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Polyphenols Concentration's Effect on the Biogas Production by Wastes Derived from Olive Oil Production

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Olive oil production gives two high polluting refuses: the semisolid Olive Pomace (OP) and the liquid Olive Mill Wastewater (OMWW). These refuses actually present either from technology or from social point of views high disposal problems. An approach able to solve these problems seems to be the Anaerobic Digestion (AD), which permits to obtain a stabilized sludge and a biogas with high content of methane. OP and OMWW contain some recalcitrant constituents as lignocelluloses and polyphenols which are able to inhibit the AD. At this aim, in the present work some physical and chemical pretreatments were accomplished by AD fermentation tests. After each pretreatment the polyphenols concentration were evaluated against no-pretreated mixture. The best results were obtained with salts additions ($FeCl_3$ and $CaCO_3$) which make the simultaneous actions of reducing the polyphenols concentration by flocculation phenomena and increasing the biodegradation of recalcitrant ligno-cellulosic materials.

1. Introduction

In Italy olive oil's production represents a very important economic activity. Over the 95% of the world production is concentrated in the Mediterranean area and Italy with about 4,650,000 tons/year of pressed olive, is the second world olive oil's producer (Battista et al., 2013). The two most abundant wastes generated from this agro-industry are a semi-solid effluent, OP and a liquid one, OMWW. Because of the low pH, the high solid content of the organic matter, OP and OMWW represent a serious environmental problem for all the Mediterranean countries. OP and OMWW contain recalcitrant substances that inhibit anaerobic digestion; in fact OP is constituted by lignin (about 37% *w/w* on dry base), cellulose and hemicelluloses (about 49 % *w/w* on dry base), that result degradable with difficultly by methanogenic microorganisms. OMWW are rich in phenolic compounds (Ricon et al., 2007) which limit the microorganisms' activity as consequence of biostatic effects (Obied et al., 2013). Polyphenolic compounds are a large and complex family of substances, characterized by the presence of large multiples phenol structural units. They are present in olive drupes, originally synthesized by the olive plants as a defence against pathogens. Due to their high solubility in water, during the extraction process pass into OMWW. The concentration of phenolic compounds in OMWW varies greatly from 0.002 to 80 g/L (Azbar et al., 2007), consequently such dephenolization process seems to be necessary to improve AD efficiency. This work concerns the evaluation of the efficacy of several pretreatments processes on a mixture constituted by OP and OMW with the aim to reduce polyphenols concentration in the fermentation medium, the pretreated mixtures were evaluated by laboratory AD tests in mesophilic condition.

2. Experimental

2.1 OP and OMWW characteristics

OP and OMWW derived from olive production factory operations which include a three phase centrifugation, in order to separate solid phase, oil and aqueous one. In Table 1 the main characteristics of OP and OMWW are reported. Bovine manure was used as inoculum in ratio of 10% *v/v*.

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Table 1: Chemical characteristics of OP and OMWW

	OP	OMWW
Density (kg/m^3)	969.5 ± 41.2	989.4 ± 5.31
pH	6.75 ± 0.05	4.86 ± 0.01
Content of TS (g/L)	331.33 ± 6.81	12.04 ± 0.02
Content of VS (g/L)	305.6 ± 6.18	7.49 ± 0.21
Low Heat Value (kJ/kg)	25,503.9 ± 51.61	n.d.
Polyphenols Content (mg gallic acid/L)	n.d.	59.18 ± 0.97

2.2 Apparatus used

The experimental apparatus used, consisted of a glass bottle of 250 mL volume, filled with a mixture approximately 1:2 of OP and OMWW and 10 mL of inoculum. The flasks were flushed with nitrogen gas for five minutes to reach anaerobic conditions. A second bottle of 500 mL was used as gasholder, full of water at pH=2 to prevent CO_2 dissolution in water; a third plastic bottle was used as water receiver in order to determine biogas production as water relocation. The three bottles were agitated at 70 rpm by a rotating shaker in a thermostatic room at 30 ± 1°C. All the tests were conducted simultaneously in triplicate to acquire sufficient certain data.

