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**EFFECT OF COBALT SUPPLEMENTATION AND FRACTIONATION ON
THE BIOLOGICAL RESPONSE IN THE BIOMETHANIZATION OF OLIVE
MILL SOLID WASTE**

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Abstract

Due to the low trace metals concentration in the Olive Mill Solid Waste (OMSW), a proposed strategy to improve its biomethanization is the supplementation of key metals to enhance the microorganism activity. Among essential trace metals, cobalt has been reported to have a crucial role in anaerobic degradation. This study evaluates the effect of cobalt supplementation to OMSW, focusing on the connection between fractionation

of cobalt in the system and the biological response. The highest biological responses was found in a range from 0.018 to 0.035 mg/L of dissolved cobalt (0.24 - 0.65 mg total cobalt/L), reaching improvements up to 23% and 30% in the methane production rate and the methane yield coefficient, respectively. It was found that the dissolved cobalt fraction is more accurately related with the biological response than the total cobalt. The total cobalt is distorted by the contribution of dissolved and non-dissolved inert fractions.

Keywords:

Trace metal requirements; Methane production rate; Improved anaerobic digestion; Microbial activity; Cobalt fractionation; Olive Mill Solid Waste

1. Introduction

About 2.8 million tons of olive oil are produced yearly worldwide, of which 48% are produced in Spain (Dermeche et al. 2013). For each ton of olive oil produced, 4 tons of Olive Mill Solid Waste (OMSW) are generated (Borja et al. 2005). The OMSW is a polluting waste resulting from the olive oil manufacturing process. In two-phase process, OMSW is a high-humidity waste with a thick sludge consistency that contains the remains of the olive fruit, including skin, seed, pulp and pieces of stones (Almansa et al. 2015). The management of OMSW through anaerobic digestion represents several benefits for its valorization. Anaerobic stabilization of this organic waste allow recovering energy due to methane production (Rincón et al. 2013). Furthermore, several authors described the possibility of using the digestate as biofertilizer after anaerobic stabilization (Tambone et al. 2009; Serrano et al., 2014).

Some organic wastes, such as OMSW, contain low concentrations of trace elements, which could limit the anaerobic digestion process (Almansa et al. 2015). Several

specific trace metals, including Co, Ni, W, Se and Mo, are essential as enzyme cofactors involved in the biochemistry of methane formation and, consequently, they are needed in a balanced anaerobic digestion process (Schattauer et al. 2011; Westerholm et al. 2015). Then, availability of trace metals is a necessary feature required for a stable anaerobic digestion process (Bayr et al. 2012; Moestedt et al. 2016). Therefore, it is important to know the appropriate concentration of each trace metal, in order to prevent limitation effects by their absence or inhibition by their excess (Pobeheim et al. 2011). Several authors reported that the chemical fractionation of the trace metal is also as important as the total concentration in the system, given that the effect over the process depends mainly on the availability of the metal for the microorganisms (Gustavsson et al. 2013).

Among the different trace metals, cobalt has been shown one of the most important for the anaerobic degradation processes, given its relevance for different reactions within the acetogenic and methanogenic steps (Fermoso et al. 2008). For example, cobalt is present in the chemical structure of CO dehydrogenase and methyl-H₄MPT:HS-CoM methyltransferase, enzymes involved in the metabolic pathways of the methanogenesis from acetate (Ferry 1999). An adequate balance of cobalt could improve the anaerobic biological response, enhancing the rate and yield of methane. Wide ranges of total cobalt concentration, from 0.05 up to 3.0 mg/kg, have been reported as recommended for anaerobic process (Pobeheim et al. 2010; Demirel and Scherer 2011). Such differences could be explained by the fact that most of the authors only report the total concentration provided, and not refer about the amount of cobalt that is bioavailable for the microorganisms.

