Anaerobic biodegradability of gallic acid found in olive mill wastewaters

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Abstract: This paper discusses the applicability of a low-cost method for evaluating the ability of anaerobic micro-organisms to decompose phenolic compounds found in olive mill wastewaters. Gallic acid (GA) was used as test substance at concentrations of 100, 500 and 1000 mg L−1. When 1000 mg L−1 GA was inoculated with anaerobic sludge, the average (of six replicates) cumulative gas volume collected over a period of 23 days was 87.6 ± 2.4 mL, while without sludge the volume was 9.3 ± 1.0 mL. The corresponding values for 500 mg L−1 GA were 48.0 ± 8.0 and 6.5 ± 1.0 mL respectively, while for 100 mg L−1 GA they were 30.8 ± 4.7 and 9.6 ± 2.3 mL respectively. These results suggest that gallic acid is readily degradable under anaerobic conditions even at concentrations as high as 1000 mg L−1 if adequate fermentation time and appropriate microbial culture are provided. The gas volume produced was found to depend linearly (r² = 0.95) on the concentration of gallic acid under the conditions studied. © 2006 Society of Chemical Industry

Keywords: gallic acid; biodegradability; anaerobic micro-organisms; sludge

INTRODUCTION

The need for appropriate management and treatment of olive mill wastewater (OMW) remains the largest single environmental problem in Mediterranean olive oil extraction countries. On the island of Crete, more than 500 000 m³ of OMW is produced annually and is currently disposed of in evaporation ponds.1 The OMW has a high polluting load with a chemical oxygen demand of up to 200 g L−1.2 The organic fraction of OMW includes sugars, tannins, polyphenols, polyalcohols, pectins and lipids, with the phenolic fraction accounting for most of the problems associated with OMW pollution. Of great concern are the antibacterial effects of high-molecular-weight phenolic compounds, which are the main drawback to OMW degradation by either aerobic or anaerobic processes.2

To address this problem, a variety of methods have been suggested for the treatment of OMW.1 However, in Crete the main problem with OMW is associated with (a) the small size of individual olive oil industries, (b) the dispersion of olive oil production mills (more than 650 on the island) over large areas and (c) the low domestic and international olive oil market price, which does not allow the implementation of technologically sound and effective OMW treatment methods.1

Anaerobic digestion can be considered as an appropriate solution to the problem, since methane utilisation for energy could compensate for the treatment cost. It is important, however, that anaerobic micro-organisms are able to decompose all compounds found in OMW, especially phenolic molecules. Borja et al.3 showed that phenolic compounds such as gallic acid, p-coumaric acid and genistic acid are present in OMW in substantial amounts. According to Robles et al.,2 the total phenolic compound concentration in OMW is 1.6 ± 0.18 g L−1, but it may vary depending on the type and origin of the effluent. A value for gallic acid concentration has only been reported in wine distillery wastewater and was in the region of 0.05 g L−1.1 Work by Borja et al.,3,4 FitzGibbon et al.5 and Mousa and Forster6 suggested that such molecules are very difficult to decompose under anaerobic conditions. It is notable that Mousa and Forster6 considered gallic acid as an inhibitor to anaerobic degradation. Because of their antibacterial effects, phenolic compounds function as microbial inhibitors. FitzGibbon et al.5 found that increasing concentrations of phenolic compounds inhibited fungal growth rates for three out of four species of fungi tested. Nevertheless, Mousa and Forster6 showed that adding glucose to a well-mixed sludge system increased both the degradation of gallic acid and the rate at which this occurred, but the mechanism by which glucose operates to counteract gallic acid inhibitory effects was not studied by the authors.7 However, it is notable that the aforementioned studies dealt with the anaerobic digestion of phenolic compounds over short retention times, e.g. between 3 and 24 h, while no information regarding
anaerobic degradation over extended periods of time was given.

In an effort to determine the adequacy of anaerobic treatment for OMW, a low-cost method would be required to evaluate the effect of the process on the degradability of phenolic and other OMW constituents. This low-cost method would have no real time constraints, allowing thorough and detailed evaluation of the process. The Miller and Wolin method allows the monitoring of the anaerobic behaviour of a molecule by measuring the gases (mainly CH4) which are produced when a solution of the compound is exposed to anaerobic microorganisms under similar anoxic conditions.

The aim of this work was (a) to test the applicability of this low-cost method suggested by Miller and Wolin for the anaerobic degradability of gallic acid, a model phenolic compound typically found in OMW, and (b) to evaluate the ability of anaerobic microorganisms to decompose gallic acid at various concentrations. Gallic acid was chosen as model compound because previous work had shown that it can seriously suppress anaerobic activity.