2.3 Analytical measurements

The cumulative gas production was recorded by the water-replacement method; while the composition of gas produced in each test was determined by an off-line gas chromatograph (Varian, CP 4900). As concerns the other analytical measurements, the following methods were used: TS, VS and density were determined according to the standard method (APHA, 1995), while the pH was measured by a pH meter CRISON (model micropH 2001). The evaluation of the polyphenols content was done by Folin- Ciocalteau method (Lee et al., 2014), which consists in a phenols compounds' oxidation. The polyphenols extraction from the samples was reached in the following way: 20 g of OP and OMWW mixture was treated firstly with 20 g of hexane and subsequently with 20 g of a mixture of methanol and water (1:2). After the separation of the aqueous phase from the oil one, 1 mL of the aqueous phase was added to 5 mL of water, 0.5 mL of the Folin- Ciocalteau reactive and 1 mL of a Na_2CO_3 saturated solution. A LAMBDA XLS UV/Vis spectrophotometer was used for the determination of the gallic acid absorbance values at 280 nm and expressed as mg/L of gallic acid (Lee et al., 2014), using a calibration curve, obtained by 10 samples with different gallic acid concentration previously arranged. For each test, the polyphenols content was measured after the pretreatment, before the fermentation test.

2.4 AD experimental procedure for CH_4 production

In all the tests a feeding mixture of OP and OMWW, having a TS content of about 10% w/w (approximately 100 g/L) was used. The mixture was obtaining by the addition of 33 g of OP and 77 g of OMWW; the concentration of 100 g/L was also used in the case of OP diluted with water, the dilution with tap water was considered a pretreatment process too.

The tests were carried out in triplicate by dividing the broth, at the end of the pretreatment step, into three parts. Each of them was inoculated with bovine manure in the ratio of 10 % *v/v*. The initial pH for all the tests were adjusted around 7 using 2N NaOH. The tests were stopped when the biogas production shut-down. The gas composition was determinate when each gasholder exhausted. To have a comparison base to evaluate the performances of the pretreated tests, no-pretreated ones with OP dilutes with OMWW and water were carried out at the same conditions. Table 2 reports a list of experimental tests carried out.

3. Pretreatment Tests

Several pretreatment processes were tested: basic pretreatment (BP), salts pretreatments using $FeSO_4$ (SP_{FeII}), $FeCl_3$ (SP_{FeIII}), $MnSO_4$ (SP_{Mn}) and $CaCO_3$ (SP_{Ca}), ultrasonic pretreatment (USP) and two combinations of the aforementioned pretreatments. The energy production under CH4 form of each test was compared with no pretreated tests (NP). All the pretreatments tests were conducted in a thermostatic chamber at 30 ± 1°C; the following sections report their main characteristics and operative conditions.

Table 2: Descriptions and abbreviations of the pretreatments tested

Pretreatments	Abbreviations
No pretreatment	
No pretreatment diluted with OMWW	NP_{OMWW}
No pretreatment diluted with water	NP_W
Basic Pretreatment	BP
Salts pretreatments	
$FeSO_4$	SP_{FeII}
$FeCl_3$	SP_{FeIII}
$MnSO_4$	SP_{Mn}
$CaCO_3$	SP_{Ca}
Ultrasonic Pretreatment	USP

3.1 Basic Pretreatment (BP)

BP test was conducted by 2N NaOH solution at pH = 12, the broth was stirred at 70 rpm for 24 hours; for the AD test, the broth was neutralized using 2N HCl solution. Among the large pallet of available chemical pretreatments (Ruggeri et al., 2012), the basic pretreatment is well recognized for its effect on lignocellulosic materials. Fan et al., 1980 suggest that the base mainly removes lignin by saponification reaction of the intermolecular esters bonds that cross-link xylane hemicelluloses and other components, such as lignin, whereas the mechanism of action appears to be due to the increase of porosity hence an increase of internal surface of the ligno-cellulosic materials available for enzymes attack as a consequence of a decrease of the degree of polymerization and of crystallinity by cracking of the structural links between the carbohydrates and lignin.