The objective of this study was to evaluate the effect of cobalt supplementation to OMSW, as a way to improve its anaerobic degradation to biomethane. This research is

mainly focused on the connection between fractionation of cobalt and the biological response presented in a digester. This study could be considered especially relevant in the treatment of different organic solid waste with deficit in trace metals, such as for OMSW, with the consequent environmental benefits.

2. Material and Methods

2.1 OMSW and anaerobic inoculum

OMSW was obtained from the experimental plant for olive oil extraction located in ‘‘Instituto de la Grasa (CSIC)’’ of Sevilla, Spain. Before its biomethanization, the OMSW was subjected to sieving (2 mm mesh size) to remove remaining olive stone pieces. The anaerobic inoculum was obtained from a full-scale anaerobic reactor treating sewage sludge from ‘‘COPERO’’ wastewater treatment plant (Seville, Spain). Chemical characterization of OMSW and the anaerobic inoculum are shown in Table 1.

2.2 Experimental design

The influence of cobalt on the response of the microorganisms involved in anaerobic degradation process was evaluated by biochemical methane potential (BMP) tests. BMP tests were performed in 250 ml Erlenmeyer flasks at 35°C (mesophilic conditions). Mechanical stirring was provided, at 300 rpm, to facilitate mass transfer. In all cases, the BMP reactors were loaded with an inoculum to substrate ratio of 2, based on volatile solids (Gustavsson et al. 2013). The BMP tests were supplemented with nine increasing concentrations of cobalt (total added cobalt concentrations: 0, 0.018, 0.030, 0.6, 2.9, 5.9, 17.7, 29.5 and 58.9 mg Co²⁺/L). These concentration values were selected in order to study a range from limiting to toxic cobalt levels. Each one of these concentrations was evaluated in triplicate. The required concentration of cobalt was achieved using stock

solutions of CoCl_2 with different concentrations (0.01, 0.10 and 1.00 M). BMP reactors were sealed and the headspace of each flask was flushed with nitrogen at the beginning of the assay. The produced biogas was passed through a 1 N NaOH solution to capture CO_2 , the remaining gas was assumed to be methane. The BMP tests were carried out the time interval required to exhaust gas production and VS removal (22-day period) and the methane production was monitored daily throughout the process.

2.3 Data fitting

Two kinetics models were applied to fit the experimental data of methane production during BMP tests: the apparent kinetic constant (k ; d^{-1}) was calculated from a First-Order exponential model (FO) (eq. 1). The maximum methane production rate (R_m ; $\text{mL CH}_4/(\text{g VS} \cdot \text{d})$) was calculated from the Transference Function (TF) (eq. 2). The application of FO model was previously reported for anaerobic processes of different substrate by several authors (Borja et al. 1995; Batstone et al. 2009), and is defined by the equation:

$$G = G_{\max} [1 - \exp(-k * t)] \quad (1)$$

where G ($\text{mL CH}_4/\text{g VS}_{\text{added}}$) is the cumulative specific methane production, $G_{\max}$ ($\text{mL CH}_4/\text{g VS}_{\text{added}}$) is the ultimate methane production, k (d^{-1}) is the specific rate constant or apparent kinetic constant and t (d) is the time.

TF model was successfully applied by several authors for anaerobic digestion of different wastes (Donoso-Bravo et al. 2010; Li et al. 2012), using the following expression:

$$G = G_{\max} * \left(1 - \exp\left[-\frac{R_m(t-\gamma)}{G_{\max}}\right]\right) \quad (2)$$

where R_m is the maximum methane production rate ($\text{mL CH}_4/(\text{g VS}_{\text{added}} \cdot \text{d})$) and γ (d) is the lag time.