**MATERIALS AND METHODS**

In this study, gallic acid (3,4,5-trihydroxybenzoic acid) was selected as a model phenolic compound of OMW. Solutions containing gallic acid (GA) at concentrations of 100, 500 and 1000 mg L\(^{-1}\) were tested in order to examine the effect of GA concentration on its decomposition by anaerobic microorganisms. Anaerobically treated sewage sludge from a municipal wastewater treatment plant (Heraklion, Greece) provided the anaerobic micro-organism inoculum. The activity of the anaerobic micro-organisms in the sludge was assessed by the use of a simple organic compound, sucrose, considered as one of the so-called anaerobic growth factors.

To 250 mL glass bottles a total volume of 155 mL of each mixture was added. For each GA concentration the following mixtures were studied: mixture I containing 155 mL of GA solution without sludge, mixture II containing 150 mL of GA solution + 5 mL of anaerobically digested sludge as the inoculum, mixture III containing 150 mL of 200 mg L\(^{-1}\) sucrose solution + 5 mL of sludge and mixture IV containing 150 mL of deionised water + 5 mL of sludge. Fresh sewage sludge was collected and used in each run. For statistical validity of the results, each sample was run in six replicates. In all mixtures the pH was corrected to a value between 7.2 and 7.5, and 1 mL of 1% resazurin redox indicator was added to each mixture to visualise pH changes. Resazurin is the most widely used indicator of reducing conditions. It turns colourless in reduced state, whereas it changes from violet to orange/red when the system becomes acidic. Next the bottles were sealed using rubber tops. With the help of a butterfly-shaped blood-sampling needle system and pressurised nitrogen gas, oxygen was removed from the bottles. Having established anaerobic conditions, the bottles were placed in a water bath in order to control and retain the bioreactor temperature at 35 °C. Using the same needle system and a 20 mL glass syringe, the volume of gas produced daily was recorded for a period of 23 days, after which gas production of all mixtures had almost ceased.

**RESULTS AND DISCUSSION**

All three GA concentrations (100, 500 and 1000 mg L\(^{-1}\)) produced large amounts of gas, the cumulative values of which for the time span of 23 days are shown in Table 1. It is evident that increasing the GA concentration increased the volume of gas produced, indicating that the anaerobic micro-organisms in the sludge were capable of biodegrading GA. Specifically, when 1000 mg L\(^{-1}\) GA with sludge was used, the average (of six replicates) cumulative gas volume collected was 87.6 ± 2.4 mL, while without sludge the volume was 9.3 ± 1.0 mL. The corresponding values for 500 mg L\(^{-1}\) GA were 48.0 ± 8.0 and 6.5 ± 1.0 mL respectively, while for 100 mg L\(^{-1}\) GA they were 30.8 ± 4.7 and 9.6 ± 2.3 mL respectively. Sludge mixed with deionised water produced 13.6 ± 5.1 mL, while the solution containing 200 mg L\(^{-1}\) sucrose and sludge produced 34.1 ± 2.7 mL.

The gas production of the anaerobically treated sludge in the water and sludge mixture which was used as control indicates that the sludge was a substrate rich in anaerobic micro-organisms, supplying also the missing nutrients to the digestion system. This good condition of the inoculum resulted in all three GA mixtures producing large amounts of gas in comparison with the mixtures where no sludge was added. Figures 1–3 show the daily gas evolution–time profiles for the three GA concentrations of 1000, 500 and 100 mg L\(^{-1}\) respectively. If the average gas production measured in the water and sludge mixture (i.e. 13.6 mL) is deducted from the respective values for the GA/sludge mixtures, then the net gas production values (i.e. corresponding to the gas produced solely by the decomposition of GA) for the 1000, 500 and 100 mg L\(^{-1}\) mixtures are 74.0, 34.4 and 17.2 mL respectively, far larger than the volumes measured in the mixtures not containing sludge (Table 1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>With sludge (ml of gas ± SE)</th>
<th>Without sludge (ml of gas ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000 mg L(^{-1}) GA</td>
<td>87.6 ± 2.4</td>
<td>9.3 ± 1.0</td>
</tr>
<tr>
<td>500 mg L(^{-1}) GA</td>
<td>48.0 ± 8.0</td>
<td>6.5 ± 1.0</td>
</tr>
<tr>
<td>100 mg L(^{-1}) GA</td>
<td>30.8 ± 4.7</td>
<td>9.6 ± 2.3</td>
</tr>
<tr>
<td>Deionised water only</td>
<td>13.6 ± 5.1</td>
<td>–</td>
</tr>
<tr>
<td>200 mg L(^{-1}) sucrose</td>
<td>34.1 ± 2.7</td>
<td>–</td>
</tr>
</tbody>
</table>