3.2 Salts Pretreatments (SP)

The OP and OMWW mixture was treated with $FeSO_4$ (SP_{FeII}), $MnSO_4 \cdot H_2O$ (SP_{Mn}), $FeCl_3$ (SP_{FeIII}) and $CaCO_3$ (SP_{Ca}) respectively; with a quantity of solid salt to reach 5 g/L of salt concentration in the medium. With the only exception of test with $CaCO_3$, the pretreatments with salts were carried out in the following way: firstly, the mixtures were treated with 2N HCl till to reach a pH = 2 and mixed for 30 minutes at 70 rpm; then the specific salt was added in order to have the desired concentration, and maintained for 24 hours at 30±1°C under agitation (70 rpm). Finally, 2N NaOH was added to reach a neutral pH. In the case of $CaCO_3$ pretreatment, the acid treatment was not used; after the salt addition, a pH of about 7 was reached using 2N NaOH.

3.3 UltraSonic Pretreatment (USP)

The ultrasonic pretreatment was carried out by an ultrasonic machine (Ultrasonic Cleaner model CP823) under the following conditions: 30°C, atmospheric pressure, a power of 1.8 kW for 30 minutes. In this case the aim was to verify whether this pretreatment is able to break down large molecules and convert them into small ones easier to be digested by microorganisms.

4. Results and comments

All tests were carried out at the same initial conditions TS = 118.93 g/L, with the only exception of the NPw test where the initial condition was TS = 116.75 g/L because of the water dilution. The reference test is represented by the NP_{OMWW}, where the mixture of OP and OMWW, was tested biologically without any physical or chemical pretreatment. Table 3 reports that the biogas production of NP_{OMWW} test was very low (0.36 NL/L) with a methane content of 51.6 % *v/v*. This result confirms the inhibiting effect of the high polyphenols concentration in the reaction medium, which was equal to 53.78 mg gallic acid /L. The pH, dropped at the end of the test to 5.90 due to the increase of volatile fatty acids (VFA) during the first phases of the AD, when hydrogen producing bacteria present a higher kinetic rate than methanogenic bacteria. Consequently the complex substances in the reaction medium were reduced in simpler molecules, mainly in short chains of organic acids (VFA), which represent the substrates for the subsequent methanogenic phase. The NP_W test was performed using only the tap water dilution as pretreatment. Table 3 shows that the simply dilution is sufficient to increase significantly the biogas production and the methane content which were 13.56 NL/L and 74.31% *v/v* respectively. As consequence of the dilution, the polyphenols initial concentration decreased till to 44.27 mg gallic acid/L and this can be viewed as mitigation on the fermentation process.

Table 3: Polyphenols concentration, VS content and methane productions

Test	VS_0 (g/L)	VS_f (g/L)	Biogas (NL/L)	% CH4 (v/v)	Y (L CH4/Kg ΔVS)	Polyphenols Content (mg gallic acid/L)
NP_{OMWW}	103.49±6.78	66.78±10.67	0.36±0.06	51.60±4.02	5.07±0.15	53.78±0.89
NP_W	98.12±4.25	55.01±6.82	13.56±0.18	74.31±1.96	175.93±22	44.27±3.34
BP	96.59±5.32	43.54±7.82	18.98±0.91	86.30±2.31	296.23±28.43	38.55±0.09
SP_{FeII}	104.1±7.21	53.55±4.21	10.74±1.31	81.88±3.22	173.96±16.37	37.80±1.88
SP_{Mn}	104.02±7.20	79.9±5.98	0.27±0.05	40.00±2.65	4.31±0.20	36.27±0.12
SP_{FeIII}	98.89±3.59	34.54±1.87	19.89±3.53	84.25±2.75	260.40±33.97	28.09±1.97
SP_{Ca}	93.12±8.02	64.95±4.54	21.57±3.10	83.02±2.36	622.72±21.25	27.15±2.08
USP	106.13±4.98	64.91±9.27	20.17±1.18	84.93±1.92	456.79±15.69	34.41±0.82