Table 2 shows the parameters obtained for each kinetic model for the different added cobalt concentrations tested, as well as the values of r^2 , Error (%) and standard error of estimate (σ_{est}) to evaluate the goodness-of-fit. Error was defined as the percentage difference between the experimental and the predicted methane yield coefficient. r^2 values were higher than 0.98 in all the cases for the two kinetics models tested (Table 2). Likewise, Error and σ_{est} , with similar and low values, also indicated a good fit over the experimental data for FO and TF, providing the indistinct use both models. The use of both models facilitates the comparison of the kinetics parameters obtained by other authors for different substrates. The kinetic parameters for each cobalt load and mathematical adjustment were calculated numerically from the experimental data obtained by non-linear regression using the software Sigma-Plot (version 10.0).

2.4 Chemical analyses

The following chemical analyses were used for the characterization of the inoculum and OMSW as well as for the effluents from each BMP test at the end of the process. The concentration of total solids (TS; g/kg), volatile solids (VS; g/kg), alkalinity (mg CaCO_3/L) and pH were carried out according to the recommendations of the Standard Methods of APHA (2005). Chemical Oxygen Demand (COD; g/kg) was determined using the method described by Raposo et al. (2008). The concentrations of different dissolved trace elements, i.e. Fe, Ni, Co, Cu, Ca, Mn, Zn, Mg, P, S and Ba, were determined by filtration of the liquid sample with a $0.2 \mu\text{m}$ filter in oxygen-free atmosphere and measured by ICP-OES (Thermo Fisher Scientific, model IRIS Advantage). Total trace elements concentrations in solid samples were determined

through the same procedure after aqua regia digestion in a microwave oven. Phenols were quantified by spectrophotometry through the Folin method, after an extraction with a methanol/water solution (80:20) (García et al. 2016). Elemental analysis to obtain the Carbon:Hydrogen:Nitrogen (C:H:N) ratio was performed by using Elemental Analyser LECO CHNS-932 (Leco Corporation, St Joseph, MI, EEUU).

3. Results and discussion

3.1 Stability of the process and removal efficiencies

Figures 1A and 1B show the mean values of the accumulated methane production throughout the experimental time for the different added cobalt concentration tested. For each tested cobalt concentration, the methane production increased exponentially up to achieve a maximum value.

The mean values obtained for dissolved cobalt, pH, biodegradability and phenol removal at the end of the BMP tests are shown in Table 3. The pH remained practically constant at values close to 7.4. These pH values are within the range that has been previously reported as optimal for the mesophilic methanogenic activity (Wheatley 1990). The biodegradability was defined as the percentage of volatile solids removed after the experimental time. All the values were in a range from 81% to 87% (Table 3). These biodegradability values were close to 76%, which was reported by Raposo et al., (2005) for anaerobic digestion of two-phase olive effluents at similar conditions. As can be seen in Table 3, the concentration of cobalt in the studied range did not affect significantly the biodegradability percentages. Phenols were largely degraded, i.e. from 82% up to 90%, for the different experiments carried out (Table 3). Phenols degradation is crucial given that such compounds have been reported as inhibitory of the microbial

activity in the anaerobic digestion process (Qiao et al. 2011). In fact, curves of methane production in the BMP test showed no turning points or lag phase, demonstrating the lack of process inhibition (Figures 1A and 1B)

3.2 Interaction between added, dissolved and total cobalt.

Figure 2 shows the relationship between added cobalt versus the dissolved and total cobalt present at the end of the BMP tests. Total cobalt has been defined as the sum of the added cobalt and cobalt already contained in the OMSW and anaerobic inoculum. Concentration of dissolved cobalt reaches a maximum value of 0.32 mg/L, at a total cobalt concentration of 29.5 mg/L (Figure 2). Further addition of cobalt to the BMP test did not result in higher dissolved cobalt concentrations. Raw materials and wastes used as substrates in anaerobic digestion, such as OMSW, are usually heterogeneous, consist of complex forms of organic and inorganic matter, which affect metals speciation (Gustavsson et al. 2013). For example, high content of hydrogen sulfide entail the precipitation of trace metals as metal sulfides, limiting the amount of bioavailable cobalt. The cobalt tends to form complexes with some organic ligands contained in soluble products excreted by microorganisms (Gonzalez-Gil et al. 2003; Gustavsson et al. 2013).