SE, standard error.
However, in line with previous work, GA mixtures exhibited a delay in gas production compared with both sucrose and deionised water mixtures. In Figs 1–3, GA (with or without sludge) gives very low (1000 mg L\(^{-1}\) mixture) or even zero (500 and 100 mg L\(^{-1}\) mixtures) gas production during the first 24 h, while, as shown in Fig. 4, sucrose results in an average value of 4.0 ± 2.1 mL. Even when just 5 mL of sludge was mixed with water, 2.8 ± 2.2 mL of gas was produced (data also shown in Fig. 4). These findings agree well with the work of Borja et al.,\(^3\) FitzGibbon et al.,\(^5\) and Mousa and Forster,\(^7\) who suggested that...
GA is difficult to degrade anaerobically in digesters with retention times below 24 h. Nevertheless, 48 h after inoculation and fermentation, all three GA mixtures produced substantial amounts of gas, providing compelling evidence that, given sufficient time in the digestion system, GA can be degraded by the anaerobic bioculture, thus overcoming the inhibitory effect reported in the literature.

The last argument is further supported by the fact that even the highest GA concentration (1000 mg L\(^{-1}\)) produced a considerable amount of gas within the experimental period, which again suggests that there was no evidence of a toxic effect of GA on the anaerobic micro-organisms found in the sludge. On the contrary, GA left exposed to these micro-organisms for a long period of time showed very good decomposition behaviour, producing gas almost constantly throughout the monitoring period. Based on these results, it can be suggested that GA can be decomposed under anaerobic conditions even at high concentrations if sufficient time and an appropriate variety of micro-organisms are provided. This finding is in good agreement with FitzGibbon et al.\(^5\) and Borja et al.,\(^3\) who showed that the growth rate of the fungus Geotrichum candidum was not affected by GA, whereas the growth of other fungi was inhibited. Anaerobically treated sludge must be considered as an adequate inoculum owing to the huge variety and number of micro-organisms that can be found in it.

Gallic acid, a carboxylated hydroxyl derivative of aromatic hydrocarbons, is considered hydrolysable in warm water. With heat it can decompose to give carbon dioxide and the highly reductive molecule 1,2,3-trihydroxybenzene (also referred to as pyrogallol, \(\text{C}_6\text{H}_3(\text{OH})_3\)) according to the reaction \(\text{C}_6\text{H}_2(\text{OH})_3\text{COOH} + \text{heat} \rightarrow \text{C}_6\text{H}_3(\text{OH})_3 + \text{CO}_2\). Pyrogallol is a highly reductive compound which tends to prevent the fermentation of complex carbohydrates into simple sugars.\(^10\) Bearing this in mind, the poor anaerobic biodegradability performance of mixtures of GA and sludge during the first 24 h of fermentation may be attributed to some soluble molecules generated by initial GA decomposition (conversion to \(\text{C}_6\text{H}_3(\text{OH})_3\)) and/or complex lipids in the sludge which are refractory and/or inhibitory to hydrolytic anaerobic micro-organisms.

Moreover, it seems that the observed delay during the first 24 h of digestion reported previously by several authors may be related to hydrolytic bacteria. Such bacteria secrete extracellular enzymes, with the aid of which the various compounds in the digestion system are broken down and liquefied.\(^10\) When considering the co-digestion of GA and sewage sludge, the liquefaction of complex polymeric substances may constitute the rate-limiting step and must be included in the digestion model. In this case, hydrolysis is expected to be mostly inhibited by GA. Enzymatic reactions involving enzyme inactivation imply the dependence of the hydrolysis rate constant on parameters such as pH, substrate and digestion retention time. It has been argued elsewhere\(^11\) that the increase in hydrolysis rate at increasing biodegradability suggests that the rate of hydrolysis of particulate organic matter is determined by the adsorption of hydrolytic enzymes to the biodegradable surface sites. This compares well with the classical addition of complexes of enzymes to improve the efficiency of anaerobic sewage sludge digestion.\(^12\)

It may be assumed that the anaerobically treated sludge used in this work is an excellent inoculum owing to the huge variety of the microbial biomass, but the drawback is that it also consists of organic complexes which contribute to the GA inhibition effect. Apparently, this study showed that decomposition does indeed take place provided that a period of more than 24 h is allowed for the system to regulate itself.

In most cases a co-substrate improves digestion and therefore biogas yields as a result of positive synergisms established in the digestion medium and the supply of missing nutrients by the co-substrate. This can

**Figure 4.** Average daily gas production for the deionised water (full symbols) and sucrose (open symbols) sludge-containing mixtures. Six replicates were averaged per sample; bars indicate standard error.
explain the increase in gas produced when sucrose was mixed with the sludge. The same explanation may be given in the case of the counteraction of glucose to the inhibitory effect of GA reported by Mousa and Forster. Glucose, a readily degradable organic molecule, acts as a source of energy for microorganisms, promoting the growth of their cell tissue and resulting in an increase in microbial biomass. Thus it may be speculated that glucose addition enhanced the microbial action to secrete external enzymes which dissolve refractory phenolic compounds more actively.

