

OMW respectively) can be explained by the initial COD (Fig. 6) the difference in cumulative biogas production (1671 ml/l and 1051 ml/l for Δ and Φ respectively) and in biogas production per COD removed (Fig. 5, 0.26 mg/ml and 0.18 mg/ml for Δ and Φ respectively) cannot be explained by the initial COD of the cultures. Interaction between the methanogenic and the other groups of microorganisms could be responsible for this difference of substrate utilization. Thus, further acclimatization with increased COD concentrations and less dilution of OMW introduced in the cultures are needed for elucidation of the interactions.

In Table 5 values of standard deviation provide information on the stability of the parameters estimated for cultures grown under the same conditions. Reproducibility of the results is a key parameter in successful anaerobic treatment and thus acclimatization factors play an important role. A replication from each one of control (Λ) treatment and treatments with electrolyzed OMW (Φ, X) failed to meet the level of the other two for cumulative biogas production, and consequently biogas to COD removed. Treatment with filtered OMW and fatty acids was the only one to exhibit reproducibility of biogas production reaching the average value of biogas to COD removal with the control culture (0.26 ml/mg COD) as shown in Fig. 5.

4. Conclusions

From the performed experiments it was observed that

- Filtration of OMW can remove 37%–58% of TPh.
- Electrolysis of undiluted OMW with 40 g/L NaCl over Ti/Ta/Pt/Ir electrode for 180 min can reduce TPh by 46% and 26%, soluble COD by 15.6% and 16.6% at 0.518 A/cm^2 and 1.034 A/cm^2 current densities respectively.

- Thermophilic growth exhibits extended lag phase comparing to the mesophilic growth.
- While biogas production is not consistent with COD removal, it is dependent on growth supplements. Both HS-Co-M and fatty acids enhanced biogas production in the presence of filtered OMW and electrolyzed OMW.
- The presence of OMW, although very diluted, affected biogas production in the two sets. Both sets of batch anaerobic experiments show that COD removal was highly correlated to the initial COD of the cultures, regardless of the nutritional differences, in the area of COD tested. The difference of % COD removed between cultures with filtered and electrochemically pretreated OMW, can be explained by the initial COD.

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Symbols

HS-Co-M — coenzyme M/2-mercaptoethanesulfonic acid
COD — Chemical oxygen demand
OMW — Olive mill wastewater
TPh — Total phenols
TS — Total solids
Sd — Standard deviation

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