The use of added cobalt concentration as a control parameter to assess the digester performance is hindered by the involved physicochemical processes, which could entail a wrong estimate of the required cobalt. It has been reported that the response of the system is significantly related to free cobalt concentration (Bartacek et al. 2012).

However, the use of this relationship is limited by the difficulties associated to free cobalt determination at low concentrations in such complex matrix (Bartacek et al. 2012). This makes necessary to study the correlation between the biological response of

the microorganism and easily measurable fractions of cobalt, such as dissolved and total.

3.3 Effect of cobalt on the methane yield coefficient and methane production rate

Figure 3 shows the values of methane yield coefficient against the total and dissolved cobalt present at the end of the BMP tests. The test without addition of cobalt, i.e. dissolved cobalt of 0.013 mg/L (0.06 mg total cobalt/L), reached a methane yield coefficient of 353 ± 31 mL/g VS (Figure 3). This value was similar to that previously reported by Rincón et al. (2013), who reached 373 ± 4 mL CH₄/g VS, for the same substrate at similar conditions. The addition of cobalt, in the studied concentration range, entailed an improvement in the methane yield coefficient from 9% up to 30%, when compared to the reactor without addition of cobalt. The highest methane yield coefficient was obtained for a dissolved cobalt concentration of 0.028 mg/L (0.35 mg total cobalt/L), reaching a value of 460 ± 40 mL/g VS.

When fitting experimental data to the FO model, the obtained k values were very similar when using concentrations of added cobalt below 17.7 mg/L (0.156 mg dissolved cobalt/L), with a mean value of 0.21 ± 0.02 d⁻¹ (Table 2). Nevertheless, k values decreased 25% when higher dissolved cobalt concentration was tested, indicating a possible toxic effect on the biological response.

Figure 4 shows the values of R_m against the dissolved and total cobalt concentration for each set of the BMP tests. Dissolved cobalt in a range from 0.018 to 0.035 mg/L (0.24 - 0.65 mg total cobalt/L) entailed a marked improvement of R_m , of around 25%, compared to the value obtained in the test without cobalt addition (0.013 and 0.06 mg/L of dissolved and total cobalt, respectively) (Figure 4). The same effect was reported by Nordell et al. (2016) which described an improvement of around 24% in the methane production rate for the anaerobic co-digestion of manure and industrial waste, when

adding of a mixture of trace elements, including cobalt at a concentration of 0.2 mg total cobalt/kg digestate. Several authors have also described an improvement in the acetogenic and methanogenic steps by the utilization of cobalt. Cobalt activates enzymes essential for the microorganisms involved in methanogenesis, such as vitamin B₁₂, coenzyme A and enzyme methyl-H₄SPT: CoM methyltransferase (Florencio et al. 1994; Pobeheim et al. 2011; Schattauer et al. 2011; Karlsson et al. 2012; Nordell et al. 2016). These enzymes are known to play a key role in degradation of volatile fatty acids (Florencio et al. 1994). The accumulation of dissolved cobalt at values higher than 0.16 mg/L (17.7 mg total cobalt/L) affected to the process decreasing R_m up to values 14% and 30% lower than the experiment without addition of cobalt and the test with the highest observed methane production rate, respectively (Figure 4).

3.4 Correlations between measured fractionation of cobalt and the biological response

The highest methane yield coefficient and methane production rate values have been observed at concentrations of dissolved cobalt from 0.018 up to 0.039 mg/L (Figure 3 and 4), which has been considered as the optimal dissolved cobalt range under the studied conditions. According to the total cobalt, the optimal range was from 0.17 up to 0.58 mg/L (Figure 3 and 4). Monitoring dissolved cobalt concentration might be more convenient than total cobalt concentration, since the optimal range of dissolved cobalt is much shorter than the range determined for total cobalt (Figure 3 and 4). At this short range, it is easier operating close to the cobalt concentration that would cause the best biological response.