As it is possible to see from Table 3, all the pretreatments tested, improved the methane yield with the only exception of the SP_{Mn} test which presents a biogas production of 0.27 NL/L lower than the reference test, NP_{OMWW}, but the polyphenols concentration decreased till to 36.27 mg gallic acid/L. The very low biogas production could be explained of considering that the pH was in the acid range (5.77). Good performances in terms of biogas production were recorded in BP, USP, SP_{FeII}I and SP_{Ca} tests. BP and USP show a biogas production of 18.98 NL/L and 20.17 NL/L respectively (Table 3), with a methane percentage over 84% *v/v*. These encouraging results are partially due to polyphenols reduction as consequence of the basic and ultrasonic pretreatments. In fact, mainly in BP test the phenolic compounds concentration remains enough high, about 44 mg gallic acid/L. This suggests that basic pretreatment mainly acts on the degradation of lignin and cellulosic materials present in the OP. In fact the methane yield was sufficient high for all the tests (Table 3). This confirms that the alkaline hydrolysis mechanism is believed to involve a saponification of the intermolecular ester bonds (Ruggeri et al., 2012) causing the swelling and inducing different effects: an increase in the internal surface area, a decrease in the degree of polymerisation, a separation of the structural links between lignin and carbohydrates i.e. a disruption of the lignin structure. Also the ultrasonic pretreatment involves the structure of OP: it contributes to destroy the complex chemical structures the lignin and the cellulose of the olive pomace, increasing the specific surface available to the bacteria' degradation but in the same time, it provokes a high polyphenols abetment. The two better results were obtained with SP_{FeIII} and SP_{Ca}: the biogas production was of 19.89 NL/L and 21.57 NL/L respectively with a methane content superior to 83% *v/v*. Table 3 shows a good reduction of the polyphenols: their concentration, in fact, is lower 30 mg gallic acid/L. SP_{FeII} and mainly SP_{FeIII} included a pre-acid cracking followed by a pretreatment with two poly-electrolytes salts, $FeSO_4$ and $FeCl_3$ respectively. Two previous researches (Ginos et al., 2005 and Azbar et al., 2007) demonstrated the benefice effect of cationic poly-electrolytes that promote the Fenton reaction of organic matter, contributing to a high increase in the VS and the TS reductions, with high removal of the polyphenolic compounds, which are colloidal substances. The metallic salts combination, with water alkalinity, generated by the presence of carbonates which produce the bicarbonate ($Ca(HCO_3)_2$), form micro-flocs which are able to adsorb and capture the colloidal substances, including the polyphenols (Masotti, 2011). Finally, The good performance of SP_{Ca} test is explainable, not only considering the ability of calcium to absorb polyphenolic compounds, but also taking into account that when the calcium carbonate concentration is about 5 g Ca^{2+}/ L, as in this case, the formation of biofilms on carbonate is able to support biomass growth (Deublin and Steinhauser, 2008). In addition Chen et al., 2008 underline that calcium is known to be essential for the growth of certain strains of methanogens and for the formation of microbial aggregates. Jackson-Moss et al., 1989 observed that Ca^{2+} concentration of up 7 g/L has no-inhibition effect on anaerobic digestion. In addition $CaCO_3$ operate as buffer agent that prevents the acidification of the reaction medium as consequence of VFA production as first step in AD process. All this aspects could explain the high yield of methane obtained with $CaCO_3$ addition. In Figure 1 are reported the increase of biogas production referred to no-treated mixture *vs*. Polyphenols reduction. A saturation phenomenon seems to be present: over 35% of polyphenols reduction there is no effect on biogas production. Unfortunately it was not possible to carried out tests without polyphenols for this kind of substrate owing the difficulties to have OP and OMWW free of polyphenols, but generally we can affirm that due to the complex structure of material, the effect of polyphenols on microorganism is to be compared with the difficulty to degrade the ligno-cellulosic materials: more less is the contents of lignocelluloses material more high is the polyphenols effects on microorganism activity; hence the elimination of polyphenol could have a beneficial effects but under a threshold which is determinate by the structural nature of substrate that needs to be biodegradated.