Regarding the third stage of the digestion mechanism, methanogenesis, it has to be mentioned that this process relies on obligate anaerobes, whose overall growth rate is slower than that of the micro-organisms responsible for the preceding stages (hydrolysis and acidogenesis). Phenolic compounds associated with complex lipids in sludge are very stable and highly inhibitory to methanogenic consortia. However, toxic phenolic compounds can be efficiently degraded, possibly through adsorption onto the bacterial biomass. Enzymes are expected to play a key role in this function as well.

Comparing the gas evolution–time profiles for the various GA- and sucrose-containing mixtures, two distinct peaks were recorded on days 2 and 4, probably as a result of the primary decomposition of both the substrate (GA or sucrose) and any other compounds found in the sludge. It is likely that at this stage the dominant gas produced in the mixtures of GA and sludge is CO₂. A possible explanation of this assumption is that, as readily degradable compounds in the sludge decompose, they provide the enzymes necessary for hydrolysis and acidogenesis of the resistant GA in the mixture. A third peak (for the 1000 and 100 mg L⁻¹ GA and sucrose mixtures) was recorded between days 8 and 10, probably mainly due to CH₄ produced from degradable compounds in the sludge and by-products (i.e. CO₂ and H₂) of the second stage of digestion (acidogenesis) of GA. The delayed methanogenesis of GA may be linked to methanogenic bacteria, which can be impaired by the complex substrate of the GA/sludge digestion system and the drop in pH from acidogenesis, which has probably started occurring from day 2. The above assumptions compare well with colour changes noted during the experiment via the resazurin indicator, which turned from purple to orange/red on day 2, verifying the slow decrease in pH as the digestion proceeded from hydrolysis to acidogenesis. As the experiment continued (from day 13 to day 21), the colour again turned to red/purple, an indication that acidification had effectively been overcome by CH₄ production. It was clear that the colour of mixtures of GA without sludge addition remained purple, suggesting that hardly any decomposition took place in the absence of inoculum.

Figure 5 shows cumulative gas production as a function of GA concentration. As can be seen, there is an almost linear relationship between gas generation and substrate concentration (correlation coefficient $r^2 = 0.95$). This relationship is of great importance for the appropriate design and operation of an anaerobic digester treating OMW, since it will allow the determination of expected gas production in relation to phenol concentration.

All experiments were carried out at 35 °C, i.e. within the mesophilic range. Of course, higher temperatures (within the thermophilic range) would have led to an increase in the growth rate of the microbial culture and thus in the rates of decomposition and gas production. However, it was not within the scope of this work to accelerate decomposition rates, but to test the applicability of a simple, low-cost method for degrading refractory compounds in OMW by anaerobic micro-organisms found in municipal wastewater.

Interestingly, the statistical analysis of the data obtained in this work showed that, despite the heterogeneity of the sludge used as inoculum source and the overall complexity of the system
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employed, the results were within confidence limits of 92–95%. This implies that the method used was well applicable for this study, while the system was well reproducible, suggesting that the biochemical pathway which plays a key role in the whole process is dominated and regulated by the various microorganisms participating, acting and counteracting.

It should be noted that no attempt was made to determine the composition of the biogas produced. During the overall digestion process, different gases would have been produced, i.e. (a) volatile fatty acids derived from protein, fat and carbohydrate components of the sludge in the hydrolysis step, (b) CO₂ and H₂ from the acidogenic step and (c) CH₄ and CO₂ from methanogenesis. Additional information on the composition of the gas phase would give a better insight into the biodegradation process, i.e. which gases are produced, in what proportions of the overall gas volume and by which specific mechanism(s).

CONCLUSIONS

The olive oil extraction industry generates resistant wastewater that is often eliminated by evaporation in open ponds or evaporation tanks. However, owing to the large volume and high pollution potential of this phenolic-rich effluent, the development of low-cost and effective processes for OMW biodegradation is an attractive approach to the industrial needs for OMW handling in-plant. Phenolic compounds associated with complex lipids in OMW are very stable and highly inhibitory to methanogenic consortia as a result of their antimicrobial activity (exerted both by the organic acid molecules and by the low pH). In this work the efficiency of anaerobic bacteria to sufficiently decompose GA was assessed in terms of biogas production.

Applying the Miller and Wolin⁸ quantitative, low-cost method, this work evaluated the effect of the process on the decomposition of GA and discussed possible degradation pathways. The data presented here show that (a) the technique can provide very useful information on the anaerobic biodegradability of compounds, especially if combined with gas chromatography for qualitative analysis of the gases produced, (b) GA readily decomposes under anaerobic conditions, suggesting that anaerobic digestion could be a possible treatment solution for OMW, and (c) prolonged digestion times of several days may be needed for the complete degradation of GA and similar compounds.

REFERENCES