It has been reported that the concentration of total cobalt is not a suitable parameter to use as indication of cobalt toxicity in the anaerobic degradation of methanol, being more recommended to monitor the dissolved fraction (Bartacek et al., 2008). In the present

study, the relation between the dissolved fraction and the biological response was also possible even though the metabolic pathways involved in the biomethanization of OMSW are much more complex than for methanol. Variations in the chemical equilibrium or in the composition of the system require redefining the optimal dissolved cobalt concentration, although the trend between the response of the microorganism and the dissolved cobalt should not be affected.

The cobalt present in the system, from anaerobic inoculum, substrate and added cobalt might be represented by four fractions (Figure 5). The distribution among the four fractions responds to its bioavailability, i.e. bioavailable or inert, and its state, i.e. dissolved or non-dissolved (Figure 5). The cobalt up-take by the microorganisms requires that cobalt is found in an assimilable form, e.g. free cobalt. There is a chemical balance between the final assimilable cobalt and dissolved bioavailable cobalt and, in turn, between this fraction and non-dissolved bioavailable cobalt (Figure 5). The measurement of bioavailable cobalt fraction should be the most accurately related to the biological response, but it implies analytical procedures that are hardly developed in the industry and even in the academic sector. For example, the determination of the limiting concentration of free cobalt is restricted because this concentration is lower than the detection limit of the available methods and also by the interferences from the complex matrix (Saracoglu et al. 2002). As shown in the present research, the measurement of the total and dissolved fraction could be used to predict the response of the microorganism to the cobalt supplementation. As suggested, the dissolved cobalt fraction is more accurately related with the biological response than the total cobalt. The total cobalt is distorted by the contribution of two inert fractions, i.e. dissolved and non-dissolved (Figure 5).

The validity of the obtained values of dissolved cobalt concentrations depends in maintaining a constant chemical environment of the system. If relevant variations happened, the relation between dissolved cobalt concentration and the response of microorganisms must be redefined, although the trends in this relation should not be affected. The use of the dissolved cobalt concentration as a parameter for optimization is especially relevant for full-scale systems. An increase in the methane production rate could allow the reduction of the hydraulic retention time, and, therefore of the reactor volume with the consequent saving on the investment costs, as well as to incrementing the energy recovery of the system.

4. Conclusions

- Anaerobic digestion of OMSW reaches high efficiencies in phenols removal and biodegradability.
- Cobalt supplementation allows improvement on methane production rate and methane yield coefficient up to 25% and 30%, respectively.
- The dissolved cobalt fraction is more accurately related with the biological response than the total cobalt.
- The validity of the obtained values of dissolved cobalt concentrations depends in maintaining a constant chemical environment of the system. If relevant variations happened, the relation between dissolved cobalt concentration and the biological response must be redefined, although the trends in this relation should not be affected.

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Figure captions

Figure 1. A) Variation of the methane production with time for total added cobalt concentrations in the range from 0 to 2.9 mg/L. B) Variation of the methane production with time for added cobalt concentrations in the range from 5.9 to 58.9 mg/L.

Figure 2. Concentration of dissolved and total cobalt against the concentration of added cobalt.

Figure 3. Variation of the maximum methane production for the different concentrations of total and dissolved cobalt.

Figure 4. Plot of the maximum methane production rate for each concentration of total and dissolved cobalt.

Figure 5. Distribution of the different cobalt fractions related to its bioavailability

Figure 1

A)

B)

Figure 2

Figure 3

Figure 4

Figure 5.