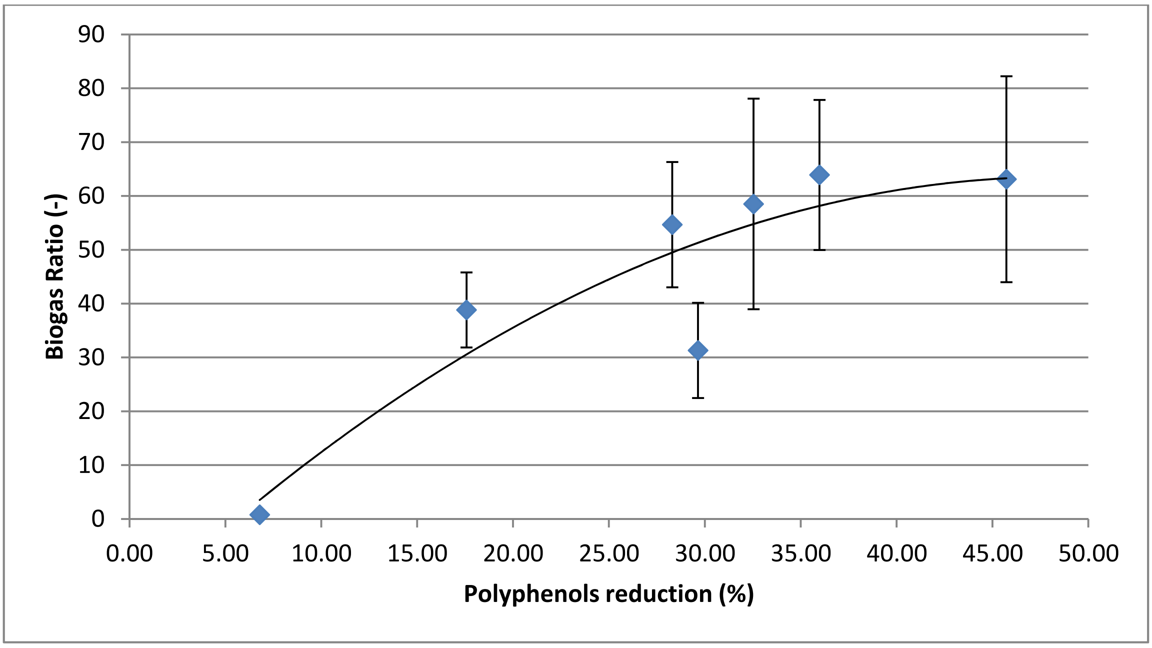

Figure 1: Polyphenols reduction effect on biogas production

5. Conclusions

The present work shows there is a relationship between biogas production and polyphenols concentration of the organic substrates: polyphenols inhibit the fermentation activity of methanogenic microorganisms as it is possible to see from the NP_{OMWW} test.

Anyway, the decrease of polyphenols content, by simple water dilution of the reaction medium, improved greatly the methane productivity (NP_W test), but the best results were obtained with chemical pretreatments of the substrates. In fact, the NaOH, poly-electrolytic salts and $CaCO_3$ addition, develops the simultaneous actions of decreasing the polyphenols concentration and destroying the ligno-cellulosicstructure of the substrates (BP test), acting as buffer action or giving important micro-nutrients which increase the bacteria metabolism (SP_{Ca} test). Lastly the reduction of polyphenols concentration seems to have a threshold effect over about 35% the biogas increase stops. This because the polyphenol effects of biostatic nature i.e. the inhibition of the membrane activities of microorganisms, contributes to lowering the possibility to biodegrade lignocelluloses structures. The elimination of the polyphenol alone has no effect on the biodegradability of substrates.

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