Table 1. Olive mill solid waste (OMSW) and anaerobic inoculum characteristics

	Anaerobic inoculum	OMSW
TS (g/kg)	21.3 ± 3.6	260.1 ± 6.8
VS (g/kg)	13.8 ± 0.1	235.6 ± 3.6
COD (g O ₂ /kg)	31 ± 3	361 ± 29
Alkalinity (mg CaCO ₃ /L)	3300	-
pH	8.1±0.2	4.96±0.06
Phenols (mg/L)	22.7± 0.5	4303± 68
Fe (mg/kg TS)	14488.2	863
Ni (mg/kg TS)	17.15	3.3
Co (mg/kg TS)	2.87	0.065
Cu (mg/kg TS)	214.2	14.7
Ca (mg/kg TS)	46911.2	2919.5
Mn (mg/kg TS)	168.3	15.35
Zn (mg/kg TS)	799.6	21.82
Mg (mg/kg TS)	6609.4	6485.3
P (mg/kg TS)	23136.4	2331.3
S (mg/kg TS)	10204.2	1499.7
Ba (mg/kg TS)	113.6	2.057
C:H:N	0.4:0.06:0.04	0.47:0.07:0.02

Table 2. Kinetics parameters obtained for the models used in the BMP tests of OMSW supplemented with cobalt.

		Added cobalt (mg/L)								
		0	0.18	0.30	0.60	2.9	5.9	17.7	29.5	58.9
F O	G _{max} (mL/g VS)	345 ± 3	373 ± 6	479 ± 6	388 ± 3	393 ± 5	413 ± 3	437 ± 4	417 ± 4	415 ± 7
	k (d ⁻¹)	0.23 ± 0.01	0.26 ± 0.01	0.20 ± 0.01	0.20 ± 0.00	0.21 ± 0.01	0.20 ± 0.00	0.19 ± 0.00	0.17 ± 0.00	0.17 ± 0.01
	r ²	0.995 6	0.988 3	0.993 9	0.997 2	0.994 2	0.997 7	0.997 7	0.997 8	0.991 8
	Error (%)	2.378	5.260	4.047	1.393	1.807	3.702	4.947	2.638	6.966
	σ _{est}	8.038 6	14.73 46	13.20 10	6.992 7	10.25 27	6.836 6	7.242 4	6.539 7	12.96 25
T F	G _{max} (mL/g VS)	343 ± 3	371 ± 5	475 ± 6	388 ± 4	393 ± 5	411 ± 3	435 ± 4	416 ± 4	410 ± 7
	R _m (mL/(g VS·d))	81 ± 2	100 ± 4	101 ± 3	76 ± 2	81 ± 2	85 ± 2	84 ± 2	70 ± 1	74 ± 3
	λ (d)	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.1
	r ²	0.995 8	0.989 4	0.995 0	0.997 2	0.994 2	0.998 0	0.997 8	0.997 8	0.993 1
	Error (%)	2.855	4.723	3.219	1.393	1.807	3.257	4.685	2.498	5.900
	σ _{est}	7.951	14.03 9	12.11 1	7.101	10.41 2	6.517	7.226	6.621	12.08 2

FO, First Order; TF, Transference Function; σ_{est}, standard error of estimate.

Table 3. Parameters analyzed in each reactor at the end of BMP.

Dissolved cobalt (mg/L)	pH	Biodegradability (%)	Phenols removal (%)
0.0127	7.43 ± 0.03	81.6	82.5
0.0179	7.41 ± 0.05	82.0	90.8
0.0275	7.39 ± 0.02	80.3	83.8
0.0388	7.45 ± 0.02	81.8	83.8
0.0634	7.43 ± 0.01	85.7	82.2
0.0655	7.46 ± 0.07	87.1	84.0
0.1576	7.41 ± 0.05	86.7	83.8
0.3125	7.40 ± 0.05	85.6	83.7
0.2986	7.54 ± 0.11	81.0	82.9

Highlights

- Biomethanization of olive mill solid waste reaches high phenols and solids removal
- Cobalt supplementation allows improvement on methane production rate up to 25%
- Cobalt supplementation allows an enhancement on methane yield coefficient up to 30%
- Dissolved Co is more accurately related with the biological response than total Co
- System variations vary the relation between dissolved Co and the biological response